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MOLECULAR AND GENETIC CHARACTERIZATION OF ALS PATIENTS

BIO-10

Dott.ssa CLAUDIA TARLARINI
Matricola: R09280

Tutor: Prof.ssa ELISABETTA GIANAZZA

Co-Tutor: Dott.ssa SILVANA PENCO

Coordinatore del Dottorato: Chiar.mo Prof. FRANCESCO BONOMI

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Abstract

Amyotrophic lateral sclerosis (ALS) is an adult-onset, rapidly progressive and ultimately fatal neurodegenerative disorder characterised by degeneration of upper and lower motor neurons. This leads to weakness, muscular atrophy and spasticity, which relentlessly progress to complete paralysis with a low survival rate beyond 5 years from symptom onset. In 10% of cases the disease is considered to be genetically transmitted (FALS) while in the remaining cases it occurs sporadically in the population (SALS). To date, cases of hereditary ALS have been attributed to mutations in more than 16 different genes, the most common being *SOD1*, *FUS*, *TARDBP* and *C9ORF72*; mutations in other genes are rare. The above genes explain 60% of the cases of familial ALS and 15% of sporadic cases.

The disease can be subdivided into bulbar (25%) and spinal-onset (75-80%) forms. Nevertheless, it is currently recognized that pathological changes are not limited to the motor system: patients with ALS may exhibit cognitive abnormalities ranging from impaired frontal executive dysfunction to overt frontotemporal dementia (FTD).

In spite of the above evidence, ALS is regarded as a complex disease in which multiple environmental and genetic risk factors contribute to disease susceptibility. Furthermore it is possible that the phenotypic variability, which is frequently detected within families, could be due to multiple genetic factors as devised in the oligogenic disease model.

In the present study we analysed 285 SALS and 17 FALS cases. Globally, our molecular analysis explained 10.3% of all ALS cases (31/302). The genes screened were *SOD1*, *TARDBP*, *FUS* and *C9ORF72*. Genomic DNA was extracted from peripheral blood through a salting out procedure; coding regions and exon-intron boundaries of *SOD1* (5 exons), *TARDBP* (1 exon), and *FUS* (5 exons) genes were amplified from genomic DNA and sequenced. Otherwise the repeat-primed PCR assay, used for *C9ORF72* gene, was performed in order to screen for the presence of the GGGGCC hexanucleotide repeats expansion in ALS patients. The repeat unit of 6 nucleotides expands up to more than several hundred times in the affected individuals. Fewer than 10 repeats are not associated with a pathological phenotype, while more than 30 repeats are associated. However we still do not know the meaning

of intermediate repeat sizes (11-29). This fact complicates the attribution of the size expansion to the pathological phenotype observed.

According to the oligogenic disease model, all patients were screened for the most common ALS-associated genes and all mutated subjects were tested also for *ANG*. The affected/unaffected family members, when available for the study, were screened for *SOD1*, *TARDBP*, *FUS* and *C9ORF72* in order to identify their genetic difference from the proband and to evaluate if this difference could explain an heterogeneous phenotypic expression of the disease.

Following these analyses we selected and described in detail 5 FALS and 2 SALS cases and one *SETX* case, with different intrafamilial phenotypic expression. In one of our *SOD1* mutation carriers, the proband manifested the disease after the delivery; together with a specific angiogenin genetic variant this condition seems to have anticipated the age of disease onset and contributed to the aggressive clinical course observed in the proband compared to other family members. We also found one case in which we observed the phenomenon of anticipation, which could be due to hormonal treatments together with the simultaneous presence of 2 mutations (*C9ORF72/ TARDBP*), as suggested in the oligogenic disease model. Indeed, the neuroprotective effects of estrogens could account for the later age at onset in women as we have tested in another family harbouring the R521C mutation. In this case a male subject developed the disease, whereas his older sister didn't show any neurological signs. In another family we observed a wide spectrum of clinical symptoms associated with A382T *TARDBP* mutation. Some family members showed cognitive impairment without signs of ALS, conversely, other relatives showed a typical ALS without any signs of dementia. In addition we have evidence of *TARDBP* mutation carriers without a neurological phenotype. Surprisingly, two subjects harbouring the mutation in homozygous state display different spectra of clinical symptoms. The screening of *SOD1*, *FUS*, *C9ORF72* and *ANG* in the family members, to identify other mutations that could explain this intrafamilial phenotypic heterogeneity, resulted negative; indicating that other genes/conditions could play a role in ALS disease.

These data support the hypothesis that phenotypic variability seen in patients carrying the same mutation, could be attributed to additional genetic and/or environmental factors, which could modify the penetrance of the disease. Furthermore we identified a

clinical subgroup of patients that develop the disease during pregnancy (child-bearing age), thus indicating an additional modifier conditions linked to hormonal changes. For all these reasons predictive or presymptomatic testing should be undertaken with caution, especially in unaffected family members. Indeed, it is impossible for the genetic test to predict clinical outcomes based on the genotypic results only. A number of uncertainties remain, due to variability in penetrance, expressivity, and modifying factors, such as gene-gene interactions and environmental influences. This adds to the debate for the ethical and psychological dilemmas about genetic testing, since still more has to be discovered related to this devastating disease.

Introduction

Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS) is a human adult-onset neurodegenerative disease, characterized by progressive weakness, muscle atrophy, spasticity, paralysis, and death, generally within 3 to 5 years after onset of symptoms. The symptoms are related to degeneration and loss of upper motor neurons in the cerebral cortex and lower motor neurons in the brain stem and spinal cord [(1)].

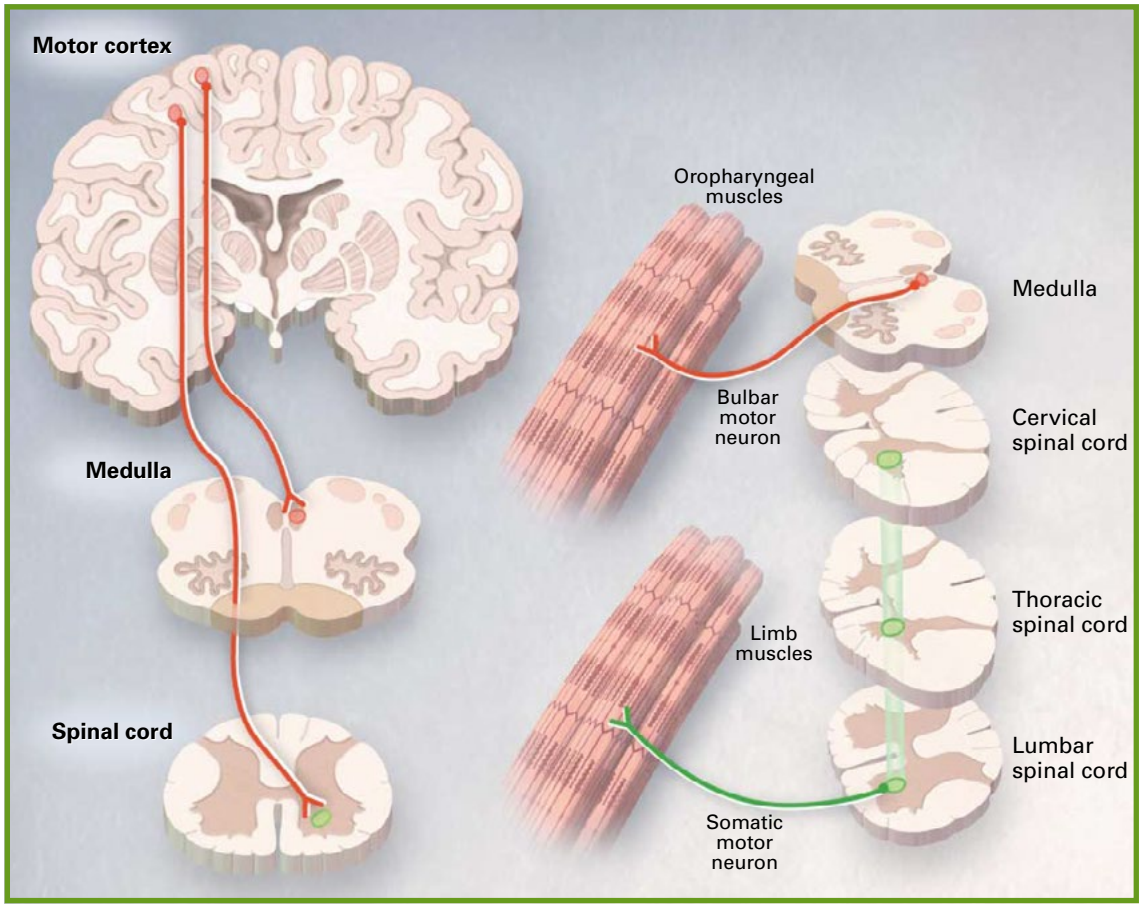


Figure 1. *Motor neurons selectively affected in ALS patients*

The French neurologist Jean-Martin Charcot described for the first time ALS in 1874. In the United States it is known as Lou Gehrig disease, by the name of the famous baseball player who, in 1930, was the first confirmed victim of this disease. ALS cases represent about 85-90% of motor neurons disease (MND), a heterogeneous group of adult-onset neurodegenerative disease.

The term “amyotrophic” refers to the atrophy of muscle fibres, which leads to weakness of affected muscles and visible fasciculations that signify disease of the lower motor

neurons. “Lateral sclerosis” refers to the hardness to palpation of the lateral columns of the spinal cord in autopsy specimens, where gliosis follows degeneration of the corticospinal tracts. The clinical results are upper motor neuron signs: overactive tendon reflexes, Hoffmann signs, clonus, and Babinski signs. ALS refers to one specific form of motor neuron disease in which there are both upper and lower motor neuron signs [1].

Recently, population-based studies have established that the incidence of ALS in Europe is fairly uniform at 2.16 per 100,000 persons/year. The mean age of onset for ALS varies between 55 and 65 years [2,3]. Few ALS patients have an early onset that causes a different progression of the disease; patients with age at onset below 41 years have survival 3 times longer than patients with an age at onset above 60 years [4].

Men have a higher incidence of disease (3.0 per 100,000 persons/year) than do women (2.4 per 100,000 persons/year), although the incidence in men and women is about the same in familial disease [5]. Explanations for this male excess have been attributed to possible protective hormonal factors in women, increased likelihood of males being exposed to such putative risk factors as trauma, physical activity and employment status [6]. After menopause the ratio M/F is around 1, suggesting a neuroprotective effect of estrogens [7,8]. Conversely, pregnancy seems to cause a fast progression of the disease [9].

Most ALS cases have no obvious family history and are referred to as sporadic ALS (**SALS**) whereas approximately 5-10% of ALS patients have a family history, with a Mendelian pattern of inheritance (**FALS**). There is also a juvenile form of disease with age at onset before 25 years (**jALS**) [10]. Familial ALS cases are indistinguishable from SALS on the basis of clinical and pathological criteria, which are very similar even if ALS patients show some degree of heterogeneity as far as symptoms, age of onset and disease duration. The mean age of onset for SALS is of about 60 year [2,3]. Only 5% of cases have an onset before the age of 30 years [3], although juvenile sporadic onset cases are being increasingly recognized [11]. The age of onset of FALS is about a decade earlier than for sporadic cases. This form affects males and females equally; patients have a shorter survival than with ALS [12]. Age of onset in FALS has a normal Gaussian distribution, whereas SALS has an age-dependent incidence.

On the basis of the clinical pattern in the initial phase of illness, different variants have been described, including bulbar, spinal and pseudoneuritic forms, flail arm syndrome, and Mill's hemiparetic type. Bulbar-onset ALS is more frequent in females and in older age groups, with tare 43% of patients over the age of 70 presenting with bulbar symptoms in comparison with 15% below the age of 30 [13]. There is a general consensus in considering older age and bulbar-onset as major negative prognostic factors [14]. The upper motor neuron phenotype appears to be a strong independent predictor of long survival [15]. Disease duration depends on the timing of involvement of respiratory muscles. Respiratory weakness may represent the onset symptom in 5% of patients or can take place later in the course of the disease, as in flail arm ALS and in upper motor neuron disease forms [15,16].

The overall median survival of patients with ALS mainly depends on site of onset of symptoms: 2-3 years for bulbar-onset cases and 3-5 years for limb onset ALS cases [17,18]. Large clinic cohort studies have shown 3 year and 5 year survival groups to be around 48% and 24% respectively, with approximately 4% surviving longer than 10 years [19], whereas 5 year survival reported in population based studies is much lower and ranges from 4 to 30% [17].

Despite many clinical trials and various advances in the understanding of ALS, there has been little success in the search for disease modifying or neuroprotective agents. Riluzole is the only approved drug that has been shown to have a modest effect on prolonging life in ALS patients [13]. The mechanism of action of riluzole is not entirely understood but is thought to include interference with N-Methyl-D-Aspartate (NMDA) receptor mediated responses, stabilization of the inactivated state of voltage dependent sodium channels, inhibition of glutamate release from pre-synaptic terminals, and increase of extracellular glutamate uptake [20].

Over 100 other neuroprotective agents have been studied in animal models and humans. Some agents that have been evaluated in phase II or III human clinical trials have shown inconclusive evidence or failed to demonstrate effectiveness for routine clinical practice.

Epidemiology

Very recently in literature many epidemiological studies have appeared about the distribution of ALS according to sex, age and geographical origin. Through these studies it was possible to classify ALS in 3 categories:

- Sporadic ALS (SALS)
- Familial ALS (FALS)
- Western Pacific ALS

Sporadic ALS

The incidence of SALS in the 1990's is reported to be between 1.5 and 2.7 per 100,000 persons/year (average 1.89) in Europe and North America, with a uniform incidence across these countries. The point prevalence in Western countries in the 1990's ranges from 2.7 to 7.4 per 100,000 (average 5.2). Furthermore the mortality rates of ALS in the 1990's range from 1.54 to 2.55 per 100,000/year [7]. Recently the annual incidence of the disease in Northern Italy, which has been reported as 2.64 per 100,000 persons/year (men, 2.97/100,000; women, 2.32/100,000), seems to be remarkably similar to the ALS distribution in all Western countries [21].

Fifteen epidemiological studies have been performed by Worm in 2001 in order to provide accurate data on the frequency of ALS in Europe and in North America. These studies have pointed out a globally and steady increase of incidence (46%) and mortality rates (57%) in the 1990s as compared to the 1960s-1970s period. This increase appears mainly due to Southern Europe countries, to female gender and to patients aged 75 years and over. These increases might be, at least in part, due to increased life expectancy in the last 40 years, to a better diagnosis since the appearance of the El Escorial criteria in the early 1990s and to a higher accuracy of death certificates collection. However this analysis doesn't allow ruling out the hypothesis of a true increase in the incidence of motor neuron disease, possibly related to other impacting factors, such environmental ones [7].

In recent years many epidemiological studies have attempted to identify environmental factors associated with SALS. The endemic occurrence of ALS in regions such as in

the high-risk areas of the Pacific cannot be fully explained by genetic factors and is stirring an animated discussion on gene-environment interactions. Attention is focused in particular on the fruits of the locally growing cycad palms; indeed, geographic incidence rates for ALS are strongly correlated with concentrations of cycasin in traditional food prepared from the toxic seed of the cycad plant [22]. Other putative environmental risk factors for ALS include pesticides and insecticides [23], and neurotoxins to which Gulf War veterans had an extended exposure [24].

Chiò *et al.* suggested that a factor related to professional football in Italy, or more factors acting together, such as heavy physical exercise, traumas or micro-traumas, have a role in determining an increased risk of ALS. ALS might be related to either the use of illegal, toxic substances assumed to improve athletic performances or to the use of therapeutic drugs in excess of recommended doses or for a long period. Finally ALS might be related to environmental toxins such as fertilizers or herbicides used on football fields [25].

A number of epidemiological studies have assessed the association of cigarette smoking with ALS incidence. Compared with never-smokers, the rate ratio (RR) of ALS for ever-smokers was 1.04 (0.80-1.34). The corresponding RR was 1.53 (1.04-2.23) in women and 0.75 (0.53-1.06) in men. Old age and female sex were associated with lower survival. Smoking was a predictor of mortality only in women. Comparing ever- versus never-smokers, RR of death was 1.31 (1.04-1.65) in women, and 0.90 (0.72-1.11) in men. In conclusion, smoking is associated with higher risk of ALS and worse survival in women but not in men [26].

The prevalence of ALS is estimated at 4-6/100,000 persons with a uniform distribution across the world. Four well-studied geographic areas with a high prevalence of ALS (clusters) are known: the Guam island in Micronesia, the Japanese Kii peninsula, the Western coast of former West Papua New Guinea, and an isolated tribe at Anguru on Groote Eylandt in the Gulf of Carpentaria (North Australia). In the Western Pacific form of ALS the prevalence is 50-100 times higher than elsewhere in the world. There is a latitude-related increase in ALS, with geographic age-adjusted incidence rates ranging from 2.0 in Israel (32° latitude) to 8.0 in the Northern Scandinavian countries (>60° latitude) [22].

The incidence of ALS varies with age and can be expressed as age-specific incidence [21,27,28]; about 80% of ALS cases are aged between 40 and 80 years, while 20% is outside this age range [29]. ALS affects people of all ages but the age-adjusted incidence rate varies greatly in different age groups. Incidence is very low in the first four decades (1.5/100,000/year), increases abruptly around age 40, reaching its peak between ages 60 and 79 (10-15/100,000/year), and decreases thereafter [30].

This phenomenon may be due to the fact that in elderly patients ALS's symptoms could be both confused with signs of aging and be masked by other diseases, such as cerebrovascular or radicular pain, nerve or muscle. Conversely, the presence of a peak in the age-specific incidence curve suggests that the disease results from a time dependent exposure to some genetic or environmental risk factors. According to this hypothesis, early onset of the disease might reflect a harsher exposure to one or more of these risk factors [31].

In all these studies except one, the age-specific incidence was greater for men than for women at all ages. However, as already mentioned, the male/female ratio for incidence (male to female ratio of around 1.5:1) was greater at younger ages. The data indicate a greater prevalence of spinal-onset disease in men and a greater prevalence of bulbar-onset disease in women. It is unclear why there should be differences in the site of onset between men and women, but it could be related to gender differences in the motor neurons in different regions [32].

There have been attempts to explain the different vulnerabilities of motor neurons in ALS. The vulnerability of bulbar motor neurons is related in part to the expression of N-Methyl-D-Aspartate (NMDA) receptor subunits [33]. Estrogen enhances NMDA receptor expression [34], so this mechanism may explain in part the phenomenon.

Males and females also differ in the proliferative ability of oligodendrocytes in the central nervous system [35]. Furthermore, males have a shorter life span than females [36], and the loss of volume in the brain with aging occurs to a greater extent in men than in women [37]. This difference in the ability to repair damage could be relevant to possible differences in the clinical course of ALS.

Average survival in ALS is widely quoted as 3-5 years. Bulbar-onset of symptoms, older age at onset and more definite El Escorial category at presentation have been

associated with reduced survival [19]. Patients with bulbar-onset disease are significantly older than those with spinal-onset, thus the negative prognostic effect of bulbar-onset is entirely covered by the older onset age of disease in these patients. Conversely Chiò *et al.* found that age and bulbar involvement were independently related to survival, which indicates that the faster progression in bulbar cases is not entirely due to older age at onset [38].

Although ventilation support can ease problems with breathing and prolong survival, it does not affect the progression of ALS; a non-invasive assisted ventilation prolongs survival and improves or maintains quality of life in people with normal to moderately impaired bulbar function [39].

However, about 10 percent of patients survive for 10 or more years. These patients usually have phenotypic variants from “classic” ALS, such as the upper motor neuron only presentation of primary lateral sclerosis, which are associated with better prognosis [18].

Familial ALS

The familial form of ALS includes families in which at least two cases are observed in relatives separated by no more than four generations. The first case of FALS was reported by Aran in 1850, when he described 4 patients affected by progressive muscular atrophy within the same family.

In retrospective epidemiological studies carried out between 1958 and 1996, 0.8-13.5% of patients with ALS reported family history. The frequency of FALS is strongly variable both in different geographical areas and within the same country.

Differences in these studies could be explained through several factors:

- inhomogeneous diagnostic criteria between different studies;
- inadequate recording of pertinent family history in the patients’ charts;
- genealogical investigations not performed;
- different subtypes of ALS in different family members and disease not recognized as one;
- reluctance of the patient to report a hereditary disease;

- loss of contact between different members of a family;
- early death to other causes of individuals in the family who transmit the gene defect;
- age of onset younger in the patients of successive generations, so that the offspring develops ALS before the parent who transmitted the gene defect (anticipation);
- incomplete disease penetrance;
- misdiagnosis of family members with ALS;
- illegitimacy.

Although most cases of ALS are sporadic, about 5-10% of cases have a family history of ALS. While most cases of familial ALS are indistinguishable from sporadic disease, others have unique phenotypes [40]. Comparative studies demonstrated some features as specific to FALS in comparison with SALS [41]:

- age of onset is about a decade earlier than for sporadic cases;
- age of onset has a normal Gaussian distribution, whereas SALS has an age-dependent incidence;
- men and women are affected equally;
- generally the disease has a longer duration;
- onset often occurs in the lumbosacral segment;
- sensitive symptoms are more frequent at the onset;
- degeneration of the posterior columns of the spinal cord, spino-cerebellar traits and dorsal nucleus of Clarke is evident in 70% of patients with familial ALS.

The age of onset of familial ALS is normally distributed with a mean of 46 years.

Survival curves for familial ALS data also demonstrate a skewed distribution with a median survival time of 24 months, with 74% surviving at 1 year, 48% at 2 years and 23% at 5 years [42].

The phenotypic heterogeneity seen in sporadic forms of ALS are also common within families: for example, within a family age of onset may vary (over 30 years) as much duration of illness (0.5 to 5 years) and signs at onset [43]. At its onset the disease is usually focal and asymmetrical (e.g. wasting of muscles of one hand), then it spreads

in a contiguous way. In most cases lower motor neuron involvement is conspicuous whereas involvement of upper motor neurons is less evident. The disease may present at first as a predominantly lower motor neuron form (progressive muscular atrophy), as a predominantly upper motor neuron form (primary lateral sclerosis), or as predominantly affecting bulbar muscles (progressive bulbar palsy) [42]. Alternatively it may develop as classical ALS.

Based on a clinical, pathological, and genetic study of 14 families [44], at least three types of familial motor neuron disease can be distinguished (Table 1):

Type I	It is characterized by rapid, progressive loss of motor function with predominantly lower motor neuron manifestations and a course lasting less than 5 years. Pathological changes are limited to the anterior horn cells and pyramidal tracts.
Type II	It is clinically identical to the first, but at autopsy additional changes are found in the posterior columns, Clarke's column, and spino-cerebellar tracts.
Type III	It is characterized by a much longer survival, usually beyond 10 and up to more than 20 years in affected family members, but is otherwise similar to type II.

Table 1. *Subclassification of FALS forms on a clinical basis*

Western Pacific ALS

As already mentioned, four geographic areas in the Western Pacific with a high prevalence of ALS are known: the Guam island and Rota in Micronesia (Mariana island), the Japanese Kii peninsula, the Western coast of former West Papua New Guinea and an isolated tribe at Anguru on Groote Eylandt in the Gulf of Carpentaria (North Australia).

Immediately after World War II, an unusual frequency of ALS was noted in Guam. Epidemiological studies indicated that the prevalence of ALS in Guam was 50-100 times the prevalence anywhere else. In 1960s incidence was about 179/100,000 for men and 60/100,000 for women.

The Chamorros of Guam call the disease *lytico-bodig*, and neuroscientists refer to it as the amyotrophic lateral sclerosis/Parkinsonism-dementia complex (ALS-PDC), because affected subjects are prone to manifest atypical Parkinsonism, dementia, motor neuron disease, or a combination of these three phenotypes.

Several theories have been proposed to explain the high prevalence of ALS/PDC among the Chamorro population [45,46]. Initially ALS and PDC were thought to represent genetic disorders [47]. These early studies demonstrated that close relatives of affected subjects were at greater risk for disease than relatives of controls. In addition, there were many families with multiple individuals in two or more generations, including some pedigrees in which four successive generations were affected. Furthermore, the identification the ALS and PDC among Filipino migrants to Guam implicated environmental factors in the pathogenesis of these disorders. The cause of ALS/PDC is unknown; therefore, it is not known if its environmental aetiology is connected to single events or repeated and cumulative. The age of exposure and latency is also unknown but it is known that the annual incidence of ALS and PDC has steadily declined since 1955 [48], and this finding must indicate that its cause ended many decades ago. The author does not know of any pathologically verified case born after 1951.

However, mathematical models constructed from over 2000 affected and non-affected residents of Guam rejected purely environmental, autosomal dominant, and autosomal recessive hypotheses of disease transmission and may indicate that both genetic predisposition and environmental exposure are necessary to develop this condition [49].

Two types of environmental hypotheses have been proposed to account for Western Pacific ALS/PDC. One is the absence of essential minerals and the other is the presence of exogenous toxin common to affected areas. The possibility that neurotoxic trace elements might be related to ALS/PDC was first proposed by Yase in 1972 [50]. Relatively high levels of aluminium and manganese and low levels of calcium and magnesium were found in samples of drinking water and soil in all three foci of ALS/PDC, including Guam, the Kii peninsula and Western New Guinea. Garruto *et al.* [51] have demonstrated deposition of aluminium in affected neurons, and in 1989 have shown that the deficiency of calcium and magnesium in the soil leads to increased uptake of aluminium as an alternative source of cations, which is the possible cause of neuronal degeneration and death [52,53]. Alternatively, the accumulation of iron with aluminium in neurons causes oxidative stress leading to degeneration of vulnerable neurons. The incidence of Guamanian ALS/PDC has been associated with prolonged exposure to an environment severely deficient in Ca^{2+} and Mg^{2+} [54].

The leading candidate as the source of an exogenous toxin used to be the toxic seed of the false sago palm, *Cycas circinalis* [55]. Cycad, used as food source in flour as well as poultice in traditional medicine by the Chamorros of Guam, is highly hepatotoxic if ingested in its raw form. However, the seeds are detoxified, through ritualistic washing, before use.

In the late 1980s Spencer *et al.* described neurological symptoms in cynomolgus monkeys that were fed large doses of beta-methyl-amino-L-alanine (BMAA) [56], an “unusual“ amino acid that is present in low quantities in cycad seeds. However, the doses required to achieve toxicity were unrealistic, and BMAA is readily removed from the seed with washing. However, Cox and Sacks have once more resurrected the cycad toxic theory by arguing for “biomagnifications” (or concentration) of toxins by consumptions of flying fox (*Pteopus ariannusmariannus*). The Chamarro people traditionally feasted on flying foxes, which consume large quantities of cycads [57]. Very recently the neurotoxic effects of cholesterol β-d-glucoside (CG) and cycad phytosteryl glucosides have been associated with increased oxidative damage of neurons [58].

Chen and colleagues reported the disappearance of amyotrophic lateral sclerosis (ALS) from Guam over the past 30 years, when one third of the Island land was used for construction of huge military bases, which coincided with rapid changes in ecology and socio economy, and with Westernisation of the life style. This slow but steady decline is believed to be the consequences of radical changes from food collection to wage-based life style and dietary improvement in recent years and removal of exogenous factors [59].

Clinical features

ALS may be suspected whenever an individual develops unexpected loss of function, or gradual, slowly progressive, painless weakness in one or more regions of the body, without changes in the ability to feel, and no other cause is immediately evident.

The clinical diagnosis of ALS in a person requires the presence of both upper and lower motor neuron features and progression of disease. Typical ALS patients show symptoms related to focal muscle weakness. In lower motor neuron (LMN) involvement, fasciculations (involuntary muscle twitching, especially in tongue and limbs) may occur early on in the disease. In addition, typical signs of LMN are atrophy, muscular hypotonia and cramps. Patients with upper motor neuron (UMN) involvement generally are hyperreflexic and stiff. Reflexes may be diminished due to LMN involvement. UMN symptoms may include spasms and sudden, uncontrolled straightening movements of the lower limbs (spasticity). Babinski's (anomaly of the cutaneous plantar reflex) and Hoffman's (same for the upper limbs) signs are variably present in the early stage of disease. These symptomatic signs reflect degeneration and death of upper and lower motor neurons in the brain and spinal cord. The degeneration of at least 30% of small interneurons in the motor cortex and spinal cord also occurs, sparing however muscles that control eye movements and urinary sphincters [(40)].

The disease can be subdivided into **bulbar**- and **spinal**-onset forms. Approximately 75-80% of patients with typical ALS have a spinal form of the disease (classical 'Charcot ALS'). They present with symptoms related to focal muscle weakness where the symptoms may start either distally or proximally in the upper limbs and lower limbs. Patients with upper limb onset have twice the likelihood for onset in the dominant arm, compared with the non-dominant arm. There is equal likelihood for presentation in either lower extremity. Persons with upper limb onset may experience reduced finger dexterity, cramping, stiffness, and weakness or wasting of intrinsic hand muscles. This may lead to difficulty with actions such as buttoning clothes, picking up small objects, or turning a key.

Patients who have lower limb onset initially may complain of tripping, stumbling, or awkwardness when running. Foot drop is common, and patients may report a "slapping" gait. Focal muscle atrophy especially involves the muscles of hands, forearms or shoulders in the upper limbs, and proximal thigh or distal foot muscles in the lower limbs. Tendon reflexes are pathologically brisk in a symmetrical manner, including finger jerks in the upper limbs and positive crossed adductor reflex in the lower limbs. Abnormal spread of tendon reflexes beyond the stimulated muscle group may be evident. The Hoffmann's and Babinski's signs may be positive [(13)].

Rarely, patients may notice focal muscle wasting before onset of weakness, and some patients may present with a spastic paraparesis. Patients may have noticed fasciculations or cramps preceding the onset of weakness or wasting for some months (or years), but rarely these are the presenting symptoms. The weakness is usually of insidious onset, and patients may notice that symptoms are exacerbated by cold weather. Although the disease is usually asymmetrical at onset, sooner or later the other limbs develop weakness and wasting, and most patients with time develop bulbar symptoms and eventually respiratory symptoms (although not necessarily in that sequence). Gradually, spasticity may develop in the weakened atrophic limbs, affecting manual dexterity and gait. During late stages of the disease patients may develop 'flexor spasms', which are involuntary spasms occurring due to excess activation of the flexor arc in a spastic limb. Occasionally encountered symptoms include bladder dysfunction (such as urgency of micturition), sensory symptoms, cognitive symptoms and multi-system involvement (e.g. dementia, Parkinsonism).

