

DOTTORATO DI RICERCA TOSCANO IN NEUROSCIENZE

XXVIII CICLO
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CEREBROSPINAL FLUID AND SERUM miRNAs IN SPORADIC AMYOTROPHIC LATERAL SCLEROSIS: POTENTIAL BIOMARKERS AND PATHOGENIC ROLE

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dedicated to Michele…

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FOREWORD

This PhD thesis is divided in seven sections: introduction, aim of the thesis, methods, results, discussion and conclusions, selected bibliography and annexes.

The annexes include all the related material that has been published to international scientific journals before and during the PhD course.

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PART I INTRODUCTION

PART I

INTRODUCTION

1

"Assumptions can be dangerous, especially in science. They usually start as the most plausible or comfortable interpretation of the available facts. But when their truth cannot be immediately tested and their flaws are not obvious, assumptions often graduate to articles of faith, and new observations are forced to fit them. Eventually, if the volume of troublesome information becomes unsustainable, the orthodoxy must collapse."

John S. Mattick

AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic Lateral Sclerosis (ALS) is an untreatable, fatal disease characterized by neurodegeneration involving primarily motor neurons in the motor cortex, brain stem and spinal cord (Maciotta et al., 2013). Increasing evidence indicates that non-neuronal neighbouring cells contribute to pathogenesis and disease progression, although motor neurons are selectively affected by degeneration and death (Ilieva et al., 2009).

Motor neuron degeneration results in progressive weakness of voluntary skeletal muscles of bulb, thorax, abdomen and limbs (Robberecht and Philips, 2013). Dysfunction of upper motor neurons (UMN) in the motor cortex leads to hyper-reflexia, extensor plantar response and increased muscle tone while dysfunction of lower motor neurons (LMN) in the brainstem and spinal cord triggers generalized weakness, hypo-reflexia, muscle atrophy, muscle cramps and fasciculation (Rowland and Shneider, 2001).

Limb involvement occurs more often than bulbar, which accounts for about 25% of ALS cases at clinical first examination. Limb symptoms in the majority of cases will

occur within 1-2 years. During the course of the disease, most cases become generalized with a combination of both LMN and UMN signs affecting spinal and brainstem regions. Patients with bulbar onset have typically slurred speech and difficulty in swallowing and the condition is designated progressive bulbar palsy (PBP) (Wijesekera and Leigh, 2009).

Mild cognitive impairment has been described in 50% of cases, with a subset of patients (about 15%) showing characteristics of frontotemporal dementia (FTD), frontotemporal lobar degeneration (FTLD) and progressive social behavioural and/or language dysfunction (Ling et al., 2013). The disease has a consistent phenotypic heterogeneity and according to newer theories ALS could not be considered a single disorder since different disorders might share a common final phenotype (Simon et al., 2014). Moreover, several ALS-related genetic alterations are also common in FTD so that the two disorders have been considered as part of a continuum of the neurodegeneration process (Paez-Colasante et al., 2015).

ALS patients' death, due mainly to respiratory failure, occurs 2-4 years after onset, however a small group of patients may have disease duration of 10 years or even more.

No objective test is capable of providing the diagnosis of ALS that remains essentially a clinical diagnosis based on clinical features, electrodiagnostic testing, and exclusion of conditions that can simulate ALS. The clinical diagnosis of ALS may be categorized into various levels of certainty by clinical and laboratory assessment based on El Escorial criteria (Brooks et al., 2000). The development of novel molecular tests to be employed in diagnosis and prognosis of disease is urgently needed to reduce the diagnostic delay and to evaluate the disease progression, in order to have the possibility of finding new therapeutic treatment for ALS (Hardiman et al., 2011).

EPIDEMIOLOGY

According to a recent critical review of the epidemiologic literature on ALS, in the European population the median (IQR) incidence rate (/100,000 population) was 2.08 (1.47–2.43), corresponding to an estimated 15,355 (10,852–17,938) cases and the median (IQR) prevalence (/100,000 population) was 5.40 (4.06–7.89), or 39,863

(29,971–58,244) prevalent cases (Burgos et al., 2013). Four European population based registers showed an increase in the incidence of ALS after the age of 40, with a peak in the late sixties or early seventies, followed by a rapid decline. The lifetime risk of developing ALS has been estimated at 1:350 for males and 1:400 for females (Logroscino et al., 2008; Smith et al., 2013). Negative prognostic indicators, arising from population-based studies, include site of disease onset, older age of onset and progression rate of respiratory, bulbar and lower limb symptoms (Chiò A et al., 2009).

The majority of cases (90%) are classified as sporadic ALS (SALS), as they are not associated with a documented family history. In retrospective epidemiological studies in about 10% of patients the disease is reported to be inherited and referred to as familial ALS (FALS), most frequently with a Mendelian inheritance that have an autosomal dominant trait with high penetrance, although pedigrees with incomplete penetrance or recessive inheritance have been also reported (Rothstein, 2009).

The mean age of onset for SALS is 56 years and for FALS is 46 years. Age of onset in FALS shows a Gaussian distribution, while SALS is characterized by an age-dependant incidence (Wijesekera and Leigh, 2009). The term juvenile ALS is used for patients with onset of disease prior to 25 (Ben Hamida et al., 1990).

The SALS and FALS are clinically indistinguishable suggesting that common pathogenesis mechanisms and pathways in the development of disease exist. The precise cause of the selective death of motor neurons remains currently elusive even if the presence of various inclusion bodies in degenerating neurones and surrounding reactive astrocytes is an established hallmark of ALS (Barbeito et al., 2004). In particular, the ubiquitinated inclusions are the most specific type of inclusions and most commonly present in ALS. They have been found in LMNs of the spinal cord and brainstem (Matsumoto et al., 1993) and in UMNs (Sasaki and Maruyama, 1994). These inclusions usually form when an increase in protein misfolding exceeds the protein degradation capacity and thereby they alter the cellular *proteostasis* (Kaniuk and Brumell, 2010). The progresses in understanding the mechanisms underlying the FALS may shed light on the pathogenic mechanisms of ALS since the majority of

mutated genes in FALS are linked to formation of cellular aggregates (Bruijn et al., 2004).

ENVIRONMENTAL FACTORS

Over the years a multitude of environmental exposure and lifestyle risk factors have been proposed as potential causes of ALS. The possible environmental factors evaluated include the intense physical activity, football, cigarette smoking, manual work, armed services and deployment, exposure to lead/solvents, pesticides and chemicals, heavy metal, electric shock, geographical clustering and cyanotoxins (Al-Chalabi and Hardiman, 2013).

For the moment unfortunately, no conclusive data are available and further studies are needed to define exogenous risk factors for ALS (Sutedja et al., 2009). An understanding of the environmental contribution to motor neuron disease is essential since this is the only easily modifiable component of risk, even in those patients with a strong family history or an identified genetic cause (Al-Chalabi and Hardiman, 2013). Interestingly, environmental exposures can result in heritable changes to genes, without altering the DNA sequence. This phenomenon is defined epigenetic and represents the most important point of convergence between genetic predisposition and environmental exposures (Paez-Colasante et al., 2015).

GENETIC COMPONENTS

The majority of ALS cases are sporadic but, more than twenty-five ALS-related genes and several additional chromosomal loci have been identified, providing fundamental insights into potential pathogenic disease mechanisms. At the moment, only for a subset of the ALS-major genes including open reading frame *C9orf72*, Superoxide dismutase-1 (*SOD1*), transactive response DNA-binding protein 43 (*TARDBP*), and fused in sarcoma translocated in liposarcoma (*FUS*) there is a wide consensus about causal role of disease. However, the exact pathogenic mechanisms of mutated forms have to be more clearly elucidated. The alterations on DNA sequences of these genes are currently analysed for genetic testing of both sporadic and familial cases, which are tested for *VCP* and *ANG* gene mutations in a subsequent step of diagnosis (Marangi and Traynor, 2015; Chiò et al., 2014).

The *SOD1* gene encodes for an enzyme composed of 153 amino acids involved in free radical scavenging and it is the first ALS-related gene described in 1983. More than 150 different mutations in *SOD1* gene have been reported to be pathogenic in about 20% of FALS cases and in 7% of sporadic patients. The thirty-year studies have only excluded the initial hypothesis that the SOD1 mutations resulted in an enzyme unable to neutralize reactive oxygen species. Currently hypotheses for the mechanism of SOD1 toxicity is that mutations cause a destabilization of secondary protein structure and promote protein oligomerization and aggregation. Several hypotheses have been proposed regarding the pathogenicity of SOD1 aggregates, including the perturbation of mitochondrial function, the alteration of axonal transport, the aberrant binding of apoptosis regulators, and the glutamate exocitotoxicity. Various lines of evidence suggest that the presence of SOD1 aggregates affects the capability of the cell to preserve the protein homeostasis (Battistini et al., 2012).

The most common genetic cause of ALS and FTD is linked to chromosome region 9p21 and this locus contains the open reading frame *C9orf72,* which encodes for the homonymous protein (C9orf72) founded in many regions of the brain, at the cytoplasmic level of the presynaptic terminal of neurons (Renton et al., 2011). The *C9orf72* gene contains in the first intron a hexanucleotide repetition (GGGGCC) and its massive expansion is considered the pathological genetic hallmark of ALS and FTD.

The massive hexanucleotide-repeat expansion is pathological over 30 repetitions, which can reach hundreds of copies in patients as well as in presintomatic carriers, is the most frequent genetic alteration inherited as autosomal dominant trait in 23-47% of FALS and 4-21% of SALS (Millecamps et al., 2012). The mechanism through which the C9orf72 triggers diseases is not completely clarified even if several evidences support the pathogenic hypothesis of alteration in RNA metabolism (Renton et al., 2011). Indeed, in the brain and spinal cord of ALS patients has shown the presence of accumulations, organized into distinct foci of mutated mRNA (DeJesus-Hernandetz et al., 2011) and of simple peptide resulting from unconventional translation of the repeated transcripts. These peptides affect transcription and translation by interfering with both mRNA splicing and ribosomal RNA biogenesis (Kwon et al., 2014). These hypotheses might suggest that hexanucleotide-repeat expansion leads

to neurodegeneration via gain of function mechanisms rather than loss of function (Sareen et al., 2013).

A direct involvement into RNA processing pathways has been reported for *TARDBP,* coding for TDP-43 protein and for *FUS*, which encodes for RNA-binding protein FUS/TLS (FUS/TLS). Both TDP-43 and FUS/TLS belong to the heterogeneous nuclear ribonucleoprotein (hnRNP) family; they are located into the cell nucleus and cytoplasm and they are involved in gene transcription and post-transcriptional modification of the newly synthesized RNA (Robberecht and Philips, 2013). Mutations in genes coding for TDP-43 and FUS/TLS have been reported in about 1-5% of familial ALS cases and in a variable percentage of sporadic patients (Robberecht and Philips, 2013). The association between mutant TDP-43, FUS/TLS and RNA molecules lead to abnormal phosphorylation, ubiquitination and then to aggregation of the translational complexes into stress granules. Both these mutated proteins generate neuronal inclusions and dystrophic neurites, as well as glial cytoplasmic inclusions (Volontè et al., 2015). Notably, TDP-43 stress granules have been also reported in several non-mutated ALS patients corroborating the hypothesis that disruption of RNA processing proceeds independently from *TARDBP* mutations (Neumann et al., 2006). These intracellular aggregates mainly contain mRNA-binding proteins with several molecules of small RNAs as microRNAs (miRNAs) that are directly involved in the mRNAs translational repression. Moreover, both TDP-43 and FUS/TLS are directly implicated in miRNA biogenesis (Kawahara and Mieda-Sato, 2012).

The alteration of RNA processing pathways is a common pathway for several other ALS-related genes and it represents a central point of this thesis (Ferraiulo et al., 2011). Various mutations have been reported in the gene coding for the Angiogenin (*ANG*) which belongs to the pancreatic ribonuclease superfamily (Greenway et al., 2006), in the gene for senataxin (*SETX*), which contains a classical seven-motif domain characteristic for RNA/DNA helicases (Chen et al., 2004; van Blitterswijk and Landers, 2010), in the gene for TATA-binding protein associated factor 15 (*TAF15*), a protein involved in the initiation of transcription by RNA polymerase II (Couthouis et al., 2011) and in Ewing sarcoma breakpoint region 1 (*EWSR1*) which is a putative RNA binding protein (Couthouis et al., 2012).

EPIGENETIC IN ALS PATHOGENESIS

Besides the genetic variations and environmental exposures, which are two aspects considered essentials in the research of pathogenic mechanisms of ALS up to now, it is also important to take into consideration a third element, which is known as epigenetic (Al-Chalabi et al., 2013).

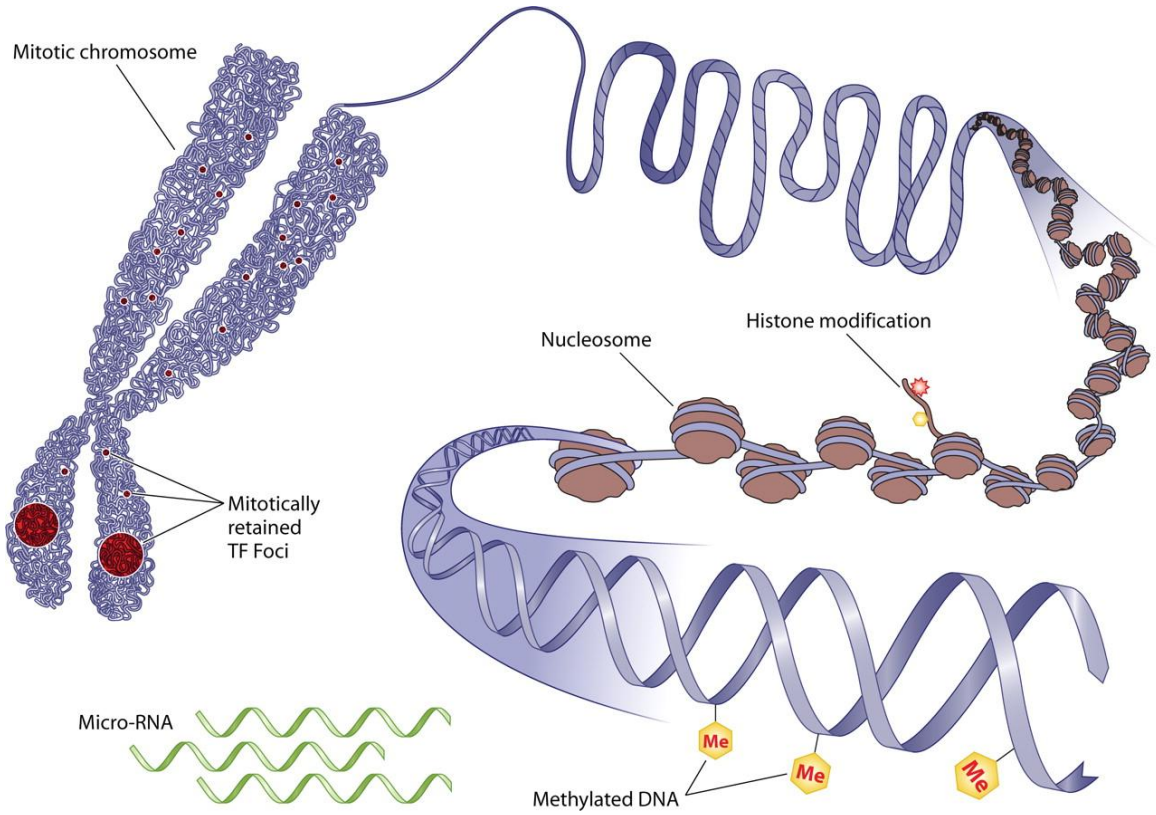
Epigenetics, from ἐπιγέννησις, could be defined as the study of heritable changes in gene expression which are not due to changes in DNA sequence but to structural adaptation of chromosomal regions or prolonged altered activity states (Eccleston et al., 2007; Bird, 2007). This definition includes two distinct points of view of epigenetic; the first one considers the methylation and histone modifications as the sole mechanisms able to maintain the epigenome excluding transcriptional effects of RNA interference (Riggs et al., 1996). The second one is more global and it includes the environmental factors as external elements, which play a key role during the development of diseases able to define the pathologic phenotype (Bird, 2007).

The epigenetic mechanisms regulate gene expression and are critical for determining and maintaining cell fate during development. Indeed, every cell of the organism contains the same DNA and epigenetic mechanisms generate the differences by programming the cell to transcribe specific tissue genes. For instance, during the neuronal development, the cells express specific genes necessary to dendritogenesis and axon growing (neuronal-specific genes) which are inaccessible to transcription in muscle or liver cells by epigenetic marks.

The behaviour, stress, diet, ageing and toxin exposures are just a few examples of the received and maintained environmental stimulus by DNA, chromatin remodelling and non-coding RNAs. The epigenetic mechanisms are the real potential convergence between genetic predisposition and environmental exposures that could explain how gene–gene and gene–environmental interactions are mediated. Moreover, the reversibility of epigenetic mechanisms is a pivotal characteristic that may play a central role for the future development of a therapy for ALS (Paez-Colasante et al., 2015).

The discovery that several ALS genes are linked to RNA biology suggests that, besides the genetic and the environmental factors, the epigenetic mechanisms play a crucial role in the development and course of ALS (Al-Chalabi et al., 2013). Indeed, DNA methylation, histone remodelling, modifications in RNA metabolism, which are the main epigenetic mechanisms have been described as deregulated both in animal models and in ALS patients (Paez-Colasante et al., 2015).

Fig. 1. Chromatin structure components and general description of epigenetic mechanisms



The picture reports in the left side the typical structure of mitotic chromosome with several mitotically retained TF Foci where the enzymatic activity is high. The smallest element of chromatin is the nucleosome, which is composed of eight units of histone proteins with 146bp of DNA twisted. On the right side there is a description of basic epigenetic mechanisms. The main epigenetic mechanisms operating on the nucleosome are DNA methylation, consisting in chemical modifications of DNA by addition or removal of methyl groups to the CpG island, and histone modifications consisting in acetylation/de-acetylation and methylation/de-methylation of histone protein. Histone acetylation or methylation can unwind the DNA, making it accessible to transcription of genes, which are usually inaccessible. Non-coding RNA molecules like microRNAs (miRNAs) are also involved in the regulation of the histone modifying enzymes activity as much as the nucleosome modifications can regulate the expression of miRNA genes (figure from "A Short Introduction to Epigenetics" from www.episona.com).

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PATHOGENIC HYPOTHESES

The pathogenesis of ALS is not clear, and the exact mechanisms, which lead to motor neurons death, are currently unknown. However, several recent findings from genetic of ALS, for instance, the identification of *TDP-43* and *FUS* mutations and, particularly, the hexanucleotide expansion on *C9orf72* provided notable information to change our thinking of the disease pathogenesis. Moreover, the subsequent studies, conducted in order to get a better insight of ALS mechanisms associated with genetic alterations, induced researchers to consider the disease as *proteinopathy* or *ribonucleopathy* (Robberecht and Philips, 2013).

In the case of *protheinopathy*, the motor neuron damage is considered a consequence of abnormal protein formations and their oligomeric complexes that disturb the normal protein homeostasis resulting in cellular stress (Saxena and Caroni, 2011). The altered *proteostasis* networks have been related to the formation of protein aggregations of mutant SOD1, valosin-containing protein (VCP), ubiquilin 2 (UBQLN2), charged multivesicular body protein 2b (CHMP2B), optineurin (OPTN) and, potentially TDP43 and FUS (Robberecht and Philips, 2013).

In the case of *ribonucleopathy*, the neuron injuries are considered related to aggregates of RNA molecules and RNA-binding proteins that alter the cellular mechanisms involving in RNA metabolism (Robberecht and Philips, 2013). In both cases, beyond the origins of these dysfunctions that could be multifactorial, through loss of function and gain of function mechanisms, the subsequent changes determine progressive cellular failures in protein degradation mechanisms and alterations of RNA processing, leading to aggregate and toxic RNA species formations.

Various cellular function interferences were described after these events at nucleus, cytoplasm and axonal level.

In nucleus disturbance of normal RNA metabolism from splicing alterations to RNA biogenesis has been widely described.

In cytoplasm of neurons several interferences with normal organelle activities have been reported such as proteasomal or autophagic protein degradation, endoplasmic

reticulum (ER) stress and Golgi and mitochondrial dysfunctions. The damages at mitochondrial levels determine morphology and functions alterations, and membrane permeability dysfunctions that lead to elevated calcium levels and decrease the activity of respiratory chain complexes I and IV implicating defective energy metabolism. The augmented concentration of calcium in the cell induces the activity of several enzymes that generate toxic Reactive Oxygen Species (ROS) (Ferraiuolo et al., 2011). The ROS cause stable oxidative damage to major cellular components such as proteins, DNA, lipids, and cell membranes (Bogdanov et al., 2000; Girotti, 1998; Shaw et al., 1995). High levels of ROS have been detected in the cerebrospinal fluid and in the spinal cord of SALS patients (Tohgi et al., 1999).

At the axonal level, protein and RNA aggregations and the subsequent impairment of axonal transport represent another widely studied pathogenic hypothesis for the ALS. Both the protein and RNA/RNA-binding proteins aggregates, which have been frequently found in spinal motor neurons of all types FALS and SALS patients, disturb normal protein homeostasis inducing cellular stress (Bendotti et al., 2012). These aggregates usually contain several different ubiquitinated proteins with a well-known intrinsic tendency to aggregate (SOD1, TDP43, FUS, and OPTN). Interestingly, FUS and TDP43 ALS-associated mutations enhance the rate of aggregation of these proteins (Johnson et al., 2009; Sun et al., 2011). Protein and RNA/RNA-binding proteins aggregates result particularly toxic for motor neurons since they could trap proteins with important cellular functions and could cause the cytoskeletal disarrangement and axonal transport dysfunction with consequent mechanical impedance.

Other neurodegenerative mechanisms associated with ALS are the activation of neuroinflammation and excitotoxicity. These mechanisms involve non-neuronal cells including microglial cells, astrocytes and oligodendrocytes.

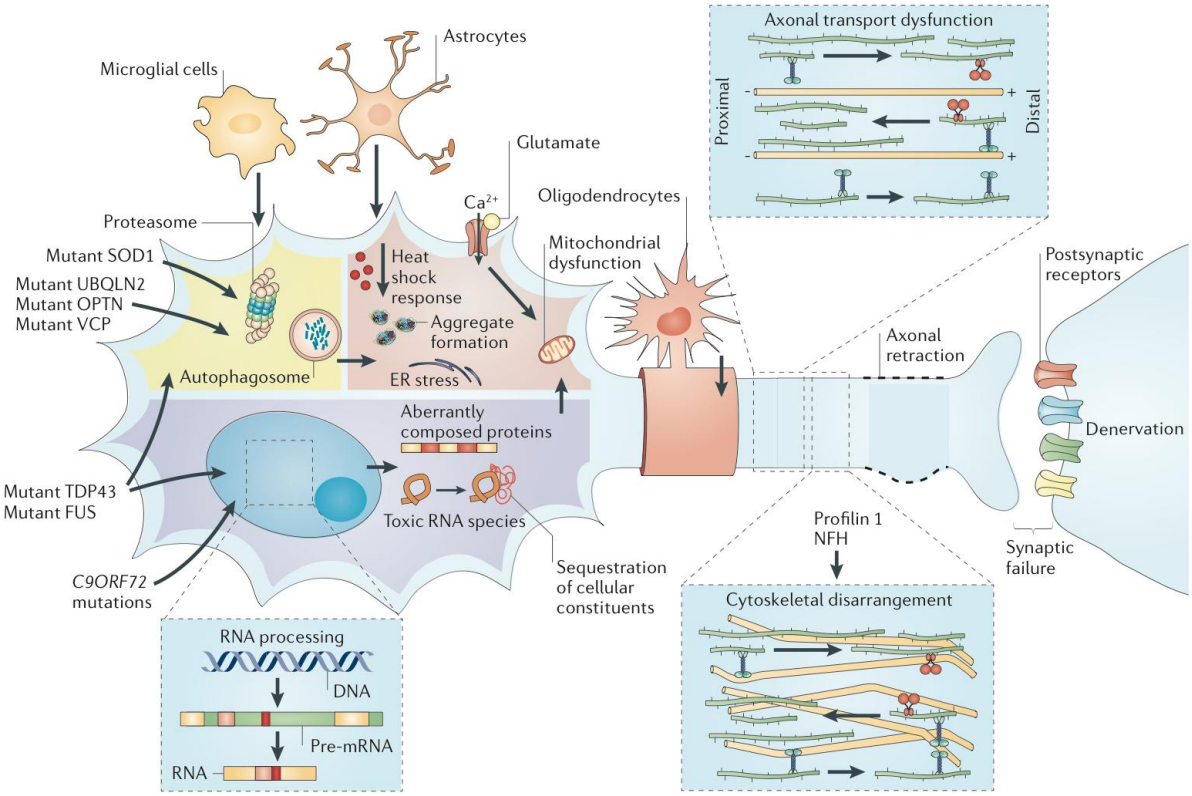
Although motor neurons are the main impaired cells in ALS pathogenesis, extensive evidences suggest that non-neuronal cells and inflammatory dysfunction play a pivotal role to the disease progression. Glial cells, oligodendrocytes, astrocytes and microglia play an important role in the ALS pathology onset and disease progression (Lee et al., 2012; Ilieva et al., 2009), initiating the process known as

neuroinflammation. Indeed, if in the one hand this process if protects the neurons through the modulation of the helpful inflammatory response slowing disease progression (Beers et al., 2011) on the other hand the constant inflammation activity becomes toxic for the neural tissue.

The first theory proposed for motor neurons degeneration in ALS based on the evidence that increased levels of glutamate were observed in the cerebrospinal fluid of patients was glutamate excitotoxicity (Rothstein et al., 1992). Excitotoxicity is the neuronal injury caused by the excess of glutamate, which induces the stimulation of the postsynaptic glutamate receptors. The augmented stimulation leads to a massive calcium influx in the cell that increases the nitric oxide formation and subsequent neuronal death (Shaw, 2005). An association between motor neurons degeneration in ALS and the loss of function of the astroglial glutamate transporter EAAT2 has been described as secondary effect rather than a primary one in ALS onset (Bendotti et al., 2001). All these cellular physiologic dysfunctions are summarized in **Fig 1**.

Although essential issues remain unsolved the progresse in our understanding of mechanisms leading to the selective motor neuron death in ALS has been substantial. Even if mutant proteins associated with FALS are expressed ubiquitously, the reason why the motor neurons are more susceptible to the their pathogenic effects is unsolved. Which are the factors that cause the vulnerability of motor neurons in SALS? When does the disease biologically start in human beings? Why do mutant proteins, which are present in individuals' life from the beginning of their existence, cause a disease after several decades? And why is ALS fatal within a couple of years? These are all aspects that have to be necessarily clarified. Moreover, better insights about the biology of motor neuron degeneration in ALS could open a window toward new treatments that make a substantial difference for patients (Robberecht and Philips, 2013).

Fig. 2. Overview of events involved in the pathogenesis of Amyotrophic Lateral Sclerosis



In the picture the main primary pathogenic changes occurring in ALS are reported and for each one the ALS-liked mutated genes are listed.

Theorizing ALS as a *proteinopathy*, alteration of proteasomal/autophagic protein degradation has been observed in association with the formation of protein aggregates of mutated superoxide dismutase 1 (Sod1), valosin-containing protein (VCP), ubiquilin 2 (UBQLN2), charged multivesicular body protein 2b (CHMP2B), optineurin (OPTN) and, potentially, TAR DNA-binding protein 43 (TDP43) and RNA-binding protein FUS (FUS).

Theorizing ALS as *ribonucleopathy*, disturbance of normal RNA metabolism and processing that produces erroneously assembled proteins and toxic RNA species have been observed in association with mutated *TARDBP* and *FUS* and expanded *C9orf72*. Some ALS-causing mutant proteins may act more downstream in this model, e.g. profilin 1 and neurofilament heavy chain (NFH) through a direct effect on the cytoskeleton and D-amino-acid oxidase may affect excitotoxicity. Axonal attraction systems and repellent systems appear to modify the processes of axonal retraction and denervation (figure from Robberecht and Philips, 2013).

2

miRNAs: Neurological Functions and Biogenesis

One of the most shocking insights from the "Human Genome Project (HGP)" was that, even though the biggest part of human genome is transcribed into RNA molecules, only 2% of the 3,234.83 Mb is translated into proteins. The consistent part of non-coding RNAs (ncRNA) is often defined the "dark matter of cells" and it appears to be biologically active in regulation of gene expression. The microRNA (miRNA) molecules constitute the most important class of nc-RNA (Goodall et al., 2013).

The recent discovery and characterization of miRNAs changed completely the dogma of molecular biology, introducing an additional and critical level of gene regulation defining a new epigenetic mechanism (Clancy, 2008). MiRNAs are short ncRNA of 18-25 nucleotides (nt) in length, central to the epigenetic processes, playing an important role as endogenous regulators of gene expression. Interestingly, the same miRNA may regulate hundreds of target mRNAs and thus may affect gene expression networks.

The number of miRNAs that have been reported in animals, plants and viruses until now is almost 16,000. More than 1,000 different miRNA molecules belong to humans and at least 20-30% of all protein-coding genes are likely controlled by miRNAs. For this reason, miRNAs can be considered as fine epigenetic regulators of mRNA transcription (Volontè et al., 2015).

In mammals, the central nervous system is a rich source of miRNAs that are essential during the brain development and in the regulation of physiology of brain. Indeed, miRNAs play a pivotal role in several brain functions such as morphogenesis, neurogenesis, neuronal differentiation, dendritic spine generation, synaptic formation and plasticity (Kosik, 2006). Furthermore, profound changes in miRNA expression at the brain level were observed in several pathological conditions both during acute and chronic illnesses (Volontè et al., 2015).

In particular, the dysfunction of miRNA expression in neurodegenerative diseases is increasing recognized in Parkinson's (PD), Alzheimer's (AD), and Huntington's diseases (HD), multiple sclerosis (MS), Rett syndrome, Fragile X and Tourette's syndromes as well as in neuropsychiatric disorders, epileptic seizures, traumatic spinal cord and brain injuries, brain cancer and ischemia, so much so that the number of miRNA-researches in ALS is increasing (Volontè et al., 2015).

Emerging evidence reveals that miRNA deregulation is deeply involved in ALS pathogenesis (Goodall et al., 2013) and many recent research efforts have investigated the function and the effects of miRNAs deregulation to motor neuron death (Droppelmann et al., 2014).

miRNAs Biogenesis

The biogenesis of miRNAs takes place in nucleus (first step) and subsequently in cytoplasm (second step). A canonical and a non-canonical pathway were described in miRNA production. However, in this thesis only the canonical miRNA pathway has been taken into consideration.

At nucleus level the first premature miRNA molecules are mainly synthesized by the RNA polymerase II (pol II) and partly by RNA polymerase III (pol III). These long primary miRNA transcripts (pri-miRNAs) are produced from genes of miRNA localized in intergenic, intronic and exonic regions. Several transcription factors and epigenetic regulators control specifically the gene expression of particular miRNA clusters. The p53, MYC and MYOD1 are examples of transcription factors that transactivate the gene expression of miR-34, miR-17 and miR-1 clusters respectively; while MYC, ZEB1, and ZEB2 regulate negatively the transcription of miR-15 and miR-200 clusters respectively. The DNA methylation and histone modification also contribute to miRNA gene expression (Ha and Kim, 2014).

The pri-miRNA molecules are substrates of the microprocessor, which is a protein complex containing the ribonuclease III Drosha and DGCR8. The microprocessor complex produces the stem loop precursor miRNAs (pre-miRNAs), which is a molecule of 70 nt in length (Gregory et al., 2004). The phosphorilation, acetylation and deacetylation of Drosha and DGCR8 have been recently observed as regulatory

post-transcriptional modifications taking part to the miRNA biogenesis. Moreover, several RNA-binding proteins were described as auxiliary factors controlling Drosha processing. The TDP-43 and FUS with p68, p72, KH-type splicing regulatory protein (KSRP) are only few examples of RNA-binding proteins interacting with Drosha and providing the stabilization and regulation of Drosha processing. Interestingly, several specific miRNAs were described as regulatory molecules of Drosha activity since they cooperate with RNA-binding proteins. The KSRP binds the terminal loop of let-7, miR21 and miR16 facilitating Drosha mediated-processing. On the contrary LIN28 binding the terminal loop of let-7 inhibits Drosha and Dicer mediated processing. Finally ADAR1 and ADAR2 editing from inosine to adenosine of certain miRNAs, like miR-142, interferes with Drosha processing (Ha and Kim, 2014).

The Exportin5 ships the pre-miRNA molecules to the cytoplasm. This protein is encoded by the *XPO5* gene and is part of a large family of karyopherins that mediate the transport of double-stranded RNA binding proteins from nucleus to cytoplasm (Brownawell and Macara, 2002). Thus, the pre-miRNAs are cleaved into 22 nt double stranded mature miRNAs (ds-miRNAs) by endoribonuclease Dicer that interacts directly with TDP-43. Several post-transcriptional modifications on TDP-43 influence the ability of this RNA-binding protein to regulate Dicer processing. Interestingly, it has been recently observed that let-7 is able to bind the mRNA of Dicer determining a negative feedback loop between Dicer and let-7 itself (Tokumaru et al., 2008).

Next, the ds-miRNAs are assembled into Argonaut protein (AGO), the most important component of RNA-induced silencing complexes (RISC) (Lieberman et al., 2003; Newman and Hammond, 2010). Four isoforms of AGO able to incorporate miRNA duplexes with preferences for these with central mismatches exist in humans (nucleotide from 8-11). From each single precursor, only one of the duplex strands will be functionally activated into AGO that directly provides double strand separation and guide/passenger strand's selection (Matranga et al., 2005). The rules for selection of guide strands from ds-miRNAs takes into consideration the thermodynamic instability of 5'terminus and the presence of a U at nucleotide in position 1 (Ha and Kim, 2014).

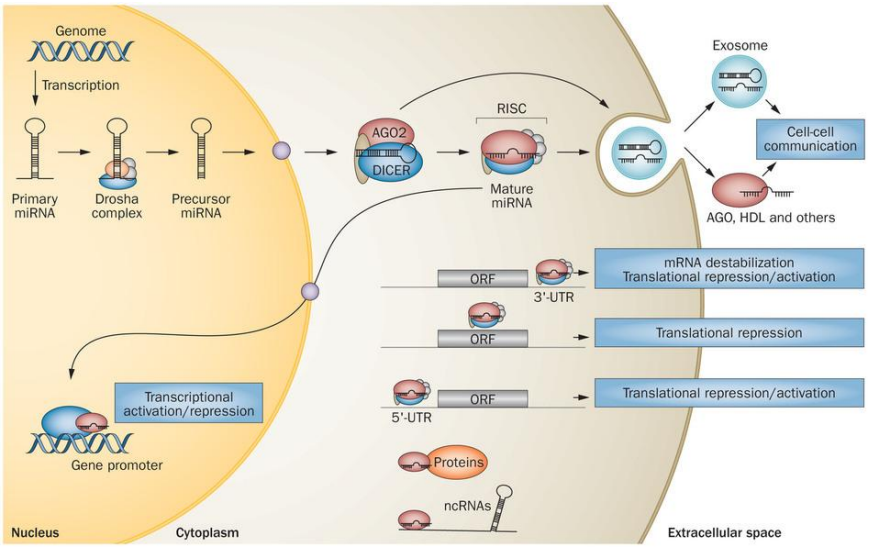
The single strand of miRNA guides the RISC complex in searching for miRNA response elements (MREs) mainly localized into the 3'-untranslated region (3'UTR) of the target mRNA to control gene expression. The AGO protein catalyses target mRNA cleavage and the following degradation of miRNAs, although translational repression is the most prevalent mechanism of action for miRNAs in humans (Liu et al., 2004).

Via base pairs recognition, miRNAs generated by both canonical and non-canonical pathways, negatively regulate the mRNA targets translation in two different ways depending on the grade of complementarity between MREs and mRNAs: the complete complementarity represses the translation, whereas the partial complementarity blocks the translation (Iorio and Croce, 2012).

Recently, the advanced RNA sequencing techniques have lead to the discovery that many miRNAs are generated via alternative mechanisms, bypassing the usual Drosha/Dicer two-step processing (Miyoshi et al., 2010). In the non-canonical pathway, better known as mirtron pathway, miRNAs biogenesis does not require either Drosha or Dicer processing and pre-miRNAs are directly loaded onto AGO2. The non-canonical pathway is usually associated with miRNAs or miRNA-like small RNAs assembly (Paez-Colasante et al., 2015).

A different expression of miRNAs between tissues or body fluids from patients and controls and from several experimental models has been observed in the majority of neurodegenerative diseases. Although the implications of miRNAs deregulation have not been completely elucidated in neurodegeneration, the dysfunction of miRNA biogenesis components examined in various experimental models of ALS and motor neurons has been reported to have severe consequences (Paez-Colasante et al., 2015).

Fig. 3. Principal events in miRNA biogenesis



In the picture the canonical pathway of miRNA biogenesis is reported. Primary miRNA transcripts (pri-miRs) are transcribed from miRNA genes by RNA polymerase II. Pri-miRs are subsequently cleaved by DROSHA with Drosha complex and 70-nt stem loop precursor miRNAs (pre-miRNAs) are generated. After pre-miRNAs are exported from the nucleus to the cytoplasm by Exportin5, DICER1 processes them and generates 22-nt mature molecules of miRNAs. The double-strand mature miRNAs (ds-miRNAs) is separated and the guide strand is subsequently loaded into the RISC complex, which contains the AGO family protein as a core component.

In the nucleus miRNA molecules regulate gene transcription interacting with transcriptional factors at gene promoter level. In the cytoplasm, mature miRNAs with RISC complex repress the mRNA translation binding the target mRNA, via base pairs recognition. Several miRNAs are also secreted to extracellular space in free form or packaged into lipid vesicles called exosomes, which seem involved in cell-to-cell communication (figure from Schwarzenbach et al., 2014).

3

DEREGULATED MIRNAS IN ALS

MIRNAS AND ALS-RELATED GENES

To better understand the function of miRNAs in motor neurons survival several studies were performed by disrupting the main components of miRNA apparatus. The essential role of miRNAs in motor neuron degeneration was uncovered by the depletion of Dicer1 in experimental model that triggered the loss of the capacity to produce mature miRNAs. The Dicer1 knockdown animal model employed in the study (MNDicer^{mut}) developed progressive locomotors defects and denervation muscle atrophy (Haramati et al., 2010).