The bulbar-onset form accounts for about 25% of ALS cases [12]. Patients with bulbar-onset ALS usually present dysarthria or, rarely, dysphagia for solid or liquids, up to the complete loss of verbal communication (anarthria) and fasciculations in the tongue. Limbs symptoms can develop almost simultaneously with bulbar symptoms and in the vast majority of cases will occur within 1-2 years. Almost all patients with bulbar symptoms develop sialorrhea (excessive drooling) due to difficulty in swallowing saliva and mild UMN type bilateral facial weakness that affects the lower part of the face. "Pseudobulbar" symptoms such as emotional instability and excessive yawning are seen in a significant number of cases. Denervation of the respiratory muscles and diaphragm is generally the fatal event and, in most cases, it occurs within 5 years of disease onset. Patients with ALS may exhibit a range of cognitive abnormalities, ranging from impaired frontal executive dysfunction, in 20-40% of patients, to FrontoTemporal Lobar Dementia (FTLD), in approximately 5% of cases [13].

About 5% of cases with ALS present with respiratory weakness without significant limb or bulbar symptoms. These patients present with two types of symptoms: respiratory failure or nocturnal hypoventilation such as dyspnea, orthopnea, disturbed sleep, morning headaches, excessive day time somnolence, anorexia, decreased concentration and irritability or mood changes [60,61]. Thus, ALS is now regarded as a multi-system degenerative disease, in which the earliest and most severe degenerative

changes tend to affect lower and usually upper motor neurons. Even within motor neuron groups, there is selective vulnerability. Certain groups of motor neurons, including those controlling eye movements and those in Onuf's nucleus in the sacral spinal cord controlling the pelvic floor muscles, tend to be spared in ALS in comparison with other groups of motor neurons [62].

In the human motor neurons uptake of calcium via glutamate receptor ion channels represents one of the key processes for triggering second messengers as well as for mediating toxic events. Indeed, sustained elevation of intracellular calcium can result in activation of a cascade of damaging biochemical events. These calcium-activated biochemical processes can injure neurons both directly and through the generation of free radicals [63].

The AMPA subtype of glutamate receptor is responsible for much of the fast excitatory transmission in mammalian CNS (central nervous system). In particular, the GluR2 subunit of this receptor renders the AMPA receptor impermeable to calcium, thus the low expression of GluR2 by human motor neurons implies that most of the surface AMPA receptors expressed by this cell group are likely to be atypical and calcium permeable. This feature suggests that human motor neurons may differ from many other groups of neurons in the human CNS in expressing a high proportion of calcium permeable AMPA receptors (Figure 2) [62]. Moreover less vulnerable motor neuron groups express parvalbumin and calbindin respectively [64]. These proteins buffer intracellular calcium and play an important role in protecting neurons from calcium-mediated injury following activation of glutamate receptors [65]. Conversely, human motor neurons that are vulnerable in ALS do not express calcium binding proteins. Thus, the combination of the low expression of the GluR2 AMPA receptor subunit and the absence of calcium buffering proteins may result in ALS susceptibility of motor neurons.

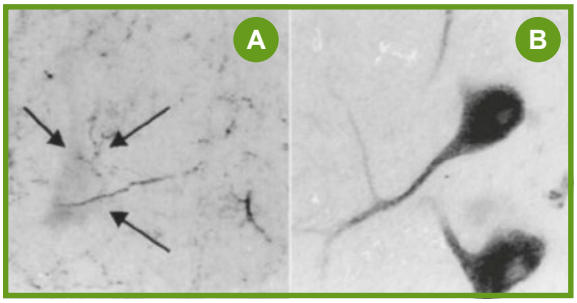


Figure 2. *Immunohistochemistry using an antibody that specifically detects the GluR2 AMPA receptor subunit.* ***A.*** *Upper motor neurons (Betz cells) show no staining.* ***B.*** *Many other neuronal groups stain strongly.*

Irrespective of the age at onset or of the presentation of the disease, ALS has always a progressive and invariably fatal course. Paralysis is progressive and leads to death within few years. Respiratory failure and other pulmonary complications or eventually insufficient caloric intake due to swallowing disorders (dysphagia) are the usual cause of death in ALS. However, patients who are kept alive by tracheostomy assisted ventilation are found to eventually develop a profound motor paralysis termed the 'totally locked-in state' (TLS), were there is paralysis of all voluntary muscles and varying degrees of oculomotor impairment [66].

The diagnosis of ALS is currently regulated according to the guidelines formulated in 1990 by the World Federation of Neurology. The El Escorial Criteria were initially developed to standardize the diagnosis and ensure inclusion of homogeneous patient population in clinical trials. The criteria were revisited in 1998 (Airlie House) and more recently in 2008 (Awaji-shima) (Table 2):

The presence of	evidence of lower motor neuron degeneration by clinical, electrophysiological, or neuropathological examination
	evidence of upper motor neuron degeneration by clinical examination
	progression of the motor syndrome within a region or to other regions, as determined by history, physical examination, or electrophysiological test
The absence of	electrophysiological and pathological evidence of other disease processes that might explain the signs of lower or upper motor neuron degeneration
	neuroimaging evidence of other disease processes that might explain the observed clinical and electrophysiological signs

Table 2. *The revisited El Escorial criteria*

Considering the four distinct body segments – bulbar, cervical, thoracic-abdominal and lower back – it is possible to classify ALS in the following categories:

El Escorial criteria (1994) [67]

- **Definite ALS:** upper and lower motor neuron signs in three regions.
- **Probable ALS:** upper and lower motor neuron signs in at least two regions with upper motor neuron signs rostral to lower motor neuron signs.
- **Possible ALS:** upper and lower motor neuron signs in one region, upper motor neuron signs alone in two or more regions, or lower motor neuron signs above upper motor neuron signs.
- **Suspected ALS:** lower motor neuron signs only in two or more regions.

Airlie House (modified) criteria (2000) [68]

- **Clinically definite ALS:** clinical evidence alone of upper and lower motor neuron signs in three regions.
- **Clinically probable ALS:** clinical evidence alone of upper and lower motor neuron signs in at least two regions with some upper motor neuron signs rostral to (above) the lower motor neuron signs.
- **Clinically probable-laboratory-supported ALS:** clinical signs of upper and lower motor neuron dysfunction in only one region, or upper motor neuron signs alone in one region with lower motor neuron signs defined by electromyography criteria in at least two limbs, together with proper application of neuroimaging and clinical laboratory protocols to exclude other causes.
- **Possible ALS:** clinical signs of upper and lower motor neuron dysfunction in only one region, or upper motor neuron signs alone in two or more regions; or lower motor neuron signs rostral to upper motor neuron signs and impossibility to prove the diagnosis of clinically probable-laboratory-supported ALS.
- **Suspected ALS:** deleted category.

Awaji-shima (modified) criteria (2008) [69]

- **Definite ALS:** defined by clinical or electrophysiological evidence by the presence of LMN as well as UMN signs in the bulbar region and at least two spinal regions or the presence of LMN and UMN signs in three spinal regions
- **Probable ALS:** defined on clinical or electrophysiological evidence by LMN and UMN signs in at least two regions with some UMN signs necessarily rostral to (above) the LMN signs
- **Possible ALS:** defined when clinical or electrophysiological signs of UMN and LMN dysfunction are found in only one region, or UMN signs are found alone in two or more regions, or LMN signs are found rostral to UMN signs; neuroimaging and clinical laboratory studies must have been performed and other diagnoses must have been excluded.

ALS and other conditions

ALS has been generally considered a paradigm of pure motor neuron disorder. Nevertheless, it is currently recognized that pathological changes are not limited to the motor systems. Patients with ALS may exhibit cognitive abnormalities ranging from impaired frontal executive dysfunction to overt FrontoTemporal Dementia (FTD) [70]. Very recently a study has shown that dementia occurs in approximately 14% of ALS patients and cognitive impairment, predominantly in the form of executive dysfunction, occurs in more than 40% of ALS patients [71].

FrontoTemporal Lobar Dementia (FTLD) affects 3-4 individuals per 100,000 per year and is characterized by abnormalities of behaviour or language. The behavioural variant is denoted as FTD, and this is the second most common cause of dementia with onset under 60 years of age. Pathologically, FTLDs are classified according to the types of inclusions found in cortical neurons in post-mortem examinations. In a large proportion of patients with FTLD, these inclusions contain TAR DNA-binding protein 43 (TDP-43), which is similar to what is found in motor neurons of patients with ALS [72]. Of the patients with FTLD, 15% also have ALS, but many more have some evidence of lower motor neuron involvement [73,74]. This suggests that ALS and FTLD are two ends of the spectrum of one disease (Figure 3).

Confirming this clinical and pathological concept, ALS and FTD share many gene defects, including *C9ORF72* (the chromosome 9 open reading frame 72), *TARDBP*, *FUS* (fused in sarcoma) and *VCP* (valosin containing protein) mutations [75,76].

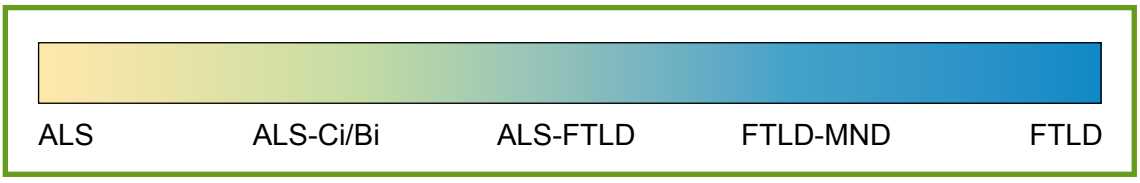


Figure 3. *Spectrum of ALS-FTLD*

Patients for which there is clinical evidence for both disorders are said to have ALS-FTLD. Many patients with ALS show some cognitive or behavioural changes but do not meet the criteria for FTLD: they are said to have ALS-Ci/Bi (ALS with cognitive or behavioural impairment). Patients with FTLD can similarly show evidence of mild motor neuron involvement (clinically or on electromyographs) without developing ALS: they are said to have FTLD-MND. Some patients have pure ALS or FTLD.

Genetics aspects of ALS

The exact molecular pathway causing motor neuron degeneration in ALS is unknown, but is likely to be a complex interplay between multiple pathogenic cellular mechanisms which may not be mutually exclusive [77,78].

To date, 16 disease loci for ALS have been reported to be associated with typical ALS or atypical motor neuron disease, and 3 additional loci both with ALS and FTD, including loci and genes for ALS with juvenile onset (jALS), such as: alsin (ALS2) located on chromosome 2q33 and senataxin (ALS4) located on chromosome 9q34 (Table 3).

Nomenclature	Frequency	Hereditarily	Disease name	Gene	Locus	Protein
ALS1	1-4% SALS 20% FALS	AD/AR	*SOD*-FALS	*SOD1*	21q22.1	Cu-Zn superoxide dismutase
ALS2	Rare	AR	Juvenile ALS type 3	*ALS2*	2p33	Alsin
ALS3	Single family	AD	FALS	/	18q21	Unknown
ALS4	Rare	AD	Distal hereditary neuropathy with signs pyramidal	*SETX*	9q34	Senataxin
ALS5	Rare	AR	Juvenile ALS	*SPG11*	15q15.1-q21.1	Spatacsin
ALS6	1% SALS 4% FALS	AD/AR	FALS	*FUS/TLS*	16q12	FUS/TLS
ALS7	Single family	AD	FALS	/	20ptel-p13	Unknown
ALS8	Rare	AD	Proximal SMA	*VAPB*	20q13	VAPB
ALS9	Rare	AD?	FALS	*ANG*	14q11.2	Angiogenin
ALS10	2% SALS 5% FALS	AD	FALS	*TARDBP* (TDP-43)	1p36	TAR DNA-binding protein
ALS11	Rare	AD	FALS	*FIG4*	6q21	Phosphoinositide-5-phosphatase
ALS12	Rare	AD/AR	FALS	*OPTN*	10p15-p14	Optineurin
ALS13	?	AD	FALS	*ATXN2*	12q24	Ataxin 2
ALS14	0.5% SALS 1% FALS	AD	FALS	*VCP*	9p13.3	Valosin containing protein
ALS15	0.5% FALS	X-linked	FALS	*UBQLN2*	Xp11.21	Ubiquilin 2
ALS16	?% SALS 1-2% FALS	AD	FALS	*PFN1*	17p3.2	Profilin 1
ALS/FTD1	Rare	AD	ALS with FTD	/	9q21-q22	Unknown

Nomenclature	Frequency	Hereditarily	Disease name	Gene	Locus	Protein
ALS/FTD2	5% SALS 35% FALS	AD	ALS with mild cognitive impairment	*C9ORF72*	9q21.2-9p13.3	C9ORF72
FTD-ALS	Rare	AD	Complex disinhibition-dementia Parkinsonism-amyotrophy	*MAPT*	17q21.1	MAPT
FTD-ALS	Rare ALS 25% familial FTD	AD	ALS with FTD, FTD	*GRN*	17q21.31	Progranulin
FTD-ALS	Rare FTD, Rare ALS	AD	FTD and ALS	*CHM2B*	3p11.2	Chromatin Modifying Protein 2B

Table 3. *Causative genes of ALS and ALS-FTD*

Many disease loci could indicate a wide genetic and allelic heterogeneity of ALS. Therefore, ALS can be inherited in an autosomal dominant, autosomal recessive, or X-linked manner. Variants of ALS have differing clinical presentation, rate of progression and prognosis; however, the age of onset seems to be independent of the type of disease transmission.

SOD1 (ALS1) is the first gene to be identified in autosomal dominant FALS pedigrees, which maps to chromosome 21q22.1. This gene encodes the antioxidant enzyme Copper Zinc (Cu-Zn) Superoxide Dismutase. In literature mutations in *SOD1* have been reported in ca. 20% of FALS and in ca. 1-4% of SALS [(40)], even if in Italy a wide range of frequency is reported for SALS (ranging from 0% to 6.3%) [(79)]. Recently, mutations in ***TARDBP*** gene (ALS10), encoding the TAR-DNA binding protein TDP-43 and located on chromosome 1p36.22, have been linked to familial and sporadic ALS. Mutations of gene *TARDBP* were first reported in FALS cases in 2008; since then, mutations in this gene have been detected with an incidence of 4-5% in FALS and up to 2% in SALS. A further ca. 4% of FALS cases and ca. 1% of SALS cases are associated to mutations in the fusion in malignant liposarcoma/traslocated in liposarcoma (***FUS/TLS***) gene (ALS6) [(80)]. Other rare mutations have been described in Alsin (***ALS2***), Senataxin (***SETX***), Vesicle Associated Membrane Protein (***VAMP***), Angiogenin (***ANG***), Phosphoinositide-5-phosphatase (***FIG4***), Progranulin (***GRN***) and recently in Paroxonasis (***PON***) and Optineurin (***OPTN***).

In addition there are several other genes potentially contributing to the development of ALS. In this broader complex genetic context, these genes are referred to as 'susceptibility genes', as mutations in these genes may only lead to ALS in the presence of other genetic or environmental risk factors. The most important are: Neurofilament Heavy Subunit (***NEFH***), Apolipoprotein E4 (***APOE***), Glial Glutamate Transporter (***EAAT2***), Vascular Endothelial Growth Factor (***VEGF***). Finally other gene mutations have been identified in ALS cases, which may influence either survival or the progression of disease: Survival Motor Neuron (***SMN***), Cylial Neurotrophic Factor (***CNFT***) or Kinesin-Associated Protein 3 (***KIFAP3***) (Table 4).

Gene	Protein product	Form of ALS	Locus	
NEFH	Neurofilament heavy	SALS/FALS	22q12.1-q13.1	Susceptibility
PRPH	Peripherin	SALS/FALS	12q12-q13	
SMN	Survival motor neuron	SALS	5q12.2-q13.3	Modifier
EAAT2	Glutamate transporter	SALS/FALS	11p13-p12	Susceptibility
GLUR2	Subunit of AMPA-R	SALS	4q32-q33	
CNTF	Cylial neurotrophic factor	FALS	11q12.2	Modifier
VEGF	Vascular endothelial growth factor	SALS/FALS	6p12	Susceptibility
APOE	Apolipoprotein E	SALS	19q13.2	Susceptibility
APEX1	Apurinic endonuclease DNA repair enzyme 1	SALS	14q11.2-q12	
HFE	Haemochromatosis	SALS	6p21.3	Susceptibility
KIFAP3	Kinesin-associated protein 3	SALS/FALS	1q24.2	Modifier
PON	Paroxonase	SALS/FALS	7q21.2-q22.1	Susceptibility
DCTN1	Dynactin 1	SALS/FALS	2p13	Modifier

Table 4. *Susceptibility and modifier genes in ALS*

Molecular genetics diagnosis

The genetic/molecular diagnosis is carried out by amplification and direct sequencing of the causative genes of ALS and/or susceptibility genes.

Major genes analysed in this study are:

- *SOD1*, *TARDBP*, *FUS* and *C9ORF72* in the familial and/or sporadic form of disease
- *TARDBP*, *FUS* and *C9ORF72* for the ALS-FTD forms of disease
- *SETX* in the juvenile form

C9ORF72 (ALS-FTD2)

A large hexanucleotide (GGGGCC) repeat expansion in the first intron of *C9ORF72* located on chromosome 9p21 is the most common mutation detected in patients with familial ALS [75,76].

C9ORF72 contains a GGGGCC hexanucleotide (G_4C_2) sequence that is located between two transcription initiation sites (Figure 4). The GGGGCC hexanucleotide repeat is located between two non-coding exons of *C9ORF72*, which encodes a completely uncharacterised protein with unknown function.

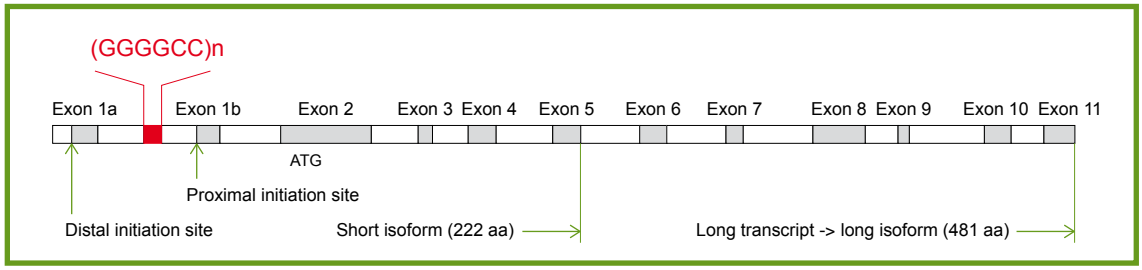


Figure 4. *C9ORF72 contains 11 exons with ATG in the second one. It generates a long (exons 2 through 11) and short (exons 2 through 5) isoform*

Two different isoforms of the protein are predicted to be generated from a total of three or more different *C9ORF72* transcripts. In most normal individuals, the GGGGCC hexanucleotide is repeated two-to-five times. It is rarely repeated more than five times and is never repeated more than 30 times. Consequently, a cut-off of 30 repeats is commonly used to differentiate between pathogenic and non pathogenic repeat sizes [76]. However, the repeat size of 30 is also present in control subjects and

therefore this generates uncertainties in the attribution of pathological significance. For sure, fewer than 10 repeats are not associated with a pathological phenotype whereas the role of intermediate repeat sizes (11-29) is not yet known. Conversely affected individuals with *C9ORF72* mutations harbour expanded alleles with repeats reaching up to hundreds and thousands.

Unexpectedly, several groups show reduced levels of at least one *C9ORF72* transcript in expanded-repeat carriers, suggesting a possible loss-of-function disease mechanism [81]. The accumulation of transcripts containing the GGGGCC repeat as nuclear RNA foci in the frontal cortex and spinal cord of *C9ORF72* mutation carriers, however, has also been demonstrated, favouring a toxic RNA gain-of-function disease mechanism in line with most other non-coding expansion disorders [75].

An abnormal expansion of this sequence is found in about 40% of families with ALS and in about 7% of patients with supposed SALS [82]. In FALS patients the mutation frequency varies between different populations, countries and regions, ranging from 0% to 18% in Asiatic countries to 46% in Finland and France; the percentage of mutated cases raises to 50-72% in families with ALS/FTD phenotype [83].

To date, less than a year after the discovery, more than 30 articles have described frequencies of *C9ORF72* repeat expansions in ALS and FTD populations, across USA, Europe, Australia, and Asia. In Figure 5, Bitterswijk *et al.* have provided a graphic representation of these frequencies. This representation suggests that *C9ORF72* mutations account for 34.2% of the familial ALS cases, 5.9% of the sporadic ALS cases, 25.9% of the familial FTD cases, 5.1% of the sporadic FTD cases, and 0.17% of the controls. Hence, these frequencies underline that *C9ORF72* repeat expansions are currently the major genetic cause of ALS and/or FTD worldwide [81,82,84].

At a neuropathological level, patients with mutations in *C9ORF72* show TDP-43-immunoreactive protein aggregates, and presence of ubiquitin-positive, TDP-43-negative inclusions in a variety of neuroanatomical regions [85]. Comparing the phenotype with that of patients carrying mutations in other ALS-related genes and of patients with unidentified genetic defects, those with *C9ORF72* expansion consistently show some specificity. The most notable one is a significantly higher frequency of cognitive impairment, which affects 40-50% of cases compared with 8-9% of non-*C9ORF72* expansion cases. Moreover in patients carrying *C9ORF72* mutations bulbar-onset seems more frequent and median survival consistently lower than in

patients carrying *TARDBP*, *SOD1* or unknown mutations. *C9ORF72* expansion is more frequent among patients with onset >61 years [86].

The penetrance of the mutation is probably 50% around the age of 60 years and nearly 100% above the age of 80 years [82]. Many questions about the genotype-phenotype correlation remain to be answered. One question concerns the intergenerational instability of the repeat. The age-of-onset has been reported to be 7 years younger in the subsequent generation, but this may reflect ascertainment bias [87]. Another question concerns a possible effect of length of expansion and of homozygosity on disease severity, and the possibility that the normal allele influences the pathogenicity of the repeats [88].

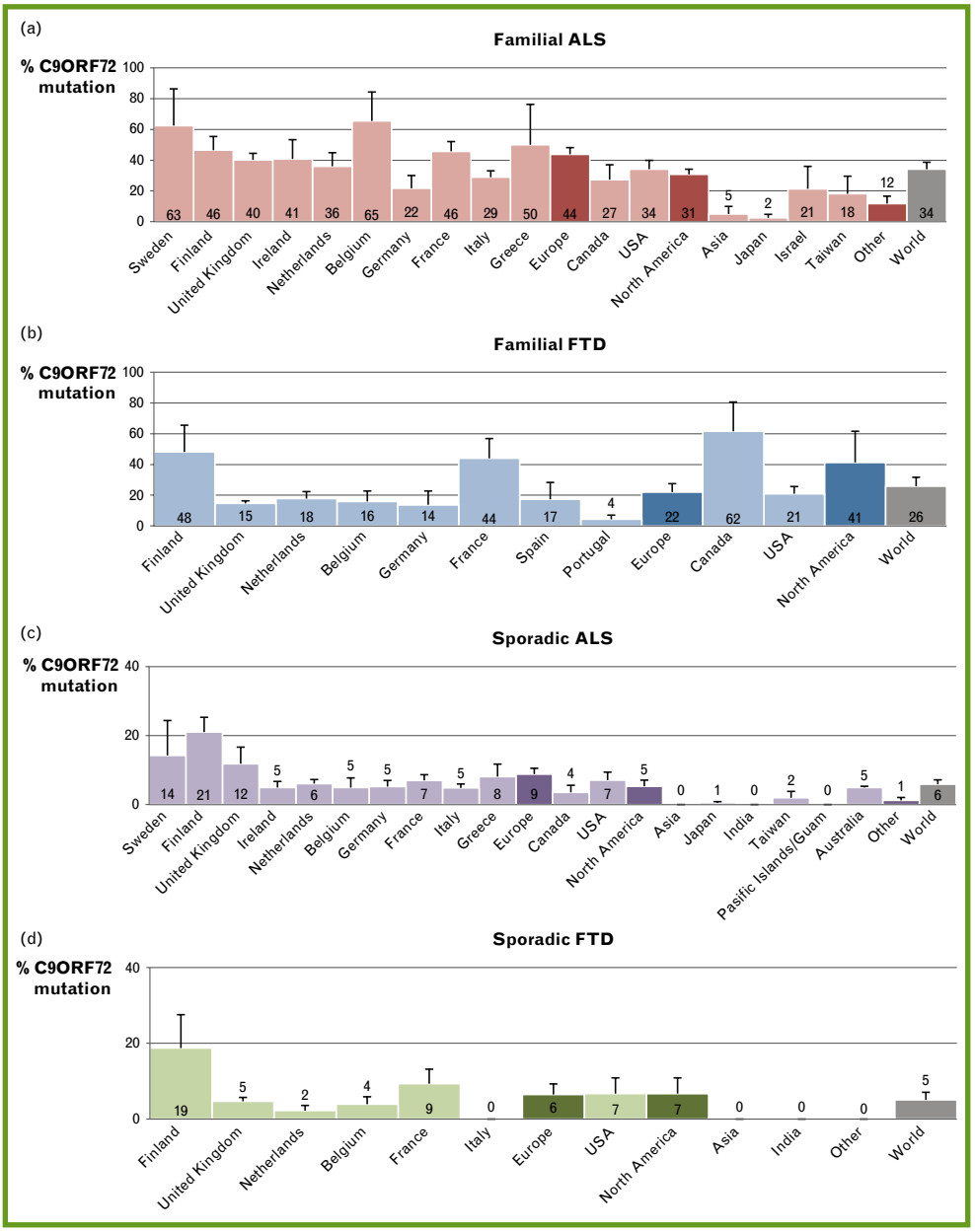


Figure 5. *C9ORF72 mutation frequencies worldwide*

SOD1 (Cu-Zn Superoxide Dismutase)

The human *SOD1* gene is located on chromosome 21q22.11, is composed of five exons and encodes for a 153 residues long protein. *SOD1* is a 32 kDa homodimeric metalloenzyme, in which each monomer consists of an eight-stranded beta-barrel and binds a copper and a zinc ion. *SOD1* is ubiquitously expressed, highly conserved and represents ca. 1% of all cytoplasmic proteins. This enzyme is highly expressed in nervous tissue, liver and erythrocytes. *SOD1* catalyses the conversion of the superoxide radical anion ($O_2^{\cdot}-$) to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) [89]. Then it is reduced to water by glutathione peroxidase and catalase.

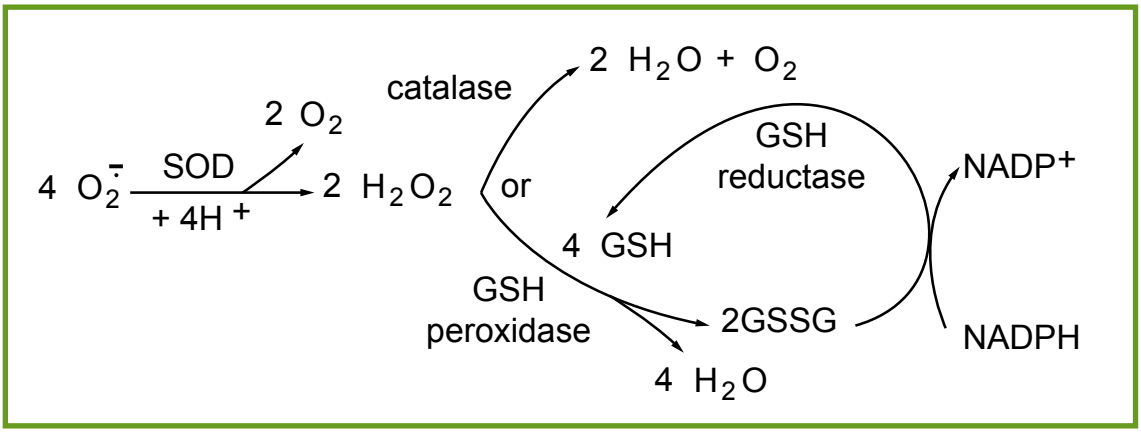


Figure 6. *Function of SOD1*

To date, more than 150 different *SOD1* mutations have been reported (http://alsod.iop.kcl.ac.uk), the vast majority of which are missense substitutions distributed throughout the five exons of the gene, although a small percentage of nonsense mutations, insertions and deletions have also been described. Of these mutations, more than 100 are missense mutations in 66 codons leading to the exchange of one amino acid for another but retaining the full-length polypeptide. About 10 mutations are nonsense mutations that alter the length of the final polypeptide. Most of these are in the 3' end of exon 4 or in exon 5. The shortest polypeptide contains 121 amino acids and the longest 156 amino acids [90].
Of particular interest is the discovery, in 2009, of an activated pseudo-exon deep inside intron 4, which results in the addition of 7 novel amino acids after exon 4, followed by a stop codon [91].

The mutations are scattered all over the molecule with preference for exons 4 and 5. Only few mutations have been found in the 24 codons of exon 3 that encode the amino acids forming the catalytic site and the zinc loop. Even more striking is the absence of mutations between Q22 and G37 in exons 1 and 2. The observations that no mutations have been found in these 14 codons, that all truncating mutations lie in exons 4 and 5, and that no null mutations have been found suggest that at least the amino terminus of the *SOD1* polypeptide is essential for the cytotoxic effect [92]. All of these mutations in *SOD1* are associated with autosomal-dominant FALS, except for two – D90A and D96N – which can cause both dominant and recessive ALS. The recessive inheritance of the D90A mutation has been reported mainly in Scandinavian families.