Subsequent studies focused on the ALS-associated proteins TDP-43 and FUS. The TDP-43 was described as a component of the nuclear microprocessor (Drosha and a larger complex of 17 polypeptides including TDP-43 and FUS as well) (Gregory et al., 2004) and a binding protein of Dicer and AGO. Interestingly, recent studies reported that TDP-43 was able to bind a subset of miRNAs in nucleus and cytoplasm (Kawahara and Mieda-Sato, 2012). Indeed, the loss of TDP-43 by its depletion affected *in vitro* the production of a specific subset of miRNAs mainly implicated in neuromuscular development (Buratti et al., 2010). Finally, it has been recently found that mutated TDP-43 aggregates into stress granules at cytoplasm level and sequesters the specific clusters of miRNAs inhibiting thus the negative regulatory function on nascent mRNAs (Honda et al, 2013).

The miR132-5p and 3p, miR143-5p and 3p, miR574-5p and 3p, miR558-3p miR663a and let-7b constituted the peculiar cluster of TDP-43 binding miRNAs (Kawahara and Mieda-Sato 2012; Buratti et al., 2010). This cluster of molecules were further analysed in serum and CSF from sporadic ALS patients. The expression levels of five out of nine TDP-43 binding miRNAs were altered in the CSF and serum. In particular, miR132-5p and 3p, miR143-5p and 3p were significantly deregulated both in serum

and in CSF, while miR574-5p and let-7b were reciprocally deregulated in CSF and serum (Freischmidt et al., 2013). Interestingly, the majority of miRNAs were upregulated in serum samples while miR132-5p and miR558-3p were 2–3 times more abundant in CSF, indicating that changes in one type of samples did not necessarily reflect miRNA levels in the other samples (Freischmidt et al., 2013). In lymphoblast cell lines from familial ALS patients, the levels of TDP-43 deregulated miRNAs have been subsequently measured and specific suppression of miR132-5p/3p, miR143-5p/3p was evident in all patients except for those with SOD1 mutations. The miR663a was exclusively deregulated in patients with FUS mutations while let7-b resulted deregulated both in those with *C9orf72* expansion or FUS mutations (Freischmidt et al., 2013).

Similarly to TDP-43, the ALS-associated protein FUS was described as a component of the microprocessor (Gregory et al., 2004). The mutated FUS was observed in aggregates in the cytoplasmic stress granules of neuronal cells (Kwiatkowski et al., 2009; Vance et al., 2009). The FUS downregulation was studied in cellular models in which a significant reduction of a specific class of molecules involved in various neuronal functions from differentiation to synaptogenesis was observed (miR-9, miR125-b, miR132 and miR143) (Morlando et al., 2012).

The global loss of miRNAs networks and specific miRNAs clusters due to biogenesis defects leads to consistent progressive cellular failures that were also observed in several studies conducted by deregulating clusters or single miRNAs. Progressive cellular failures at the axonal and neuromuscular junction levels and an increased in the susceptibility to excitotoxicity and neuroinflammation were reported in these works and briefly summarized in subsequent paragraphs.

miRNA Deregulation and Neurofilament

A valid biomarker of the ALS disease has to reflect the status of damaged tissue or cells and the most promising molecular biomarker for ALS has currently been represented by neurofilaments, which constitute the neuronal cytoskeleton and provide the structural support to the axon. Recent studies reported a significant increase in NF-L (neurofilaments-light-chain) and pNF-H (phosphorilated-

neurophilament-heavy-chain) in both serum and CSF from ALS patients (Weydt et al., 2015; Steinacker et al., 2015). The increase in human biofluid of these intracellular proteins reflects the neuronal damage in ALS associated with deregulation of neuronal proteins and it seems to be really specific for motor neurons that have particularly developed axons.

A possible explanation of the significant increase in neurofilaments in ALS could be found in miR-9 downregulation that was reported in induced pluripotent stem cells (iPSC)-derived from neurons of FTD/ALS patients with mutated TDP-43 (Zhang et al., 2013). *In silico* analysis revealed that miR-9 has one and nine binding sites (MREs) in the 3'UTR of NF-L and NF-H respectively (Haramati et al., 2010). Thus, direct inhibition of NF-H gene expression by miR-9 was confirmed in cellular model and, even if no luciferase assay was performed to validate the result, it is possible to hypothesize that the increase in neurofilaments levels in motor neuron degeneration could be linked to the loss of miR-9 (Haramati et al., 2010).

Interestingly, the most important study of miRNAs deregulation in human spinal biopsies from ALS patients identified a set of specific miRNAs that directly controls neurofilaments gene expression. An overall downregulation of miRNAs in patients compared to controls was reported for miR146a*, 524-5p, 582-3p, b1336 and b2404 (Campos-Melo et al., 2013). All the miRNAs presented MREs in mRNA of NF-L at the 3'UTR. In particular, a significant downregulation of miRb-1336 and miRb-2404, that usually stabilizes NF-L, was reported. The two-downregulated miRNAs were validated and a consequent reduction of translation of NF-L mRNA followed by neuromuscular junction pathology was observed (Ishtiaq et al., 2014).

These results demonstrate that the neurofilaments gene expression is finely regulated at posttranscriptional level by a cluster of specific miRNA molecules furnishing a direct, not necessary causal link with the increase of neurofilaments levels in human biopsies and CSF (Volontè et al., 2015).

miRNA Deregulation and Increased Susceptibility to Excitotoxicity

An intense activity of cell-to-cell communication was observed in the motor neuronal microenvironment (Raposo and Stoorvogel, 2013). In particular, the damaged motor

neurons release miRNA and mRNA molecules outside the cells in the form of intercellular messages after they are packaged into small membrane vesicles called exosomes. Experimental evidences reported the presence of specific miRNAs into exosomes released by neurons and picked up by astrocytes that modulate the glutamate path by internalized RNA molecules (Morel et al., 2013).

Both in ALS patients and in the spinal cord biopsies from SOD1 mouse model a decrease in the levels of glutamate transporter 1 (GLT1) coded by excitatory amino acid transporter 2 (*EAAT2*) gene was described (Robberecth and Philips 2013). This reduction of *EAAT2* expression has been recently associated with the identification of miR124a. This specific miRNA is normally released by neurons into exosomes and taken up by astrocytes to reduce the toxic effects of glutamate. Indeed, the *EAAT2* gene expression increased indirectly inside the astrocytes thanks to the miR124a. This specific miRNA is likely to down regulate the expression of inhibitors of transcription of *EAAT2*. Moreover, the *in vivo* injections of synthetic miR124a, into spinal cord of SOD1 mouse models, lead to an increase by 30% in the *EAAT2* expression. Thus, as this work described, the upregulation of miR124a is indirectly linked to the upregulation of GLT1 resulting in a protection of neurons against the toxic effect of glutamate (Morel et al., 2013).

MIRNA DEREGULATION AND NEUROINFLAMMATION

Microglial cells are other important cellular components of motor neuronal microenvironment. These cells produce and release higher levels of inflammatory cytokines and specific miRNAs including miR29, miR133a, miR155, miR221, miR223 or miR652, all having a well-known functional role in inflammation (Roy et al., 2015). Indeed, in the context of ALS pathogenesis and disease progression a pivotal role is played by cells of the immune system resident in the brain such as activated microglial cells and T-cells activated.

A specific miRNA signature was identified in a study that consisted in miRNA profiling conducted in CD14+CD16- monocytes from blood samples of ALS patients and in microglia and LY6C^{hi} monocytes from mutant SOD1 mice. The cluster of deregulated miRNAs reported in the study revealed a unique inflammatory miRNA

signature that included miR27a, 155, 146a and miR32-3p for human and murine monocytes, and let7, miR15-b, 16, 132, 146a, 155, 451 and miR223 only for murine monocytes (Butovsky et al., 2012).

It is known that in inflammatory response a key role is played by TGF1-β cytokine since it appears to block the activation of lymphocytes and monocytes. The miR21 and 106b, that target TGF1-β, resulted upregulated in blood of ALS patients (Butovsky et al., 2012). Similarly, miR155 that promotes macrophage inflammatory response and increase pro-inflammatory cytokine levels inhibiting TGF1-β production was reported as upregulated (Koval et al., 2013).

Another study has been recently conducted to characterize the functional connections among miRNAs and targets deregulation in the context of ALS-neuroinflammation. In order to achieve this objective, activated microglia from brain tissue of SOD1 mice were employed as *in vitro* model. The upregulation of miR22, miR125b, miR146b and miR365 was reported. Among the deregulated miRNAs, enormous interest was raised by miR365 and miR125b since they target IL-6 and the signal transducer and activator of transcription 3 (STAT3) respectively. The downregulation of the IL-6 and STAT3 pathways favoured proinflammatory signal by determining an increase in the tumour necrosis factor (TNF). Interestingly, the increasing of TNF supports reciprocally the upregulation of miR125b and establishes a kind of vicious cycle that is likely to culminate in abnormal TNF release. This result suggests that the deregulation of miR365 and miR125b could directly influence the pathologic-inflammatory signature of cytokine in ALS (Parisi et al., 2013).

MIRNA DEREGULATION AND MUSCLE DENERVATION

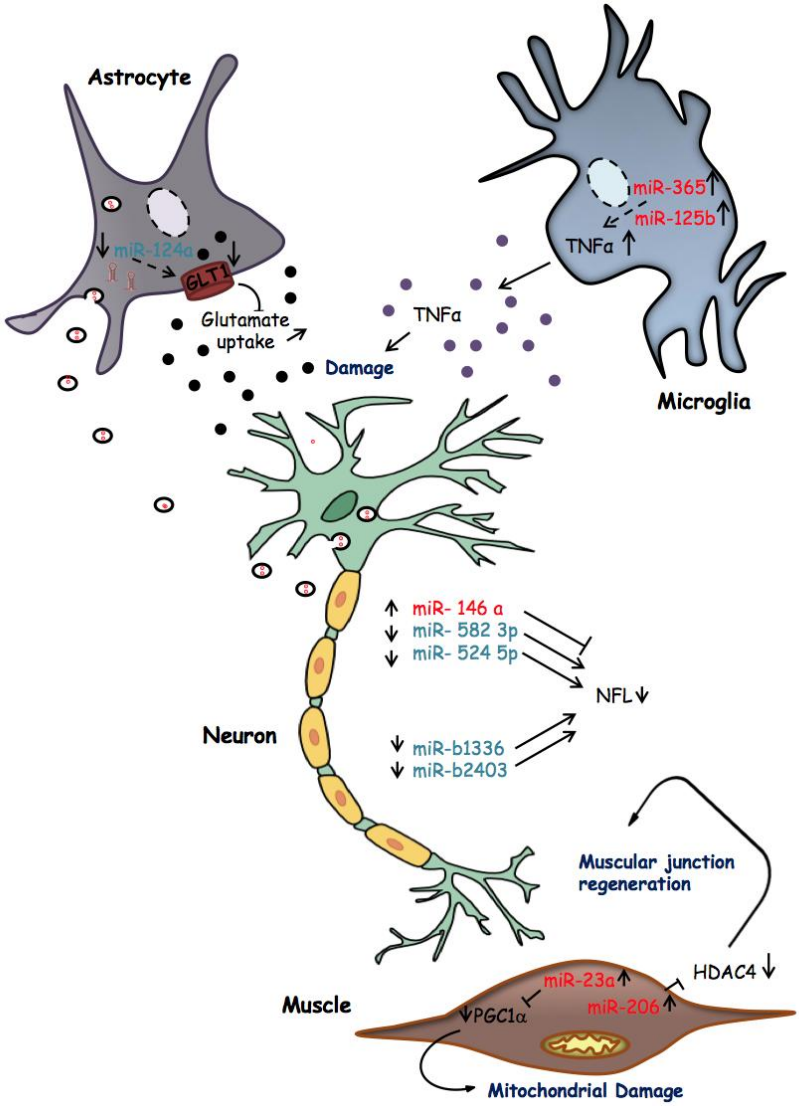
In the perspective to better insight the changes on miRNA profiling in consequence of denervation, typical figure of ALS at neuromuscular junction, a comparison between normal and denervated tissue from animal model was performed. Moreover, the bidirectional signalling between neurons and skeletal muscle fibers was studied and the obtained results were confirmed in skeletal muscle biopsies from ALS patients.

In muscle biopsies from lower limbs of SOD1 mice model and normal adult mice the miRNA profiling analysis revealed a significant upregulation of miR206. This is a

muscle specific miRNA with a pivotal role in miofibers development and plasticity (McCarthy, 2008). Subsequent studies, conducted in a mouse model deficient for miR206$^{-/-}$, revealed that miR206 genetic ablation did not alter the formation of neuromuscular synapses during development. However, deficiency of miR206 in the SOD1 mice accelerated the disease progression, most probably because the muscular miRNA plays a key role in the nerve-muscle communication and for this reason it is essential in the re-innervation process arousing after nerve damage. Subsequent molecular experiments reported that miR206 was necessary for the correct regeneration of neuromuscular synapses after acute nerve injury and mediated its effects at least in part through the histone deacetylase 4 (HADAC4) and fibroblast growth factor (FGF) signalling pathways (Williams et al., 2009).

Another study of miRNA expression conducted in human skeletal muscle biopsies and comparing ALS patients and healthy controls confirmed a significant increase in miR206 and revealed the contemporary upregulation of a miRNAs 23a, 29b and 455. Among these, miR23a resulted particularly interesting since it represses the translation in a 3'UTR dependent manner of the suppressor of the activity of peroxisome-proliferator activated receptor-g co-cactivator (PGC)-1alpha. This protein, indeed, has been described as directly involved in mitochondrial biogenesis and function as well as in the increment of several miRNAs potentially implicated in skeletal muscle and neuromuscular junction regeneration (Russell et al., 2013).

Fig. 4 miRNAs and their targets deregulated in ALS



Neuronal damages in ALS have been usually associated with deregulation of neuronal *proteostasis*, release of toxic molecules from glial cells and dysfunctions of neuromuscular junction. In motor neurons, the down regulation of NFL has been described in association with the deregulation of five miRNA (up-regulated in red, down regulated in blue). To avoid toxic glutamate release, neurons secrete vesicles containing miR124a, which is uptakes by astrocytes and indirectly enhances GLT1 levels. During pathological conditions miR124a and GLT1 down regulation in astrocytes have been observed with consequent glutamate toxicity. In addition, in the neuroinflammation context, microglia produce higher levels of inflammatory TNFa, trough indirect targeting of increased miR125b and miR365. In muscle cells, miR206 has been observed up regulated in response to neuromuscular junction damage and acts through HDAC4 suppression to cells regeneration. On the contrary, miR23a up-regulation inhibits PGC1alpha with impairment of mitochondrial function in muscle and damage escalation (figure from Volontè et al., 2015).

4

miRNAs as Peripheral Biomarkers

MiRNA molecules are present in human body fluids in a remarkably stable form. Recent evidence indicates that miRNAs can reveal changes in the cells and tissue of origin. Although blood is the handiest and most easily accessible human body fluid, in the case of CNS disorders, miRNAs detected in CSF seem to better reflect brain physiological and pathological conditions, representing a more sensitive marker of changes than those revealed in blood or other body fluids (Rao et al., 2013).

In the last few years, miRNAs have aroused great interest as potential biomarkers in neurological disorders. In the light of the results achieved for various neurodegenerative disorders spread from Multiple Sclerosis, Alzheimer's disease and Huntington's diseases (Cloutier et al., 2015), several studies were conducted in blood from ALS patients in order to find a specific signature of neurodegeneration and different miRNAs were proposed as potential biomarkers of disease. Different sources were investigated as blood cells, serum and plasma with different miRNA profiling techniques (microarray or qRT-PCR) obtaining not always congruent results.

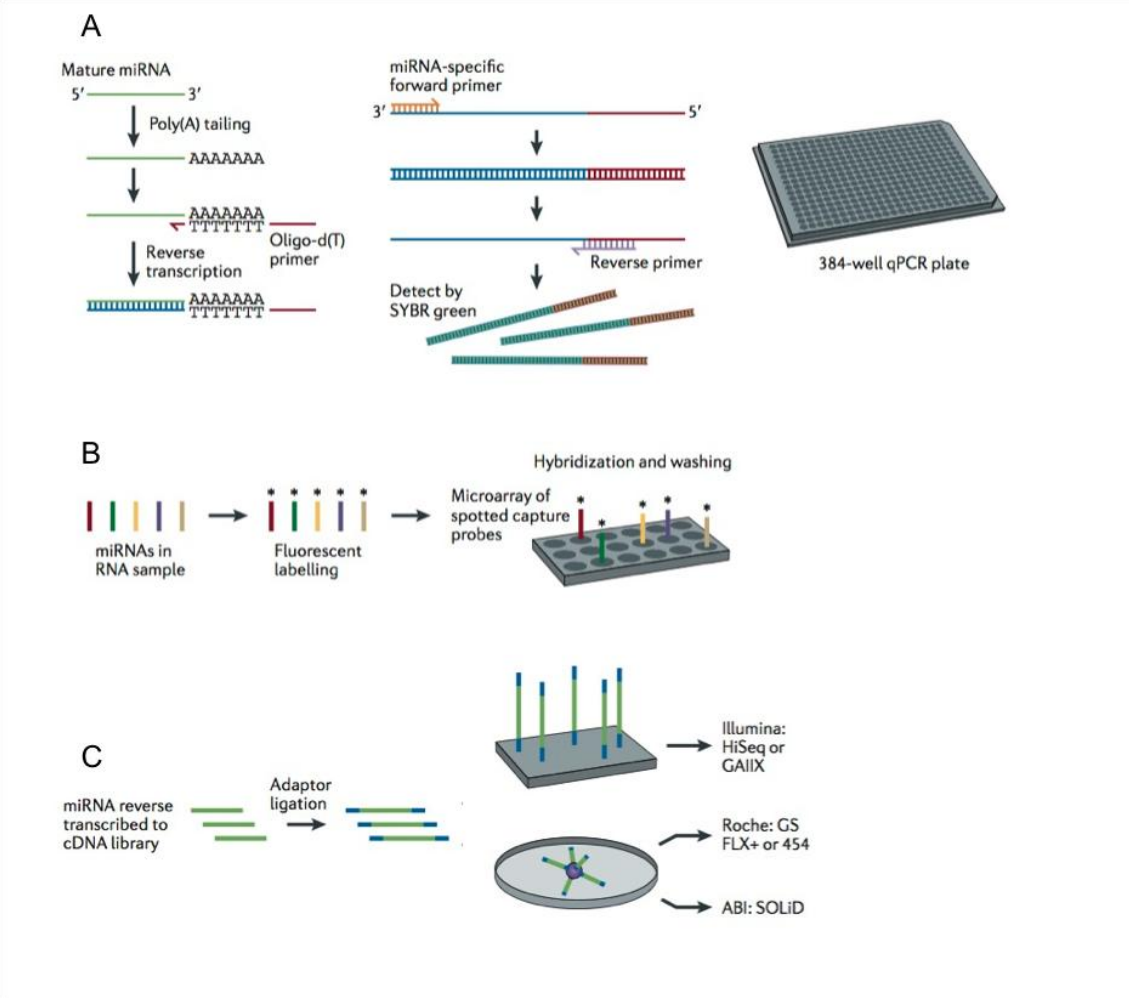
Different Approaches for Identification of miRNA Biomarkers

The most promising approach for identification of miRNA biomarkers is miRNA profiling, defined as the measurement of the relative abundance of a group of miRNAs detectable in cell-free biofluids (Pritchard et al., 2012). It has become evident that the use of a complex set of biomarkers, rather than the use of a single marker, may improve the accuracy, specificity and sensitivity of the analysis and contain more exhaustive diagnostic information (Keller et al., 2009). Quantitative reverse transcription PCR (qRT-PCR), miRNA microarray and RNA sequencing (RNA-seq) are the better known and characterized approaches for miRNA profiling (Pritchard et al.,

2012). In **Fig. 5** vantages and disadvantages of the three different approaches are reported.