The majority of mutations in *SOD1* are missense mutations and there is no clear correlation with the clinical expression of the disease (genotype-phenotype correlation). This inter- and intra-familial variability precludes precise predictions on the course of the disease. Clinical characteristics, age at onset, site of onset and survival vary greatly between and among specific mutations [22]. A notable exception is represented by A4V, the mutation most frequently observed in ALS1 pedigrees, which is consistently associated with a high penetrance, younger age at onset, prevalence of lower motor neuron signs, and a very rapid disease course, usually less than 12 months [93].

Some other of the *SOD1* mutations is often associated with ‘early onset’ (G37R, L38V), ‘shorter survival’ (A4V) or relatively ‘benign’ forms of ALS (G37R, G41D, G93C, and H46R). However, these associations must be interpreted with caution when used to give a prognosis in individual cases [22]. In Italy the following mutations have been described: A4V, G12R, G41S, V47F, L84F, G93D, A95T, I113T [94] and L144F [95].

In 2005 Battistini *et al.* [96] showed the results of a referred based multi-centre study on the distribution of *SOD1* gene mutations in the largest cohort of Italian ALS patients. Two hundred and sixty-four patients (39 FALS and 225 SALS) of Italian origin were studied. In 7 out of 39 FALS patients the authors found an association between type of *SOD1* gene mutations and phenotype. For example the G41S mutation was identified in four patients belonging to four unrelated families. The clinical phenotype of the four probands was quite uniform with early UMN and LMN involvement in one or both lower limbs, rapidly spreading to upper limbs, appearance of bulbar signs within 1 year, and death a few months later. The patient carrying the G12R mutation reported signs of

lower motor neuron involvement. The disease course was slowly progressive with bulbar symptoms developed in late stage and death after 7 years from disease onset. For the L144F mutation a great variability of age at onset and disease duration was observed among affected relatives, while clinical presentation, prominently affecting the lower limbs, was quite uniform [96].

The exact mechanism by which *SOD1* mutations lead to ALS pathology is unknown although numerous hypotheses have been proposed to explain *SOD1*-mediated toxicity, such as misfolded proteins aggregation, oxidative stress, mitochondrial dysfunction, endoplasmic reticulum stress, glutamate excitotoxicity, inflammation and microglial activation, and axonal transport abnormalities [80]. Much information about the role of *SOD1* in the pathogenesis of ALS comes from animal models and the *SOD1*^{G93A} mice are the most commonly used models for ALS studies. *SOD1* transgenic animal models recapitulate many of the features of ALS, including motor neuron degeneration, paralysis and premature death [97]. Interestingly, *SOD1* knock-out mice do not develop ALS, which supports the notion that toxic gain of function may be responsible for the motor neuron degeneration in *SOD1*^{G93A} mice since enzymatic activity is retained in *SOD1*^{G93A} mutation [80]. Most evidence indicates that FALS-linked mutant *SOD1* gain a toxic function, which may stem from mutant-specific characteristics such as enhanced aggregation propensity, the formation of aberrant protein interactions, or intracellular mislocalisation. The accumulation of *SOD1*-rich proteinaceous deposits in the spinal cord led to the hypothesis that *SOD1* mutants are, or become, unstable and misfold to form high-molecular-weight aggregates that are selectively toxic to motor neurons. Immunohistochemical studies have localized these inclusions predominantly to motor neurons and, in some cases, astrocystes (Figure 7) [98]. Other toxic effects of *SOD1* mutations may involve increased vulnerability to excitotoxic mechanisms by selective inactivation of the glutamate transporter, EAAT1, or by mitochondrial degeneration [22].

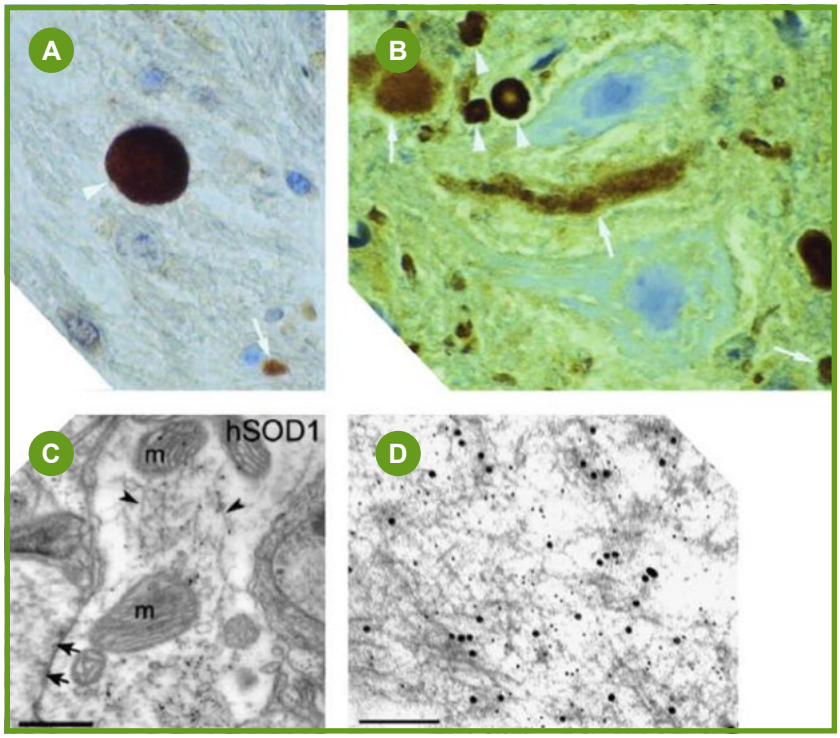


Figure 7. ***A.*** *An inclusion from the spinal cord of a FALS patient, expressing a SOD1 mutation (white arrowheads).* ***B.*** *SOD1 positive inclusions from the spinal cords of end-stage transgenic mice (white arrowheads).* ***C.*** *Fibrillar nature of inclusions in mice. The letter "m" denotes mitochondria.* ***D.*** *Fibrillar aggregates in mice.*

TARDBP (TAR-DNA Binding Protein)

In 2006, the transactive response DNA binding protein 43 (TDP-43) was recognized as a primary protein component of intracellular ubiquitinated inclusions in most cases of ALS and frontotemporal lobar degeneration with ubiquitin-positive inclusions (FTLD-U) [(72)]. Now it is clear that inclusions are present in both sporadic and familial cases of ALS and FTLD-U with or without MND.

Human *TARDBP* gene is located on chromosome 1p36.22 and encodes for the 43KDa TAR DNA-binding protein (TDP-43). This protein is 414 amino acids long and is encoded by six exons. It contains two RNA-recognition motifs (RRM1 and 2) and a C-terminal glycine-rich region that may mediate interactions with other proteins [(99)]. TDP-43 has a bipartite nuclear localization signal (NLS) between the N terminus and RRM1, and one nuclear export signal (NES) in RRM2. TDP-43, belonging to ribonucleoprotein family, is predominantly nuclear, although the protein is synthesized in the cytoplasmic compartment and is capable of shuttling between nucleus and

cytoplasm. It is involved in a variety of functions in nucleus processes, including gene transcription, RNA splicing, microRNA processing and stabilization as well as transport of mRNA.

Almost all the *TARDBP* mutations identified in ALS patients are located in exon 6, which encodes the C terminal glycine-rich part of the protein. Most are missense mutations, but there are a few deletion mutations that give rise to a protein truncated at the very C terminal [100]. Although originally identified as binding to the polypyrimidine-rich sequence of the transactive response element of the HIV virus, TDP-43 has subsequently been shown to have roles in regulating alternative splicing of numerous genes including *FUS*, *VCP*, Progranulin, and other transcripts encoding neurodegenerative disease-associated proteins as well as many other RNA processing genes [101].

Collectively, there are 70 distinct *TARDBP* point mutations or variants identified to date, including 28 of the above-mentioned missense mutations, two benign missense mutations, one nonsense mutation, six synonymous mutations, seven mutations in the 5'-untranslated region (UTR), 21 intronic mutations, and five mutations in the 3'-UTR region of *TARDBP* [102]. These mutations all seem to show an autosomal dominant pattern of inheritance. The majority of these mutations occur in highly conserved amino acids lying in exon 6 of *TARDBP*, which encodes ca. 60% of the TDP-43 protein and more than 70% of the entire mRNA transcript. In the light of these observations, it is clear that exon 6 and its encoded glycine-rich domain are critical components of the TDP-43 proteins [102].

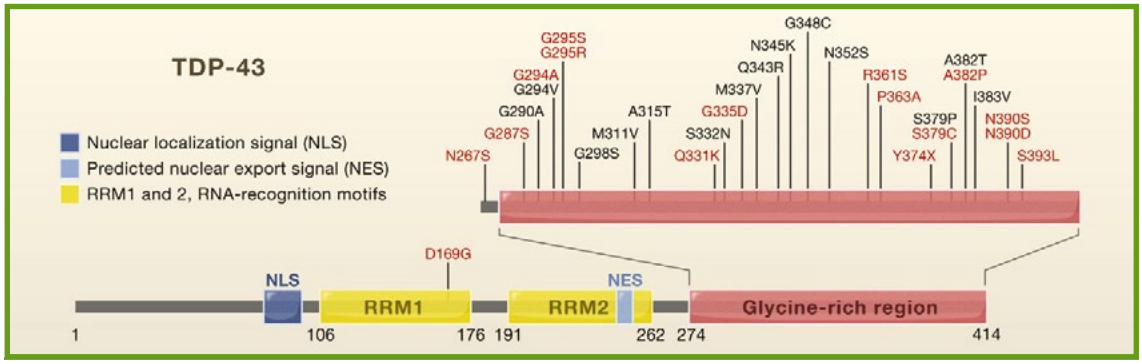


Figure 8. *TDP-43 gene structure*

It was suggested that specific mutations in TDP-43 may alter the phosphorylation state of TDP-43 or increase TDP-43 aggregation. Thus, it is plausible that three missense mutations, i.e. S379C, S379P and S393L, are pathogenic because they abolish phosphorylation target sites for casein kinase I [103,104], thereby implying that reduced or impaired phosphorylation of TDP-43 at these sites may play a mechanistic role in the onset or progression of ALS. Conversely, 9 other pathogenic missense mutations that replace glycine-rich domain amino acids with serine or threonine would be predicted to increase the hyperphosphorylation state of TDP-43 [103,105,106]. Further, the Q331K and N345K mutations could be pathogenic as a result of creating novel targets for ubiquitination, whereas the G348C and S379C mutations may be predisposing to ALS by increasing the propensity for TDP-43 to aggregate through disulfide bond formation [105].

The majority of ALS patients harbouring missense mutations in the *TARDBP* gene are characterized by a spinal-onset of disease in both upper and lower motor neurons. Bulbar muscle symptoms are less frequent at the onset of the disease; however, several ALS patients who presented with upper and lower motor neuron involvement developed bulbar symptoms at follow up examination. Of note, a unique *TARDBP* missense mutation (Ala382Thr) has been found in approximately one third of all ALS cases in the Island of Sardinia, a genetic isolate phylogenetically distinct from other European populations [107].

Of the patients with ALS, 15% have FTD with TDP-43-positive inclusions in cortical neurons, whereas at least 50% have evidence for subtler cognitive and/or behavioural dysfunction [108]. Some carriers of the *TARDBP* mutation presenting FTLD show evidence of mild motor neuron involvement without developing ALS (FTD-MND). Therefore patient with *TARDBP* mutation develop typical ALS with some variability within families in the site and age of onset. Chiò *et al.* in 2010 identified 3 index cases of familial ALS carrying the p.Ala382Thr missense mutation of the *TARDBP* gene and with clinical, neuroimaging, and neuropsychological features of FTLD [109].

Corrado *et al.* had previously described the same mutation in one patient with ALS who displayed no apparent cognitive impairment [103]. Finally, a *TARDBP* missense mutation has now been described in one patient with sporadic FTLD-TDP, who presented with behavioural impairment but no clinical signs of MND [110,111].

Since most *TARDBP* mutations are private (within a single family), it is difficult to establish clear genotype-phenotype correlation. It has been suggested that A382T, which is the most commonly observed variant, may be associated with predominantly lower motor neuron disease, with an asymmetrical onset in the distal muscles of the limbs, subsequently spreading to proximal muscles and with relative sparing of the bulbar muscles [103]. Moreover, the fact that the same genetic mutation can cause both ALS and FTLD-TDP suggests that these two clinical entities might be considered as two ends of a spectrum of TDP-43 proteinopathies.

FUS/TLS (Fusion in malignant liposarcoma/traslocated in liposarcoma)

The identification of TDP-43 in ALS pathogenesis fuelled the discovery, recently reported by Kwiatkowski *et al.* and Vance *et al.* [112,113], of additional ALS mutations in a gene encoding another DNA/RNA-binding protein called *FUS/TLS*. They identified novel variants in the *FUS* gene as the disease causing-mutations in ALS families. Mutations in *FUS* gene are identified based on a linkage analysis in a large Cape Verde family with autosomal recessive ALS [112] and in numerous British kindreds with autosomal dominant ALS [113].

The locus for ALS6 has been mapped to region 16p11.2 containing the *FUS* gene. The *FUS/TLS* protein is 526 amino acids long and is encoded by 15 exons. It is characterized by an N-terminal domain enriched in glutamine, glycine, serine, and tyrosine residues (QGSY region), a glycine-rich region, an RNA-recognition motif (RRM), multiple RGG repeats implicated in RNA binding, a C-terminal zinc finger motif, and a highly conserved C-terminal region [99].

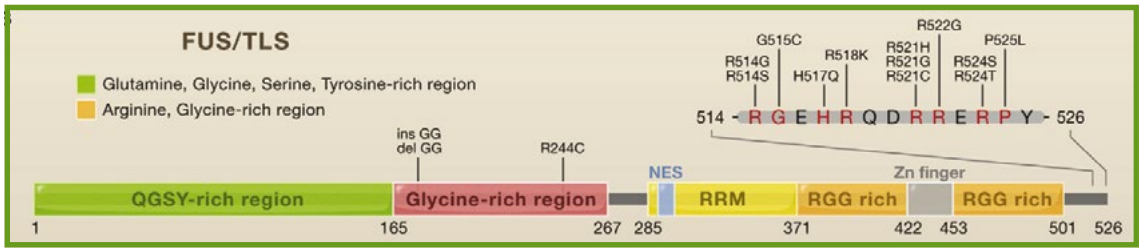


Figure 9. *FUS/TLS gene structure*

The FUS protein resembles TDP-43, and has been implicated in alternative splicing, genomic maintenance, and transcription factor regulation [114]. Like TDP-43, FUS/TLS is almost ubiquitously expressed. It is mainly localized in the nucleus, but cytoplasmic accumulation has been detected in most cell types.

Analysis of the brains and spinal cords of ALS patients with *FUS/TLS* mutations revealed normal staining of FUS/TLS in the nuclei of many neurons and glial cells but aggregates of FUS/TLS in neuron cytoplasm. Cytoplasmic inclusions containing the FUS/TLS protein are absent in normal individuals, in ALS patients with *SOD1* mutations, and in sporadic ALS patients who presumably are positive for TDP-43 aggregates. Importantly, TDP-43-positive inclusions are absent in ALS patients with *FUS/TLS* mutations, implying that neurodegenerative processes driven by *FUS/TLS* mutations are independent of TDP-43 aggregation [99].

Mutations in *FUS* cause ALS and almost all are dominant, although the first one was identified in a Cape Verdian recessive pedigree (H517Q) [112]. Up to now, more than 50 different mutations have been described, the vast majority of which are missense substitutions and the rest are frameshift or nonsense mutations. To date only 2 insertion/deletion mutations in the G-rich region of *FUS* have been described [112]. Several missense mutations were revealed predominantly in exon 14 and 15, which encode the C-terminus, the commonest being Arg521Cys [113]. Forty-two mutations in exons 3, 5, 6, 14 and 15 have now been reported. Although genotype-phenotype correlations are not possible for the majority of *FUS* mutations, it has been suggested that mutations of arginine 521, in particular R521C, may result in an uncommon phenotype characterized by a symmetrical proximal spinal-onset, with early involvement of the axial muscles [115].

FUS mutations have been found in patients with ALS who may or may not also have FTLD, but not unequivocally in patients with ‘pure’ FTLD [116]. Van Langenhove *et al.* identified one patient with FTLD with a novel missense mutation, M254V, which was absent in 638 control individuals [117].

In the brain and spinal cord of patients with *FUS* mutations, *FUS*-positive inclusions are present [112,113]. The mutations in *FUS* gene P525L, c.1554−1557delACAG and, to a lesser degree, R495X are associated with an aggressive phenotype of ALS, which affects patients in the first or second decade of life and is fatal after 1-2 years [118,119].

Chiò in 2009 analysed 52 index cases from seven Italian regions with non-*SOD1* and non-*TARDBP* FALS to define the spectrum and frequency of *FUS/TLS* mutations in Italian population. He found two multi-generational families carrying mutations in the *FUS* gene – one heterozygous missense mutation in a family of Northern Italian origin (R514S), and another heterozygous missense mutation in a family of Sicilian origin (P525L). The clinical phenotype was similar within each of the families, with a predominantly upper limb onset in the family carrying the p.R514S mutation (young mean age at onset and rapid progression) and bulbar-onset, with very young age and a rapid course in the family carrying the p.P525L mutation, confirming that this particular *FUS* mutation results in a highly aggressive form of ALS [120].

SETX (Senataxin)

ALS4, initially mapped to chromosome 9q34, has subsequently been found to be caused by mutations in the *SETX* gene [121]. The gene is composed of 26 exons and encodes for the 2.677-residue long protein Senataxin.

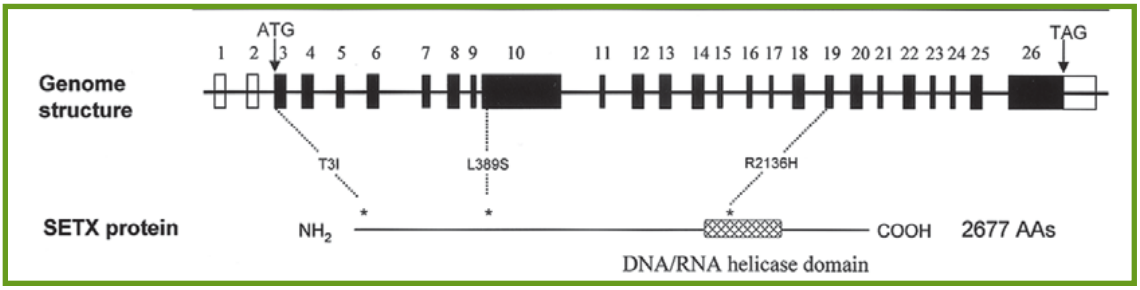


Figure 10. *Exon organization and protein of SETX respectively*

The Senataxin protein shares extensive homologies with the fungal Sen1p proteins. Saccharomyces cerevisiae Sen1p is involved in splicing and termination of tRNA, small nuclear RNA and small nucleolar RNA and has RNA helicase activity encoded by its C-terminal domain [122]. The C terminus of Senataxin contains a classical seven-motif domain found in the superfamily I of helicases, suggesting that it is a putative DNA/RNA helicase. The C-terminal domain of Senataxin also shares extensive homology with RENT1/Upf1 and IGHMB2, two members of the superfamily I of helicases.

RENT1/Upf1 has been shown to be involved in nonsense-mediated RNA decay and possesses both DNA and RNA helicase activity with a 5'-3' polarity of unwinding. IGHMB2, dysfunctional in spinal muscular atrophy (SMA) with respiratory distress,

has been described as a DNA binding protein with transcriptional transactivating properties [123].

Senataxin is a ubiquitously expressed DNA/RNA helicase, possibly involved in repairing DNA double-strand breaks following oxidative stress [124]. Moreover, it has been shown to bind RNA polymerase II and other proteins involved in mRNA transcription and processing, suggesting that it is involved in transcriptional regulation [123].

Interestingly, *SETX* mutations, mostly leading to a premature termination of the protein product, have been described in two different pathologies: ataxia-ocular apraxia-2 (AOA2) with an autosomal recessive pattern of inheritance and juvenile ALS (jALS). This jALS is an autosomal dominant disorder characterized by a childhood- or adolescent-onset and slow disease progression. Patients usually develop symmetrical weakness and atrophy of the distal muscles of the limbs in their second decade. The phenotype is characterized by the involvement of the corticospinal tracts (pyramidal signs associated with degeneration of motor neurons in the brain and spinal cord), while bulbar and respiratory muscles are consistently spared [125].

To date 3 different missense mutations (L389S, R2136H and T3I) in the *SETX* gene have been detected in 3 unrelated families. All affected individuals from 3 pedigrees were heterozygous for the missense substitution, confirming the autosomal dominant pattern of inheritance [121]. Recently Zhao *et al.* identified a novel variation, Thr1118Ile, in a 42-year-old individual with sporadic ALS. This study shows that Senataxin might be associated with sporadic ALS and that the possible clinical phenotypes of Senataxin mutations may be wider than originally considered [126].

Angiogenin (*ANG*)

The *ANG* gene, on chromosome 14q11, is composed of two exons, of which only one coding. *ANG*, a 123-residue protein, is a potent inducer of neovascularisation *in vivo* and a member of the pancreatic ribonuclease A (RNase A) superfamily [127].

The *ANG* protein plays a role in inhibiting of protein translation by cleaving tRNA and helps in rRNA biogenesis and cellular proliferation [128]. *ANG* is synthesized with a signal peptide of 24 amino acids that is cleaved to form the mature protein. The RNase activity of *ANG* is important for its biological activity, and *ANG* variants with

decreased RNase activity invariably have reduced angiogenic activity [129].

ANG is expressed in the neuroaxis; however, its mechanism of action is yet to be established. In endothelium, *ANG* organizes cell formation into tube-like structures, induces secondary messengers and supports endothelial cell adhesion and spreading. In steps that are critical for angiogenesis, *ANG* is internalized by endothelial cells and transported to the nucleolus [128], where it stimulates rRNA transcription, a rate-limiting step in ribosome biogenesis, protein translation and cell growth. *ANG* shares a metabolic pathway with vascular endothelial growth factor (*VEGF*), which is implicated in ALS [127]. *ANG* mediates neovascularisation and promotes neurite outgrowth during early embryonic development. Mutations in *ANG* gene cause loss of ribonucleolytic activity and nuclear translocation activity [130].

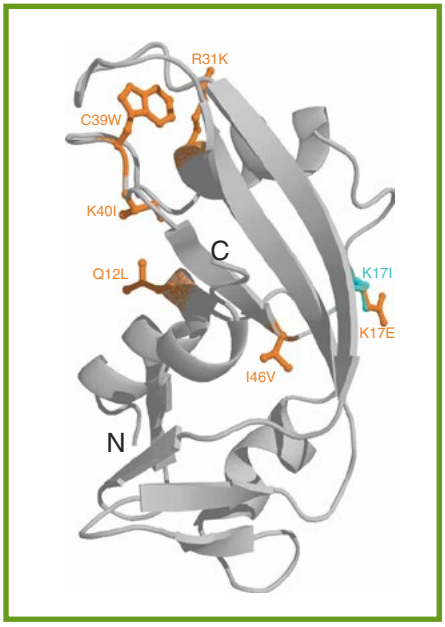


Figure 11. *Three-dimensional structure of ANG showing the modeled mutations Q12L, K17E, K17I, R31K, C39W, K40I and I46V in ball-and-stick representation*

In 2006 Greenway *et al.* identified 5 missense mutations in 11 Scottish or Irish individuals with ALS that affect functionally important residues (Q12L, K17I, K17E, C39W and K40I), evolutionarily highly conserved in *ANG*, RNase or both (Figure 11) [127]. Lys40 in *ANG* is a critical residue involved in catalysis. Molecular modelling of the K40I substitution suggests that several key interactions are lost owing to mutation, predicting loss of ribonucleolytic activity. C39W causes substantial structural change owing to loss of disulfide bridge formation with Cys92. Disruption of this disulfide bridge is likely to affect protein folding, resulting in lower ribonucleolytic activity. Gln12 interacts with the active site residue Lys40 in the native structure and the Q12L substitution would disrupt this interaction and make the protein enzymatically less active. It is unlikely that variants K17I or K17E have a substantial effect on the structure, because of Lys17 in *ANG* is distant from the active site, in a loop on the surface of the molecule. However it was previously demonstrated that a conserved region containing Lys17 is involved in the activity of *ANG* against intact

ribosomes. This suggests that isoleucine or glutamate variants of Lys17 may alter *ANG* activity [127,131].

As for two others variants found in the cohort of patients: the R31K variant may have a role in nuclear translocation but would cause only minor perturbation in the structure, whereas Ile46 is not fully conserved, and replacement of this residue by valine is predicted to cause little structural change.

Finally Greenway found *ANG* mutations as a clear susceptibility factor for the development of ALS, particularly in individuals of Irish and Scottish descent. Greenway in the 2004 have also reported an association of ALS with the rs11701 (G110G) SNP in the Irish and Scottish population with ALS, although no association was observed in the populations from the US, England, Sweden [132] or Italy [133].

Only the study of Conforti *et al.* reveals an association between FALS and rs11701 SNP in *ANG* gene, but not in SALS patients. Further investigations on the relationship between angiogenic genes and ALS are needed [134].

Aim of the study

Amyotrophic lateral sclerosis (ALS) is a complex disorder. Both genetic and environmental factors are known to contribute to it. The discovery of mutations in *SOD1* as causative of familial ALS [135], in 1993, started the molecular genetics era of ALS research. Over the last 20 years an increasing number of causative genes have been identified, revealing a high degree of genetic heterogeneity. The pathogenic hexanucleotide repeat expansion in chromosome 9 open reading frame 72 (*C9ORF72*) is currently known as the most relevant genetic risk factor for ALS.

The combination of a genetic risk factor with a large effect with another mutation with incomplete penetrance fits well the oligogenic disease model. An oligogenic aetiology should be considered in genetic counselling, especially of unaffected family members.

For all these reasons, aim of the present study is the molecular characterization of the *C9ORF72*, *SOD1*, *TARDBP* and *FUS* genes in a group of Italian ALS patients, with a definite/probable/possible disease diagnosis.

All the enrolled ALS patients were divided in familial ALS (FALS) and sporadic ALS (SALS). FALS patients underwent first genetic counselling. All mutated subjects were communicated the result of the genetic test within a multidisciplinary context (neurologist, psychologist, geneticist). The at risk family members of ALS mutated subjects were also evaluated in a multidisciplinary context, to assess their purpose to undergo genetic testing.

Frequency of *C9ORF72*, *SOD1*, *TARDBP* and *FUS* mutations in our Italian cohort of patients was evaluated in order to verify data collected by other Italian studies. Moreover we studied all the ALS associated genes in order to confirm the oligogenic model on our cohort of patients.

In addition, for some patients with specific phenotype features, we analysed specific genes (*SETX*, *GRN*, *VCP*). To explore the phenotypic heterogeneity within the families, we also investigated the possible relationship between the ALS causative gene mutation and other modifier genes reported to be involved in ALS pathogenesis, such as *ANG, PON1, PON2, VEGF, MTHFR, APOE, HFE*.

Material & methods

Patients

Our cohort of ALS patients attended the NEuroMuscular Omnicenter (NEMO), a specialized centre for the study and treatment of neuromuscular diseases. This centre is located within one of the most important hospitals in Italy, Niguarda Ca' Granda Hospital, which is a national and international benchmark for many diseases, including rare diseases.

ALS diagnosis requires a set of neurological analyses repeated over time and a number of different medical examinations to exclude other neurological disorders (differential diagnosis).

The laboratory and clinical studies below are helpful in confirming the diagnosis, and ruling out other possible diseases.

- **Biochemical tests:** blood tests for people suspected of having ALS are routine laboratory tests. Usually ALS patients will not show any abnormalities within their blood, but blood tests are conducted to show that no other disease is present that is being mistaken for ALS. A blood test will check blood cell count, hormone levels, as well as liver and kidney function and detect if any infections are present. In ALS CK (creatine kinase) may be normal or moderately elevated (2-3 times normal) indicating the impairment of muscle.
- **Electromyography:** the signs of fibrillation or positive waves (together referred to as spontaneous activity) and a decreased recruitment in a muscle, qualified as lower motor neuron findings, can be used to upgrade the probability of the diagnosis of ALS.
- **Magnetic resonance imaging (MRI) of the spine:** this test uses powerful magnets and radio waves to produce a detailed view of the nervous system. Multi-focal spine disease can mimic the findings of ALS and therefore should be ruled out with an anatomical study.
- **Muscle biopsy:** it is not routinely performed, but is done in selected cases in which the diagnosis is problematic. In ALS, a muscle biopsy will show neurogenic atrophy. It is useful to determine if the patient has a muscle disorder (myopathy), such as myositis or muscular dystrophy, rather than ALS.

- **Spinal tap (lumbar puncture):** it is rarely necessary to exclude other neurological disorders such as primary lateral sclerosis, multi-focal motor neuropathy, Kennedy disease, myasthenia gravis and spinal muscular atrophy. A lumbar puncture can help diagnose serious infections, such as meningitis; disorders of the central nervous system, such as Guillain-Barrè syndrome and multiple sclerosis; or cancers of the brain or spinal cord.

Therefore the diagnosis of ALS is based primarily on clinical presentation, with supportive data from electrodiagnostic, imaging and laboratory studies.

Our cohort of ALS cases includes 302 subjects with definite/probable/possible diagnosis of ALS according to the previously described El Escorial criteria.
The selected ALS patients are 17 FALS (5.6%) and 285 SALS (94.4%), respectively.
All patients included in this study were subjected to a specific integrated procedure of genetic counselling. The main steps are summarized in Figure 12.