Several miRNA profiling in biomarker discovery employed the quantitative reverse transcription PCR (qRT-PCR) in TaqMan qRT-PCR or in SYBR-green-based qRT-PCR. The reverse transcription reaction differs between the approaches, indeed the TaqMan assay usually uses stem–loop probes that are specific to the 3'end of the miRNA, while in the SYBR-green assay the miRNA molecules are typically polyadenylated at the 3'end, and oligo-d(T) is used as a reverse transcription primer. For TaqMan assay, amplicons are generated by using miRNA-specific forward primer and a reverse primer. The DNA polymerase, proceeding along the template, hydrolyses the probe and fluorescent dye is free from the quencher, resulting in detectable light emission. A miRNA-specific forward primer and a reverse oligo-d(T) primer are employed for SYBR-green assay. The universal reverse primer anneals to the 3'portion of the miRNA sequence and the poly(A) tail enables PCR amplification with dsDNA-intercalating SYBR green dye as the detector.

Fig. 5 Vantages and disadvantages of miRNA-profiling approaches



A. The qRT-PCR technique presents more advantages than disadvantages. In particular, it is an established, sensitive and specific method that allows the direct quantification of molecules; it allows the amplification starting from a very low quantity of RNA (<ng) and the data analyses are relatively rapid and of easy interpretation; the technique is cheaper than the others and both TaqMan and SYBR-green-based qRT-PCR are available in 'array' format. This is particularly important for the miRNA profiling studies since it is possible to quantify a specific group of well-known and best-characterized miRNA molecules that are deeply described into various biological pathways. The main limitations of the technique are that novel miRNAs cannot be identified and the number of samples processed per day is not very high. **B.** MiRNA microarray is an established method, fairly low-cost and high-throughput. However, it has lower specificity compared to qRT-PCR or RNA sequencing, the difficulty to use for absolute quantification and the inability to identify novel miRNAs. **C.** The RNA sequencing (RNA-seq) is the only approach able to detect novel miRNAs with high accuracy in distinguishing miRNAs presenting very similar sequences. The main disadvantages of this technique are the substantial computational support for data analysis, the inability for absolute quantification and the high cost of the instrumentation and reagents compared to the other techniques (modified figure from Pritchard et al., 2012).

ALS miRNA Biomarkers Studies in Blood

Starting from different populations of blood cells two important studies were conducted in order to identify a specific pattern of deregulated miRNAs in ALS. The first study (Butovsky et al., 2012) selected a specific population of blood-sorted monocytes (CD14+CD16-) to quantify the deregulated miRNAs by qRT-PCR previously identified in monocytes cells of ALS-model. The deregulated miRNAs selected in SOD1 mice included let-7, miR15b, miR16, miR27a, miR34a, miR132, miR146a, miR155, miR223, and miR451. In human cohorts, the 664 miRNAs analysed were quantified in 8 samples from ALS patients, 8 from patients with multiple sclerosis and 8 from healthy controls. Half of miRNAs that constituted the specific signature in SOD1 mice was also observed in ALS. The human miRNAs signature included miR27a, miR155, miR146a, and miR532-3p, with miR27a highly upregulated in ALS and not expressed in healthy controls and subjects with multiple sclerosis. The second study, instead, analysed the miRNA expression profiled by qRT-PCR in circulating leucocytes from SALS patients. The comparison between patients and controls revealed a group of seven miRNAs downregulated (miR451, 1275, 328-5p, 638, 149 and 665) and miR338-3p upregulated (de Felice et al., 2012). Subsequent studies expanded the quantification analysis of miR338-3p (Shioya et al., 2010) to serum, CSF and brain tissue samples from ALS patients, and the upregulation was confirmed in all the examined samples (de Felice et al., 2014).

MiRNA molecules have the peculiarity of circulating in several body fluids in cell-free forms, since they are resistant to RNAse activity, and packaged into micro vesicles. Moreover, miRNAs usually reflect the healthy condition of origin tissue. In the light of these considerations three recent miRNA profiling studies were performed: two from serum and one from plasma samples of ALS patients. The first study compared the miRNAs levels in serum samples of FALS patients, asymptomatic mutation carriers and healthy controls. A homogenous miRNA signature was found in FALS independently from the underlying disease gene. In particular, the pre-manifest ALS mutation carriers presented 24 significantly downregulated microRNAs up to at least two decades before the supposed time of disease onset and more than 90% of the downregulated microRNAs in mutation carriers overlapped with the patients with FALS (Freischmidt et al., 2014). The second study characterized a group of selected

circulating miRNAs in 18 serum samples from SALS patients and 16 serum samples from controls. Researchers showed that different miRNAs were present among FALS and SALS cases and that miRNA signature of sporadic ALS was highly heterogeneous. However, two miRNAs, miR1234-3p and miR1825, resulted significantly downregulated in SALS and these miRNAs were proposed as biomarkers for SALS (Freischmidt A et al., 2015). The same research group conducted both studies employing the microarray technique to profile the serum-miRNAs. The third recent miRNA profiling was performed on 16 plasma samples from SALS patients and 10 from healthy controls. The experimental plan was divided in two phases: the discovery step with a discovery cohort analysed by microarray and the validation step conducted by qRT-PCR in a validation cohort of 48 SALS patients, 47 healthy controls and 30 disease controls. A total of three miRNAs resulted significantly upregulated (miR4258, 663b and 4649-5p) and six were downregulated (miR26b-5p, 4299, 4419, 3187-5p, 4496 and let-7f-5p) in the discovery cohort. Following validation experiments confirmed the significant upregulation of miR4649-5p, and the significant downregulation of miR4299. This couple of miRNAs were suggested as potential biomarkers for ALS (Takahashi et al., 2015). Beyond the potential role as biomarkers it has been observed that the most notable target gene of these miRNAs was EPH receptor A4 (ephrin type-A receptor 4) (*EPHA4*), which was reported to be a disease modifier of ALS (Van Hoecke et al., 2012).

A specific peripheral miRNA-deregulation was described in four other works. In the first study, in which ALS patients were enrolled as control group (Gandhi R et al., 2013), the miRNA levels in monocytes from ALS patients (n=15), RRMS (n=50), SPMS (n=51) and healthy controls (n=32) were analysed by qRT-PCR. The aim of the study consisted in the selection of deregulated molecules to develop immune biomarkers to monitor multiple sclerosis (RRMS - relapsing–remitting multiple sclerosis- and SPMS –secondary progressive multiple sclerosis-). Among the 16-deregulated miRNAs between MS and ALS samples, the miR-92 and let-7 resulted differentially expressed in RRMS but no differences between SPMS and ALS were reported, suggesting that similar processes may occur in SPMS and ALS. In the second study, nine different TDP-43 binding miRNAs were amplified by qRT-PCR and miR132-5p, miR132-3p, miR143-5p, miR143-3p and let7b-5p resulted specifically deregulated in serum of

ALS patients (Freischmidt et al., 2013). The third study (Toivonen et al., 2014), instead, consisted in the validation experiment for miR206 by qRT-PCR. The miR206, previously shown as upregulated in SOD1 mouse model during the initial time after denervation (Williams et al., 2009), resulted also increased in a group of 12 serum samples from ALS patients and suggested as a potential biomarker of neuromuscular damage corroborating the results previously reported by Williams and colleagues. Lastly, in a recent work the quantification of a muscle-specific set of miRNAs (miR-206, miR-1, miR-133a/b, miR-27a) has been performed by qRT-PCR in serum from SALS. The expression levels of miR-206 and miR-133 resulted significantly increased while miR-27a was significantly reduced as compared to controls (Tasca et al., 2016).

ALS MIRNA BIOMARKERS STUDIES IN CSF

Expression levels of miRNAs in CSF was investigated in MS (Haghikia et al., 2012), AD (Cogswell et al., 2008; Müller et al., 2014; Burgos et al., 2014; Denk et al., 2015) and Parkinson's Disease (PD) (Burgos et al., 2014), with promising results.

To our knowledge no CSF miRNA profiling experiments have been published for ALS in the Medical Literature Analysis and Retrieval System Online (MEDLINE) database. The miRNA quantification by qRT-PCR on CSF sample from ALS patients was reported for TDP-43 binding miRNAs studies (Freischmidt et al., 2013), and for miR338-3p to verify the miRNA deregulation in CSF samples from ALS patients (de Felice et al., 2014). Thus, Freischmidt and colleagues revealed a significant deregulation of miR132-5p and 3p, miR143-5p and 3p, miR574-5p and let7b-5p in CSF from ALS and de Felice and colleagues confirmed the miR338-3p upregulation in CSF from ALS.

However, it is worth noticing that conducting by generalized research on the Internet indicating as keywords "CSF-microRNA-profiling-Amyotrophic-Lateral-Sclerosis" was found a report entitled "Profiling of miRNAs in Cerebrospinal Fluid from Patients with Amyotrophic Lateral Sclerosis" conducted by Machida and colleagues (Machida et al., 2015). The study was performed on CSF samples from 23 ALS patients and 10 normal controls using microarray technique (3D-GENE, Torray). The profiling analysis identified twenty-nine ALS-miRNAs, of which eleven were detected only in

ALS patients, twenty-five were upregulated and four downregulated (data not shown) in ALS patients. They suggested that the combination use of five miRNAs (miR10a, miR516b, miR24-2*, miR122* and miR4762-3p) exclusively detected in CSF from ALS patients are able to best differentiate healthy controls from patients with a sensitivity of 73,9% and specificity of 100%.

PART II

AIM OF THE THESIS

At present there is no diagnostic laboratory test for ALS and the variability of clinical presentation often makes early diagnosis difficult. Thus, the scientific research on ALS points to the identification of specific biomarkers, which can recognize ALS before the symptom manifestation and/or discriminate ALS from other pathologies, providing a valuable tool in differential diagnosis.

The identification of a specific miRNA profile in ALS that may have a strong impact on the biomarker research field and that may provide insight into the disease pathogenesis has been the main objective of this work of thesis.

In detail, the PhD research has been focused on the two following specific topics:

- miRNA profiling of Cerebrospinal Fluid pooled samples and serum pooled samples from ALS patients and healthy controls by qRT-PCR,

- validation of the miRNAs selected in CSF and serum profiling in each single sample from ALS patients and controls included in the pools.

PART III

METHODS

1

PATIENT DATA AND SAMPLES

CSF samples from 24 sALS and Serum samples from 24 sALS patients were available for the present study. ALS diagnosis was performed according to World Federation of Neurology El Escorial Revised criteria, and patients with no evidence of a family history of the disease were classified as sporadic (Brooks et al., 2000). The Sanger sequencing and repeat-primed PCR methods were employed to screen case and control samples for the presence of *SOD1*, *TARDBP-43* and *FUS* mutations (Chiò et al., 2014) and the GGGGCC hexanucleotide repeat expansion in *C9orf72* (DeJesus-Hernandez et al., 2011) respectively.

Both for CSF and for Serum samples, among the 24 patients included in the study, 8 were positive for *C9orf72* expansion. The unaffected control groups consisted of 24 age- and sex-matched CSF samples from subjects who underwent lumbar puncture for neurological or microbiological diagnostic purposes and were negative for all the performed tests. The serum control group included samples from 24 age- and sex-matched healthy voluntary subjects for which were excluded neurological disorders. At the enrolment time, all participants did not have manifested neurodegenerative disorders, infections of hepatitis B virus (HBV), hepatitis C virus (HCV), human immunodeficiency virus (HIV) or human T-cell leukemia virus type 1 (HTLV-I). The Characteristics of ALS patients and controls enrolled for CSF study are reported in **Tab. 1** while in **Tab. 2** there are the characteristics of patients and controls for Serum study.

The CSF, free of blood contamination, was centrifuged (1600 xg, 4°C, 15 min), frozen within 40 min of collection and stored at -80°C until use. The serum samples not haemolysed, drawn using vacuum systems, were centrifuged (2000 xg, 4°C, 10 min), the supernatant was aliquoted (0.5 ml) and stored immediately at -80°C until use. All procedures from withdrawal to storage of both CSF and serum samples were

performed according to the Guidelines for CSF and blood Biobanking for Biomarker Research (Teunissen et al., 2014).

miRNA profiling was performed in a total of six pooled CSF samples and six pooled Serum samples (total volume 200 µl), consisting of three pools derived from ALS patients and three pools from control subjects. Each pool was composed of an equal volume (25µl) of four CSF or serum samples from females and four CSF or serum samples from males. The subsequent miRNA validation experiments were carried out using 200µl of CSF or serum from each individual.

Written consent was obtained from each participant. This study was approved by the local ethics committee in accordance with the ethical standards of the Declaration of Helsinki and was carried out according to the international Good Laboratory Practice (GLP) and Good Clinical Practice (GCP) standards.

Tab. 1 Characteristics of ALS patients and controls enrolled for CSF study

	Patients w/o mutation	***C9orf72* patients**	**Control subjects**
Nº of subjects	16	8	24
Gender	8M/8F	4M/4F	12M/12F
Age mean ± SD (years)	63.94±8.45	60.50±8.50	58.25±5.04
Age at onset	59.0±14.4	57.55±12.6	
Bulbar onset	25.0%	25.0%	
Disease duration (months)	41.5±20.5	47.0±19.7	

Tab. 2 Characteristics of ALS patients and controls enrolled for Serum study

	Patients w/o mutation	***C9orf72* patients**	**Control subjects**
Nº of subjects	16	8	24
Gender	8M/8F	4M/4F	12M/12F
Age mean ± SD (years)	64.25±14.99	62.20±9.01	54.38±8.44
Age at onset	61.15±10.2	58.55±11.5	
Bulbar onset	25.0%	25.0%	
Disease duration (months)	40.5±15.7	47.5±20.2	

2

RNA EXTRACTION AND REVERSE TRANSCRIPTION

Total RNA from CSF was isolated employing the miRNeasy Mini kit (Qiagen) while miRNeasy Serum/Plasma kit (Qiagen) was employed for total RNA extraction from serum samples. Synthetic exogenous Ce-miR-39 miRNA (Cel-miR-39-3p) from *Cenorabditis Elegans* was added into each sample according to the manufacturer's recommendations. Purified RNA was stored at –80°C in RNase-free water. We quantified total isolated RNA using a spectrophotometer and evaluated the RNA quality by O.D.260/280 ratio. Standard procedures were employed to ensure the quality and the reproducibility of the pre-analytical step.

Isolated RNA was used as starting material for reverse transcription (RT) employing the miScript II RT kit (Qiagen) that provides the polyadenylation of mature miRNAs and reverse transcription into cDNA using oligo-dT primers. Following the manufacturer's recommendations, 4,7 µl of isolated RNA were added to the reverse-transcription reaction, for each RNA samples isolated from 200 µl of starting materials (CSF or Serum samples). The components of RT reaction master mix reported in **Tab 3.1** and cycling conditions of RT reaction in **Tab 3.2**. The cDNA samples obtained by reverse transcription were stored undiluted at -20°C and thereafter diluted by adding 90µl of RNase-free water to each 20 µl, prior to real-time PCR.

In order to check the quality of performed procedures we used miScript miRNA QC PCR Array (Qiagen) that ensures the selection of only high-quality samples, employable in subsequent experiments, by testing the quality of RNA isolation and cDNA preparation. A total of 32 cDNA samples were analysed on one 384-well miScript miRNA QC PCR Array utilizing 5 µl of cDNA appropriately diluted.

Tab. 3.1 RT reaction master mix components

Component	Volume/reaction
5x miScript HiSpec Buffer	4 µl
10x miScript Nucleics Mix	2 µl
RNase-free water	7.3 µl
miScript Reverse Transcriptase Mix	2 µl
Template RNA	4.7 µl
Total volume	20 µl

Tab. 3.2 Cycling conditions of RT reaction

Step	Time	Temperature
Incubation	60 min	37°C
Incubation	5 min	95°C

3

MIRNA PROFILING BY QPCR

cDNA samples prepared by RT reaction from RNA of pools were utilized as the template for real-time quantitative PCR (qPCR) analysis, performed in the 7900HT Fast Real-Time PCR System (Life Technologies) using the human-miFinder 384HC miRNA PCR array (Qiagen), which profiles the expression of the 372 most abundantly expressed and best-characterized miRNAs in miRBase (www.mirbase.org). According to the manufacturer's recommendations, a final volume of 10µl containing cDNA properly diluted and SYBR Green-based real-time PCR (Qiagen) was dispensed on human-miFinder PCR array. The components of qPCR reaction master mix are reported in **Tab. 4.1** and the qPCR amplification conditions in **Tab 4.2.**

In CSF and Serum-miRNA profiling, after setting the baseline and the threshold, exponential processes (Ct-values) were converted to linear comparisons relative to the control group. Thus, the Ct-values were normalized to the Cel-miR-39-3p miRNA using relative expression quantification ($2^{-\Delta\Delta Ct}$) method and it is the most utilized exogenous control for the normalization in miRNA profiling study.

Free software miScript miRNA PCR Array Data Analysis (Qiagen), available at http://pcrdataanalysis.sabiosciences.com/mirna, was utilized for the analysis of

qPCR results of miRNA profiling experiments. Only miRNAs with Ct <35 and with a high efficiency amplification plot were taken into consideration for subsequent analysis. The ALS patients and control groups were compared using Student t-test and the cut off for the *p-value* was set at 0.05.

Tab. 4.1 Real-time PCR reaction mixture components for human-miFinder 384HC miRNA PCR array

Component	384-well Array
2x QuantiTect SYBR Green PCR Master Mix	2050 µl
10x miScript Universal Primer	410 µl
RNase-free water	1540 µl
Template cDNA	100 µl
Total volume	4100 µl

Tab. 4.2 Cycling conditions used to program the thermal cycler to perform qRT-PCR.

Step	Time	Temperature
PCR Initial activation step	15 min	95°C*
3-step cycling		
Denaturation	15 s	94°C
Annealing	30 s	55°C
Extension	30 s	70°C§
Cycle number	40	

*HotStarTaq DNA Polymerase is activated by this heating step; § fluorescence data collection.

4

miRNA ENDOGENOUS CONTROLS SELECTION

Relative expression quantification ($2^{-\Delta\Delta Ct}$) method was performed also in miRNA validation experiments employing both exogenous and endogenous controls (EC). The EC is one or a cluster of endogenous RNA molecules resulted the most stable and reproducible across different samples and experiments.

For accurate results in relative quantification it is necessary to normalize the amount of target miRNA by using a suitable EC. Indeed, the normalization with EC corrects for factors that could lead to inaccurate quantification, including variation in RNA

content, possible RNA degradation, presence of inhibitors in the RNA samples, and differences in sample handling. Normalization also allows the comparison among results from different experiments and samples.

Thus, free data analysis by the web interface RefFinder was employed to identify the best endogenous controls from a cluster of miRNAs selected in CSF and Serum profiling. This software integrates the major computational programs (geNorm, Normfinder, BestKeeper and comparative ΔΔCt method) to compare and rank the tested candidate reference genes on each single patient and control. Based on the rankings from each program, it assigns an appropriate weight to an individual gene and calculated the geometric mean of their weights for the overall final ranking using Recommended Comprehensive Ranking (RCR) method (Xie et al., 2012).

5

MIRNA VALIDATION

Validation experiments of miRNAs selected in the profiling experiments were performed in triplicate by SYBR Green-based real-time PCR, analysing individually CSF and Serum samples obtained from each subject. The exogenous and the selected endogenous controls were used in the validation experiments to normalize miRNA expression values.