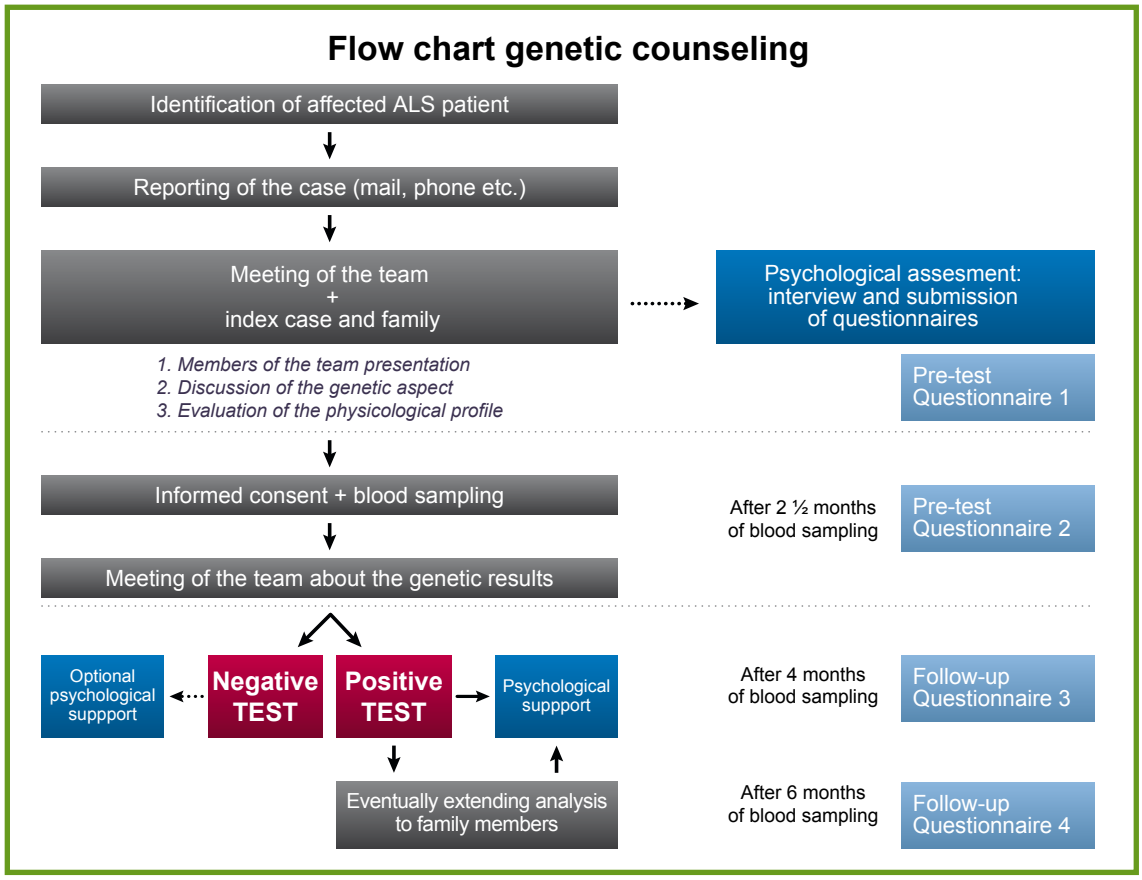


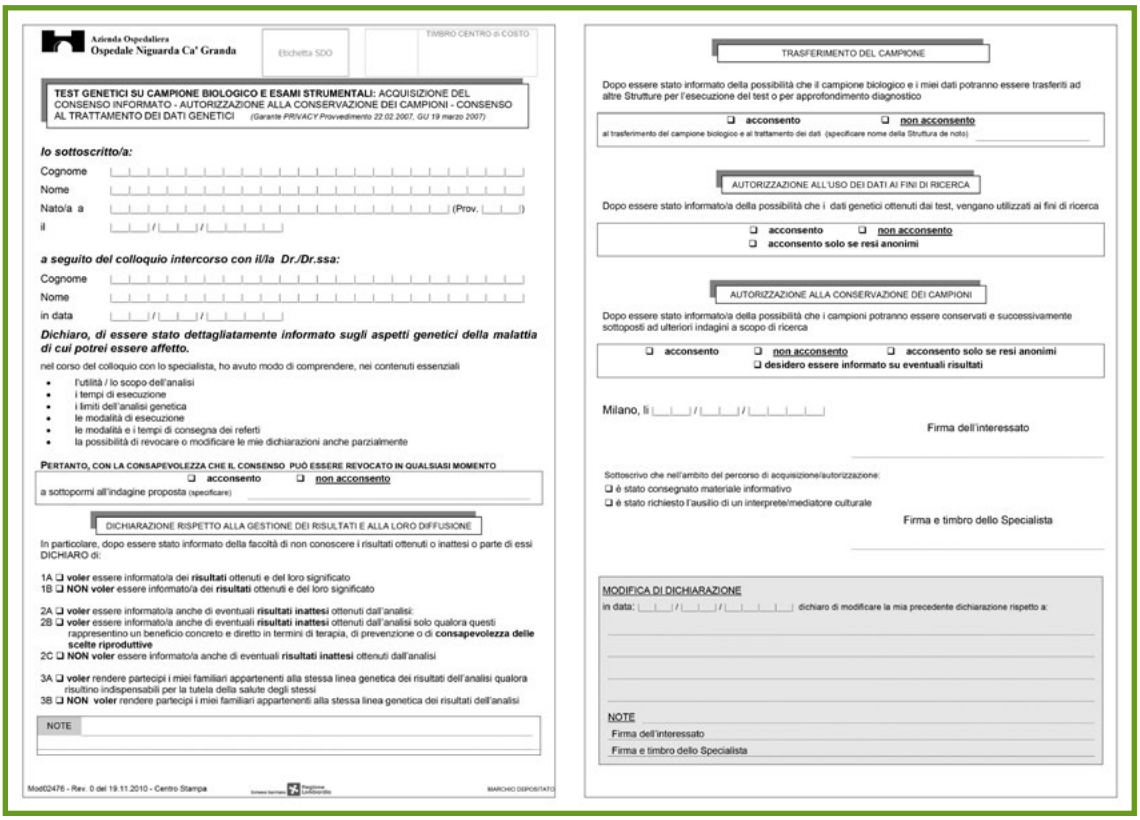
Figure 12. *This flow-chart set up by our team in Niguarda Ca' Granda Hospital shows the steps to arrive at optimal clinical definition and management of ALS patients*

Genetic counselling

Genetic counselling is a communication process related to the occurrence or risk of an inherited disease. Genetic counselling aims at educating individuals about disease, including the nature of inheritance; and at facilitating understanding of genetic testing options for confirmation of disease. Assessing family history is a key component of genetic counselling. The geneticist should obtain a detailed three-generation pedigree that captures the presence of FTD, ALS, other dementias and Parkinsonism or other neurological or non-neurological disorders. The pedigree should include ages and site of disease onset, diagnosis and ages at death. Particularly important is to identify precisely the geographical origin of the family. Therefore pre-test genetic counselling should help individuals to appreciate the risks, benefits and limitations of testing.

Informed consent

Every proband and all the family members who decided to participate in this study were offered genetic test and counselling, and all signed the informed consent approved by the ethics committee of our hospital (Figure 13).



Azienda Ospedaliera
Ospedale Niguarda Ca' Granda

Etichetta SDO

TIMBRO CENTRO di COSTO

TEST GENETICI SU CAMPIONE BIOLOGICO E ESAMI STRUMENTALI: ACQUISIZIONE DEL CONSENSO INFORMATO - AUTORIZZAZIONE ALLA CONSERVAZIONE DEI CAMPIONI - CONSENSO AL TRATTAMENTO DEI DATI GENETICI *(Garante PRIVACY Provvedimento 22.02.2007, GU 19 marzo 2007)*

Io sottoscritto/a:

Cognome
Nome
Nato/a a (Prov.)
il

a seguito del colloquio intercorso con il/la Dr./Dr.ssa:

Cognome
Nome
in data

Dichiaro, di essere stato dettagliatamente informato sugli aspetti genetici della malattia di cui potrei essere affetto.

nel corso del colloquio con lo specialista, ho avuto modo di comprendere, nei contenuti essenziali

- l'utilità / lo scopo dell'analisi
- i tempi di esecuzione
- i limiti dell'analisi genetica
- le modalità di esecuzione
- le modalità e i tempi di consegna dei referti
- la possibilità di revocare o modificare le mie dichiarazioni anche parzialmente

PERTANTO, CON LA CONSAPEVOLEZZA CHE IL CONSENSO PUÒ ESSERE REVOCATO IN QUALSIASI MOMENTO

❑ **acconsento** ❑ **non acconsento**

a sottopormi all'indagine proposta (specificare)

DICHIARAZIONE RISPETTO ALLA GESTIONE DEI RISULTATI E ALLA LORO DIFFUSIONE

In particolare, dopo essere stato informato della facoltà di non conoscere i risultati ottenuti o inattesi o parte di essi DICHIARO di:

1A ❑ **voler** essere informato/a dei **risultati** ottenuti e del loro significato
1B ❑ **NON voler** essere informato/a dei **risultati** ottenuti e del loro significato

2A ❑ **voler** essere informato/a anche di eventuali **risultati inattesi** ottenuti dall'analisi:
2B ❑ **voler** essere informato/a anche di eventuali **risultati inattesi** ottenuti dall'analisi solo qualora questi rappresentino un beneficio concreto e diretto in termini di terapia, di prevenzione o di **consapevolezza delle scelte riproduttive**
2C ❑ **NON voler** essere informato/a anche di eventuali **risultati inattesi** ottenuti dall'analisi

3A ❑ **voler** rendere partecipi i miei familiari appartenenti alla stessa linea genetica dei risultati dell'analisi qualora risultino indispensabili per la tutela della salute degli stessi
3B ❑ **NON voler** rendere partecipi i miei familiari appartenenti alla stessa linea genetica dei risultati dell'analisi

NOTE

Mod02476 - Rev. 0 del 19.11.2010 - Centro Stampa

MARCHIO DEPOSITATO

TRASFERIMENTO DEL CAMPIONE

Dopo essere stato informato della possibilità che il campione biologico e i miei dati potranno essere trasferiti ad altre Strutture per l'esecuzione del test o per approfondimento diagnostico

❑ **acconsento** ❑ **non acconsento**

al trasferimento del campione biologico e al trattamento dei dati (specificare nome della Struttura de noto)

AUTORIZZAZIONE ALL'USO DEI DATI AI FINI DI RICERCA

Dopo essere stato informato/a della possibilità che i dati genetici ottenuti dai test, vengano utilizzati ai fini di ricerca

❑ **acconsento** ❑ **non acconsento**
❑ **acconsento solo se resi anonimi**

AUTORIZZAZIONE ALLA CONSERVAZIONE DEI CAMPIONI

Dopo essere stato informato/a della possibilità che i campioni potranno essere conservati e successivamente sottoposti ad ulteriori indagini a scopo di ricerca

❑ **acconsento** ❑ **non acconsento** ❑ **acconsento solo se resi anonimi**
❑ **desidero essere informato su eventuali risultati**

Milano, li

Firma dell'interessato

Sottoscrivo che nell'ambito del percorso di acquisizione/autorizzazione:
❑ è stato consegnato materiale informativo
❑ è stato richiesto l'ausilio di un interprete/mediatore culturale

Firma e timbro dello Specialista

MODIFICA DI DICHIARAZIONE

in data: dichiaro di modificare la mia precedente dichiarazione rispetto a:

NOTE
Firma dell'interessato
Firma e timbro dello Specialista

Figure 13. *Informed consent*

DNA extraction from whole blood

DNA extraction with salting-out procedure

DNA was extracted from whole blood of all the ALS patients with the salting-out procedure [136]. This method avoided using the hazardous organic solvents phenol and chloroform and involved salting out of the cellular proteins by dehydration and precipitation with a saturated NaCl solution. Average yields were similar to those obtained with the phenol-chloroform extraction procedure (50-200 µg), and the quality of DNA was excellent.

The procedure consisted of the following steps:

1. each whole blood sample was thawed; 2 to 4 mL of whole blood were placed in a 50 mL tube together with 45 mL of lysis buffer (20 mM Tris/HCl, 5 mM EDTA, pH 7.8-8) stored at 4 °C;
2. a centrifugation at 3500 rpm for 20 minutes at 4 °C was performed;
3. the surnatant was discarded and the pellet was resuspended in 40 mL of lysis buffer;
4. a centrifugation at 3500 rpm for 20 minutes at 4 °C was performed;
5. steps 3) and 4) were repeated until the pellet was clear;
6. the pellet was resuspended in 10 mL of lysis buffer; 400 µL of 10% SDS (0.4 % in the final volume) and 150 µL of 10 mg/mL proteinase K were added; the tube was shaken vigorously till foam formation;
7. the tube was incubated overnight at 37 °C;
8. 3.3 mL of 5 M NaCl were added and the solution was shaken;
9. a centrifugation at 3500 rpm for 15 minutes at room temperature was performed;
10. the pellet was carefully transferred into a new 50 mL tube containing 30 mL of 99% ethanol stored at -20 °C;
11. the tube was gently inverted several times until DNA precipitated; precipitated DNA strands were removed with a plastic spatula, washed with 70% ethanol (stored at -20 °C) and transferred in a 1.5 mL tube;
12. after ethanol evaporation, DNA was suspended in 150 to 200 µL of dd H_2O.

DNA obtained with this procedure was stored at 4 °C until its use.

DNA extraction with QIAGEN kit

DNA was re-extracted from whole blood of mutated subjects using the QIAamp® DNA Blood Mini Kit (QIAGEN); the procedure comprised 4 steps and was automated on the QIAcube (QIAGEN) (Figure 14), an instrument that uses advanced technology to process QIAGEN spin columns, enabling seamless integration of automated, low-throughput sample preparation. Up to 12 samples could be processed per run. The principle of the QIAcube extractor is based on the lysis and elimination of red blood cells and on the subsequent extraction of genomic DNA from leukocytes through cells lysis by using an anionic detergent. Contaminating RNA is removed by enzymatic treatment, while other contaminants, such as proteins, are eliminated by separation on pre-packed columns.

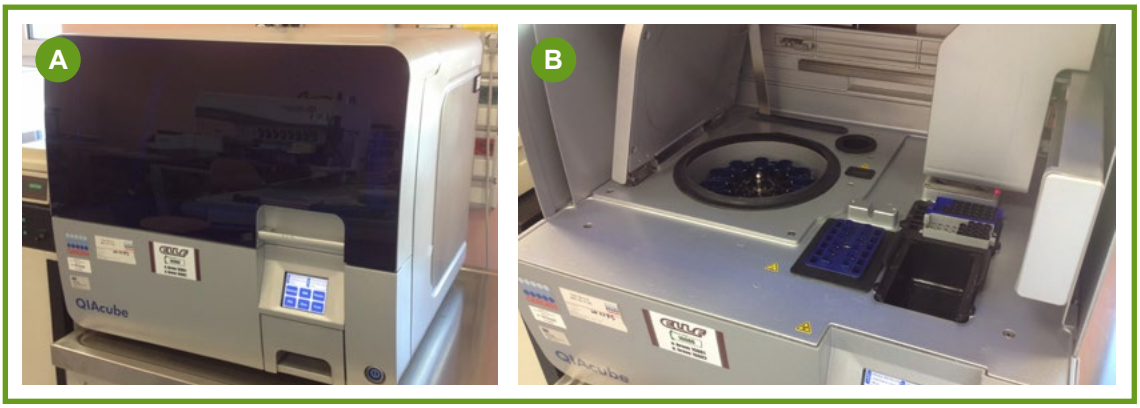


Figure 14. QIAcube instrument. ***A.*** *The outside.* ***B.*** *The inside.*

Sample preparation using QIAcube followed the same 4 steps as the manual spin procedure (Figure 15). Each whole blood sample was thawed and 200 µL were used for DNA extraction in order to obtain about 3-12 µg of DNA. Briefly, optimized buffers lysed samples, stabilized nucleic acids and enhanced selective DNA adsorption to the QIAamp membrane. Alcohol was added and lysates were loaded onto the spin columns. DNA was adsorbed onto the silica membrane during a brief centrifugation. Salt and pH conditions in the lysate ensured that proteins and other contaminants, which could inhibit PCR and downstream enzymatic reactions, were not retained on the membrane. DNA bound to the membrane was washed in two centrifugation steps by using two different wash buffers; wash conditions ensured the complete removal of any residual contaminants without affecting DNA binding. Purified DNA was then eluted from the spin column in a concentrated form in a low salt buffer (100 µL final volume).

The concentration of DNA extracted following the two different procedures described before was checked by spectrophotometry; DNA purity was assessed through λ260/λ280 ratio (expected ratio ca. 1.75). The integrity of each DNA sample was verified through electrophoresis on a 0.5% agarose gel. In order to prepare a 100 mL solution, 0.5 g of agarose were weighed and added to 100 mL of TAE buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA); the solution was then brought to the boil until it was limpid. After cooling, 8 µL of GelRed™ 10000X (Biotium) were added; this red fluorescent nucleic acid dye can be excited with a common 300 nm UV transilluminator. One microlitre of each sample genomic DNA was mixed with 7 µL of H_2O and 2 µL of a gel loading buffer (GelPilot DNA Loading Dye 5X, QIAGEN), and the samples were loaded into the gel wells. For sizing of genomic DNA, a DNA ladder (1 kb Molecular Ruler, BIORAD), previously diluted 1:4, was always added in each electrophoretic gel, in a position adjacent to that of the DNA samples under verification. 1 kb DNA ladder contained fragments of 15, 7, 6, 5, 4, 3, 2, 1 kb. DNA was stored at 4 °C until its use.

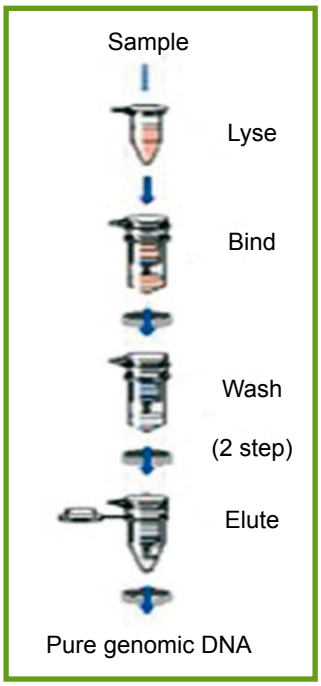


Figure 15. *Schematic representation of the spin procedure for DNA extraction by using the QIAamp® DNA Blood Mini Kit*

DNA amplification

Extracted DNA was amplified by means of Polymerase Chain Reaction (PCR). Primers were designed to amplify the 5 exons and the corresponding intron/exon boundaries of *SOD1* and *FUS* genes. Moreover primers were designed for exon 6 of *TARDBP* and for promoter, exon 1 and exon 2 of *ANG* gene and their intron/exon boundaries. Specific protocols were developed in our laboratory in order to amplify the largest number of exons with the same protocol. Because of their length, both promoter and exon 2 of *ANG* gene as well as *TARDBP* exon were divided into two parts and amplified with the same PCR reaction. In some case other genes were screened (*SETX*, *GRN*, *VCP*) by using previously described primers [126,137,138].

Table 5 shows the length of PCR products and some characteristics of the primers (sequence, melting temperature) used in each PCR amplification.

	Exon	Primer forward	Primer reverse	PCR products (bp)	Tm (°C)
SOD1	**1**	5'-GCG-ATT-GGT-TTG-GGG-CCA-GAG-3'	5'-ACC-CGC-CTC-CAT-GCA-AAA-GGT-TGC-3'	259	57
	2	5'-ACT-CTC-TCC-AAC-TTT-GCA-CTT-3'	5'-CGT-TTA-GGG-GCT-ACT-CTA-CT-3'	261	57
	3	5'-CAT-AAT-TTA-GCT-TTT-TTT-TTC-TTC-TTC-3'	5'-ACG-GAA-TTA-TCT-TAG-GCA-CAT-A-3'	258	57
	4	5'-GCA-TCA-GCC-CTA-ATC-CAT-CTG-3'	5'-CGC-GAC-TAA-CAA-TCA-AAG-TGA-A-3'	276	57
	5	5'-AGT-GAT-TAC-TTG-ACA-GCC-CAA-AG-3'	5'-CTA-CAG-CTA-GCA-GGA-TAA-CAG-ATG-3'	257	57

	Exon	Primer forward	Primer reverse	PCR products (bp)	Tm (°C)
TARDBP	**6a**	5'-TGC-TTG-TAA-TCT-AAG-TTT-TGT-TGC- 3'	5-GCT-GGC-TGG-CTA-ACA-TGC-3'	401	56
	6b	5'-GG-TGG-GAT-GAA-CTT-TGG-TG-3'	5'-TGC-TGA-ATA-TAC-TCC-ACA-CTG-AAC-3'	457	56

	Exon	Primer forward	Primer reverse	PCR products (bp)	Tm (°C)
FUS	**5**	5'-TGT-TGG-GTA-CAG-AGA-ATG-GAC-TCC-AC-3'	5'-AAA-TGG-GCT-GCA-GAC-AAA-GCT-G-3'	314	60
	6	5'-CCT-GGC-ACT-TGT-CAA-ACC-TT-3'	5'-GCA-CTA-GGG-ACT-GGC-TTC-AG-3'	414	60
	13/14	5'-GTC-ACC-GTA-GTT-TCT-TCC-TAG-TTC-TAG-3'	5'-AAC-CCT-GTT-ATC-CTA-TGG-CCT-CTG-T-3'	511	60
	15	5'-GAC-CCA-CTT-GAG-ATA-AGA-TAC-TCG-CT-3'	5'-TCT-ACC-TTC-CTG-ATC-GGG-ACA-TCG-A-3'	389	60

	Exon	Primer forward	Primer reverse	PCR products (bp)	Tm (°C)
ANG	**Pro1**	5'-GCT-GTC-GAC-CAG-TGT-CAA-GA-3'	5'-GTG-CCA-GCC-TCT-GCG-TAG-T-3'	320	55
	Pro2	5'-GCG-GAA-AAG-AGA-AAT-TCA-GA-3'	5'-GGG-AAA-CTG-GCT-TCT-GCT-3'	357	55
	1	5'-CGG-TCC-CAA-CTA-CGC-AGA-3'	5'-GAG-TCC-CAA-GCT-GCC-TCT-AC-3'	343	55
	2A	5'-GCA-AAG-CTC-CTG-TCC-TTT-TG-3'	5'-CGC-TTG-TTG-CCA-TGA-ATA-AA-3'	318	55
	2B	5'-GAA-AGC-ATC-ATG-AGG-AGA-CG-3'	5'-GAA-TGT-TGC-CAC-CAC-TGT-TC-3'	378	55

Abbreviations: bp, base pairs; Tm, melting temperature.

Table 5. *PCR products lengths, primer sequences and melting temperatures (Tm)*

All reagents used for PCR amplifications were provided by Euroclone. Each PCR reaction was performed in a total volume of 12.5 µL (PCR mixture).

PCR mixture used to amplify 5 exons of *SOD1* gene as well as exon 6 of *TARDBP* gene and *ANG* gene contained:

- 4.475 µL H_2O
- 2.25 µL 50% Gly
- 1.25 µL PCR reaction Buffer 10x (Mg++ free)
- 0.375 µL 50 mM $MgCl_2$
- 1 µL 2.5 mM dNTPs
- 1 µL 10 µM primer forward
- 1 µL 10 µM primer reverse
- 0.15 µL Red®Taq 5U/µL DNA Polymerase
- 1.0 µL genomic DNA

PCR mixture used to amplify 5 exons of *FUS* gene contained:

- 7.225 µL H_2O
- 1.25 µL PCR reaction Buffer 10x (Mg++ free)
- 0.375 µL 50 mM MgCl2
- 1 µL 2.5 mM dNTPs
- 1 µL 10 µM primer forward
- 1 µL 10 µM primer reverse
- 0.15 µL Red®Taq 5U/µL DNA Polymerase
- 0.5 µL genomic DNA

A single program on a thermal cycler (GeneAmp® PCR System 9700, Applied Biosystems) was used for all the PCR amplifications:

- 1 cycle at 96 °C for 10 minutes, to ensure the complete denaturation of template DNA and the activation of the polymerase;
- 35 cycles, each one composed of: 95 °C for 30 seconds (DNA denaturation) – 55/56/57/60 °C for 60 seconds (primers annealing) – 72 °C for 60 seconds (DNA extension);
- 1 cycle at 72 °C for 5 minutes to fill-in the protruding ends of reaction products.

The annealing temperature changed depending on the gene and the exon to be amplified.

For each amplification protocol, a negative control was used: it consisted of a PCR mixture aliquot with no DNA added. Negative control had to give no PCR products and was indicative of the presence or absence of contamination in the reaction. Once the amplification was completed, agarose gels were prepared in order to test the quality of the PCR products.

Agarose gel could be prepared at different percentages to allow the separation of PCR products on the basis of their lengths, expressed as base pairs. For the confirmation of our PCR products, a 2% agarose gel was sufficient (gel preparation is described in the section “DNA extraction with QIAGEN kit”). Three microlitres of amplified DNA were mixed with 2 µL of the gel-loading buffer previously diluted 1:2, and the samples were loaded into the gel wells. For sizing the PCR products (217 to 314 bp), a DNA ladder (GeneRuler™ 100 bp DNA Ladder, Genenco) was always used; it contained the following 11 discrete fragments: 1031, 900, 800, 700, 600, 500, 400, 300, 200, 100 and 80 bp.

The amplification protocol for *SOD1*, *FUS*, and *ANG* was performed using Biomek NX^{P}. Biomek NX^{P} (Beckman Coulter) puts every aspect of liquid handling, including pipetting, dilution, dispensing and integration, into a single, automated system that is as powerful and flexible as it is efficient and economical (Figure 16).

Figure 16. *Biomek NX^{P} (Beckman Coulter)*

Biomek NX^{P} offers proven pipetting performance for low-volume reaction setup and assay miniaturization, as well as accurate and repeatable results extending into the submicrolitre range. Since many functions are combined into a single step, setup is fast and effortless.

Automation of reaction setup can also eliminate both human error and contamination associated with manual processing, while assuring high quality results. The reliability of

this instrument, repeatable pipetting, tube to plate capability and an efficient automation combine to provide an ideal automation solution for the amplification protocol. Moreover its low-volume capability helps miniaturize reaction setups and save on costly reagents.

Biomek NX^{P} combined with robust Biomek Software is used to manage and program the instrument (Figure 17). This software provides the ability to customize the interface setting for every PCR step, as type of tip to use, source and destination of reagents and it is able to configure methods with drag-and-drop ease. The users have only to prepare the worktop with tips, reagents, tubes, primers, DNA and plate of destination in the correct position.

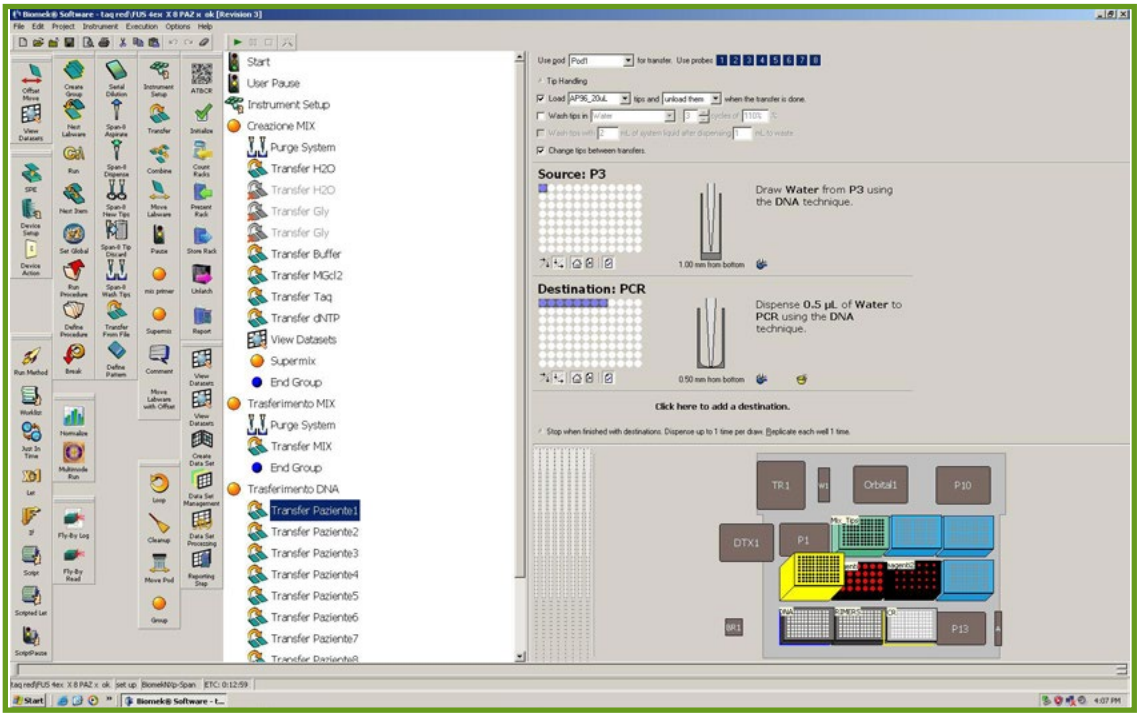


Figure 17. *Software interface of Biomek NX^{P}*

Purification of PCR products

Automated

Amplicons have to be purified to eliminate excess oligos, nucleotides, salts, enzymes and other contaminants that may inhibit subsequent reactions. The purification was performed using AMPure® PCR Purification Kit (Agencourt® Bioscience Corporation) (Figure 18) and automating the process through Biomek 3000 (Beckman Coulter). The Agencourt AMPure method utilizes Solid Phase Reversible Immobilization (SPRI) magnetic bead-based technology, which requires no centrifugation or filtration.

The Agencourt AMPure purification process binds PCR amplicons to para-magnetic particles and draws them out of solution, allowing contaminants such as primers, primer dimers, salts, and dNTPs to be easily rinsed away providing purified PCR product ideal for downstream genomics applications. The para-magnetic particles are constituted for 40% of magnetite and have uniform size (1 μm); their negative charge binds PCR products 100 bp and larger. Placed in a magnetic field, the amplicons remain bound to the magnetic beads in the plate wells, while the washes are performed and the contaminants removed. At the end the purified DNA is eluted with dd H_2O and transferred to another plate.