In validation experiments the relative quantification was carried out using Data Assist v3.0 (Life Technologies). The Ct values of each mature miRNA were normalized to Ct value of both the exogenous and endogenous controls $\Delta^{Ct} = Ct^{miRNA} - Ct^{EEC}$. The normalized miRNA-Ct value (Δ^{Ct}) of our samples was compared to the Δ^{Ct} value of a calibrator according the formula $\Delta\Delta^{Ct} = \Delta^{Ct\ Sample} - \Delta^{Ct\ Calibrator}$. The control group mean was used as the calibrator in the calculation of $\Delta\Delta^{Ct}$. The fold change was calculated with the following formula $2^{-\Delta\Delta Ct}$. The resulting value was the relative expression

quantification of each miRNA obtained by comparing patients and controls. MiRNAs were considered downregulated for fold change values ≤0.67 and upregulated for values >1.5 and the miRNA molecules with Ct >35 were excluded from subsequent data analyses.

6

STATISTICAL ANALYSIS FOR miRNA VALIDATION

All statistical analyses were performed with SPSS (Version 18; SPSS Inc) and GraphPad Prism (Version 5.0). The Gaussian distribution of data was assessed by the Shapiro–Wilk test. Statistical differences were verified by Student's unpaired two-tailed t-test in the case of normal distributions, or two-tailed Mann–Whitney U test in the case of non-normal distributions. Analysis was performed for every miRNA, and ratios between different miRNAs were examined to identify any specific characteristics in ALS. The ratio between miRNAs was calculated as $2^{-\Delta Ct}$ (ΔCt= Ct of upregulated miRNA - Ct of downregulated miRNA), as described by Sheinerman et al., 2013.

Receiver operating characteristic (ROC) curves were plotted to evaluate the power of miRNAs (singles or in combination) to differentiate ALS patients from controls. The ROC curves are summarized into a single metric known as the: Area Under the Curve (AUC). The AUC is an effective and combined measure of sensitivity and specificity that describes the inherent validity of diagnostic tests and a guide for assessing the utility of a biomarker based on its AUC is as follows: 0.9–1.0 = excellent; 0.8–0.9 = good; 0.7–0.8 = fair; 0.6–0.7 = poor; 0.5–0.6 = fail (Xia et al., 2013).

Differences among groups of patients, stratified based on *C9orf72* expansion and clinical features (gender, site of onset), were evaluated by Student's unpaired two-tailed t-test or two-tailed Mann–Whitney U test in the case of normal distributions or

non-normal distributions, respectively. Spearman's rho (*r*) was calculated to find correlation between miRNA expression level and age at onset. Association of each single miRNA expression level with disease duration was estimated using the Kaplan-Meier method and compared by the log-rank. Patients were divided into high miRNA expression group (miRNA levels greater than the median) or low miRNA expression group (miRNA levels less than the median). *p-values* smaller than 0.05 were considered statistically significant.

PART IV

RESULTS

1

miRNA PROFILING AND IDENTIFICATION OF DIFFERENTIALLY EXPRESSED miRNAs

CSF PROFILING

The CSF profiling permitted the amplification by real-time PCR of 35 (9.4%) and 39 (10.4%) miRNAs in CSF pools from ALS patients and control subjects respectively, for a total of 42 out of 372 (11%) miRNAs positively detected in our sample cohort. No detectable traces or traces with Ct >35 were identified for 330 miRNA molecules. Furthermore, our results showed an overall down-regulation of miRNAs in CSF from ALS patients compared to controls; indeed the majority of deregulated miRNAs were downregulated. The results of real-time PCR amplification of CSF-miRNA profiling are reported in **Fig. 6A**.

From miRNA profiling analysis, we selected thirteen downregulated miRNAs (let7a-5p, let7b-5p, let7f-5p, miR15b-5p, miR21-5p, miR122-3p, miR127-3p, miR148a-3p, miR150-5p, miR183-3p, miR195a-5p, miR204-5p, miR373-5p) and one upregulated miRNA (miR181a-5p), using a fold change threshold >1.5. Among these deregulated miRNAs, three (let7-a, miR195a-5p and miR21-5p) were significantly downregulated with a *p-value* of 0.0023, 0.039 and 0.030, respectively. The changes in the other eleven miRNAs did not reach statistical significance even though they were up- or down-regulated with a fold change >1.5. The results of CSF-miRNA profiling statistical analysis are graphed in the *Volcano Plot* reported in **Fig. 7A**.

SERUM PROFILING

In the Serum profiling, 223 (59.9%) miRNAs from ALS pools of patients and 218 (58.6%) miRNAs from pools of control subjects were amplified, for a total of 227 out of 372 (61%) miRNAs positively detected in our sample cohort. No detectable traces

or traces with Ct >35 were identified for 145 miRNA molecules. Contrary to the CSF profiling, an overall up-regulation of miRNAs was observed in serum from ALS patients compared to controls, as shown in **Fig. 6B**.

Serum miRNA profiling analysis permitted the selection of eleven upregulated miRNAs (let7b-5p, let7f-5p, miR15b-5p, miR16-5p, miR27a, miR30c-5p, miR122-3p, miR197-3p, miR223-3p, miR328-3p, miR373-5p) and one downregulated miRNA (miR125b-5p), using a fold change threshold >1.5. No miRNAs reached statistical significance in serum profiling. The *Volcano Plot* reported in **Fig. 7B** graphs the results from statistical analysis for serum profiling.

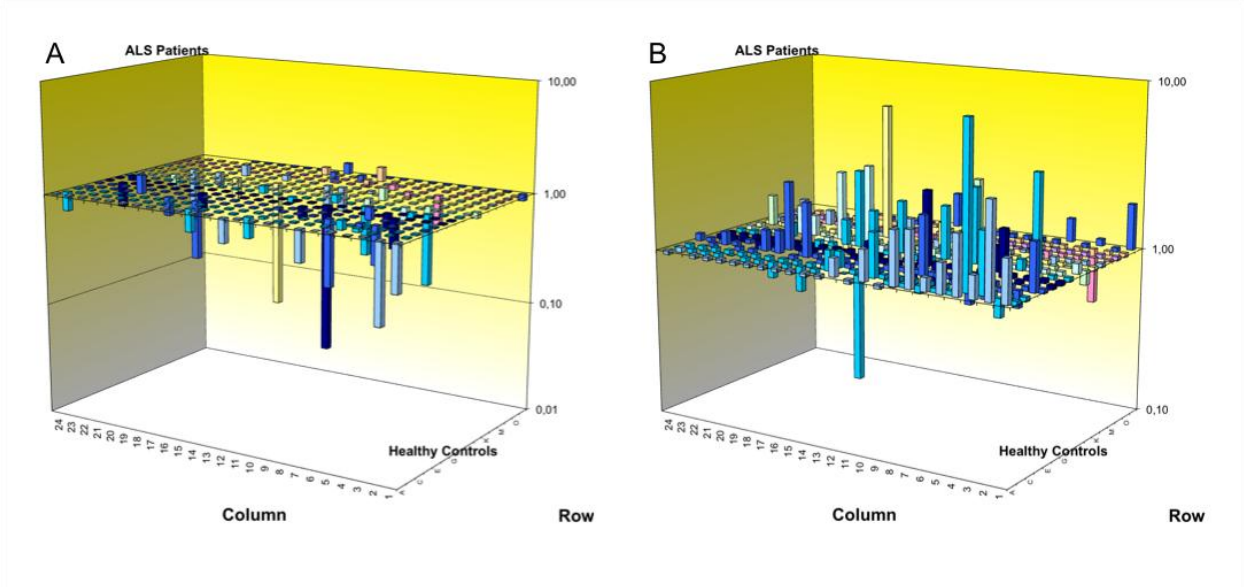


Fig. 6 The *3D Profile* of fold difference in expression of each miRNA between patients and controls in the 384-well format of the PCR Array. The graph indicated by **A** is referred to CSF miRNA profiling while **B** regards serum miRNA profiling. The *3D Profile* graphs the fold difference in miRNAs expression between patients and controls. Columns pointing up (with z-axis value>1) indicate a miRNA up-regulation and columns pointing down (with z-axis value<1) indicate a miRNA down-regulation.

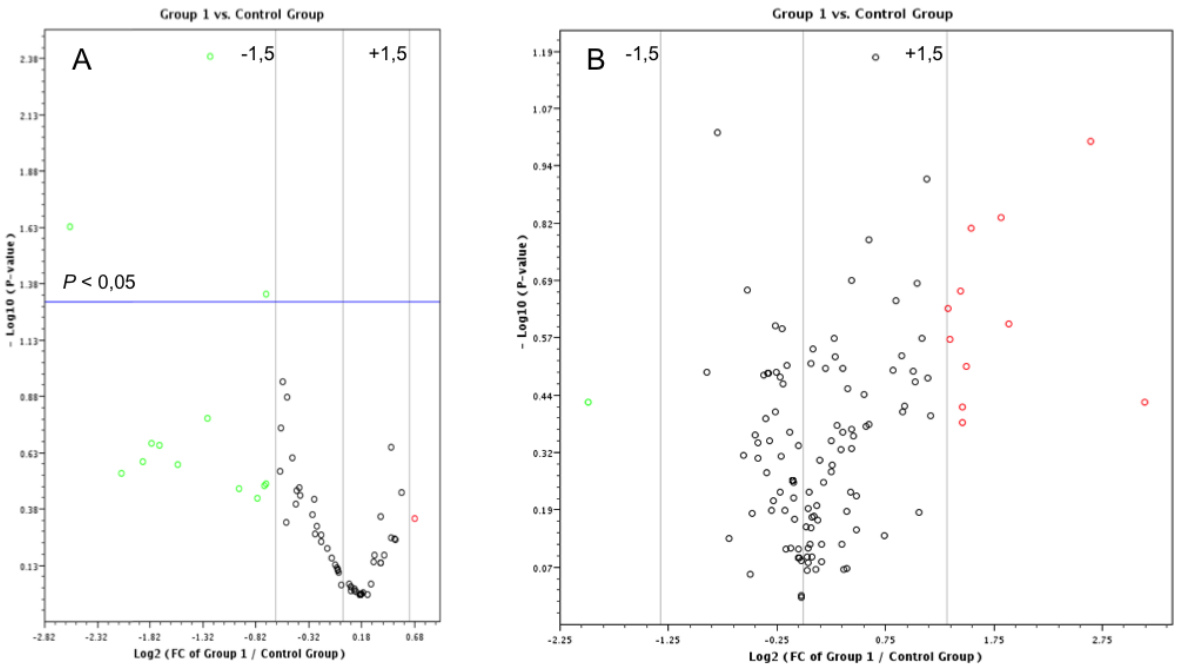


Fig. 7 *Volcano Plot* of $\log_2$ of the fold change for each miRNA and its *p* value. In **A** is reported the *Volcano Plot* from CSF-miRNA profiling results and in **B** the Plot from Serum profiling. The *p*-value is calculated based on the Student's t-test of the replicate $2^{(-\Delta Ct)}$ values for each miRNA in the control group and patient group. The central line in the graph indicates a fold change of 1. Vertical sliders indicate miRNAs as either up- or down- regulated with a fold change >1.5. The spots beyond right vertical slider indicate the upregulated miRNAs and the spots beyond the left slider the downregulated miRNAs. The horizontal line, parallel to the x-axis, indicates the desired threshold for the *p-value* of the Student's t-test, defined <0.05. The Volcano Plot was generated by the software miScript miRNA PCR Array Data Analysis (Qiagen).

2

miRNA ENDOGENOUS CONTROL SELECTION FOR VALIDATION EXPERIMENTS

After miRNA profiling for CSF and serum, we selected the best candidate miRNAs to use as endogenous controls for normalization in Validation phase. From the CSF profiling the miR608 and miR328-3p were selected by RefFinder from a panel of four potential endogenous references, since their expression showed high stability and reproducibility across different samples and experiments **(Fig. 8A)**. The same software selected miR608 and miR489-3p as the best endogenous controls from serum profiling into a cluster of six potential endogenous controls **(Fig. 8B)**. Both for the CSF and serum normalization, in validation experiments the Synthetic exogenous Cel-miR-39-3p was also employed as control.

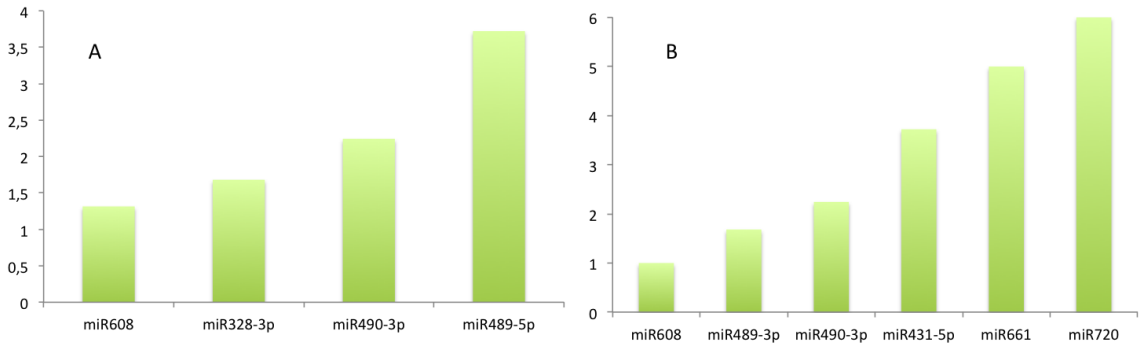


Fig. 8 Endogenous controls selection with RefFinder. In **A** is reported the bar diagram for Endogenus controls selection for CSF, in **B** is reported the bar diagram for Serum. The graph reports the overall final ranking of the candidate reference miRNAs, using Recommended Comprehensive Ranking (RCR) method. The RCR method measures the stability using the Geometric mean method. A lower Geometric mean value denotes more stable expression. The x-axis reports the candidate reference miRNAs and the y-axis shows the Geometric mean value.

3

EXPRESSION PROFILES OF SELECTED CSF MIRNAS

The fourteen deregulated miRNAs were validated in CSF samples obtained from each subject by qPCR. These miRNAs with a fold change ≤0.67 were considered downregulated and miRNAs with a fold change >1.5 were considered upregulated. The deregulation observed in the profiling was confirmed, with overall miRNA downregulation and only miR181a-5p upregulated **(Fig. 9)**. Among the fourteen selected molecules, eight miRNAs were significantly deregulated in ALS patients compared to controls. In particular, changes in miR21-5p, miR195-5p and let7a-5p, which were significantly downregulated in miRNA profiling, were confirmed in the validation experiments, and a significant downregulation was also reported for miR148-3p, miR15b-5p, let7b-5p and let7f-5p. The miRNA181a-5p was confirmed as upregulated in CSF from ALS patients (**Fig. 10**). No significant differences were evidenced for the other six miRNAs included in the validation experiments.

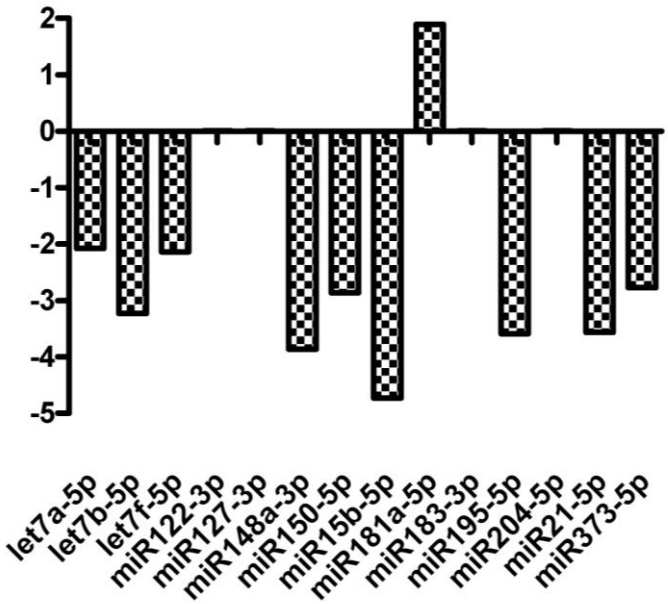


Fig 9. Results of miRNA validation experiments of CSF. Bar diagram shows the relative expression levels of miRNAs selected with profiling and validated in each single CSF sample. The y-axis log2 of relative expression levels is reported, considering the control group as calibrator. The data obtained in the profiling were confirmed for the majority of the selected miRNAs.

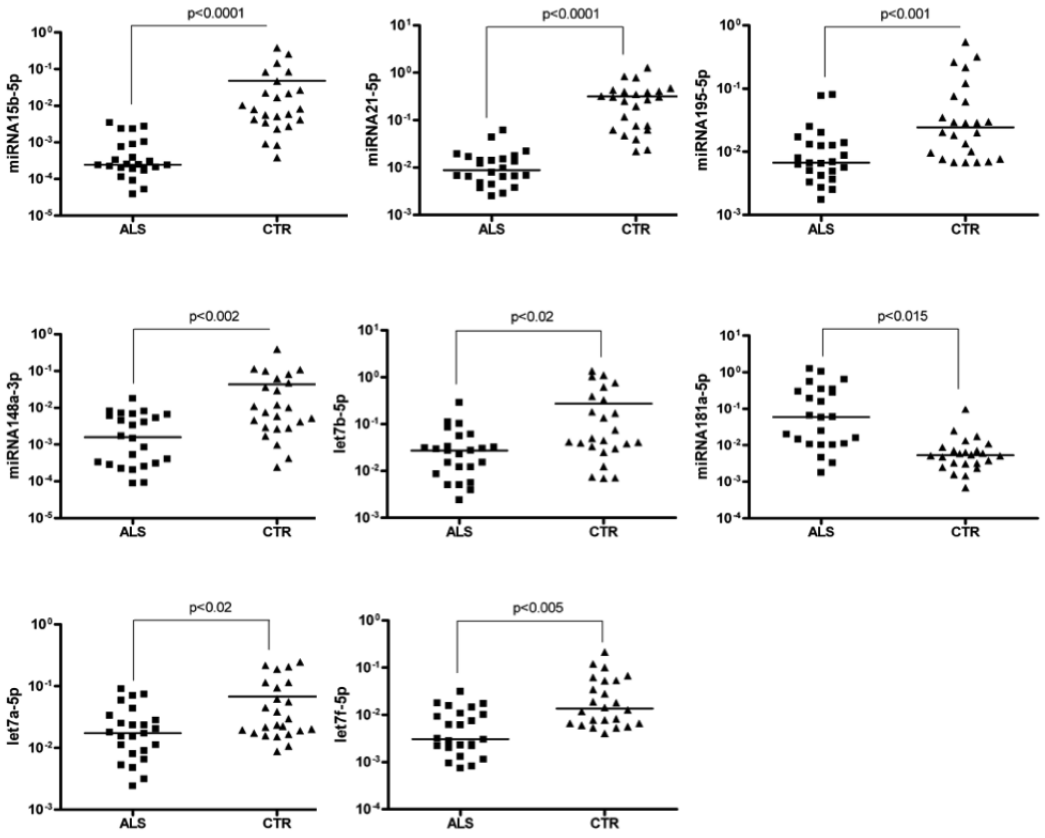


Fig. 10 Scatter graphs of results of validation experiments for the significantly deregulated CSF miRNAs. The *p-values* were calculated using two-tailed Mann–Whitney U test. Relative expression of deregulated miRNAs in CSF from ALS patients (ALS) and controls (CTR) normalized by $2^{-\Delta Ct}$ are graphed. Medians are indicated as horizontal lines.

The levels of the eight deregulated miRNAs were analysed in the ALS patient group to evaluate possible correlations with *C9orf72* expansion and clinical variables. The *C9orf72* repeat expansion, which represents the most frequent genetic alteration in ALS, was present in eight out of the twenty-four patients included in the study. No significant differences in miRNA expression levels were observed between ALS patients with or without the expansion. Statistical analyses failed to find any association of miRNA levels with site of onset, age at onset and disease duration. No statistically significant differences were found between males and females (**Tab. 5**).