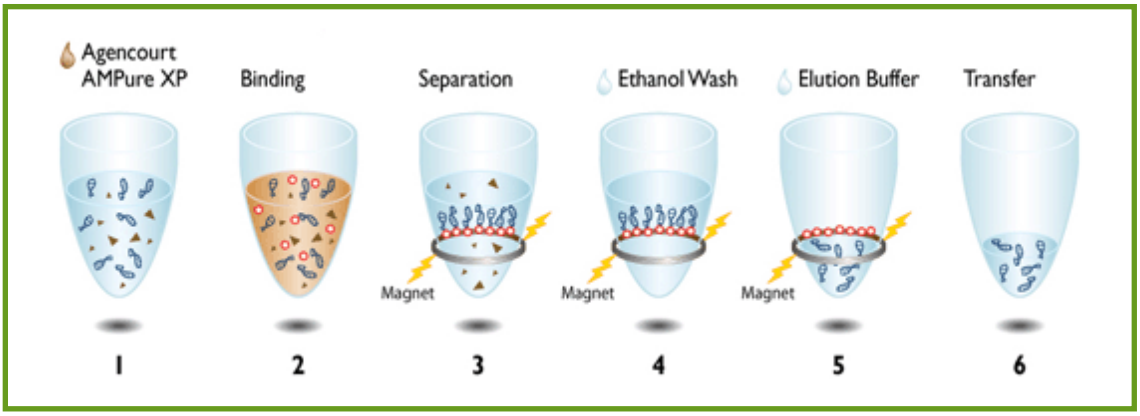


Figure 18. Schematic representation of PCR purification

The AMPure procedure is performed in 8 steps:

1. mix AMPure reagent with the PCR reaction mixture. Add 1.8 µL AMPure per 1.0 µL of PCR product;
2. bind PCR products to para-magnetic beads and incubate for 3-5 minutes;
3. move the plate on the magnetic support, incubate for 5-10 minutes to separate beads from solution;
4. aspirate the cleared solution from the reaction plate and discard;
5. dispense 200 µL of 85% ethanol to each well of the reaction plate and incubate for 30 seconds at room temperature; aspirate out the ethanol and discard; repeat for a total of two washes;
6. place the reaction plate on bench top to air-dry; the plate should be left for 10-20 minutes to allow complete evaporation of residual ethanol that may inhibit the subsequent reactions;
7. add 40 µL of dd H_2O to each well of the reaction plate and mix in order to elute purified PCR product from the beads;
8. transfer the purified products to a new plate.

This process has been automated and optimized by using the Biomek 3000 (Beckman Coulter) (Figure 19). This instrument is an automated laboratory workstation fully compatible with Agencourt AMPure.

Figure 19. *Biomek 3000 (Beckman Coulter)*

As for the Biomek NX^P, Biomek 3000 automates many processes. This instrument has a mechanical arm that moves on 3 axes (X, Y, Z) mono and multichannel pipettes (P20, MP20, P200, MP200, P1000) besides the gripper tool that picks up and dislocates the plates. On the worktop there is space to accommodate the magnetic support as well as containers for reagents, plates, tips and waste. User-friendly software facilitates tailoring each protocol to specific requirements, or building new protocols from scratch. The user must have the material correctly placed on the worktop, as indicated on the program, and start it.

Manual

The purification of PCR products was performed by using the QIAquick® PCR Purification Kit (QIAGEN) that combines the convenience of spin-column technology with the selective binding properties of a uniquely designed silica membrane. DNA adsorbs to the silica membrane in the presence of high concentrations of salts, while unwanted primers and impurities, such as enzymes, unincorporated nucleotides, agarose, oils and detergents pass through the column. Contaminants are efficiently washed away, and the pure DNA is eluted with Tris buffer.

For the PCR products purification, the protocol described below was followed (Figure 20):

1. 5 volumes of PBI buffer (binding buffer) were added to 1 volume of the PCR sample and mixed;
2. a QIAquick spin column was placed in a 2 mL collection tube;
3. the sample was applied to the spin column and centrifuged at 14000 rpm for 60 seconds;
4. the eluate was discarded and the spin column was placed back in the same tube;
5. 0.75 mL of PE buffer (washing buffer) was added to the column and a centrifugation at 14000 rpm for 60 seconds was performed;
6. after discarding the eluate and placing back the spin column in the same tube, a centrifugation at 14000 rpm for additional 60 seconds was carried out;
7. the column was placed in a clean 1.5 mL centrifuge tube;
8. 30 µL of EB buffer (elution buffer) were added to the centre of the membrane and, after an incubation time of about 60 seconds, a centrifugation at 14000 rpm for 60 seconds was performed.

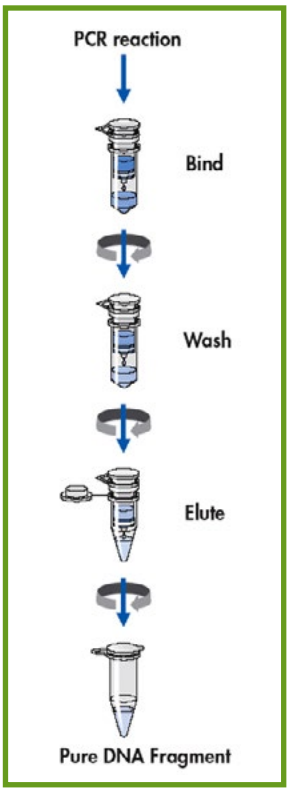


Figure 20. *Schematic representation of the spin procedure for PCR products purification by using the QIAquick® PCR Purification Kit.*

Purified DNA fragments were quantified in order to settle the suitable quantity of DNA to use in the subsequent sequencing reaction. DNA yield was determined by 2% agarose

gel analysis: the same procedure described in “Confirmation of PCR Products” was used. The amount of loaded DNA sample was estimated by visual comparison of the band intensity with that of the standards (GeneRuler™ 100 bp DNA Ladder, Genenco); DNA quantity for a correct sequencing reaction is 10 ng per 100 bp. Purified PCR products were stored at 4 °C until their use.

Sequencing analysis

Sequencing a DNA fragment amplified by PCR allows its nucleotide sequence to be determined, individualizing the possible presence of a variation. The most commonly used approach is the dideoxy chain termination method (Sanger method) [139]. The key principle of the Sanger method is the use of dideoxynucleotide triphosphates (ddNTPs) as DNA chain terminators: they are analogues of the normal dNTPs, but differ in that they lack a hydroxyl group at the 3’ carbon position as well as at the 2’ carbon. Lacking this group, any ddNTP that is incorporated into a growing DNA chain cannot participate in phosphodiester bonding at its 3’ carbon atom, thereby causing abrupt termination of chain synthesis. Competition for incorporation into the growing DNA chain between a ddNTP and its normal dNTP analogue results in a population of fragments of different lengths [140]. The technique requires a single-stranded DNA template, a DNA primer and a sequencing kit containing DNA polymerase, dNTPs and the four ddNTPs labelled with fluorescent dyes, each of which with different wavelengths of fluorescence and emission.
The newly synthesized and labelled DNA fragments are heat denatured and separated by size by capillary electrophoresis (CE).

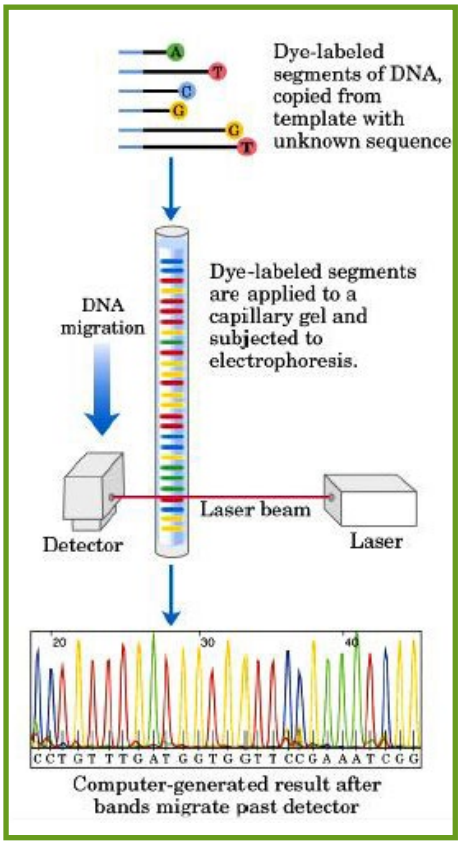


Figure 21. Schematic representation of the automated DNA sequencing with capillary electrophoresis (www.appliedbiosystems.com)

During CE, the DNA fragments are electrokinetically injected into capillaries filled with a high-resolution polymer. High voltage is applied so that the negatively charged DNA fragments move through the polymer toward the positive electrode. CE can resolve DNA molecules that differ in molecular weight by only one nucleotide. Shortly before reaching the positive electrode, the fluorescently labelled DNA fragments move through the path of a laser beam that causes the dyes on the fragments to fluoresce. An optical detection device detects the fluorescence; specific software converts the fluorescence signal to digital data and records them (Figure 21). Since each dye emits light at a different wavelength when excited by the laser, all four colours, and therefore, all four bases, can be detected and distinguished in one capillary injection (Figure 22).

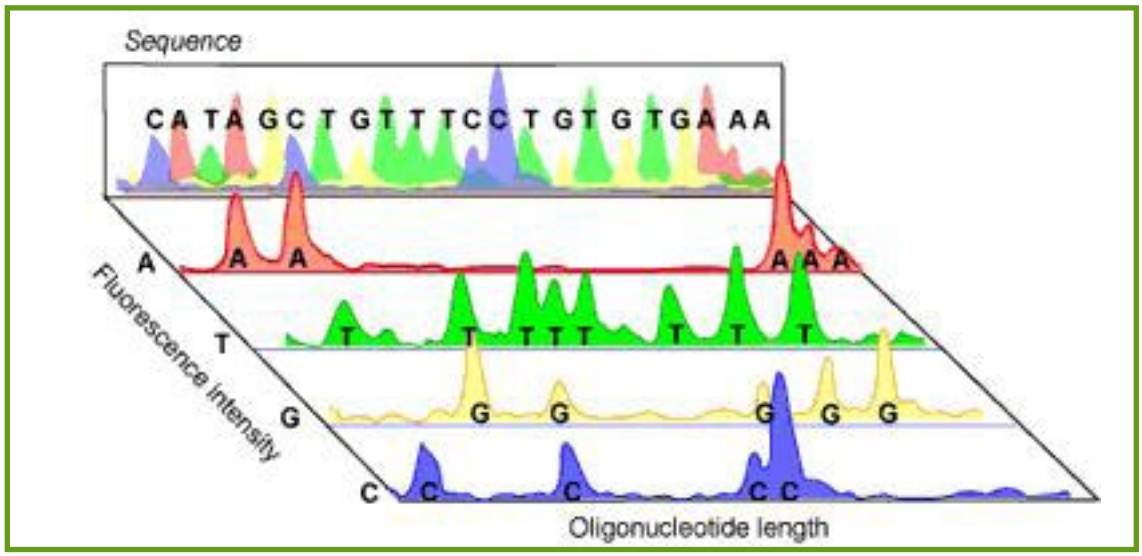


Figure 22. *Electropherogram (www.appliedbiosystems.com)*

Automated and manual sequencing reaction protocol

The sequencing reaction was performed by using the BigDye Term Cycle Seq Kit (Applied Biosystems). Version 1.1 was used for *SOD1* and *ANG* gene, while version 3.1 for *FUS* gene.

When automated on Biomek 3000, each protocol involved the use of:

- 1 µL of 10 µM primer forward or reverse;
- 1 µL of DNA sequencing Mix;
- 2 µL of BigDye® Terminator v1.1 5X Sequencing Buffer;

- DNA quantity established following the procedure “Confirmation of PCR products” (usually 1 µL of amplified DNA was used);
- H_2O to a final volume of 10 µL.

Only for *TARDBP* gene the sequencing reaction protocol was performed in a manual mode, possibly in a dark room, using BigDye v3.1.

Each protocol involved the use of:

- 1 µL of 10 µM primer forward or reverse;
- 2 µL of DNA sequencing Mix;
- DNA quantity established following the procedure “Confirmation of PCR products (usually 2 to 3 µL of amplified DNA were used);
- H_2O to a final volume of 20 µL.

Each exon of each gene was sequenced on both strands; therefore two sequencing reactions, one with primer forward and the other with primer reverse, were performed.

For the sequencing reaction with BigDye v3.1, the following program on the thermal cycler was performed:

- 1 step at 96 °C for 1 minute;
- 25 cycles, each one composed of: 96 °C for 10 seconds (DNA denaturation) – 50 °C for 5 seconds (primer annealing) – 60 °C for 4 minutes (DNA extension).

For the sequencing reaction with BigDye v1.1 the program is devoid of the first step.

Purification of sequencing reaction products

Automated

It was necessary to purify the sequencing reaction products in order to remove unincorporated dye terminators and all the other reagents and by-products. Removal of unincorporated dye-terminators is required prior to capillary electrophoresis, because they can cause a “background noise” which makes the electropherograms unreadable.

The CleanSEQ Sequencing Reaction Clean-Up has proven an efficient and highly effective dye-terminator removal system with the ABI 3730 sequencer. This system utilizes Agencourt's patented SPRI™ para-magnetic bead technology as in the AMPure purification process previously described.

Also in this case, the process is automated through Biomek 3000 (Beckman Coulter). The protocol can be performed directly in the thermal cycler plate. CleanSEQ contains magnetic particles in an optimized binding buffer to selectively capture sequencing extension products, whereas unincorporated dyes, nucleotides, salts and contaminants are removed using a simple washing procedure.

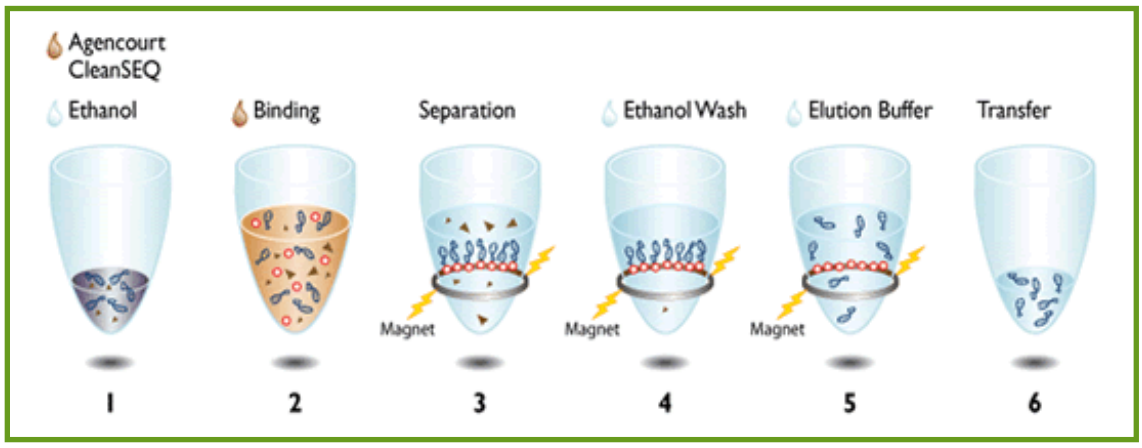


Figure 23. Schematic representation of CleanSEQ purification

The CleanSEQ procedure is performed in 7 steps:

1. gently shake the CleanSEQ bottle to re-suspend any magnetic particles that may have settled. Add 10 µL of CleanSEQ to the reaction plate;
2. add 42 µL of 85% ethanol to the reaction plate; pipette the mix 7 times;
3. place the reaction plate onto magnetic support for 3 minutes to separate beads from solutions;
4. aspirate the cleared solution from the reaction plate and discard;
5. dispense 100 µL of 85% ethanol and incubate at room temperature for at least 30 seconds, then aspirate out the ethanol and discard; repeat for a total of 2 washes;
6. let the reaction plate air-dry for 10 minutes at room temperature;
7. add 40 µL of dd H_2O and incubate the plate for 5 minutes at room temperature and then transfer samples into a new clean reaction plate.

Manual

The DyeEx™ 2.0 Spin Kit (QIAGEN), providing the combination of gel-filtration technology with a pre-hydrated microspin format, was used. This procedure is based on gel filtration chromatography that separates molecules according to molecular weight. The kit uses gel-filtration material consisting of spheres with uniform pores; when sequencing reactions are applied onto columns, dye terminators diffuse into the pores and are retained in the gel-filtration material, while DNA fragments are eluted (Figure 24).

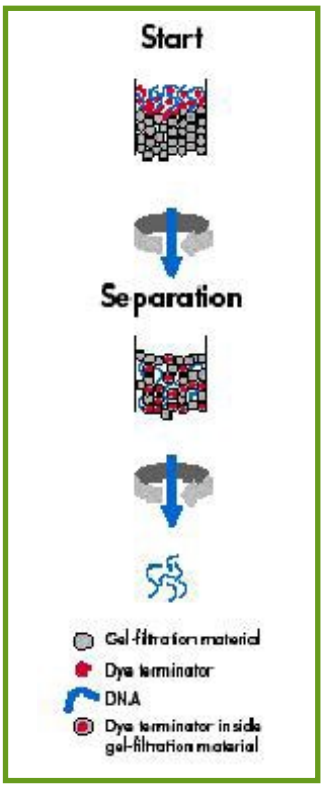


Figure 24. *Separation principle of DyeEx™*

For the purification of sequencing reaction products, the protocol described below was followed:

1. the spin column was gently vortexed to resuspended the resin;
2. the cap of the column was loosened a quarter turn to avoid a vacuum inside the spin column, the bottom closure was snapped off, and the spin column was then placed in a 2 mL collection tube;
3. a centrifugation at 2700 rpm for 3 minutes was performed;
4. the spin column was carefully transferred to a clean centrifuge tube and the sequencing reaction (20 µL) was slowly applied to the gel bed;
5. a centrifugation at 2700 rpm for 3 minutes was performed;
6. the spin column was removed from the microcentrifuge tube: the eluate contained the purified DNA.

After sequence products purification, the samples were loaded on ABI PRISM 3730 (Applied Biosystems).

Sequencing

Samples preparation

For DNA sequencing a 48-capillary 3730 DNA Analyzer (Applied Biosystems) instrument was used. Once purified, samples containing the sequencing reactions were prepared on a special plate for multi-channel electrophoresis runs. This plate was formed by 96 wells; purified sequences were added in alternate rows since the 48 capillaries enter simultaneously in different wells. Two microlitres of purified products together with 8 µL of deionized formamide were placed in each well. The plate was covered with a rubber mat and centrifuged to bring the samples down to the bottom of the wells. Samples were denatured at 95 °C for 2 minutes on a thermal cycler and then incubated at -20 °C for at least 5 minutes.

Samples loading

Before loading the plate in the instrument, the worksheet showing the order of samples was entered in the computer. The first step was to appoint the plate with the “bar code” present on its side, together with the date of the transaction. The worksheet is a table in which rows represent the name of wells and columns the name of the sample, the protocol type and the reading type to be used. The plate was then inserted in the instrument: the 48 samples placed in alternating columns were analysed first.

Software for sequence analysis

After the electrophoretic run and the acquisition of data by the analyser, each DNA fragment sequence could be viewed on the computer screen: the four bases, labelled with different fluorophores, were detected and represented as peaks of four colours (green for adenine, blue for cytosine, black for guanine and red for thymine). Raw data analysis was performed by using the “Sequencing Analysis” software (Figure 25), designed to base-call, trim, display, edit, and print DNA sequencing data generated from the genetic analyser; this step allowed to check if the procedure was successful or if the electrophoretic run had to be repeated. Moreover, this software provided multiple metrics (sample score, read length, signal-to-noise values) that help pinpoint the cause of data failure.

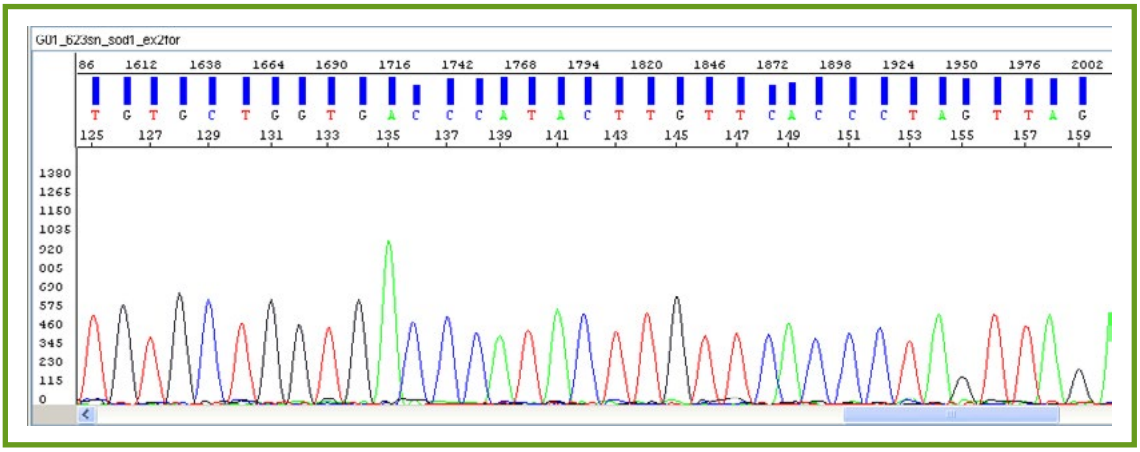


Figure 25. "Sequencing Analysis" interface: the software was used to observe DNA sequences

By using the SeqScape® software (Figure 26), all the variations (deletions, insertions, mismatches, heterozygous bases, and heterozygous insertion/deletions) between the reference genomic sequence and each subject sample sequence were detected. Software examines the quality of each forward and reverse trace and forms an accurate consensus call. Variations between the consensus and the reference sequence are reported as mutations in the Mutations report. All analysis in SeqScape® software occurred in a project; before creating a project, a project template containing a reference data group, analysis defaults and display settings was created. Once a project was created, the project template was used recurrently.

As for the "Sequencing Analysis" software, the sequence was represented by a succession of peaks characterized by four different colours, one for each nucleotide. A peak overlap (identified by letter N) showed the presence of two different nucleotides in the same position and, therefore, the presence of a variant in the heterozygous state. If the alteration was present in the homozygous state, a different peak in comparison with the reference sequence was found: however, the change was signalled by the software. The existence of an insertion or a deletion in the sample was characterized by the presence of a complicated pattern of overlapping peaks from the site of the alteration because of a different positioning between the two strands. The presence of any genetic variant was always verified also on the complementary strand (3' → 5').

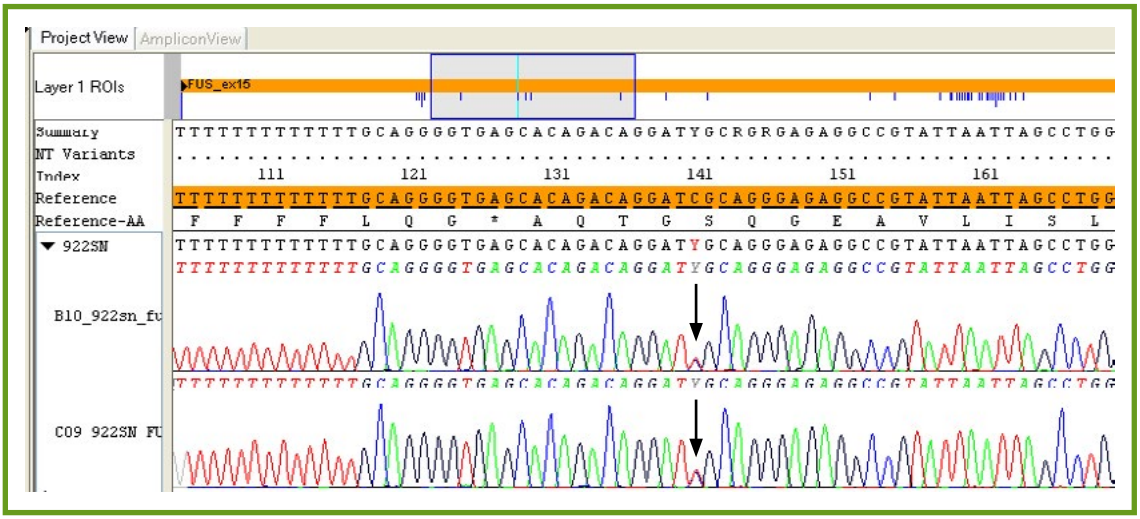


Figure 26. *SeqScape® interface: the software was used to identify polymorphisms/mutations by comparing the DNA fragment of interest with the reference sequence; the arrows represent a mutation found in the heterozygous state*

C9ORF72 analysis

The GGGGCC hexanucleotide repeat in *C9ORF72* was analysed by a specific protocol quite different from the usual process based on amplification, purification and sequencing of the samples described above.

The pathogenic repeat expansion may not be amplified by a conventional PCR, and therefore DeJesus-Hernandez and colleagues in 2011 [75] developed a repeat-primed PCR assay, resulting in the identification of the pathogenic repeat expansion. All reagents used for PCR assay were provided by Roche. Each PCR reaction was performed in a total volume of 14 µL (PCR mixture) and involved the use of:

- Roche Fast Start mix (1X)
- Primer Mix
- 7%DMSO
- Q solution (1X)
- 0.18 mM Deaza GTP
- 0.9 mM MgCl2
- DNA

The primer mix contains 3 different primers (Table 6) and 25 µL of H_2O in a total volume of 50 µL.

Name	Sequence	Comment
RP-PCR-F1 (10 µL)	6-FAM-AGTCGCTAGAGGCGAAAGC	FAM fluorophore
RP-PCR-R (5 µL)	TACGCATCCCAGTTTGAGACGGGGGCCGGGGCCGGGGCCGGGG	
RP-PCR-Anchor (10 µL)	TACGCATCCCAGTTTGAGACG	

Table 6. *Primers for repeat primed PCR*

The repeated primer PCR reaction using the genotyping primers previously reported was performed on a thermal cycler using an already described, optimized cycling program [141]. The PCR products were analysed without any purification or sequencing, on an ABI PRISM 3730 DNA Analyser and visualized using GeneMapper software.

The special plate for multi-channel electrophoresis runs was used. Two microlitres of PCR products together with 7.5 µL of deionized formamide and 0.5 µL of Liz500 size standard were placed in each well of the plate. The samples were denatured at 95 °C for 3 minutes on a thermal cycler and then immediately transferred at -20 °C for at least 5 minutes.

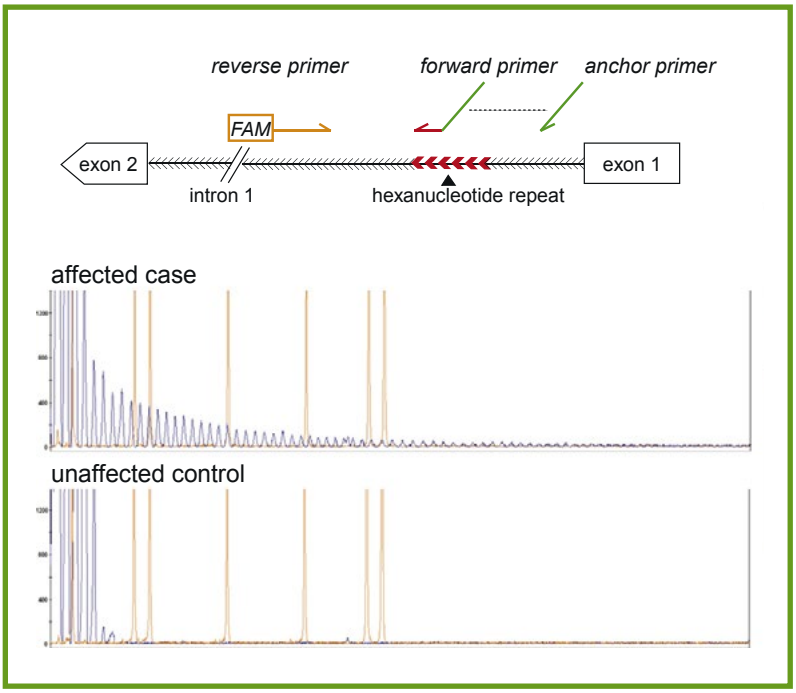


Figure 27. *A graphical representation of primer binding for repeat-primed PCR analysis is shown in the upper panel. In the lower panel, capillary-based sequence traces of the repeat-primed PCR are shown. Orange lines indicate the size markers, and the vertical axis represents fluorescence intensity. A typical saw tooth tail pattern that extends beyond the 300 bp marker with a 6 bp periodicity is observed in the case carrying the GGGGCC repeat expansion [76].*

Results

Our cohort of patients includes 285 SALS patients (94.4%) and 17 FALS patients (5.6%) respectively.

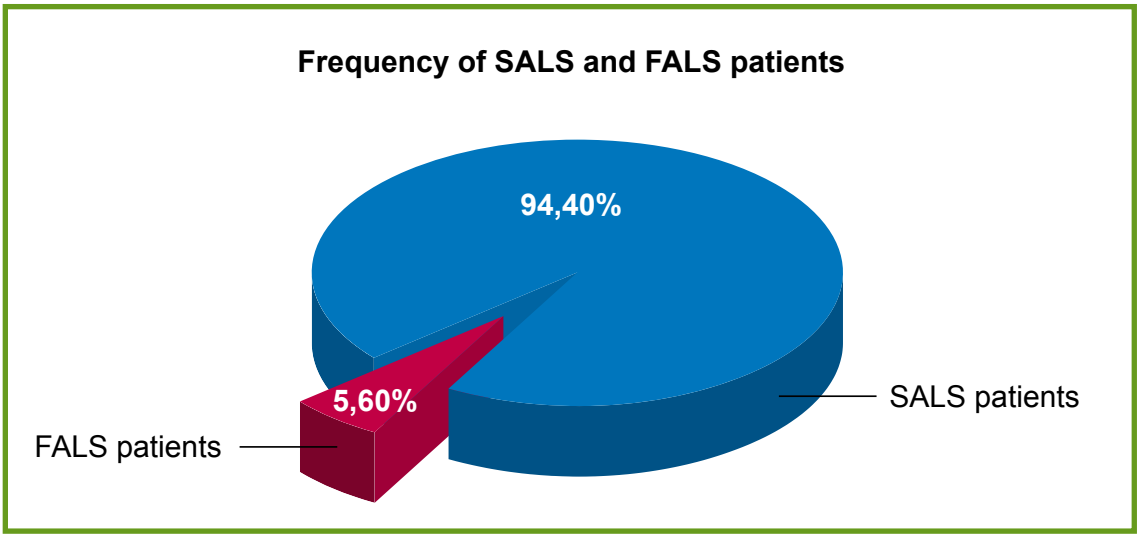


Figure 28. *Percentage distribution of SALS and FALS in our cohort of patients*

In our cohort we identified, overall, 15 cases of *C9ORF72* pathological repeat expansion. We considered positive to this genetic test only samples characterized by an electropherogram containing 19, or more, peaks with an exponential decay; the electropherograms of samples without repeat expansion are on the contrary characterized by an abrupt cut-off.

The characterization of ALS causative genes (*SOD1*, *TARDBP*, *FUS*) through sequencing allowed us the identification of 18 genetic variants in SALS and FALS cases.

Genetic analysis of FALS patients

Direct sequencing analysis of *SOD1*, *TARDBP* and *FUS* genes in 17 FALS patients allowed the identification of 9 different genetic variants. Specifically, we detected:

- 1 mutation in *SOD1* gene identified in 2 distinct patients;
- 3 different mutations in *TARDBP* gene identified in 5 distinct patients;
- 2 different mutations in *FUS* gene identified in 2 distinct patients.

As for the *C9ORF72* assay, 4 patients showed a pathological repeats expansion.

Our results about FALS patients are summarized in Table 7.

Gene	Number of genetic variants	%
SOD1	2/17	11.8
TARDBP	5/17	29.4
FUS	2/17	11.8
C9ORF72	4/17	23.5

Table 7. *Distribution of identified genetic variants among ALS causative genes in FALS patients in our cohort*

ALS causative mutations have been found in 76.5% of FALS cases.

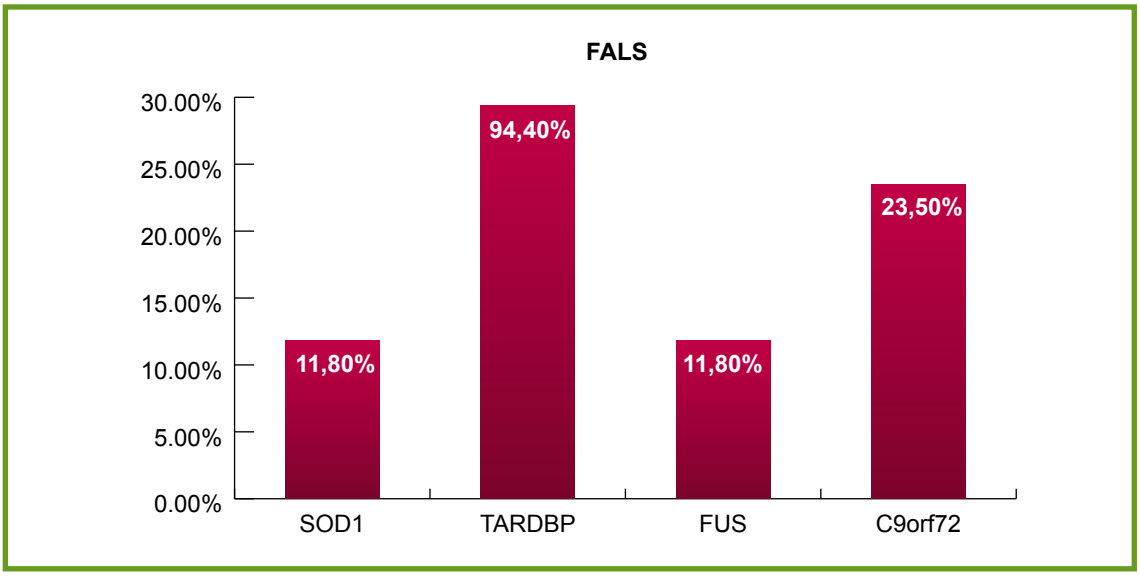


Figure 29. *Schematic representation of FALS mutations distribution*

FALS cases include 9 (53%) male subjects and 8 (47%) female subjects, respectively. Specifically, the same mutation in *SOD1* (G93D) and *TARDBP* (A382T) was detected in 2 females. Conversely the carriers of G294V *TARDBP* are both males and females.

Gene	Male (9)	Female (8)
SOD1	-	2 (25%)
TARDBP	2 (22.2%)	3 (37.5%)
FUS	1 (11.1%)	1 (12.5%)
C9ORF72	-	4 (50%)

Table 8. *Distribution of mutations between sexes*

In 2 FALS female patients analysed, *FUS* and *TARDBP* mutated genes were present together with *C9ORF72* pathological expansion. The first patient harbouring *FUS/C9ORF72* mutations has a family history of dementia, whereas the *TARDBP/C9ORF72* patient has a family history of ALS.

Gene	Gender	Age at onset	Site of onset	Mutation/repeats number	Geographical origin
SOD1	F	33	Lower limb	G93D	Caltanissetta (Sicily)
	F	18	Lower limb	G93D	Caltanissetta (Sicily)
TARDBP	M	69	Bulbar	G294V	Catania (Sicily)
	M	62	Upper limb	G295S	Florence (Tuscany)
	F	54	Lower limb	G294V	Catania (Sicily)
	F	63	Bulbar	A382T	Nuoro (Sardinia)
FUS	M	49	Lower limb	R521C	Piacenza (Emilia)
C9ORF72	F	48	Lower limb	28-40	Macerata (Marche)
	F	36	Lower limb	25-30	Nuoro (Sardinia)
C9ORF72+FUS	F	69	Bulbar	>30 + R491C	Catania (Sicily)
C9ORF72+TARDBP	F	44	Upper limb	>30 + A382T	Sassari (Sardinia)

Table 9. *Summary of mutations and clinical characteristics of FALS patients in our cohort*

Genetic analysis of SALS patients

Direct sequencing analysis of *SOD1*, *TARDBP* and *FUS* genes in 285 SALS cases allowed the identification of 9 different genetic variants. Specifically, we detected:

- 2 different mutations in *SOD1* gene found in 2 distinct patients;
- 3 different mutations in *TARDBP* gene found in 4 distinct patients;
- 3 different mutations in *FUS* gene found in 3 distinct patients.

Moreover, we identified 11 *C9ORF72* pathological repeat expansions. These results are summarized in Table 10.

Gene	Number of genetic variants	%
SOD1	2/285	0.7
TARDBP	4/285	1.4
FUS	3/285	1.1
C9ORF72	11/282	3.9

Table 10. *Distribution of identified genetic variants among ALS causative genes in SALS patients in our cohort*

Overall, the mutations detected in our cohort of patients explain 7.1% of SALS cases.

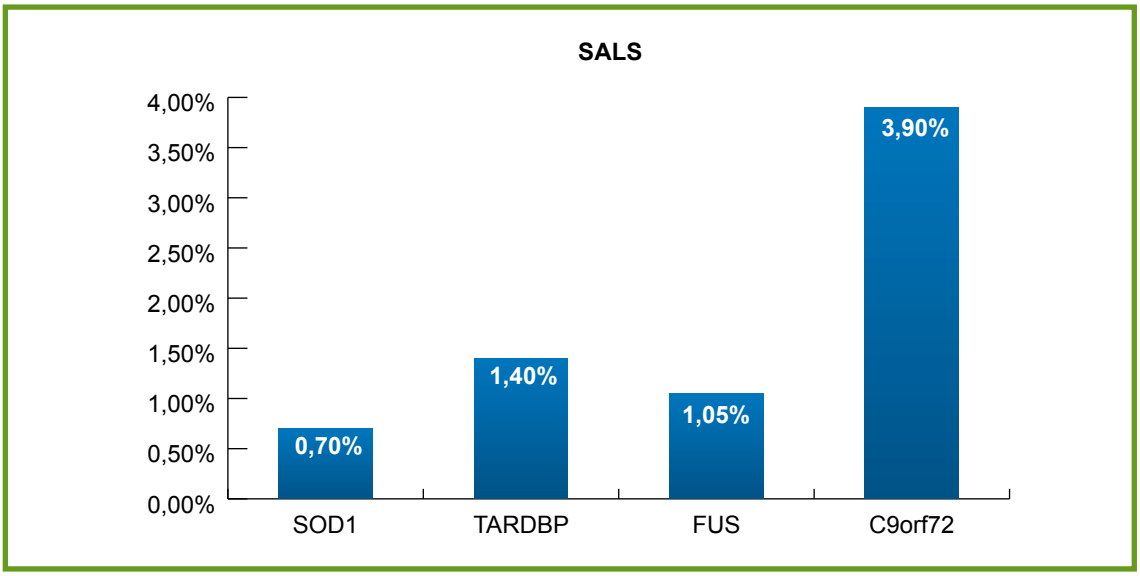


Figure 30. *Schematic representation of SALS mutations distribution*

SALS cases include 160 (56.1%) male subjects and 125 (43.9%) female subjects, respectively. The distribution of mutations between sexes is summarized in Table 11.

Gene	Males (160)	Females (125)
SOD1	1 (0.6%)	1 (0.8%)
TARDBP	2 (1.3%)	2 (1.6%)
FUS	-	3 (2.4%)
C9ORF72	4 (2.5%)	7 (5.7%)

Table 11. *Distribution of mutations between sexes*

C9ORF72 analysis of 3 SALS patients (1 male and 2 females) was impossible because amplification did not occur.

Gene	Gender	Age at onset	Site of onset	Mutation/repeats number	Geographical origin
SOD1	M	46	Upper limb	N65S	Fano (Marche)
	F	43	Lower limb	T137A	Zambrone (Calabria)
TARDBP	M	60	Upper limb	A382T	Margno (Lombardy)
	M	51	Upper limb	A382T	Cagliari (Sardinia)
	F	46	Upper limb	G368S	Lodi (Lombardy)
	F	69	Lower limb	G294V	Chiaramonte (Sicily)
FUS	F	37	Respiratory	R522G	Naples (Campania)
	F	33	Bulbar	P525L	Catania (Sicily)
	F	44	Upper limb	R521C	Bellano (Lombardy)
C9ORF72	M	73	Bulbar	19	Milan (Lombardy)
	M	72	Upper limb	21	Bresso (Lombardy)
	M	49	Lower limb	25-35	Seulo (Sardinia)
	M	67	Lower limb	19	Pontinia (Lazio)
	F	71	Lower limb	19	Milan (Lombardy)
	F	60	Lower limb	>30	Milan (Lombardy)
	F	55	Lower limb	>30	San Pietro di Caridà (Calabria)
	F	70	Lower limb	>30	Cremona (Lombardy)
	F	43	Bulbar	>30	Scerni (Abruzzo)
	F	62	Upper limb	>30	Foggia (Puglia)
	F	59	Lower limb	>30	Palestro (Piedmont)

Table 12. *Summary of mutations and clinical characteristics of SALS patients in our cohort*

Selected familial clinical cases

We report the five most interesting FALS cases that we analysed genetically. All the selected patients show heterozygous missense mutations. We report also the results for relatives who decided to undergo molecular genetic testing. We analysed sequentially *SOD1*, *TARDBP*, *FUS* and *C9ORF72* both in the proband and in all of the mutated proband's relatives.

Family 1

We reported the family harbouring the G93D mutation in exon 4 of *SOD1* (Figure 31). This mutation has been already reported in literature [142,143].

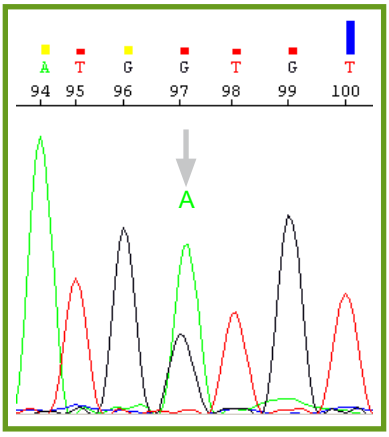


Figure 31. *Electropherogram of the G93D mutation (c.281 G>A) in the SOD1 gene. The arrow indicates the presence of mutation.*

Organism	Amino acid		
H.sapiens	**74**	**GPKDEERHVGDLGNVTADKDGVADVSIEDSVISLSGDHCIIGRTLVVHEK**	**123**
C.elegans	95	GPKSEIRHVGDLGNVEAGADGVAKIKLTDTLVTLYGPNTVVGRSMVVHAG	144
S.pombe	74	DRTAAVRHVGDLGNLESDAQGNIKTTFSDSVISLFGANSIIGRTIVIHAG	123
S.cerevisiae	74	APTDEVRHVGDMGNVKTDENGVAKGSFKDSLIKLIGPTSVVGRSVVIHAG	123
D.rerio	74	GPTDSVRHVGDLGNVTADASGVAKIEIEDAMLTLSGQHSIIGRTMVIHEK	123
G.gallus	74	GPKDADRHVGDLGNVTA-KGGVAEVEIEDSVISLTGPHCIIGRTMVVHAK	122
C.lupus	73	GPKDQERHVGDLGNVTAGKDGVAIVSIEDSLIALSGDYSIIGRTMVVHEK	122
B.taurus	72	GPKDEERHVGDLGNVTADKNGVAIVDIVDPLISLSGEYSIIGRTMVVHEK	121
M.musculus	74	GPADEERHVGDLGNVTAGKDGVANVSIEDRVISLSGEHSIIGRTMVVHEK	123
R.norvegicus	74	GPADEERHVGDLGNVAAGKDGVANVSIEDRVISLSGEHSIIGRTMVVHEK	123
M.mulatta	74	GPKDEERHVGDLGNVTAGKDGVAKVSFEDSVISLSGDHSIIGRTLVVHEK	123
P.troglodytes	74	GPKDEERHVGDLGNVTADKDGVADVSIEDSVISLSGDHCIIGRTLVVHEK	123
D.melanogaster	72	APVDENRHLGDLGNIEATGDCPTKVNITDSKITLFGADSIIGRTVVVHAD	121

Table 13. *Amino acid homology at codon 93 of the SOD1 protein in different species*

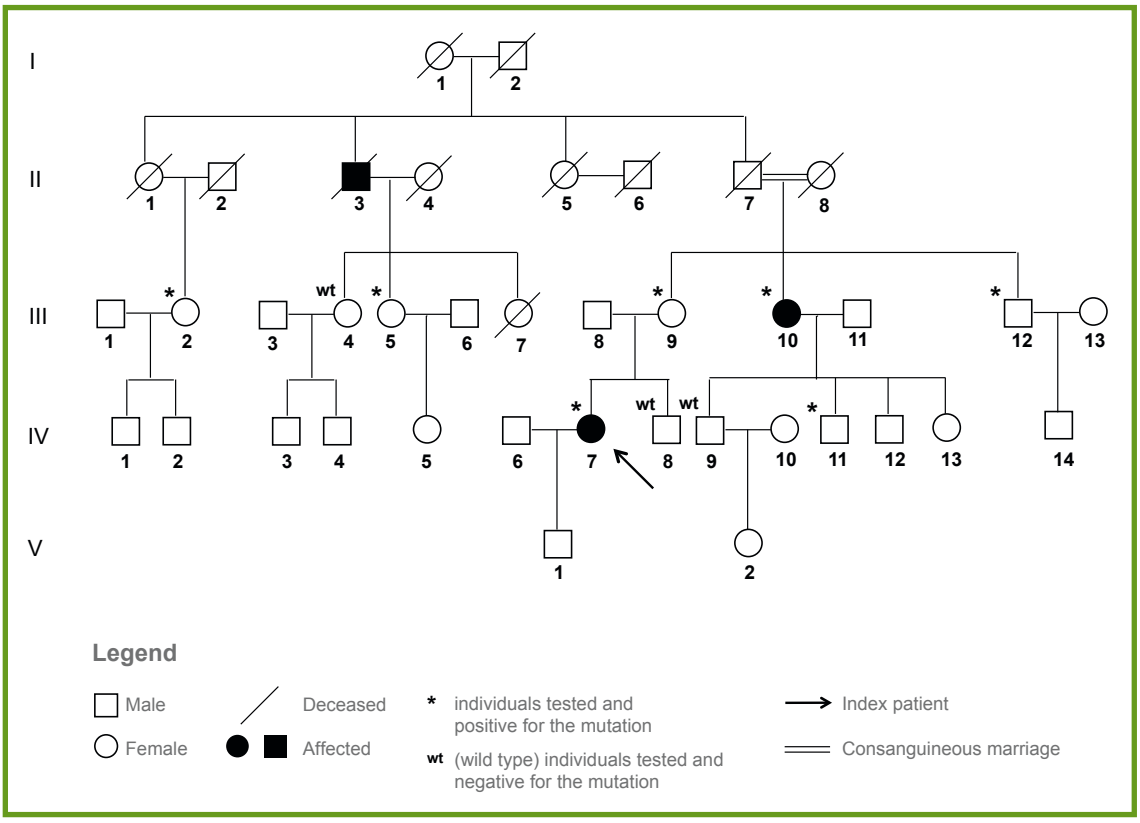


Figure 32. *Pedigree of family 1 with a G93D mutation in the SOD1 gene*

The index case is a 33-year-old woman, unipara, presented with subacute, severe, left lower limb pain the day after a normal delivery, followed by relentless progressive weakness and wasting of the left leg; at birth, the newborn was healthy. Later, the patient developed progressive worsening of her weakness with involvement of the four limbs and the bulbar district, and after 2 years had to face severe respiratory failure. Five years from onset, the patient is alive, wheelchair-bound, in ventilatory assistance by tracheostomy and in enteral nutrition by gastrostomy. The patient was admitted at NEMO and her neurological examination revealed severe weakness – ranging from grade 1/5 to 2/5 according to the Medical Research Council (MRC) scale – of both upper and lower limb muscles; all deep tendon reflexes were reduced, and plantar reflexes were flexor bilaterally. Hoffmann sign was negative bilaterally, and jaw jerk reflexes were normal. MRI of the spine was negative. Needle electromyography (EMG) showed increased insertional activity, spontaneous activity (fibrillation potentials and positive sharp waves), increased amplitude, and long duration motor unit action potentials, associated with reduced recruitment in all limbs. Fasciculation potentials were diffusely present. The study was interpreted as consistent with lower motor

neuronopathy. Comprehensive laboratory tests yielded no abnormal results, except for a mild increase of creatine kinase (CK) level (175 U/L; reference range, 30-150 U/L).

The family of the index case, which included her 55-year-old mother (III:9), her 60-year-old father (III:8), and a 26-year-old brother (IV:8), had positive history for motor neuron disease (III:10 and II:3); no clinical information was available for the proband's grandfather (II:7). Following the identification of the G93D mutation in the proband, other family members decided to undergo the genetic analysis within a multidisciplinary context. The results of genetic testing are summarized in Table 14.

Subjects	*SOD1* result
IV:7	G93D
IV:11	G93D
III:2	G93D
III:5	G93D
III:9	G93D
III:10	G93D
III:12	G93D
IV:8	G93G
IV:9	G93G
III:4	G93G

Table 14. *Results of molecular analysis in all the tested family members (affected and unaffected) for SOD1 gene*

All mutated subjects were clinically investigated and only a 60-year-old female (III:10) was found affected. Neurological examination of the patient revealed diffuse fasciculations in both upper and lower limbs with bilateral muscular weakness (grade 3/5 on the MRC scale) and atrophy of both hands; tendon reflexes in upper and lower limbs were reduced. Hoffmann, Babinski, or Chaddock signs were absent. Laboratory tests revealed a mild CK level increase (160 U/L). EMG of tibialis anterior, biceps brachii, and first dorsal interosseous showed bilaterally a neurogenic pattern: high frequency large motor unit potentials, sporadic denervation (fibrillation potentials, positive waves), and fasciculations. Transcranial magnetic stimulation of the motor cortex revealed normal central conduction time. Forced vital capacity was 96% of predicted. Clinical characteristics of all G93D *SOD1* mutation carriers are summarized in Table 15.

Subjects	Clinical characteristics
III:2	58 year-old; female; asymptomatic; normal neurological examination; mild increase of CK serum level (160 U/L; reference range, 30-150 U/L)
III:5	45 year-old; female; cramps and fasciculations; normal neurological examination; normal CK serum level
III:9	58 year-old; female; cramps and fasciculations; normal neurological examination; mild increase of CK serum level (158 U/L)
III:10	60 year-old; female; hand weakness and hypotrophy; mild increase of CK serum level (162 U/L)
III:12	51 year-old; male; asymptomatic; normal neurological examination; normal CK serum level
IV:7 (proband)	38 year-old; female; tetraplegic, in ventilatory assistance by tracheostomy and in enteral nutrition by gastrostomy; mild increase of CK serum level (175 U/L)
IV:11	27 year-old; male; asymptomatic; normal neurological examination; normal CK serum level

Table 15. *Clinical characteristics of G93D SOD1 mutation carriers*

Family 2

We described a family with the A382T mutation in the *TARDBP* gene (Figure 33).

This mutation has already been reported by different authors [106,109].

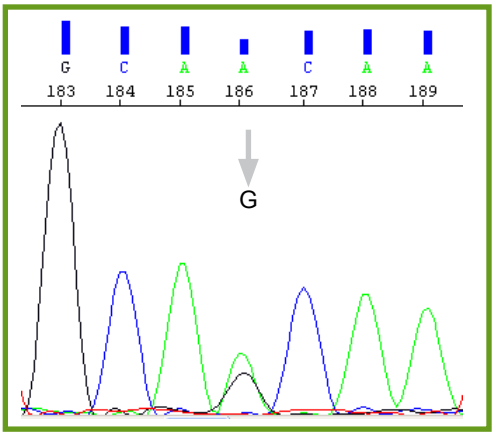


Figure 33. *Electropherogram of the A382T mutation in the TARDBP gene (exon 6). G nucleotide is wild type whereas A nucleotide corresponds to the mutated allele (c. 1144 G>A). The arrow indicates the presence of mutation.*

Organism	Amino acid	
H.sapiens	**381 AAIGWGSASNAGSGS-GFNGGFGSSMDSKS----------SGWGM---**	**414**
D.rerio	378 AALGWGTGSNSGAASAGFNSSFGSSMESKS----------SGWGM---	412
G.gallus	381 AAIGWGSASNAGSSS-GFNGGFGSSMDSKS----------SGWGM---	414
P.troglodytes	381 AAIGWGSASNAGSGS-GFNGGFGSSMDSKS----------SGWGM---	414
M.mulatta	381 AAIGWGSASNAGSGS-GFNGGFGSSMDSKS----------SGWGM---	414
M.musculus	381 APLGWGSASNAGSGS-GFNGGFGSSMDSKS----------SGWGM---	414
C.lupus	381 AAIGWGSASNAGSGS-GFNGGFGSSMDSKS----------SGWGM---	414
B.taurus	381 AAIGWGSASNAGSGS-GFNGGFGSSMDSKS----------SGWGM---	414

Table 16. *Amino acid homology at codon 382 of the TDP-43 protein in different species*

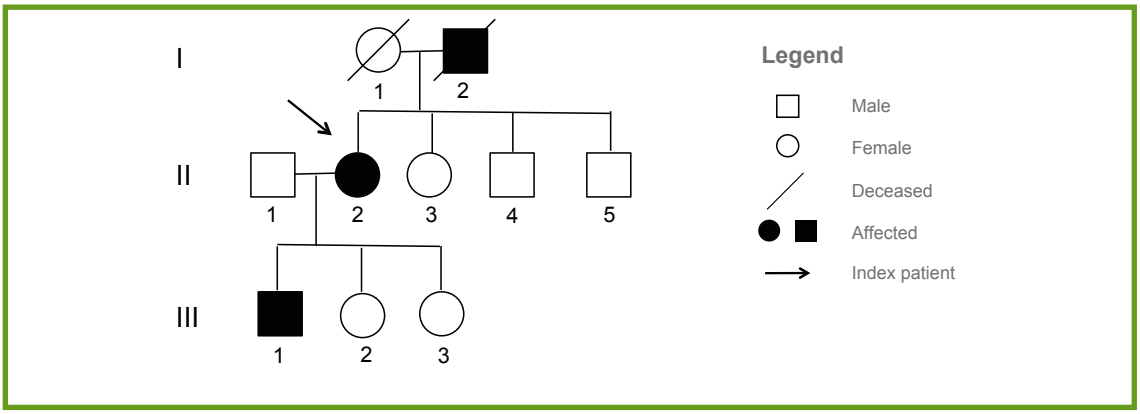


Figure 34. *Pedigree of the family with the A382T mutation in TARDBP*

The index case (II:2) is a Sardinian 63-year-old woman with signs of multi-district involvement of the upper and the lower motor neurons. Diagnosis of ALS was made according to El Escorial criteria. This patient initially presented difficulty in slurred speech and in swallowing liquids, with a slowly worsening course. Over the following months she developed muscle weakness at the right upper limb associated with hypotrophy of the hand's muscles. Recently she showed emotional instability, muscle cramps and diffuse fasciculations in all four limbs.

The patient's family pedigree showed a positive history of motor neuron disease. The father of the proband (I:2) died of ALS when 79-year old. Her son (III:1) is 40 years old and was diagnosed with a spinal form of ALS since age 35. Following positive genetic test for the presence of the A382T mutation in the proband, we had liked to test for the presence of the same mutation the other affected family members. Unfortunately the proband's father (I:2) was already died, whereas the affected proband's son (III:1) as well as the other unaffected daughters were not tested because they live in Sardinia and were not interested in genetic testing at the time we offered it.

Family 3

We reported the family harbouring the G294V mutation in exon 6 of *TARDBP* (Figure 35). This mutation has been already described in a familial case of ALS [(103)].

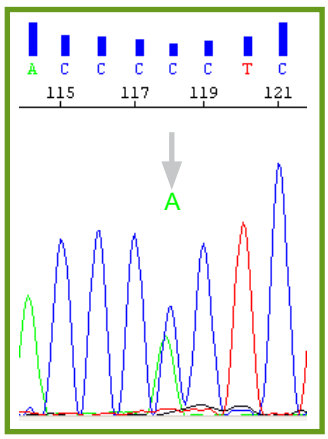


Figure 35. *Electropherogram of G294V mutation (c.881 G>T) in the TARDBP gene. Sequence analysis (reverse strand) of the PCR product showed the mutated T (forward strand). The arrow indicates the presence of mutation.*

Organism	Amino acid		
H.sapiens	**268**	**RQ-LERSGRFGGN----------PGGFG---NQGGFGNSRGGGAGLGNN**	**302**
C.elegans	344	QS----------	345
D.rerio	274	RQMMERAGRFGNG----------FGGQG---FAGSRSNMGGGGGGSSSS	309
G.gallus	268	RQ-LERGGRFGGN----------PGGFG---NQGGFGNSRGGGGGLGNN	302
P.troglodytes	268	RQ-LERSGRFGGN----------PGGFG---NQGGFGNSRGGGAGLGNN	302
M.mulatta	268	RQ-LERSGRFGGN----------PGGFG---NQGGFGNSRGGGAGLGNN	302
M.musculus	268	RQ-LERSGRFGGN----------PGGFG---NQGGFGNSRGGGAGLGNN	302
C.lupus	268	RQ-LERSGRFGGN----------PGGFG---NQGGFGNSRGGGAGLGNN	302
B.taurus	268	RQ-LERSGRFGGN----------PGGFG---NQGGFGNSRGGGAGLGNN	302

Table 17. Amino acid homology at codon 294 of the TDP-43 protein in different species

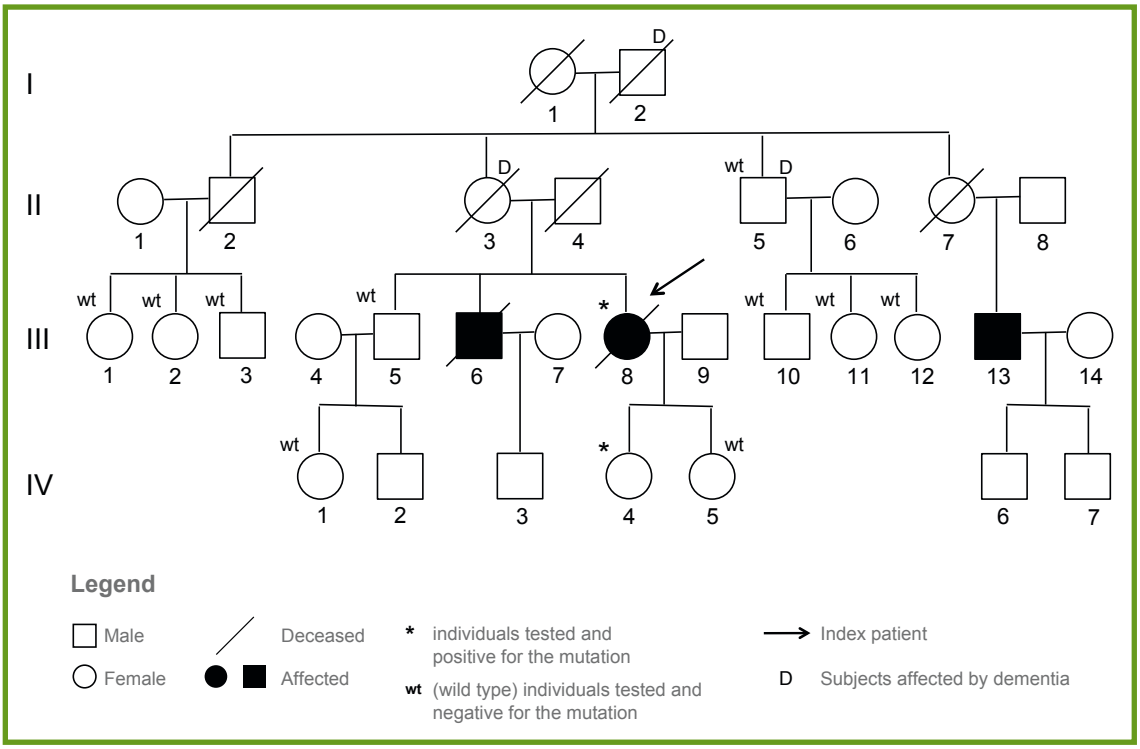


Figure 36. Pedigree of family 3 with G294V TARDBP mutation

The proband (III:8) was a woman who developed ALS with spinal-onset when she was 54 years old and died 2 years later. This patient presented fasciculations initially to the abdomen and afterwards to the lower limbs, associated with progressive weakness. A few months later she developed difficulty in slurred speech and change in the tone of her voice. No cognitive involvement was observed.