The Receiver Operator Characteristic (ROC) curve analyses revealed that all the eight significant miRNAs could be fair/good potential biomarkers for ALS diagnosis giving the following areas under the ROC curve (AUC): let7a-5p (0.70) let7b-5p (0.76) let7f-5p (0.73) miR148-3p (0.78) miR195-5p (0.79). However, among these miRNAs, the upregulated miR181a-5p and the downregulated miR21-5p and miR15b-5p showed the highest diagnostic accuracy (**Fig. 12**). The miR181a-5p levels gave an AUC of 0.81 (95%CI: 0.677– 0.953). At the cutoff value of 6.16, the optimal sensitivity and specificity were 87% and 70% respectively. The downregulated miR21-5p and miR15b-5p gave areas under the ROC of 0.87 (95%CI: 0.761-0.996) and 0.87 (95%CI: 0.757-0.982) respectively. At the cutoff value of 4.01 for miR21-5p, the optimal sensitivity and specificity were 95% and 74%, respectively, and at the cutoff value of 8.17 for miR15b-5p the optimal sensitivity and specificity were 95% and 74%, respectively.

The three miRNAs with the highest diagnostic accuracy, revealed by ROC curve, were analysed in combination. Values obtained from ratios between the upregulated miRNA and the two-downregulated miRNAs were examined comparing patients and controls. A significant deregulation was reported for the analyses of both miR181a-5p/miR21-5p ($p<0.0001$) and miR181a-5p/miR15b-5p ($p<0.0001$). The ROC curve analysis of miR181a-5p/miR21-5p ratio showed an area under the curve of 0.917 (95%CI: 0.836-0.999). At the cutoff value of 0.45, the optimal sensitivity and specificity were 90% and 87% respectively. The miR181a-5p/miR15b-5p ratio gave an area under the ROC of 0.922 (95%CI: 0.844-0.996). The optimal sensitivity and specificity were 85% and 91% respectively, at the cutoff value of 29.8 (**Fig. 12**).

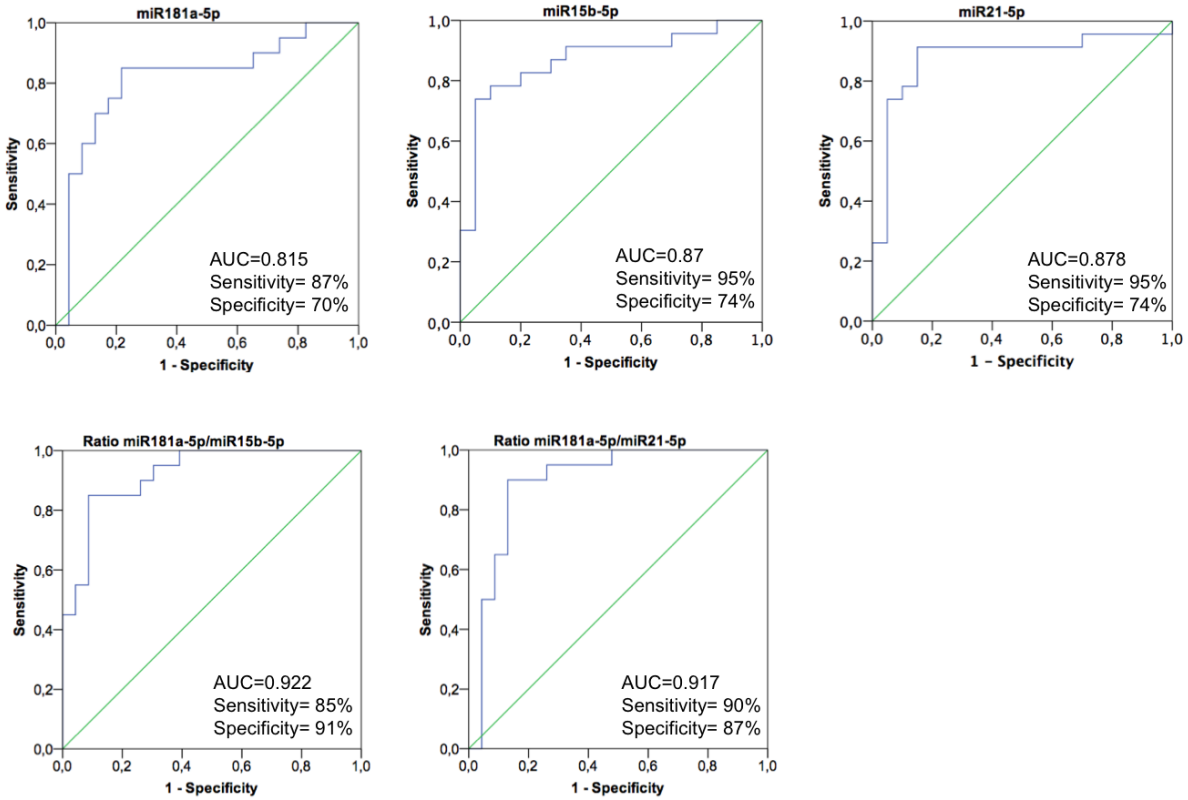


Fig. 12 Receiver operating characteristic (ROC) curve for the upregulated miRNA (miR181a-5p) and the two-downregulated miRNAs (miR15b-5p and miR21-5p), which showed the highest significant differences in validation. For miR181a-5p, the normalized expression level ($2^{-\Delta\Delta Ct}$) was selected as test variable and for the miR21-5p and miR15b-5p the logarithm of the normalized expression level was used. In the case of analyses in combination, ROC curve was obtained for the ratios between miRNAs, calculated as $2^{-\Delta Ct}$ (ΔCt= Ct miR181a-5p - Ct miR21-5p and Ct miR181a-5p - Ct miR15b-5p). AUC (area under the ROC curve), sensitivity and specificity are reported for each ROC curve.

4

EXPRESSION PROFILES OF SELECTED SERUM MIRNAS

The twelve deregulated miRNAs were validated in serum samples obtained from each subject by qPCR. Similarly to CSF validation experiments, miRNAs with a fold change ≤0.67 were considered downregulated and miRNAs with a fold change >1.5 were considered upregulated. The group of miRNAs resulted deregulated from the serum profiling was totally confirmed, with an overall miRNA upregulation and only miR125b-5p downregulated (**Fig. 13**).

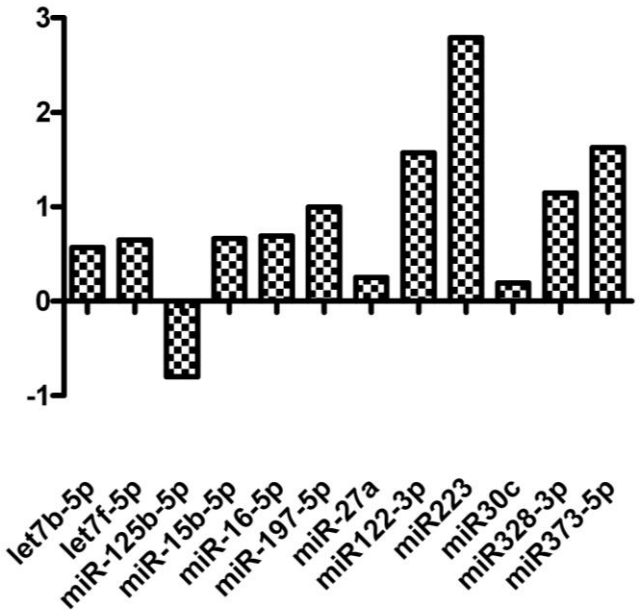


Fig 13. Results of miRNA validation for Serum-miRNAs. Bar diagram shows the relative expression levels of miRNAs selected with profiling and validated in each single serum sample. The y-axis log2 of relative expression levels is reported, considering the control group as calibrator. The data obtained in the profiling were confirmed for the majority of the selected miRNAs.

Among the twelve selected molecules, three miRNAs were significantly deregulated in ALS patients compared to controls (**Fig. 14**): the miR125b-5p was significantly downregulated in ALS patients and a significant upregulation was reported for miR328-3p and miR373-5p. No significant differences were evidenced for the other nine miRNAs included in the validation experiments.

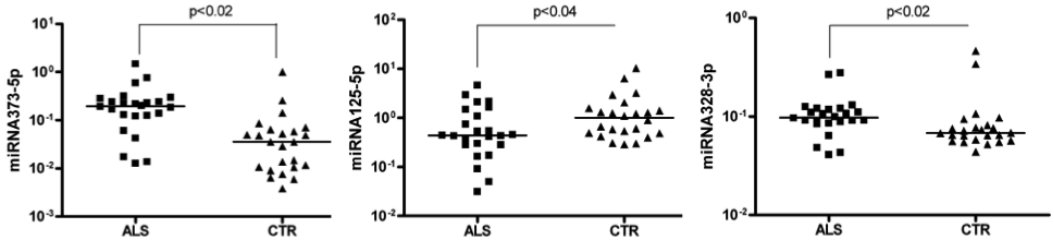


Fig. 14 Scatter graphs of results of validation experiments for the significantly deregulated serum miRNAs. The *p-values* were calculated using two-tailed Mann–Whitney U test. Relative expression of deregulated miRNAs in serum from ALS patients (ALS) and controls (CTR) normalized by $2^{-\Delta Ct}$ are graphed. Medians are indicated as horizontal lines.

The levels of the three deregulated miRNAs were analysed in the ALS patients' cohort to evaluate potential correlations with *C9orf72* expansion and clinical variables. However, no significant differences in miRNA expression levels were observed between ALS patients with or without the expansion. Moreover, statistical analyses did not find any association between miRNA levels and gender, site of onset, age at onset and disease duration (**Tab. 6**).

The Receiver Operator Characteristic (ROC) curve analyses revealed that the three significant miRNAs could be potential biomarkers for ALS diagnosis even of they need of validation in a bigger cohort of patients and controls (**Fig. 15**). The downregulated miR125b-5p levels gave an AUC of 0.66 (95%CI: 0.496– 0.829). At the cutoff value of 0.17, the optimal sensitivity and specificity were 70% and 78% respectively. The upregulated miRNAs: miR328-3p and miR373-5p gave AUCs of 0.74 (95%CI: 0.587-0.909) and 0.79 (95%CI: 0.698-0.946) respectively. At the cutoff value of 0.82 for miR328-3p, the optimal sensitivity and specificity were 85% and 80%, respectively, and at the cutoff value of 1.04 for miR373-5p the optimal sensitivity and specificity were 76% and 80%, respectively.

Since any serum-miRNA showed AUCs superior to 0.8 we decided to extend the analysis to a larger group of patients and controls.

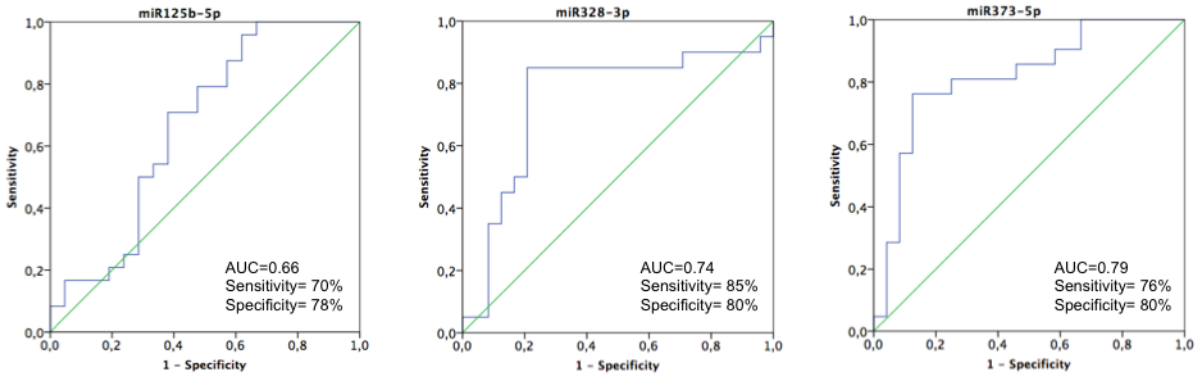


Fig. 15 Receiver operating characteristic (ROC) curve for the downregulated miRNA (miR125b-5p) and the two-upregulated miRNAs (miR328-3p and miR373-5p). For miR125b-5p, the logarithm of the normalized expression level ($2^{-\Delta\Delta Ct}$) was selected as test variable and for the miR328-3p and miR373-5p the normalized expression level was used. AUC (area under the ROC curve), sensitivity and specificity are reported for each ROC curve.

Tab. 5 Correlations between miRNAs level and C9orf72 expansion, and ALS clinical variables in CSF

miRNA	*C9orf7* *	Disease duration	Site of onset	Gender	Age at onset
Let7a-5p	0.750	0.240	0.266	0.436	0.769
Let7b-5p	0.750	0.379	0.267	0.631	0.384
Let7f-5p	0.750	0.253	0.349	0.971	0.658
miR15b-5p	1.000	0.070	0.497	0.684	0.270
miR21-5p	0.437	0.515	0.197	0.481	0.156
miR148a-3p	0.617	0.242	0.230	0.052	0.076
miR181a-5p	1.000	0.542	0.933	0.393	0.137
miR195-5p	0.211	0.745	0.211	0.796	0.286

Tab. 6 Correlations between miRNAs level and C9orf72 expansion, and ALS clinical variables in Serum

miRNA	*C9orf7* *	Disease duration	Site of onset	Gender	Age at onset
miR328-3p	0.750	0.318	0.250	0.853	0.406
miR373-5p	1.000	0.638	0.927	0.247	0.206
miR125b-5p	1.000	0.407	0.925	0.912	0.259

* patients were dichotomized in carriers and non-carriers of the C9orf72 expansion. For each column p-values are reported

PART V

DISCUSSION AND CONCLUSION

1

DISCUSSION

EXPERIMENTAL STRATEGY

In this work of thesis, a qualitative and quantitative study of miRNAs in CSF and serum from SALS patients was carried out in order to identify a specific signature of miRNAs as biomarkers for ALS employable in clinical practices and to better understand the epigenetic role of these ncRNA molecules in the pathogenesis of motor neuron disease.

The expression of a panel of 372 miRNAs, representing the most abundantly expressed and best-characterized miRNAs in miRNA-databases for both the biofluids, was profiled. A total of 48 samples (24 from sporadic ALS patients and 24 controls) for CSF and serum, respectively, were analysed applying qRT-PCR in two phases: initial screening by miRNA-PCR array, using pooled samples, and subsequent validation of selected molecules in each single sample. Among the various techniques for miRNA profiling we chose the quantitative reverse transcription PCR (qRT-PCR) since it is the most specific and sensitive (Pritchard et al., 2012). This technique provides the immediate availability of quantitative data using nanograms of RNA and gives the possibility to study a consistent number of specific miRNAs with well-known target mRNAs and molecular functions (Pritchard et al., 2012; Pacifici et al., 2014). As in several miRNA-profiling studies, the initial screening was performed in pooled samples. This approach, using a sensitive assay such as qRT-PCR, offers a competitive and cost-effective tool for identification of ALS miRNAs.

The study provides the first description of CSF miRNA profiling in ALS identifying a pattern of miRNAs significantly deregulated in ALS. In the literature only two reports have been published on miRNAs in CSF from ALS patients to date; they however used different approaches: the screening of a specific set of miRNAs binding TDP-43

(Freischmidt et al., 2013) and the analysis of one selected miRNA, found over-expressed in ALS blood leukocytes (De Felice et al., 2014).

The serum analysis performed in this PhD project is the first miRNA profiling for SALS conducted by qRT-PCR using a specific PCR array, bypassing the miRNA molecules selection on large-scale employing microarray or RNA-seq. Two serum miRNA profiling studies in ALS have already been realized; the first one was conducted in FALS and the second one in SALS by the same research group. In both studies the miRNA profiling was performed using Affymetrix GeneChip miRNA 3.0 Arrays (microarray analysis). A strikingly homogenous miRNA profile in FALS, independently from the underlying disease gene, was reported in the first study, in which a comprehensive miRNAs expression profiling of serum from FALS, asymptomatic mutation carriers and healthy control subjects was performed (Freischmidt et al., 2014). In the second study, instead, a significant downregulation of two miRNAs (miR1234-3p and miR1825) was observed in SALS compared to controls. Therefore, after validation experiments conducted by qRT-PCR, the two deregulated miRNAs were proposed as valid biomarkers of ALS by the authors (Freischmidt A et al., 2015). Four other reports have been published on miRNAs quantification levels in serum from ALS patients, but they have not been performed by miRNAs profiling. Indeed, the studies are quantification analyses of different selected miRNAs, specifically selected: a set of miRNAs binding TDP-43 (Freischmidt et al., 2013), the miR338-3p found over-expressed in ALS blood leukocytes (De Felice et al., 2014), the miR206 resulted upregulated in SOD1 mouse model (Toivonen et al., 2014) and a muscle-specific set of miRNAs (miR-206, miR-1, miR-133a/b, miR-27a) (Tasca et al., 2016).

The approach used in the present study to identify deregulated miRNAs can present some limitations. We have employed qRT-PCR, which is a highly sensitive technique, but cannot identify novel miRNAs. Thus, it is possible that other informative miRNAs exist in CSF or serum. On the other hand, validation experiments showed that this method is not only sensitive and specific but also reproducible and accurate to detect miRNAs in CSF and serum. In addition, all the steps of the qRT-PCR have been monitored by quality control procedures, to ensure the reliability of the results. A

second challenge in miRNA quantification is data normalization, that is a key point for an objective evaluation of expression levels. For miRNA analysis, no consensus internal controls have been established yet. We used two endogenous reference miRNAs selected as the most stable and reproducible across different samples and experiments for each biofluid: miRNA608 and miR328-3p were employed for CSF, while miRNA608 and miR489-3p were used for serum. In addition, an exogenous control (Cel-miR-39-3p), widely reported in the literature, has been employed for the miRNA relative quantification. The analyses performed using the three reference miRNAs led to the same results.

CEREBROSPINAL FLUID AND SERUM MIRNAS IN SALS PATIENTS

The CSF-profiling allowed the detection of 42 out of 372 miRNAs. The percentage of miRNAs detected in our study (11%) was congruent with that recently reported by Denk and colleagues (15%) who used qRT-PCR to profile 384 different miRNAs in CSF from AD patients (Denk et al., 2015). A total of fourteen deregulated miRNAs were selected from the profiling: thirteen that were downregulated (let7a-5p, let7b-5p, let7f-5p, miR15b-5p, miR21-5p, miR122-3p, miR127-3p, miR148a-3p, miR150-5p, miR183-3p, miR195a-5p, miR204-5p, miR373-5p) and one upregulated (miR181a-5p). We observed an overall downregulation of miRNAs in ALS samples; this result is consistent with other studies reporting that the majority of deregulated miRNAs in tissues from ALS patients and ALS models are downregulated (Paez-Colasante et al., 2015).

In order to confirm these results, the expression levels of the selected miRNAs were analysed in each single sample included in the pool, using qRT-PCR. A group of eight out of the twelve miRNAs selected by miRNA profiling were confirmed as significantly deregulated: seven were downregulated (let7a-5p, let7b-5p, let7f-5p, miR15b-5p, miR21-5p, miR148a-3p, miR195a-5p) and one upregulated (miR181a-5p). We observed an interesting overlapping of six deregulated miRNAs in our dataset (miR181a-5p, miR21-5p, miR148a-3p and let7a-5p, let7b-5p and let7f-5p) with those described by Burgos and colleagues, who listed the 50 most abundant miRNAs detectable in CSF (Burgos et al., 2013). This observation suggests that the majority of

miRNAs selected in this study should be specific for nervous tissue and have a brain origin.