The patient's family pedigree showed a positive history both for motor neuron disease and for dementia. Previously, in 2009, the proband's brother (III:6) developed a

bulbar form of ALS and died the following year. Moreover the mother of the proband was affected by FTD and other relatives were affected by dementia (I:2; II:3; II:5). Other family members underwent genetic testing and only the proband's daughter (IV:4) resulted to be heterozygous for the G294V mutation; however, due to her young age (<30-year-old), she currently presents no neurological symptoms.

Family 4

We described a family with the R521C mutation in the *FUS* gene (Figure 37). This mutation has already been reported by different authors [99].

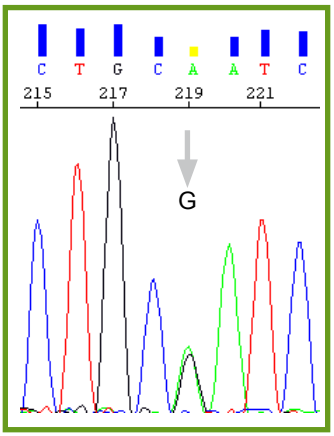


Figure 37. *Electropherogram of the R521C mutation in the FUS gene (c.1561 C>T). This mutation was identified in exon 15. Sequence analysis (reverse strand) of the PCR product showed the mutated T (forward strand). The arrow indicates the presence of mutation.*

Organism	Amino acid		
H.sapiens	**491**	**RGGFRGGRG------GGDRGGFGPGKMD--SRGEHRQD--RRERPY**	**526**
D.rerio	507	RGGFRGGR-------GGDRGGFGPGKMD--SRGDHRHD--RRDRPY	541
G.gallus	470	RGNFRGGR-------GGERGGFGPGKMD--SRGDHRQD--RRERPY	504
B.taurus	477	RGGFRGGRG------GGDRGGFGPGKMD--SRGEHRQD--RRERPY	512
M.musculus	483	RGGFRGGRG------GGDRGGFGPGKMD--SRGEHRQD--RRERPY	518
R.norvegicus	483	RGGFRGGRG------GGDRGGFGPGKMD--SRGEHRQD--RRERPY	518

Table 18. *Amino acid homology at codon 521 of the FUS protein in different species*

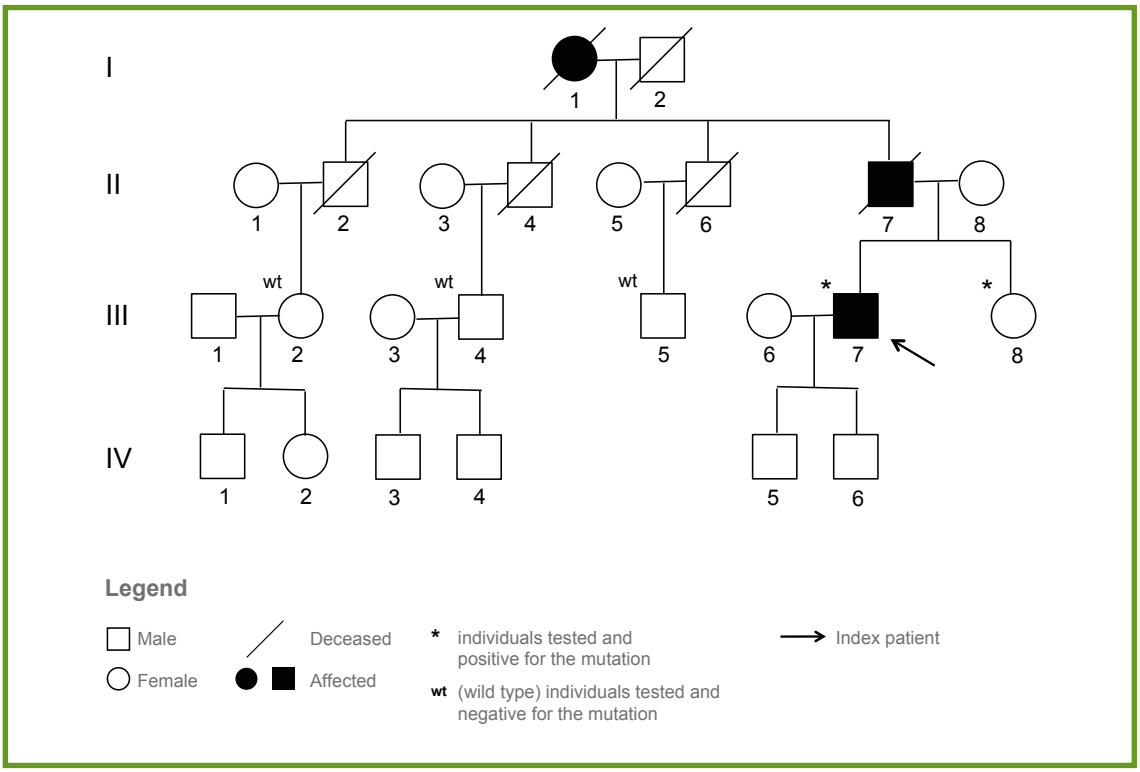


Figure 38. *Pedigree of the family with the R521C mutation in the FUS gene*

At first neurological examination, the index case, a 49-year-old man, showed weakness at the left lower limb. Laboratory tests revealed a sharp increase in CK level (900 U/L). Needle electromyography (EMG) demonstrated a widespread distribution of axonal suffering compatible with neuropathy. Very recently the proband showed progressive weakness of all four limbs. No bulbar signs were present, and in particular we did not observe slurred speech or difficulty in swallowing liquids and foods. Diagnosis of ALS was made according to El Escorial criteria.

The father of the proband (II:7) was affected by ALS and died when he was 58 years old. The family members who decided to undergo genetic testing were: III:2; III:4; III:5 and III:8. Only the proband's sister (III:8) resulted positive at the genetic test but without neurological clinical signs, although she is older than her affected brother.

Family 5

We described a family with the A382T mutation in the *TARDBP* gene (Figure 39). As mentioned above, this mutation has already been reported by different authors [106,109]. Moreover the proband has a pathological repeat expansion in the

C9ORF72 gene. The amino acid homology among different species for position 382 in *TARDBP* gene is presented in Table 16.

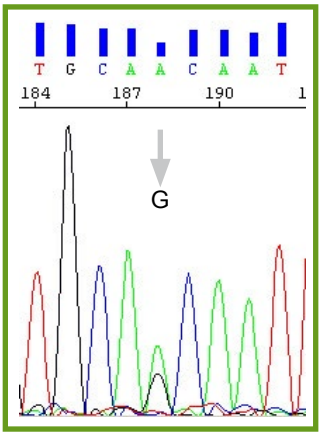


Figure 39. *Electropherogram of the A382T mutation in the TARDBP gene (exon 6). G nucleotide is wild type whereas A nucleotide corresponds to the mutated allele (c. 1144 G>A). The arrow indicates the presence of mutation.*

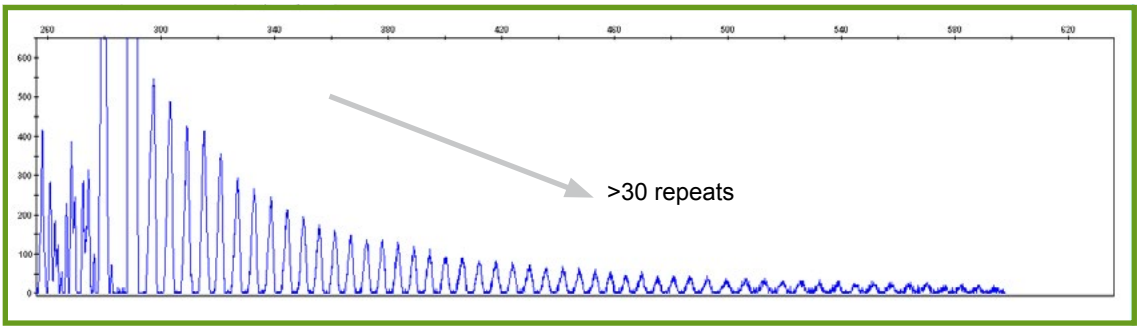


Figure 40. *Electropherogram with more than 30 peaks and an exponential decay*

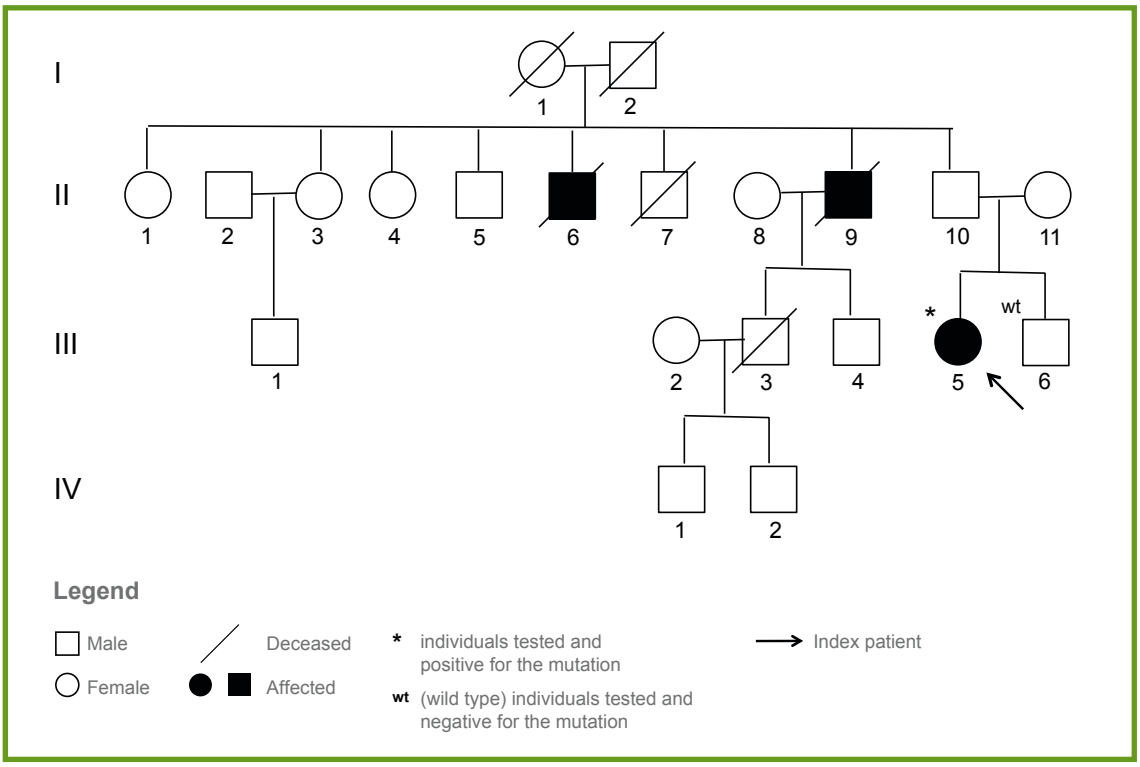


Figure 41. *Pedigree of the family with the A382T mutation in TARDBP*

The index case (III:5) is a Sardinian 44-year-old woman who developed a spinal form of ALS starting with hypotrophy of the right hand. Needle electromyography (EMG) demonstrated neurogenic abnormalities, with aspects of denervation and reinnervation in various districts, consistent with a widespread suffering of the lower motor neurons. Few months later diagnosis of ALS was made. It should be noted that in 2008 the proband was submitted to hormonal treatments for in vitro fertilizations (FIVET).

The patient's family pedigree showed a positive history of motor neuron disease. Both II:6 and II:9 had a spinal-onset of disease with a fast progressive course. They died when they were 62 and 54 years old, respectively, after a disease duration of about 3 years. They were both non-competitive soccer players. The proband's father (II:10) has refused to undergo genetic testing. Currently he suffers from hypertension and hypercholesterolaemia, but he does not present neurological signs. Only III:6 was tested and resulted negative both for the A382T *TARDBP* mutation and for *C9ORF72* pathological repeats expansion. The remaining living siblings of the proband's father (II:1; II:3; II:4; II:5) were not tested because they live in Sardinia and refused to undergo genetic testing since they referred to be in healthy status.

Selected sporadic clinical cases

We report the two most interesting SALS cases that were genetically analysed.
The first family has two subjects presenting the p.A382T mutation in the homozygous state: the index patient with an 8-year history of sporadic Parkinson's disease who developed ALS and FTLD 6 years later, and his brother without neurological signs and symptoms. The amino acid homology among different species for position 382 in *TARDBP* gene is presented in Table 16. The second case is an ALS patient carrying a *de novo* missense mutation in the *FUS* gene (R522G), who developed the disease during her pregnancy.

Family 6

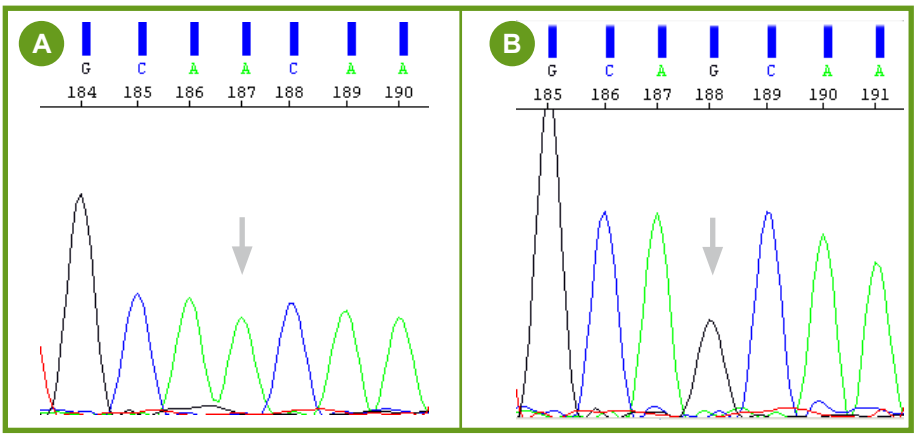


Figure 42. Electropherogram that allows the identification of the p.A382T (c. 1144 G>A) mutation in the homozygous state. ***A.*** *The arrow indicates the presence of mutation in the homozygous state (p.T382T).* ***B.*** *The arrow points to the wild type sequence.*

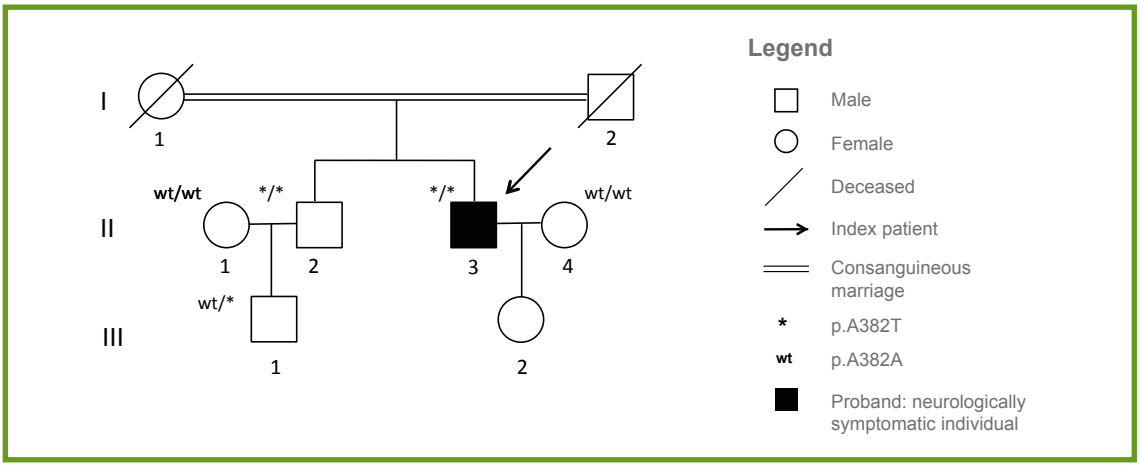


Figure 43. Pedigree of family 6

A 61-year-old man (II:3) complained of progressive stiffness and slowness of movement of the left arm associated with bradykinesia and impairment of the voice that became soft and monotonous. The diagnosis of akinetic rigid dominant Parkinson's disease was made. After 6 years, he developed progressive weakness of the lower limbs with gait impairment and frequent falls. Needle electromyography (EMG) demonstrated chronic and active denervation in muscles of the lower limbs; a nerve conduction study showed mild amplitude reduction without conduction blocks at both legs. Results of the tests were interpreted as consistent with possible inflammatory axonal polyneuropathy. Later, the patient developed progressive worsening of the weakness in the lower limbs and involvement of both upper limbs;

the neurological examination revealed hypophonia, rigidity, and distal weakness of both upper-limb muscles and hypotonia associated with diffuse weakness of both lower-limb muscles, which were also hypotrophic; tendon reflexes were brisk in the upper limbs and reduced in the lower limbs. Further neurophysiological analysis showed widespread suffering of lower motor neurons in the four limbs. A diagnosis of ALS was made. Finally, the patient developed progressive dyspnea requiring non-invasive ventilatory support and dysphagia (for solids and liquids). Eight years after onset, the patient developed behavioural (apathy) and psychiatric (hallucination) symptoms. Neuropsychological examination showed pathological scores consistent with behavioural variant FTLD (bvFTLD). One month later, the patient died due to acute respiratory failure.

Genetic screening of family members prompted us to clinically evaluate the mutation carriers; the 67-year-old proband's brother (II:2) did not show any neurological signs. The two heterozygous mutation carriers are III:1, a 31-year-old healthy man without any neurological signs or symptoms, and III:2, a 36-year-old woman unable to hear or speak after meningitis infection at 3 years of age. The father and mother of the index case were reported to be first cousins; they died at ages 67 and 54 of a liver cirrhosis and a tongue tumour, respectively; their DNA was not available for genetic testing, and they were reported to have had no signs of motor neuron dysfunction, extrapyramidal disorder, or cognitive impairment.

Family 7

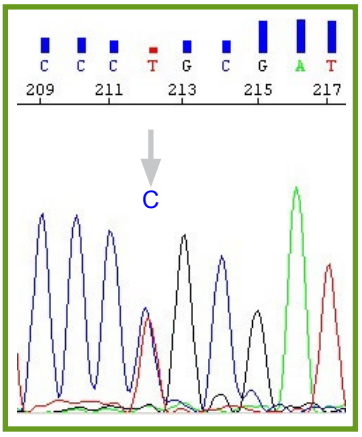


Figure 44. *Electropherogram of R522G mutation in the FUS gene (c.1564 A>G). Sequence analysis (reverse strand) of the PCR product showed the mutated G (forward strand). The arrow indicates the presence of mutation.*

Organism	Amino acid		
H.sapiens	**491**	**RGGFRGGRG------GGDRGGFGPGKMD--SRGEHRQD--RRERPY**	**526**
D.rerio	507	RGGFRGGR-------GGDRGGFGPGKMD--SRGDHRHD--RRDRPY	541
G.gallus	470	RGNFRGGR-------GGERGGFGPGKMD--SRGDHRQD--RRERPY	504
B.taurus	477	RGGFRGGRG------GGDRGGFGPGKMD--SRGEHRQD--RRERPY	512
M.musculus	483	RGGFRGGRG------GGDRGGFGPGKMD--SRGEHRQD--RRERPY	518
R.norvegicus	483	RGGFRGGRG------GGDRGGFGPGKMD--SRGEHRQD--RRERPY	518

Table 19. Amino acid homology at codon 522 of the FUS protein in different species.

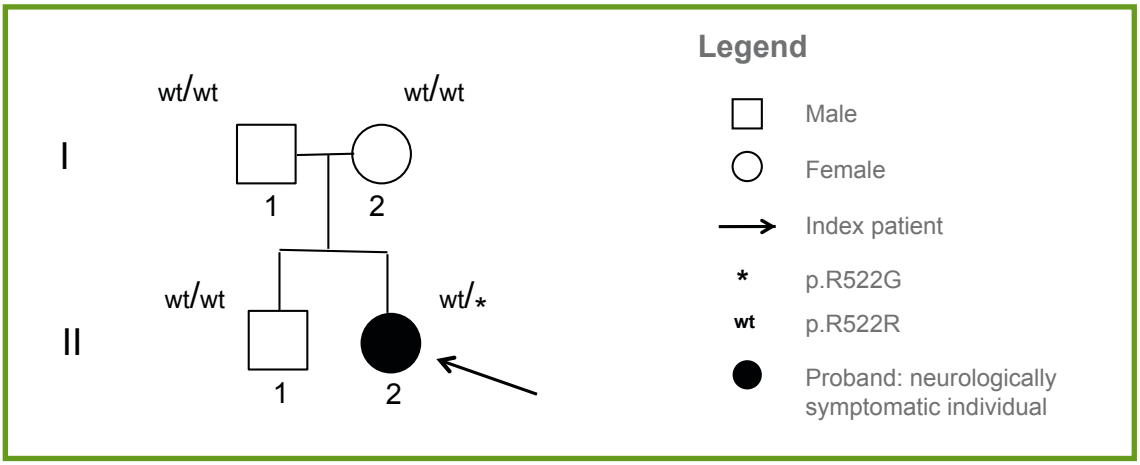


Figure 45. Pedigree of family 7

A 37-year-old female during the last trimester of pregnancy complained of dyspnea, especially when she was lying. One year later she developed feeling of breathlessness in aloud reading and soon after presented difficulty in slurred speech and in swallowing solids. Needle electromyography (EMG) was consistent with ALS diagnosis emphasizing the occurrence of denervation potentials at the right deltoid muscle and at the bilateral brachial biceps muscles. Two years after onset, the patient is alive, in non-invasive ventilatory assistance and in enteral nutrition by gastrostomy.

The proband's parents (I:1) as well as proband's brother (I:2) are healthy. Neither the father nor the mother carry the *FUS* mutation identified in the proband, nor any other mutation of genes related to ALS. The proband's brother (II.1) was tested and resulted negative. Accordingly this is one of the few genetically proven cases of *FUS* mutation in a patient with true sporadic ALS (*de novo* mutation).

A senataxin case

The clinical features of one patient in our cohort suggested screening for mutation the *SETX* gene. Sequencing all the 26 exons and intron/exon boundaries of the *SETX* gene lead to the identification of a heterozygous missense mutation, L389S (c.1166 T>C) in exon 10 (Figure 46). This mutation was previously reported in settings different from the Italian populations [121].

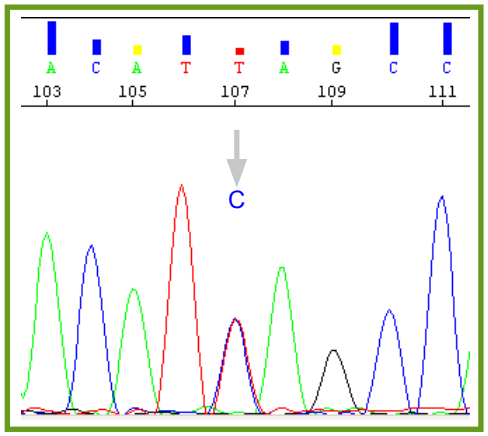


Figure 46. *Electropherogram of L389S mutation in the SETX gene. The arrow indicates the presence of mutation.*

Organism	Amino acid			
H.sapiens	**344**	**S-YLDDMVTCSQIVYNYNPEKTKKDS--GWRTAICPDYCPNMYEEMETLA**	**390**
D.rerio	311	PEYDDNVVTCSQMVYDCYASKKQGHAPGSSNSGVGSNYV--IYDDMQSLV	358
G.gallus	345	TDYDDEMVTCSQIVYTCNTEKPQKDT--GWKTAICPDYCPNMYEDMQTLA	392
M.musculus	344	P-HFDDMVTCSQIVYNFNPEKTKKDS--GWRSAICPDYCPNMYEEMETLA	390
R.norvegicus	344	P-HFDDMVTCSQIVYNYNPEKTKKDS--GWRSAICPDYCPNMYEEMETLA	390
M.mulatta	344	S-YLDDMVTCSQIVYNYNPEKTKKDS--GWRTAICPDYCPNMYEEMETLA	390
P.troglodytes	344	S-YLDDMVTCSQIVYNYNPEKTKKDS--GWRTAICPDYCPNMYEEMETLA	390
C.lupus	344	S-YSDDMVTCSQIVYNYNPEKTKKDS--GWRSAICPDYCPNMYEEMETLA	390
B.taurus	344	S-YLDDMVTCSQIVYNYNPEKTKKDS--GWRSAICPDYCPNMYEEMETLA	390

Table 20. *Amino acid homology at codon 389 of the SETX protein in different species*

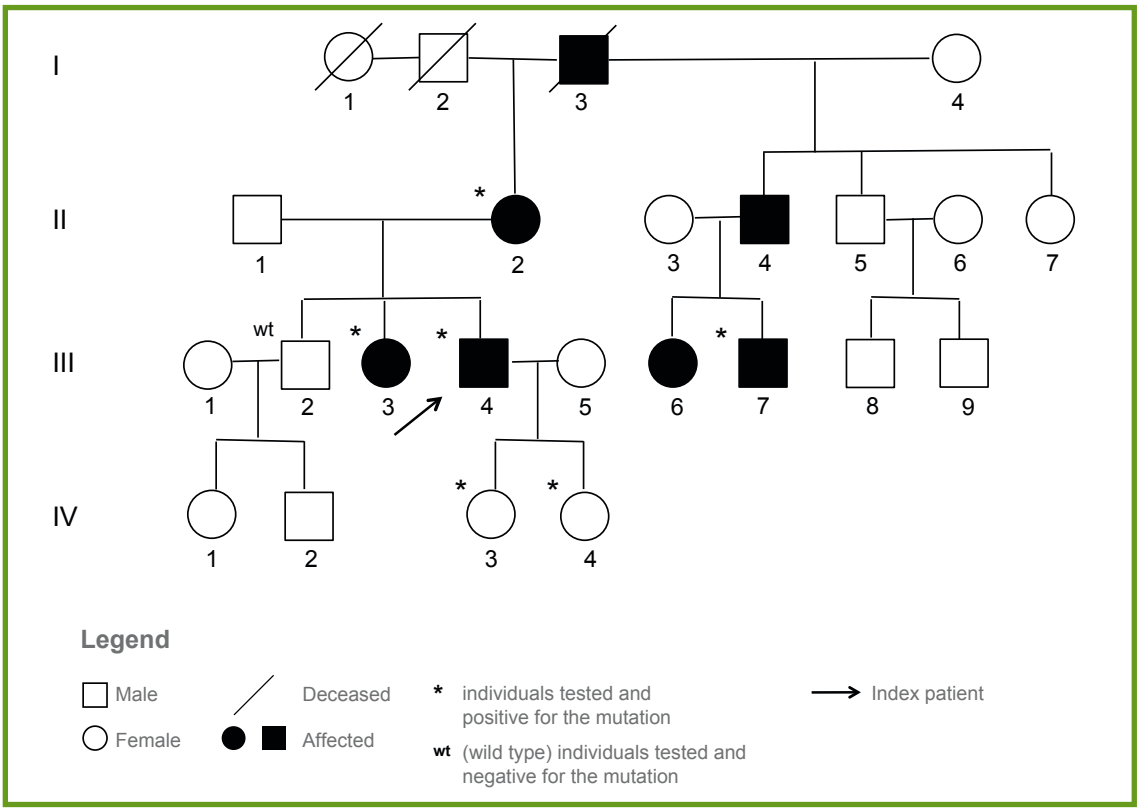


Figure 47. *Pedigree of the family with the L389S mutation in senataxin*

A 24-year-old man complained of weakness of the right hand and leg during nine months. The first neurological examination showed weakness of the right hand and distal right leg, and brisk tendon reflexes. No fasciculation was visible; no cramp was reported. Needle EMG demonstrated fibrillation potentials and increased amplitude of motor unit action potentials (MUAPs) in distal muscles of the right limbs. A nerve conduction study showed multiple conduction blocks at right ulnar and peroneal nerves (amplitude reduction by 55% in the arm and by 67% in the leg). The study was interpreted as consistent with multi-focal motor neuropathy (MMN). In 1998, the man developed a progressive worsening with involvement of all four limbs. The patient's condition deteriorated and neurological examination revealed distal weakness of both upper and lower limb muscles; all tendon reflexes were brisk and plantar reflexes were extensor bilaterally; Hoffmann sign was positive bilaterally. MRI of the spine was negative. Nerve conduction studies of the four limbs did not detect conduction blocks. EMG showed spontaneous activity (fibrillation potentials and positive sharp waves), increased amplitude and long duration of MUAPs, associated with reduced recruitment in all limbs.

A wider investigation of familial history let us know that four relatives, living in Germany, were affected by motor syndromes with juvenile onset. One uncle (II:4) as well as one cousin (III:6) were not available for examination or *SETX* mutation screening and refused to undergo genetic testing. It was impossible to test the maternal grandfather (I:3), because he was already dead. Conversely III:7 decided to send us a blood sample for genetic testing. We tested also the proband's mother (II:2), who has progressive walking impairment, as well as the proband's sister, who has just begun to develop lower motor neuron signs (III:3). All the available affected relatives resulted positive to the test. The proband's siblings (IV:3; IV:4) resulted to be heterozygous for the L389S mutation; however due to their early age they actually presents no neurological symptoms.

Genetic counselling

The results of the genetic tests were communicated to all subjects carrying a mutation within a multidisciplinary context, in order to help the proband and their relatives understand the uncertainties that may remain after clinical genetic testing.

At this time predictive or presymptomatic testing should be undertaken with caution. Specifically, families should consider the limitations of genetic testing, including the fact that the mutation in ALS causative genes cannot predict exact age of onset or symptom expressivity. In the context of diagnostic testing, detecting a mutation will resolve neither issues of reduced penetrance nor clinical heterogeneity.

Discussion

General considerations

Our cohort of ALS patients is represented by 302 subjects. Globally, our molecular analysis explained 10.3% of ALS cases (31/302) with 64.7% (11/17) of familial cases and 7.1% (20/285) of sporadic cases. The highest frequency of positive cases was obtained in *C9ORF72* (5%), followed by *TARDBP* (2.9%), *FUS* (1.65%) and *SOD1* (1.32%).

The clinical phenotype of FALS cases is usually indistinguishable from SALS. However, in comparison with SALS, FALS is characterized by an equal male: female sex ratio, an earlier age at onset, and, generally, a longer disease duration. The first signs of the disease often occur in the lumbosacral segment, and atypical symptoms may be present at onset [22].

In our mutated population we have found a slight difference in the age of onset between SALS cases (mean age 55.5, range between 33 and 73) and FALS cases (mean age 49.5 range between 18 and 69) as well as, in FALS patients, a higher rate of lower limbs onset (6) compared to other types of onset (3 bulbar and 2 upper limb). These results are consistent with literature data [89]. Conversely in our FALS cohort we have found more female (8) than male (3) mutated patients.

There is considerable evidence of a genetic contribution to familial and sporadic forms of ALS and the observation that Mendelian genes are found mutated in subjects with no family history is the most direct evidence for this. We provide here an account on the genes tested in this study.

C9ORF72

In our SALS population we found 3.9% of *C9ORF72* hexanucleotide repeats expansion carriers, who represent our most common mutation. This finding is in agreement with what recently reported in literature regarding Italian population. Indeed, Sabatelli and colleagues analysed a large cohort of 1757 Italian SALS cases identifying 69 mutation carriers (4%) [144].

The identification of the *C9ORF72* expansion in apparently sporadic ALS may have different explanations, including poor diagnosis in the past, lack of knowledge of

family history, mutation carriers in previous generations dying of other diseases prior to developing motor neuron degeneration, reduced penetrance of the gene, and even varying phenotypic manifestations among mutation carriers within the same family [145].

The expansion of the hexanucleotide repeat in *C9ORF72* gene was recently identified as a major cause of both FALS (23%-47%) and FTD (12%-29%) cases. These percentages derived from the study of DeJesus-Hernandez [75] and colleagues who analysed a cohort of 34 FALS patients and 374 FTD patients derived from three well-characterized patient series diagnosed at the Mayo Clinic Florida (MCF) as well as from the study of Byrne *et al.* [146] who analysed 386 SALS and 49 FALS in an Irish population. In the same years Renton and colleagues [76] analysed 290 SALS and 112 FALS in a Finnish population and confirmed these figures (46.4% FALS and 21% SALS). To further determine the frequency of the hexanucleotide expansion in outbred European populations, they screened a cohort of 268 FALS from North America (n=198), Germany (n=41), and Italy (n=29), finding that 102 affected individuals (38.1%) carried the same hexanucleotide GGGGCC repeated expansion within the *C9ORF72* gene. The range of *C9ORF72* frequencies depends on the origin of the population analysed [75,76,146]; in our cohort *C9ORF72* mutated patients account for 23.5% of FALS.

Specifically in Italy, Ratti and colleagues conducted a mutational analysis in a very large cohort of 259 FALS and 1275 SALS Italian patients with ALS and ALS-FTD and found that mutations in *C9ORF72* account for 23.9% (62/259) of FALS and 5.2% (66/1275) of SALS cases, respectively [147].

In addition this study revealed that mutational frequency of *C9ORF72* was higher in FALS (50%) and SALS (10%) patients presenting with concomitant FTD signs [146]. In our population cognitive impairment was present in 14/302 (4.6%) SALS and none of FALS patients; therefore we couldn't confirm the recent findings that a family history of neurodegenerative disease and of cognitive/behavioural impairment makes an individual at higher risk of carrying a repeat expansion in *C9ORF72*. Moreover, we detected just one *C9ORF72* repeats expansion carrier among our 14 SALS patients with Parkinson's disease and behavioural variant FTLD. A possible overlap with Parkinsonism is also suggested by the peculiar phenotype of *C9ORF72* repeats expansion carriers, which often includes extrapyramidal features and a positive family

history for Parkinson's disease [147]. All these observations suggest that *C9ORF72* repeats expansion may contribute to the pathogenesis of a broad spectrum of neurodegenerative diseases.

Together with cognitive impairment, bulbar-onset seems more frequent in patients carrying *C9ORF72* mutations than in patients carrying *TARDBP*, *SOD1* or unknown mutations. Moreover *C9ORF72* expansion is reported to be more frequent among patients with disease onset >61 years [86]. Our results do not confirm these data because our *C9ORF72* mutated patients developed mainly lower limbs onset (9/15) compared with other types of onset and just half of our *C9ORF72* carriers are older than 60 years.

One of the limits to the clinical utility of *C9ORF72* gene testing is the lack of knowledge on the minimum number of repeats required to confer a pathological phenotype. The repeat unit is six nucleotides long, and in the affected individuals the unit expands up to more than several hundred times. It is not clear which is the normal range of repeat size in healthy individuals; for sure, fewer than 10 repeats are not associated with a pathological phenotype, while more than 30 repeats are. However the significance of intermediate repeat sizes (11-29) is still unknown.
For this reason, in our study, we decided to consider as pathological also the patients with intermediate repeats.

Regarding the repeats size it is necessary to remember that cytosine (C) methylation in CpG dinucleotides is an important epigenetic modification that could lead to gene expression silencing as a result of CpG's hypermethylation. Indeed a direct correlation between CpG methylation and repeat size was already observed in other neurological disorders (e.g. Friedrich ataxia), in which the degree of methylation gradually increases along with the numbers of nucleotide repeats.

A second problem relates to the repeat-primed PCR method that we used to screen ALS and FTD patients. Even though this method is fast and cost-effective, it does not provide an accurate estimate of the number of repeats. Alternatively, Southern blot analysis should be used to estimate the number of repeats in expanded repeats carriers; however, its application appears to be laborious and too much expensive.

Finally, somatic heterogeneity is likely to be common in repeat expansion carriers, resulting in varying repeat sizes in different tissues from a single patient. As a result, repeats sizes determined using DNA extracted from whole blood, that we used for our samples, may not adequately reflect the *C9ORF72* repeat sizes in a patient's brain or spinal cord tissue and may hamper genotype-phenotype correlations.

SOD1

In our SALS cohort we identified 0.7% of *SOD1* mutation carriers. This frequency of *SOD1* mutation in SALS patients is lower than what evaluated by other groups in Italian population. Specifically Gellera and colleagues reported 2/44 *SOD1* mutated carriers who account for 4.5% in the population studied [(94)]. Conversely our data are in agreement with results from another Italian study, conducted by Battistini on a large cohort of 225 SALS patients that included no *SOD1* mutated carrier [(96)]. However, it is interesting to note that a wide variability in the *SOD1* mutation carriers frequency has been reported within the same Italian SALS population (ranging from 0% to 6.3%) [(79,94,148)].

This difference could be due to bias in the patient's selection. Indeed, it is possible that SALS are misdiagnosed, so some of them could have been FALS cases instead. This may occur for a variety of reasons including decreased penetrance, poor diagnosis of ALS in the elderly in previous generations, or incomplete family history. Another possibility is that apparently sporadic ALS with *SOD1* mutations may be due to *de novo* mutations. It is difficult to determine whether the *SOD1* mutations identified were due to *de novo* mutations because seldom DNA samples were available from the parents of the SALS cases: given that ALS is predominantly a disease of middle and older age groups, the proband's parents are alive any not longer.

The frequency of *SOD1* mutations among familial cases in our cohort is 11.8%. This rate is similar to figures based on Chiò's study made on 22 FALS cases (13.6% of all FALS) [(79)], likely because we have a similar sample size.

Generally, in patients with *SOD1* mutations cognitive impairment is very rare (0-2.6%) and bulbar-onset is observed less frequently (7-12%) than in non-*SOD1* patients [(31)]; age of disease onset is generally higher than in *FUS* patients but is lower than in other non-*SOD1* patients. Our population confirms these literature data about cognitive

impairment as well as bulbar-onset. Indeed we don't have any *SOD1* mutation carriers with cognitive impairment and our *SOD1* mutated patients all have a spinal-onset of disease (3 lower limbs and 1 upper limbs). Otherwise, age at disease onset in our cohort of FALS patients is lower than in *FUS* mutation carriers; this is probably due to the G93D mutation found in our patients, which is associated to a specific mild phenotype. Indeed, carriers with long survival time generally survive more than 20 years after the onset of the disease. In one of our *SOD1* carriers, who developed the disease during pregnancy, we have also found angiogenin genetic variant (IVS1+27 variant in heterozygous state C/T) [149]. Likewise we could hypothesize that there could be other modifier genes or hormonal condition that influenced the phenotypic expression.

TARDBP

In our SALS population we identified 1.4% of *TARDBP* mutation carriers. This rate is higher than mutational frequency reported in other Italian studies. Ticozzi and colleagues [150] screened 208 FALS and 188 SALS cases for mutations in exon 6 of the *TARDBP* gene. Their observation that *TARDBP* mutations account for ca. 0.5% of all SALS patients are not in agreement with another screening that analysed 541 SALS and 125 FALS patients, respectively, and reported mutations in ca. 2% of sporadic cases [103]. The discrepancy may reflect the different Italian geographic origin of the patients together with the larger number of ALS cases analysed by Corrado and colleagues. Overall, we can conclude that our proportion of SALS *TARDBP* mutated cases is closer to the results from Corrado *et al.* [103].

Our cohort includes 17 FALS patients with a rate of 29.4% (5/17) *TARDBP* mutation carriers. This incidence is completely different from that in previous studies, which screened 208 and 125 FALS patients, respectively, and found 13 (6.3%) and 6 (4.8%) *TARDBP* mutation carriers, respectively [103,150].

This apparent higher incidence of *TARDBP* mutations in our Italian FALS patients with respect to other studies may be influenced by the small sample size as well as by the presence of 2 mutated Sardinian and Sicilian patients. Indeed, very recently, it has been found that a single missense mutation in *TARDBP* gene (A382T) accounts for approximately one-third of all ALS cases in the Sardinian population. Furthermore,

these ALS patients shared a high-risk haplotype across the *TARDBP* locus, indicating that they had a common ancestor and were part of a larger kindred [107]. Since Sicily is as geographically isolated as Sardinia, the incidence of *TARDBP* mutation carriers could be higher than in the rest of Italy.

Regarding the clinical phenotype, cognitive impairment has been observed in 31% of patients with mutations in *TARDBP* [151]. In our cohort of 9 *TARDBP* mutated patients, we found only one patient with bvFTLD; this accounts for 11.1% of all *TARDBP* cases. Millecamps and colleagues supposed the occurrence of *TARDBP* mutations is rare in patients with age of onset >61 years [86], but this assumption has not been confirmed in our as well as in other studies [103]. Moreover, with a single exception, all of the missense mutations identified in this gene are clustered in the glycine-rich C-terminal region encoded by exon 6. Hence, we decided to analyse only this exon in all ALS patients.

FUS

In our study both the rate of SALS *FUS* mutation carriers (1.05%) and the mean age at disease onset (38 years) are very similar to data reported in the study of Sproviero [152]. Indeed, Sproviero and colleagues reported a molecular screening of the *FUS/TLS* gene in 327 sporadic ALS patients from Southern Italy with a mutational frequency of 1.25%. The mean age at disease onset in the ALS mutated patients was significantly lower (range: 26-53; average 39 years) than in the whole cohort (56.77 years), confirming that mutations in *FUS* are associated with an earlier onset of the disease in comparison with the general mean age of approximately 60 years reported for ALS [40].

Moreover as far as our population is concerned, there was a slight observed female bias among SALS patients with *FUS* mutations, but this was not statistically significant (p=0.083 calculated with Fisher's test).

In our FALS cohort we found 11.08% of *FUS* mutation carriers. The frequency of *FUS* mutation in FALS patients is higher than what evaluated by other groups [115,120]. In the Chiò's study mutations in the *FUS* gene accounted for 3.8% of 52 non-*SOD1* and non-TDP-43 FALS index cases. In the same year, in a cohort of 94 FALS non-*SOD1* and non-TDP-43 mutated patients, Ticozzi estimated that ca. 4% of FALS cases are caused by mutation in the *FUS* gene.

The higher incidence of *FUS* mutations in our 10 non-*SOD1* and non-TDP-43 mutated FALS cases may be due to the small sample size, which could not reflect the situation in the population at large. In addition, in our FALS cohort we do not found a difference in the male/female distribution of *FUS* mutations as reported by others [115,120].

Although genotype-phenotype correlations are not possible for the majority of *FUS* mutations, it has been suggested that substitutions of arginine 521, in particular the replacement with a cysteine (R521C), may result in an uncommon phenotype characterized by a symmetrical proximal spinal-onset, with early involvement of the axial muscles [115]. These observations are in agreement with our population, in which we found both in 1 FALS and 1 SALS case with this mutation. These cases have a spinal-onset of disease with an early age at onset and the characteristic phenotype. In *FUS* mutation carriers age of disease onset is consistently lower than in other FALS mutated cases, but this findings not confirmed in our cohort, because 2 G93D *SOD1* mutations carriers in the FALS cohort have a younger onset form of disease (18- and 33-year-old). Moreover it is important to note that the FALS patient with the presence of *FUS/C9ORF72* mutation simultaneously has an older age at onset (69-year-old). This case needs further investigation to correlate a combination genotype with specific clinical phenotype (possible presence of other modifier genes).

Senataxin

The high degree of clinical and genetic heterogeneity in the various forms of juvenile ALS can make differential diagnosis difficult. Other genetic disorders that may mimic juvenile ALS phenotypes include: spinal muscular atrophy, hereditary spastic paraplegia, and the hereditary motor neuronopathies/motor forms of Charcot-Marie-Tooth disease.

We report the first Italian ALS patient with *SETX* autosomal dominant mutation. In this case the late diagnosis of ALS has been established as a consequence of the clinical and neurophysiologic picture that were initially consistent with a distal motor neuropathy. Indeed, the increment of deep tendon reflexes was not sufficient to rule out that hypothesis, since 25% of patients with multi-focal motor neuropathy reveal hyperreflexia [153]. However, the progression of muscle wasting, the EMG findings of

acute denervation, and the discovery of a positive familial history of motor syndrome induced us to go further in genetic investigation, and to test for senataxin gene.

We described the L389S mutation in the *SETX* gene, which has been previously associated with ALS [121]. In our *SETX* family we found different polymorphisms in the proband compared with the family members. This intrafamilial variability together with other modifier genes could explain the different phenotypic expression of the proband [154].

Geographical distribution of mutations

We decided to look for a regional distribution of mutations in our cohort of SALS and FALS cases.

Our SALS population of mutated patients (20) includes patients originating from different Italian regions. Specifically 9 are from Lombardy, 2 from Sardinia, 2 from Sicily and 2 from Calabria. The remaining 5 SALS mutation carriers came from Piedmont, Lazio, Abruzzo, Campania and Marche. Differently, our FALS population of mutated patients (11) originates mainly from Southern or Central Italian regions. Specifically, we have 5 Sicilians and 3 Sardinians. The remaining 3 mutation carries came from Tuscany, Emilia and Marche respectively.

The *C9ORF72* repeats expansion seems to be more frequent in the Northern than in the Southern part of the country, at least in SALS patients. This observation derives from the presence of 11 mutated patients, of whom 6 come from Lombardy and 1 from Piedmont and the others from Abruzzo, Calabria, Lazio and Sardinia. Conversely, in the FALS cases we do not observe the same geographical distribution: 2 originate from Marche and Sardinia, while the remaining 2 cases (with coexistent *TARDBP* or *FUS* mutation) come from Sicily and Sardinia respectively.

Lattante and colleagues suggested a likely regional distribution of the frequency of the *C9ORF72* analysing 480 SALS and 48 FALS patients from the Central or Southern regions of Italy [155]. The percentage of SALS with *C9ORF72* expansion (2.5%) is considerably lower than that reported by another study conducted on North-Eastern Italian regions [156]. Indeed Bertolin and colleagues analysed 307 SALS and 46 FALS cases and observed 4.5% of *C9ORF72* pathological repeats expansion in SALS cases.

Regarding FALS cases, we did not find significant differences (9/48 rate 18.8% vs. 10/46 rate 21.7%). Considering together the presence of *C9ORF72* expansions both in FALS and SALS, this difference between Northern and Southern Italian regions has been confirmed by Chiò and colleagues who analysed 475 patients originating from Piedmont and Valle d'Aosta [151]. They found 32/475 cases with *C9ORF72* mutations that account for 6.7% of cases.

Regarding *SOD1* we found 4 cases originating from Southern Italy. This finding confirms what previously reported in others Italian studies, even though we have a small sample size of *SOD1* mutation carriers. These studies identified a higher frequency of *SOD1* G93D carriers in Sicily compared with other Italian regions [31]. For the *FUS* mutations carriers we found 4 patients mutated with a uniform geographical distribution. Hence, for *SOD1* and *FUS* genes no specific geographical association seems to exist.

In our ALS cohort we found a higher frequency mutated patients for *TARDBP* (2.9%) than for the others two genes. This frequency is very similar to that observed by Lattante in her study. This similarity is probably due to the population studied. Indeed our *TARDBP* mutated carriers as well as Lattante's population [155] come mainly from Southern Italy (6/9). These observations have been confirmed by Chiò, who screened for *TARDBP* gene 135 Sardinian patients with ALS and found 39 (28.7%) carrying the A382T missense mutation [107].

In conclusion, the frequency of mutations in ALS associated genes has been evaluated in different epidemiological studies carried out all over the world, whereas it is important to assess the frequency of mutations in a specific population – in our case, specifically in the Italian population. In fact, the frequency of mutations of a gene can be different in different countries or even in different regions of the same country.

Genotype-phenotype correlation

We reported a detailed profile of clinical and genetics findings of some Italian families carrying *SOD1*, *TARDBP* or *FUS* mutations with different intrafamilial phenotypic expression. Based on our cohort, we can draw some conclusions.

The G93D mutation was identified in a 33-year-old pregnant woman who showed an unusually fast progression of the disease compared to the paucisymptomatic presentation associated with this mutation in the two previously described Italian families [142,143]. Moreover the remaining mutation carriers did not show the aggressive clinical course displayed by the proband.

Hence, to explore the phenotypic heterogeneity within the proband family, we investigated the possible relationship between the G93D *SOD1* mutation and some modifier genes reported to be involved in ALS pathogenesis. Various genes/genetic variants could have been tested. Since the involvement of angiogenic factors in ALS, albeit controversial, is still attractive [157], we selected *ANG* and *VEGF* SNPs for the analysis. Then we looked for *HFE* gene variants implicated in the oxidative stress and reported to be associated in some populations with motor neuron degeneration [158]. Moreover, we decided to investigate *PON* genes, albeit association studies between *PON* genetic variants and the risk for SALS in different populations reported conflicting results [159,160]. ALS subjects have been also reported to have a higher median homocysteine (Hcy) levels; therefore MTHFR C677T variant was selected because linked to an increased level of Hcy [161]. Higher Hcy levels seem to also be a possible marker of disease progression [162]. Finally, alleles E2, E3, and E4 of APOE gene were included in our analysis because of their association to some neurodegenerative disorders, such as ALS [163].

Results of our study did not identify haplotypes specific to G93D mutation carriers or to wild-type subjects. As unique characteristic of the proband (IV:7), we identified the *ANG* IVS1+27 variant in heterozygous state C/T. To date, little information about the role of *ANG* variants in a *SOD1* mutated genetic context are available. In the present family, the proband (IV:7) manifested the disease after the delivery, a situation that could inherently result in a hypoxic condition: *ANG*, like *VEGF*, is expressed in response to hypoxia [164,165] and plays a neuroprotective role in motor neurons [166]. Thus, we might speculate that the patient's genetic framework, including the G93D mutation and the IVS1+27 variation, facilitated the anticipation of onset age and contributed to the aggressive clinical course observed in the proband [149].

The A382T *TARDBP* mutation was identified in a 63-year old Sardinian woman. In this case the proband showed a bulbar-onset of ALS with a relatively fast

progressive course of the disease. In this family we observed the phenomenon of anticipation. This condition occurs when the signs and symptoms of some genetic conditions tend to become more severe and appear at an earlier age as the disorder is passed from one generation to the next. Indeed the proband's father died of ALS when 79-year old whereas the proband's son showed ALS long before the mother manifested the first signs of disease. Anticipation is most often seen with certain genetic disorders of the nervous system, such as Huntington disease, but has never been described in *TARDBP* mutation carriers. The A382T mutation has been recently described in 3 ALS Italian families, with variable age and site of onset among the family members. Patients with ALS carrying A382T *TARDBP* mutation may develop FTLD [(109)], but so far we did not observe dementia in this family.

We identified another *TARDBP* mutation, A294V, in a 54-year old woman. Her family has a wide spectrum of clinical symptoms. Some family members showed cognitive impairment without signs of ALS (II:3, II:5) conversely other relatives showed a typical ALS without any signs of dementia (III:6, III:8, III:13).

The proband's daughter resulted heterozygous for A294V, but she actually doesn't present any neurological signs, probably because she is very young (<30 years). However we are not able to predict whether this subject will develop ALS in the future or the clinical features of the disease.

These two cases of *TARDBP* mutations support the hypothesis that a mutation is not the only factor that determines the clinical course of the disease. Other factors must also contribute to phenotype, and it is not yet possible to predict the evolution of patients based only on the presence of the mutation or on the age at onset or the rate of progression in other family members.

The R521C *FUS* mutation was identified in a 49-year old man. Out of 4 family members analysed, only the proband's sister (III:8) resulted positive to the test. She is 4 years older than her affected brother, but she doesn't show any neurological signs. This different clinical phenotype with the same mutation could be explained by a reduced disease penetrance, evident among most affected families with the R521C mutation [(167)]. Conversely we could explain these phenotypic differences through the neuroprotective effects of estrogen that could account for the later age at onset in women. Indeed it has been demonstrated that post-menopausal women, who

are in an estrogen-deprived state, are at risk for neurodegenerative diseases [168]. Moreover estrogen loss from natural or surgical menopause has been associated with a decline in cognitive function [169] that is reversed by estrogen therapy.

Unfortunately we couldn't test the paternal grandmother (I:1) as well as proband's father (II:7) because they were already dead. The paternal grandmother was probably affected by ALS, because she showed an inability to support her head, likely a dropped-head syndrome, before her last pregnancy. This syndrome, together with a young age at onset, is a frequent feature in the R521C mutation carriers [167].

Considerable intrafamilial phenotypic differences were observed in some families carrying various mutations in the *SOD1*, *TARDBP* and *FUS* genes. Age and site of onset varied between members of a family. The disease duration could also differ, as shown in families carrying the G294V TDP-43 mutation. These data support the hypothesis that a mutation is not the only factor that determines the clinical course of the disease and introduce the oligogenic disease model.

Oligogenic disease model

Blitterswijk and colleagues report on screening ALS subjects for mutations in five pathogenic genes: *TARDBP*, *FUS*, *SOD1*, *ANG*, and *C9ORF72*. The authors find that a significant proportion of subjects have more than one causative mutation in ALS-associated genes. They detect *FUS* and *TARDBP* mutations in combination with *ANG* mutations, and *C9ORF72* repeats expansion with *TARDBP*, *SOD1* and *FUS* mutations. The combination of a genetic risk factor with a large effect with another mutation with incomplete penetrance fits well the oligogenic disease model [170].

In our cohort we screened 8 index cases and 12 family members for *SOD1*, *TARDBP*, *FUS*, *C9ORF72* and *ANG* and we found a second mutation in two familial cases. We reported a double mutation carrier belonging to family 5, she is a Sardinian woman showing an anticipation of the onset of disease (44-year-old) compared with affected family members; this clinical feature could be explained by the presence of two mutations simultaneously. One of them is A382T *TARDBP* mutation that accounts for one-third of ALS in Sardinian population, whereas the second one is a pathological repeats expansion in the *C9ORF72* gene (>30).

Studying the pedigree of this proband, we observed that the ALS phenotype is associated with the paternal line, even if the proband's father has no neurological signs – he should be a A382T obligated carrier. We had the opportunity to study both the brother and the mother of the proband, but not yet the father.

The proband's brother was negative for *TARDBP* mutations, as well as the mother, and we investigated the presence of repeats expansion in *C9ORF72* gene in this subjects. Indeed the penetrance of the hexanucleotide repeat may be variable, as already reported in some families [146], and/or dependent on the presence of other protective/ adverse genetic factors. However the proband's brother, as well as the proband's mother, resulted negative for presence of pathological repeats expansion (<2).

Moreover we could also speculate that the anticipation presentation of the proband disease could be explained also by hormone treatments which she has undergone for in vitro fertilization. Indeed the suppression of estrogens, required before beginning an in vitro fertilization, could be harmful, because of their neuroprotective role. In addition, also the progesterone levels, modified in the hormonal therapy, play an important role in stimulating neuron growth and enhancing the re-myelination of nerve fibres. Therefore these hormonal imbalances, together with the double mutations *TARDBP/C9ORF72*, could explain the anticipated presentation in the proband.

In addition we have data on *TARDBP* mutation carriers not related to neurological phenotype. Indeed we recently found a first family with two subjects presenting the p.A382T mutation in the homozygous state (family 6) [171]. This mutation is the most frequently observed *TARDBP* mutation, but so far it was always found in heterozygous state with only one homozygous case recently reported in Sardinia (Italy) [107]. Sardinian patients carrying this mutation present with a heterogeneous spectrum of ALS phenotypes, ranging from pure ALS to ALS associated with a wide range of cognitive impairment.

This family originates from an isolated geographical place in Northern Italy, Valsassina. Surprisingly, the two subjects harbouring the mutation in homozygous state display different spectra of clinical symptoms: the proband suffers from neurological disease, whereas his brother doesn't manifest any neurological signs. Two other family members are carriers of the same mutation in heterozygous state, without neurological signs and symptoms. The clinical differences within this family make it impossible

for the genetic test to predict clinical outcomes based on genotypic results only. Therefore we screened *SOD1*, *FUS*, *C9ORF72* and *ANG* in the family members, to identify other mutations that could explain this intrafamilial phenotypic heterogeneity. The genetic testing of these ALS-associated genes resulted negative. In this case we are not able to explain the different phenotype in two siblings with the same A382T mutation, but it is always necessary to consider the variability of penetrance, expressivity and modifying factors, such as gene-gene interactions, as proposed in the oligogenic ALS model.

ALS and pregnancy

ALS during pregnancy has been reported since 1967 [172-174]. This association up to now has been considered rare and possibly coincidental as no pathogenic relationship between the disease and pregnancy has been demonstrated. However, hormonal modifications of pregnancy and, specifically, the reduction of estrogens are reported as possible risk factors, which may increase the susceptibility to ALS during pregnancy [173]. Moreover, considering that FALS often have an earlier disease onset, it could be expected that women in reproductive age carry ALS-associated mutations [41].

In our cohort of patients we identified a subgroup of women that developed ALS during or immediately after pregnancy (1 month) that we define as child-bearing age. In one of them (family 1) we performed a genetic characterization of ALS not only for causative genes but also for susceptibility/modifier factors (see genotype-phenotype session). The proband showed an unexpected fast-progressing course of the disease compared to the G93D carriers within her family and the two families previously described. Unlike the rest of her family, the proband had the *ANG* variant IVS1+27 in heterozygous state C/T [149]. In addition, estrogens are known to have a potential neuroprotective effect, and, despite data in ALS are scarce and contradictory, a decrement of estrogen levels, seen in pregnancy, could unmask ALS, especially in patients lacking the complete ability to inactivate superoxide radicals as a result of mutations in the *SOD1* gene [175].

The second woman who developed ALS in child-bearing age was affected by the first symptoms of disease during the last trimester of pregnancy (family 7). The index

case is a R522G *FUS* mutation carrier, her parents are referred to be healthy; neither harbour the mutation; maternity and paternity was confirmed by definite genetic proof, with a confidence interval greater than 99.99%. From this evidence, she has a *de novo* mutation. Hence we proved the presence of a *FUS* mutation in a patient with true sporadic ALS. Because the DNA samples of the relatives of sporadic *FUS* mutation carriers has been unavailable in most studies it was difficult to distinguish between familial patients with reduced penetrance and true sporadic patients [176].

Much evidence suggests that mutations in *FUS* are associated with earlier onset of ALS than the general mean age of approximately 60 years [177]. Moreover the frequency of *de novo* mutations has been often reported for *FUS* gene, whereas it is rarely reported for *SOD1* and *TARDBP* genes [178,179].

The exact mechanism giving rise this type of mutations is not known, though certain spots in the genome are thought to be particularly susceptible to mutation (i.e., “mutational hotspots”), presumably because of the surrounding sequence. Finally in this case we have both the presence of a *de novo* mutation and the pregnancy condition with a reduced level of the neuroprotective estrogens, which could anticipate the clinical manifestation of ALS. After 2 years from the onset of the disease, the proband is still alive, so she doesn’t seem to show the rapid clinical progression of disease typical of *FUS* mutated patients.

Conclusions

In conclusion, in our cohort of 285 SALS and 17 FALS we globally detect 31 mutation carriers representing 10.3% of the investigated sample. Several clinical phenotypes of ALS may be identified, but no clear boundaries are observed among them, as they represent points on a spectrum. Indeed, each one of the ALS-causing genes (*SOD1*, *TARDBP*, *FUS* and *C9ORF72*) can induce various phenotypes. For example, a *C9ORF72* expansion mutation can give rise to an ALS phenotype in some family members, a frontotemporal dementia phenotype in others, and Parkinsonism or even psychosis in others. This phenotypic variability could be identified also in patients carrying the same mutation. These considerations indicate that other factors must also contribute to the phenotype, and that it is not yet possible to predict the evolution of disease patients based on the presence of the mutation only. Recently, in the oligogenic disease model, it has been confirmed that the combination of a genetic risk factor with a large effect and another mutation with incomplete penetrance could modify the phenotypic expression in ALS patients.

Together with the oligogenic disease model other conditions such as pregnancy are related to the phenotypic variability. Indeed, estrogens are known to have a potential neuroprotective effect, and a decrement of estrogens, typical during pregnancy, could unmask ALS, especially in patients lacking the complete ability to inactivate superoxide radicals as a result of *SOD1* mutations.

All these observations should be considered during genetic counselling.
Families should consider the limitations of genetic testing, including the fact that the mutation in ALS-causative genes cannot predict exact age at onset of disease or symptoms expressivity. Indeed, it is necessary to consider, for example, the variability of penetrance, expressivity and modifying factors, such as gene-gene interactions, as proposed in the oligogenic ALS model.

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