The selected miRNAs have not been identified as significantly deregulated in the two previously published studies (Freischmidt et al., 2013; De Felice et al., 2014). A possible explanation for this discrepancy could be the different strategies used to identify the deregulated miRNAs. Only for let7b-5p is there a partial overlapping with Freischmidt and colleagues' study, which reported a decrease of let7b in CSF from ALS patients, even though it did not reach statistical significance (Freischmidt et al., 2013). Regarding other neurodegenerative diseases, two studies reported a significant downregulation in expression levels of both miR15b-5p and miR181a-5p in CSF from AD patients (Cogswell et al., 2008; Burgos et al., 2014). Thus, in CSF from ALS patients, the levels of miR15b-5p seem to follow the same trend described in AD patients, whereas the deregulation of miR181a-5p shows an opposite tendency.

The serum-profiling permitted the detection of 227 out of 372 miRNAs and the percentage of miRNAs detected in our study was 61%. A group of eleven upregulated miRNAs (let7b-5p, let7f-5p, miR15b-5p, miR16-5p, miR27a, miR30c-5p, miR122-3p, miR197-3p, miR223-3p, miR328-3p, miR373-5p) and one downregulated miRNA (miR125b-5p) were selected from the profiling. In this case, we observed an overall upregulation of miRNAs in SALS. Among the miRNAs reported in our study, three have been already described as significantly deregulated in patients with ALS. In particular, oppositely to our results, miR27a has been previously reported as significantly downregulated in serum from ALS patients compared to controls by Tasca and colleagues (Tasca et al., 2016). On the other side, our result was congruent with Butovsky and colleagues' study, which described a significant upregulation of miR27a in peripheral monocytes from ALS patients (Butovsky et al., 2012). In the same way, let7b-5p, and let7f-5p, which were upregulated in our study, resulted significantly downregulated in serum from SALS by Freischmidt and colleagues' study (Freischmidt et al., 2013) and in plasma from SALS by Takahashi and coleagues' research (Takahashi et al., 2015) respectively. However, a significant increase of let7b-5p and let7f-5p has been observed in monocytes from ALS patients (Butovsky et al., 2012). Possible reasons for these incongruities could be linked to several

technical aspects and lie in the different strategies used to identify the deregulated miRNAs. Since our results are congruent with these reported by Butovsky and colleagues, who analysed the ALS-miRNA of monocytes, a possible contamination in our serum samples from fragments or vesicles of monocytes cannot be excluded.

To verify the observed deregulation of the twelve miRNAs, their expression levels were analysed in each single serum sample included in the pool, using qRT-PCR. Among the selected molecules, only three miRNAs were significantly deregulated in ALS patients compared to controls; in particular, the miR125b-5p was downregulated and miR328-3p and miR373-5p were upregulated. These miRNAs have not been previously described in association with neurodegenerative diseases but they correlate with several tumours. In particular, it is worth noticing that a significant downregulation of miR328 has been previously observed in serum from patients with glioblastoma, showing an opposite tendency compared to our result (Wu et al., 2012). This observation is consistent with the results obtained for CSF that are discussed below.

Why are miRNA present in the serum and what could be the implications of these changes in miRNA profiles for diagnosis and therapy? These are important open questions since the role of miRNA in serum has not been completely clarified. It has been hypothesized that serum miRNAs could have cytoplasmic origin: they are packaged into exosomes and secreted into the intercellular space. Serum exosomes are vesicles circulating throughout the body that, picked up by cells via targeted or non-targeted ways, deliver packages of miRNAs, which are able to change gene expression inside that cell (Creemers et al., 2012). Alternatively, miRNAs could be released from cells undergoing apoptosis or necrosis (Valadi et al., 2007). Independently from their origin, serum miRNAs are promising molecules able to give important indications about mechanisms involved in neurodegeneration.

In the present study, no associations were found between miRNA levels and ALS clinical variables either for CSF or for serum. Moreover, no significant differences were present when miRNA levels of patients carrying the *C9orf72* expansion were compared with those of patients without the expansion. Although the analysis was performed in a relatively small number of patients, these data suggest a possible

mutation-independent deregulation of CSF and serum miRNAs. This is in line with the results obtained in a recent study of miRNA in the serum of sporadic and familial ALS patients, in which deregulated miRNAs revealed the same trend in patients with or without mutations in the major ALS genes, including *C9orf72* expansion. It is hypothesized the presence of common pathogenic denominators connecting defects in sporadic ALS and in ALS associated with mutation in several different genes (Freischmidt et al., 2014).

HYPOTHESES ABOUT THE ROLE OF CSF-MIRNAS

The results obtained from CSF-miRNAs analyses were more promising than these got from serum. Indeed, the ROC curve analyses of CSF-miRNAs showed a higher diagnostic sensitivity and specificity than those of serum-miRNAs. Among the significantly deregulated CSF-miRNAs, miR181a-5p, miR15b-5p and miR21-5p, which showed the highest sensitivity and specificity in differentiating ALS from age-matched controls, have been selected for the subsequent analysis. Interestingly, the sensitivity and specificity of these miRNAs were increased when they were paired combining the upregulated miRNA with the downregulated miRNAs (miR181a-5p/miR15b-5p and miR181a-5p/miR21-5p). Therefore, miR15b-5p, miR21-5p and miR181a-5p might be strong candidates for new ALS biomarkers. Furthermore, the observation that miR181a-5p was downregulated in CSF from AD patients increased the application potential of this miRNA as specific marker of ALS.

Beside their role as potential biomarkers, the deregulated miRNAs could reflect the biology of the tissue of origin, providing important insight into disease processes responsible for motor neuron degeneration. The majority of miRNAs analyzed in this study are molecules with well-know functions and the information about their potential role derives mainly from studies of brain tumours. For example, miR15-5p has been widely described in cancer and a significant upregulation has been reported in the CSF of patients with gliomablastoma (Teplyuk et al., 2012) and glioma (Baraniskin et al., 2012). Abundant data have recently shown a very tight connection between miR21 and miR15, particularly in brain tumours. The expression levels of miR21 have been described as upregulated in CSF from patients with glioma,

glioblastoma and diffuse large B-cell lymphoma (PCNSL) (Sheinerman and Umansky, 2013; Baraniskin et al., 2011). In addition, the use of miR21 in combination with miR15 has been proposed as specific signature for glioma, improving the diagnostic accuracy (Baraniskin et al., 2012). Further studies reported a significant upregulation of miR21 expression levels in CSF from patients with glioblastoma (Akers et al., 2013; Akers et al., 2015), supporting the hypothesis that miR21 could be a marker of glial cell proliferation (Garg et al., 2015). Finally, miR181a-5p has been reported as downregulated in neuroblastoma, glioblastoma and glioma (Gibert et al., 2014; Conti et al., 2009; Shi et al., 2008).

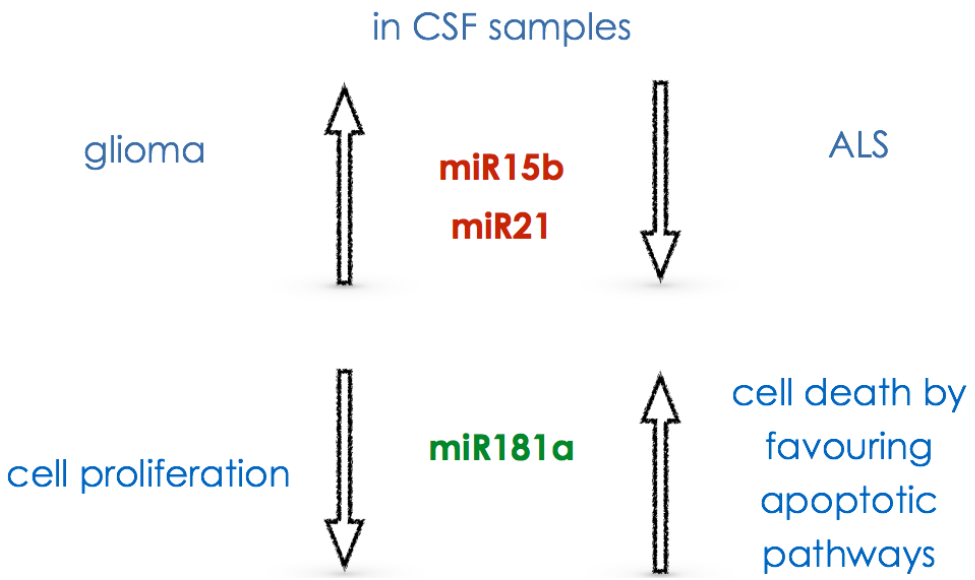
It is worth noting that in brain tumours the deregulation of these three miRNAs displays a trend opposite of that observed in ALS in our study, where miR181a-5p is upregulated and miR21-5p and miR15b-5p are downregulated.

It has been proposed that in the glioma, glioblastoma and astrocytoma carcinogenesis these three miRNAs may act in combination as promoters of glial cell proliferation (Conti et al., 2009; Shi et al., 2008; Baraniskin et al., 2012). In particular, miR21-5p is one of the first miRNAs to be described as an oncomir, since it works as an oncogene and its overexpression leads to tumour growth, inhibiting the expression of tumour suppressor genes. It has empirically been observed that miR21 leads to the downregulation of PDCD4 and modulates the networks of p53 and transforming growth factor β (Papagiannakopoulos et al., 2008). The role of miR-15b in gliomas seems to be less defined, since its expression has been reported to be down- or up-regulated by different research groups. However, recent evidence suggests the oncogenic potential of this miRNA in glioma tumorigenesis and malignant progression (Pang et al., 2015). Finally, mi181a-5p has been suggested as an anti-oncomir, which acts as a tumour suppressor in normal tissues. Thus, its downregulation leads to cancerous growth increasing the expression of oncogenes in astrocytic tumours. In vitro experiments suggested that miR181a-5p triggers growth inhibition, apoptosis, and inhibits invasion (Conti et a.l., 2009).

In the light of this evidence, it can be hypothesized that, whereas the contemporary downregulation of miR181a-5p and upregulation of miR21-5p and miR15b-5p are associated with apoptotic mechanism inhibition and cell proliferation, the

contemporary upregulation of miR181a-5p and downregulation of miR21-5p and miR15b-5p we find could be linked to cell death by favouring apoptotic pathways. This deregulation could directly involve microglia, which in turn may contribute to neuronal damage. In particular, it has been demonstrated that hypoxia, a common condition in ALS, induces the upregulation of the Fas ligand (FasL) and the simultaneous downregulation of miR- 21 in microglia, influencing neuronal apoptosis. Moreover, the ectopic expression of miR-21 partially protects neurons from cell death caused by hypoxia-activated microglia (Zhang et al., 2012). Thus, deregulated miRNAs could be able to modulate microglia response during inflammation after hypoxia, to alter communication between microglia and neurons, and finally to promote motor neuron degeneration (**Fig. 16**).

Fig. 16 CSF-miRNAs and their trend in brain tumours and in ALS



The picture reports a schematic representation of the opposite deregulation of miR15b, miR21 and miR181a in CSF samples from patients with brain tumours and patients with Amyotrophic Lateral Sclerosis. The contemporary downregulation of miR181a-5p and upregulation of miR21-5p and miR15b-5p in glioma and glioblastoma is associated with apoptotic mechanism inhibition and cell proliferation, while the combined upregulation of miR181a-5p and downregulation of miR21-5p and miR15b-5p in ALS could be linked to cell death by favouring apoptotic pathways.

2

CONCLUSION

The analyses of miRNAs in CSF and serum permitted the identification of deregulated molecules in the biofluids from ALS patients.

The selected miRNAs in serum are promising but they need further confirmation experiments in a larger cohort of patients and controls.

In CSF, considering the high diagnostic value of combined miR181a-5p, miR15b-5p and miR21-5p analyses in this pilot study, we provide initial evidence that identified miRNAs could represent promising biomarkers for ALS. If replicated in a larger cohort of patients, these molecules may represent a valuable diagnostic tool.

In this study CSF and serum miRNA levels of ALS patients have been compared to those of control subjects not affected by neurological disorders. It will be of fundamental importance to extend the comparison to patients affected by other neurodegenerative diseases, to evaluate the specificity of deregulated miRNAs as ALS biomarkers.

In perspective, these miRNAs may be used as prognostic biomarkers and as indicator of disease progression, to facilitate clinical management of this disease.

Moreover, beside of the potential role of miRNAs as biomarkers, the finding of a deregulated miRNA expression in patients with ALS may provide important insights about the pathogenesis of the disease and eventually contribute to develop potential future therapeutic approaches.

PART VI

SELECTED BIBLIOGRAPHY

Akers JC, Ramakrishnan V, Kim R, Skog J, Nakano I, Pingle S, Kalinina J, Hua W, Kesari S, Mao Y, Breakefield XO, Hochberg FH, Van Meir EG, Carter BS, Chen CC. MiR-21 in the extracellular vesicles (EVs) of cerebrospinal fluid (CSF): a platform for glioblastoma biomarker development. PLoS One. 2013; 8:e78115.

Akers JC, Ramakrishnan V, Kim R, Phillips S, Kaimal V, Mao Y, Hua W, Yang I, Fu CC, Nolan J, Nakano I, Yang Y, Beaulieu M, Carter BS, Chen CC. miRNA contents of cerebrospinal fluid extracellular vesicles in glioblastoma patients. J Neurooncol. 2015; 123:205-16.

Al-Chalabi A, Hardiman O. The epidemiology of ALS: a conspiracy of genes, environment and time. Nat Rev Neurol. 2013; 9:617-28.

Al-Chalabi A, Kwak S, Mehler M, Rouleau G, Siddique T, Strong M, Leigh PN. Genetic and epigenetic studies of amyotrophic lateral sclerosis. Amyotroph Lateral Scler Frontotemporal Degener. 2013; 14 Suppl 1:44-52.

Baraniskin A, Kuhnhenn J, Schlegel U, Chan A, Deckert M, Gold R, Maghnouj A, Zöllner H, Reinacher-Schick A, Schmiegel W, Hahn SA, Schroers R. Identification of microRNAs in the cerebrospinal fluid as marker for primary diffuse large B-cell lymphoma of the central nervous system. Blood. 2011; 117:3140-6.

Baraniskin A, Kuhnhenn J, Schlegel U, Maghnouj A, Zöllner H, Schmiegel W, Hahn S, Schroers R. Identification of microRNAs in the cerebrospinal fluid as biomarker for the diagnosis of glioma. Neuro Oncol. 2012; 14:29-33.

Barbeito LH, Pehar M, Cassina P, Vargas MR, Peluffo H, Viera L, Estévez AG, Beckman JS. A role for astrocytes in motor neuron loss in amyotrophic lateral sclerosis. Brain Res Brain Res Rev. 2004; 47:263-74.

Battistini S, Benigni M, Ricci C, Rossi A. SOD1 mutations in Amyotrophic Lateral Sclerosis. Eur Neurol J. 2012; 4:33-43.

Beers DR, Henkel JS, Zhao W, Wang J, Huang A, Wen S, Liao B, Appel SH. Endogenous regulatory T lymphocytes ameliorate amyotrophic lateral sclerosis in mice and correlate with disease progression in patients with amyotrophic lateral sclerosis. Brain. 2011; 134:1293-314.

Ben Hamida M, Hentati F, Ben Hamida C. Hereditary motor system diseases (chronic juvenile amyotrophic lateral sclerosis). Conditions combining a bilateral pyramidal syndrome with limb and bulbar amyotrophy. Brain. 1990; 113:347-63.

Bendotti C, Tortarolo M, Suchak SK, Calvaresi N, Carvelli L, Bastone A, Rizzi M, Rattray M, Mennini T. Transgenic SOD1 G93A mice develop reduced GLT-1 in spinal cord without alterations in cerebrospinal fluid glutamate levels. J Neurochem. 2001; 79:737-46.

Bendotti C, Marino M, Cheroni C, Fontana E, Crippa V, Poletti A, De Biasi S. Dysfunction of constitutive and inducible ubiquitin-proteasome system in amyotrophic lateral sclerosis: implication for protein aggregation and immune response. Prog Neurobiol. 2012; 97:101-26.

Bird A. Perceptions of epigenetics. Nature. 2007; 447:396-8.

Bogdanov M, Brown RH, Matson W, Smart R, Hayden D, O'Donnell H, Flint Beal M, Cudkowicz M. Increased oxidative damage to DNA in ALS patients. Free Radic Biol Med. 2000; 29:652-8.

Brooks BR, Miller RG, Swash M, Munsat TL; World Federation of Neurology Research Group on Motor Neuron Diseases. El Escorial revisited: revised criteria for the diagnosis of amyotrophic lateral sclerosis. Amyotroph Lateral Scler Other Motor Neuron Disord. 2000; 1:293-9.

Brownawell AM, Macara IG. Exportin-5, a novel karyopherin, mediates nuclear export of double-stranded RNA binding proteins. J Cell Biol. 2002; 156:53-64.

Bruijn LI, Miller TM, Cleveland DW. Unraveling the mechanisms involved in motor neuron degeneration in ALS. Annu Rev Neurosci. 2004; 27:723-49.

Buratti E, De Conti L, Stuani C, Romano M, Baralle M, Baralle F. Nuclear factor TDP-43 can affect selected microRNA levels. FEBS J. 2010; 277:2268-81.

Burgos K, Malenica I, Metpally R, Courtright A, Rakela B, Beach T, Shill H, Adler C, Sabbagh M, Villa S, Tembe W, Chiò A, Logroscino G, Traynor BJ, Collins J, Simeone JC, Goldstein LA, White LA. Global epidemiology of amyotrophic lateral sclerosis: a systematic review of the published literature. Neuroepidemiology. 2013; 41:118-30.

Burgos KL, Javaherian A, Bomprezzi R, Ghaffari L, Rhodes S, Courtright A, Tembe W, Kim S, Metpally R, Van Keuren-Jensen K. Identification of extracellular miRNA in human cerebrospinal fluid by next-generation sequencing. RNA. 2013; 19:712-22.

Burgos K, Malenica I, Metpally R, Courtright A, Rakela B, Beach T, Shill H, Adler C, Sabbagh M, Villa S, Tembe W, Craig D, Van Keuren-Jensen K. Profiles of extracellular miRNA in cerebrospinal fluid and serum from patients with Alzheimer's and Parkinson's diseases correlate with disease status and features of pathology. PLoS One. 2014; 9:e94839.

Butovsky O, Siddiqui S, Gabriely G, Lanser AJ, Dake B, Murugaiyan G, Doykan CE, Wu PM, Gali RR, Iyer LK, Lawson R, Berry J, Krichevsky AM, Cudkowicz ME, Weiner HL. Modulating inflammatory monocytes with a unique microRNA gene signature ameliorates murine ALS. J Clin Invest. 2012; 122:3063-87.

Campos-Melo D, Droppelmann CA, He Z, Volkening K, Strong MJ. Altered microRNA expression profile in Amyotrophic Lateral

Sclerosis: a role in the regulation of NFL mRNA levels. Mol Brain. 2013; 6:26.

Chen YZ, Bennett CL, Huynh HM, Blair IP, Puls I, Irobi J, Dierick I, Abel A, Kennerson ML, Rabin BA, Nicholson GA, Auer-Grumbach M, Wagner K, De Jonghe P, Griffin JW, Fischbeck KH, Timmerman V, Cornblath DR, Chance PF. DNA/RNA helicase gene mutations in a form of juvenile amyotrophic lateral sclerosis (ALS4). Am J Hum Genet. 2004; 74:1128-35.

Chiò A, Logroscino G, Hardiman O, Swingler R, Mitchell D, Beghi E, Traynor BG; Eurals Consortium. Prognostic factors in ALS: A critical review. Amyotroph Lateral Scler. 2009; 10:310-23.

Chiò A, Battistini S, Calvo A, Caponnetto C, Conforti FL, Corbo M, Giannini F, Mandrioli J, Mora G, Sabatelli M; ITALSGEN Consortium, Ajmone C, Mastro E, Pain D, Mandich P, Penco S, Restagno G, Zollino M, Surbone A. Genetic counselling in ALS: facts, uncertainties and clinical suggestions. J Neurol Neurosurg Psychiatry. 2014; 85:478-85.

Clancy S. RNA Functions. Nature Education 2008; 1:102.

Cloutier F, Marrero A, O'Connell C, Morin P Jr. MicroRNAs as potential circulating biomarkers for amyotrophic lateral sclerosis. J Mol Neurosci. 2015; 56:102-12.

Cogswell JP, Ward J, Taylor IA, Waters M, Shi Y, Cannon B, Kelnar K, Kemppainen J, Brown D, Chen C, Prinjha RK, Richardson JC, Saunders AM, Roses AD, Richards CA. Identification of miRNA changes in Alzheimer's disease brain and CSF yields putative biomarkers and insights into disease pathways. J Alzheimers Dis. 2008; 14:27-41.

Conti A, Aguennouz M, La Torre D, Tomasello C, Cardali S, Angileri FF, Maio F, Cama A, Germanò A, Vita G, Tomasello F. miR-21 and 221 upregulation and miR-181b downregulation in human grade II-IV astrocytic tumors. J Neurooncol. 2009; 93:325-32.

Couthouis J, Hart MP, Shorter J, DeJesus-Hernandez M, Erion R, Oristano R, Liu AX, Ramos D, Jethava N, Hosangadi D, Epstein J, Chiang A, Diaz Z, Nakaya T, Ibrahim F, Kim HJ, Solski JA, Williams KL, Mojsilovic-Petrovic J, Ingre C, Boylan K, Graff-Radford NR, Dickson DW, Clay-Falcone D, Elman L, McCluskey L, Greene R, Kalb RG, Lee VM, Trojanowski JQ, Ludolph A, Robberecht W, Andersen PM, Nicholson GA, Blair IP, King OD, Bonini NM, Van Deerlin V, Rademakers R, Mourelatos Z, Gitler AD. A yeast functional screen predicts new candidate ALS disease genes. Proc Natl Acad Sci U S A. 2011; 108:20881-90.

Couthouis J, Hart MP, Erion R, King OD, Diaz Z, Nakaya T, Ibrahim F, Kim HJ, Mojsilovic-Petrovic J, Panossian S, Kim CE, Frackelton EC, Solski JA, Williams KL, Clay-Falcone D, Elman L, McCluskey L, Greene R, Hakonarson H, Kalb RG, Lee VM, Trojanowski JQ, Nicholson GA, Blair IP, Bonini NM, Van Deerlin VM, Mourelatos Z, Shorter J, Gitler AD. Evaluating the role of

the FUS/TLS-related gene EWSR1 in amyotrophic lateral sclerosis. Hum Mol Genet. 2012; 21:2899-911.

Creemers EE, Tijsen AJ, Pinto YM. Circulating microRNAs: novel biomarkers and extracellular communicators in cardiovascular disease? Circ Res. 2012; 110:483-95.

De Felice B, Guida M, Guida M, Coppola C, De Mieri G, Cotrufo R. A miRNA signature in leukocytes from sporadic amyotrophic lateral sclerosis. Gene. 2012; 508:35-40.

De Felice B, Annunziata A, Fiorentino G, Borra M, Biffali E, Coppola C, Cotrufo R, Brettschneider J, Giordana ML, Dalmay T, Wheeler G, D'Alessandro R. miR-338-3p is over-expressed in blood, CFS, serum and spinal cord from sporadic amyotrophic lateral sclerosis patients. Neurogenetics. 2014; 15:243-53.

DeJesus-Hernandez M, Mackenzie IR, Boeve BF, Boxer AL, Baker M, Rutherford NJ, Nicholson AM, Finch NA, Flynn H, Adamson J, Kouri N, Wojtas A, Sengdy P, Hsiung GY, Karydas A, Seeley WW, Josephs KA, Coppola G, Geschwind DH, Wszolek ZK, Feldman H, Knopman DS, Petersen RC, Miller BL, Dickson DW, Boylan KB, Graff-Radford NR, Rademakers R. Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS. Neuron. 2011; 72:245-56.

Denk J, Boelmans K, Siegismund C, Lassner D, Arlt S, Jahn H. MicroRNA Profiling of CSF Reveals Potential Biomarkers to Detect Alzheimer`s Disease. PLoSOne. 2015; 10:e0126423.

Droppelmann CA, Campos-Melo D, Ishtiaq M, Volkening K, Strong MJ. RNA metabolism in ALS: when normal processes become pathological. Amyotroph Lateral Scler Frontotemporal Degener. 2014; 15:321-36.

Eccleston A, DeWitt N, Gunter C, Marte B, Nath B. Introduction Epigenetics. Nature 2007; 447: 395.

Ferraiuolo L, Kirby J, Grierson AJ, Sendtner M, Shaw PJ. Molecular pathways of motor neuron injury in amyotrophic lateral sclerosis. Nat Rev Neurol. 2011; 7:616-30.

Freischmidt A, Müller K, Ludolph AC, Weishaupt JH. Systemic dysregulation of TDP-43 binding microRNAs in amyotrophic lateral sclerosis. Acta Neuropathol Commun. 2013; 1:42.

Freischmidt A, Müller K, Zondler L, Weydt P, Volk AE, Božič AL, Walter M, Bonin M, Mayer B, von Arnim CA, Otto M, Dieterich C, Holzmann K, Andersen PM, Ludolph AC, Danzer KM, Weishaupt JH. Serum microRNAs in patients with genetic amyotrophic lateral sclerosis and pre-manifest mutation carriers. Brain. 2014; 137:2938-50.

Freischmidt A, Müller K, Zondler L, Weydt P, Mayer B, von Arnim CA, Hübers A, Dorst J, Otto M, Holzmann K, Ludolph AC, Danzer KM, Weishaupt JH. Serum microRNAs in

sporadic amyotrophic lateral sclerosis. Neurobiol Aging. 2015; 36:2660.e15-20.

Gandhi R, Healy B, Gholipour T, Egorova S, Musallam A, Hussain MS, Nejad P, Patel B, Hei H, Khoury S, Quintana F, Kivisakk P, Chitnis T, Weiner HL. Circulating microRNAs as biomarkers for disease staging in multiple sclerosis. Ann Neurol. 2013; 73:729-40.

Garg N, Vijayakumar T, Bakhshinyan D, Venugopal C, Singh SK. MicroRNA Regulation of Brain Tumour Initiating Cells in Central Nervous System Tumours. Stem Cells Int. 2015; 141793.

Gibert B, Delloye-Bourgeois C, Gattolliat CH, Meurette O, Le Guernevel S, Fombonne J, Ducarouge B, Lavial F, Bouhallier F, Creveaux M, Negulescu AM, Bénard J, Janoueix-Lerosey I, Harel-Bellan A, Delattre O, Mehlen P. Regulation by miR181 family of the dependence receptor CDON tumor suppressive activity in neuroblastoma. J Natl Cancer Inst. 2014; 106.

Girotti AW. Lipid hydroperoxide generation, turnover, and effector action in biological systems. Journal of Lipid Research. 1998; 39:1529–42.

Goodall EF, Heath PR, Bandmann O, Kirby J, Shaw PJ. Neuronal dark matter: the emerging role of microRNAs in neurodegeneration. Front Cell Neurosci. 2013; 7:178.

Greenway MJ, Andersen PM, Russ C, Ennis S, Cashman S, Donaghy C, Patterson V, Swingler R, Kieran D, Prehn J, Morrison KE, Green A, Acharya KR, Brown RH Jr, Hardiman O. ANG mutations segregate with familial and 'sporadic' amyotrophic lateral sclerosis. Nat Genet. 2006; 38:411-3.

Gregory RI, Yan KP, Amuthan G, Chendrimada T, Doratotaj B, Cooch N, Shiekhattar R. The Microprocessor complex mediates the genesis of microRNAs. Nature. 2004; 432:235-40.

Ha M, Kim VN. Regulation of microRNA biogenesis. Nat Rev Mol Cell Biol. 2014; 15:509-24.

Haghikia A, Haghikia A, Hellwig K, Baraniskin A, Holzmann A, Décard BF, Thum T, Gold R. Regulated microRNAs in the CSF of patients with multiple sclerosis: a case-control study. Neurology. 2012; 79:2166-70.

Haramati S, Chapnik E, Sztainberg Y, Eilam R, Zwang R, Gershoni N, McGlinn E, Heiser PW, Wills AM, Wirguin I, Rubin LL, Misawa H, Tabin CJ, Brown R Jr, Chen A, Hornstein E. miRNA malfunction causes spinal motor neuron disease. Proc Natl Acad Sci U S A. 2010; 107:13111-6.

Hardiman O, van den Berg LH, Kiernan MC. Clinical diagnosis and management of amyotrophic lateral sclerosis. Nat Rev Neurol. 2011; 7:639-49.

Honda D, Ishigaki S, Iguchi Y, Fujioka Y, Udagawa T, Masuda A, Ohno K, Katsuno M, Sobue G. The ALS/FTLD-related RNA-binding proteins TDP-43 and FUS have

common downstream RNA targets in cortical neurons. FEBS Open Bio. 2013 Nov 20;4:1-10.

Ilieva H, Polymenidou M, Cleveland DW. Non-cell autonomous toxicity in neurodegenerative disorders: ALS and beyond. J Cell Biol. 2009; 187: 761-72.

Iorio MV, Croce CM. MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review. EMBO Mol Med. 2012; 4:143-59.

Ishtiaq M, Campos-Melo D, Volkening K, Strong MJ. Analysis of novel NEFL mRNA targeting microRNAs in amyotrophic lateral sclerosis. PLoS One. 2014; 9:e85653.

Johnson BS, Snead D, Lee JJ, McCaffery JM, Shorter J, Gitler AD. TDP-43 is intrinsically aggregation-prone, and amyotrophic lateral sclerosis-linked mutations accelerate aggregation and increase toxicity. J Biol Chem. 2009; 284:20329-39.

Kaniuk NA, Brumell JH. Examining ubiquitinated protein aggregates in tissue sections. Methods Mol Biol. 2010;648:175-82.

Kawahara Y, Mieda-Sato A. TDP-43 promotes microRNA biogenesis as a component of the Drosha and Dicer complexes. Proc Natl Acad Sci U S A. 2012; 109:3347-52.

Keller A, Leidinger P, Lange J, Borries A, Schroers H, Scheffler M, Lenhof HP, Ruprecht K, Meese E. Multiple sclerosis: microRNA expression profiles accurately differentiate patients with relapsing-remitting disease from healthy controls. PLoS One. 2009; 4:e7440.

Kosik KS. The neuronal microRNA system. Nat Rev Neurosci. 2006; 7:911-20.

Koval ED, Shaner C, Zhang P, du Maine X, Fischer K, Tay J, Chau BN, Wu GF, Miller TM. Method for widespread microRNA-155 inhibition prolongs survival in ALS-model mice. Hum Mol Genet. 2013; 22:4127-35.

Kwiatkowski TJ Jr, Bosco DA, Leclerc AL, Tamrazian E, Vanderburg CR, Russ C, Davis A, Gilchrist J, Kasarskis EJ, Munsat T, Valdmanis P, Rouleau GA, Hosler BA, Cortelli P, de Jong PJ, Yoshinaga Y, Haines JL, Pericak-Vance MA, Yan J, Ticozzi N, Siddique T, McKenna-Yasek D, Sapp PC, Horvitz HR, Landers JE, Brown RH Jr. Mutations in the FUS/TLS gene on chromosome 16 cause familial amyotrophic lateral sclerosis. Science. 2009; 323:1205-8.

Kwon I, Xiang S, Kato M, Wu L, Theodoropoulos P, Wang T, Kim J, Yun J, Xie Y, McKnight SL. Poly-dipeptides encoded by the C9orf72 repeats bind nucleoli, impede RNA biogenesis, and kill cells. Science. 2014; 345:1139-45.

Lee Y, Morrison BM, Li Y, Lengacher S, Farah MH, Hoffman PN, Liu Y, Tsingalia A, Jin L, Zhang PW, Pellerin L, Magistretti PJ, Rothstein JD. Oligodendroglia

metabolically support axons and contribute to neurodegeneration. Nature. 2012; 487:443-8.

Lieberman J, Song E, Lee SK, Shankar P. Interfering with disease: opportunities and roadblocks to harnessing RNA interference. Trends Mol Med. 2003; 9:397-403.

Ling SC, Polymenidou M, Cleveland DW. Converging mechanisms in ALS and FTD: disrupted RNA and protein homeostasis. Neuron. 2013; 79:416-38.

Liu J, Carmell MA, Rivas FV, Marsden CG, Thomson JM, Song JJ, Hammond SM, Joshua-Tor L, Hannon GJ. Argonaute2 is the catalytic engine of mammalian RNAi. Science. 2004; 305:1437-41.

Logroscino G, Traynor BJ, Hardiman O, Chio' A, Couratier P, Mitchell JD, Swingler RJ, Beghi E; EURALS. Descriptive epidemiology of amyotrophic lateral sclerosis: new evidence and unsolved issues. J Neurol Neurosurg Psychiatry. 2008; 79:6-11.

Machida A, Ohkubo T, Tsunoda A, Matsuo H, Takanori Y, Mizusawa H. Prifiling of miRNAs in Cerebrospinal Fluid from patients with Amyotrophic Lateral Sclerosis. Neurology. 2014; 82: S26.005.

Maciotta S, Meregalli M, Torrente Y. The involvement of microRNAs in neurodegenerative diseases. Front Cell Neurosci. 2013; 7:265.

Marangi G, Traynor BJ. Genetic causes of amyotrophic lateral sclerosis: new genetic analysis methodologies entailing new opportunities and challenges. Brain Res. 2015; 1607:75-93.

Matranga C, Tomari Y, Shin C, Bartel DP, Zamore PD. Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. Cell. 2005; 123:607-20.

Matsumoto S, Goto S, Kusaka H, Imai T, Murakami N, Hashizume Y, Okazaki H, Hirano A. Ubiquitin-positive inclusion in anterior horn cells in subgroups of motor neuron diseases: a comparative study of adult-onset amyotrophic lateral sclerosis, juvenile amyotrophic lateral sclerosis and Werdnig-Hoffmann disease. J Neurol Sci. 1993; 115:208-13.

Mattick JS. The hidden genetic program of complex organisms. Sci Am. 2004; 291:60-7.

McCarthy JJ. MicroRNA-206: the skeletal muscle-specific myomiR. Biochim Biophys Acta. 2008; 1779:682-91.

Millecamps S, Boillée S, Le Ber I, Seilhean D, Teyssou E, Giraudeau M, Moigneu C, Vandenberghe N, Danel-Brunaud V, Corcia P, Pradat PF, Le Forestier N, Lacomblez L, Bruneteau G, Camu W, Brice A, Cazeneuve C, Leguern E, Meininger V, Salachas F. Phenotype difference between ALS patients with expanded repeats in C9ORF72 and patients with mutations in

other ALS-related genes. J Med Genet. 2012; 49:258-63.

Miyoshi K, Miyoshi T, Siomi H. Many ways to generate microRNA-like small RNAs: non-canonical pathways for microRNA production. Mol Genet Genomics. 2010; 284:95-103.

Morel L, Regan M, Higashimori H, Ng SK, Esau C, Vidensky S, Rothstein J, Yang Y. Neuronal exosomal miRNA-dependent translational regulation of astroglial glutamate transporter GLT1. J Biol Chem. 2013; 288:7105-16.

Morlando M, Dini Modigliani S, Torrelli G, Rosa A, Di Carlo V, Caffarelli E, Bozzoni I. FUS stimulates microRNA biogenesis by facilitating co-transcriptional Drosha recruitment. EMBO J. 2012; 31:4502-10.

Müller M, Kuiperij HB, Claassen JA, Küsters B, Verbeek MM. MicroRNAs in Alzheimer's disease: differential expression in hippocampus and cell-free cerebrospinal fluid. Neurobiol Aging. 2014; 35:152-8.

Neumann M, Sampathu DM, Kwong LK, Truax AC, Micsenyi MC, Chou TT, Bruce J, Schuck T, Grossman M, Clark CM, McCluskey LF, Miller BL, Masliah E, Mackenzie IR, Feldman H, Feiden W, Kretzschmar HA, Trojanowski JQ, Lee VM. Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Science. 2006; 314:130-3.

Newman MA, Hammond SM. Emerging paradigms of regulated microRNA processing. Genes Dev. 2010; 24:1086-92.

Pacifici M, Delbue S, Kadri F, Peruzzi F. Cerebrospinal fluid MicroRNA profiling using quantitative real time PCR. J Vis Exp. 2014; e51172.

Paez-Colasante X, Figueroa-Romero C, Sakowski SA, Goutman SA, Feldman EL. Amyotrophic lateral sclerosis: mechanisms and therapeutics in the epigenomic era. Nat Rev Neurol. 2015; 11:266-79.

Pang C, Guan Y, Zhao K, Chen L, Bao Y, Cui R, Li G, Wang Y. Up-regulation of microRNA-15b correlates with unfavorable prognosis and malignant progression of human glioma. Int J Clin Exp Pathol. 2015; 8:4943-52.

Papagiannakopoulos T, Shapiro A, Kosik KS. MicroRNA-21 targets a network of key tumor-suppressive pathways in glioblastoma cells. Cancer. Res. 2008; 68:8164-72.

Parisi C, Arisi I, D'Ambrosi N, Storti AE, Brandi R, D'Onofrio M, Volonté C. Dysregulated microRNAs in amyotrophic lateral sclerosis microglia modulate genes linked to neuroinflammation. Cell Death Dis. 2013; 4:e959.

Pritchard CC, Cheng HH, Tewari M. MicroRNA profiling: approaches and considerations. Nat Rev Genet. 2012; 13:358-69.

Rao P, Benito E, Fischer A. MicroRNAs as biomarkers for CNS disease. Front Mol Neurosci. 2013; 6:39.

Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. J Cell Biol. 2013; 200:373-83.

Renton AE, Majounie E, Waite A, Simón-Sánchez J, Rollinson S, Gibbs JR, Schymick JC, Laaksovirta H, van Swieten JC, Myllykangas L, Kalimo H, Paetau A, Abramzon Y, Remes AM, Kaganovich A, Scholz SW, Duckworth J, Ding J, Harmer DW, Hernandez DG, Johnson JO, Mok K, Ryten M, Trabzuni D, Guerreiro RJ, Orrell RW, Neal J, Murray A, Pearson J, Jansen IE, Sondervan D, Seelaar H, Blake D, Young K, Halliwell N, Callister JB, Toulson G, Richardson A, Gerhard A, Snowden J, Mann D, Neary D, Nalls MA, Peuralinna T, Jansson L, Isoviita VM, Kaivorinne AL, Hölttä-Vuori M, Ikonen E, Sulkava R, Benatar M, Wuu J, Chiò A, Restagno G, Borghero G, Sabatelli M; ITALSGEN Consortium, Heckerman D, Rogaeva E, Zinman L, Rothstein JD, Sendtner M, Drepper C, Eichler EE, Alkan C, Abdullaev Z, Pack SD, Dutra A, Pak E, Hardy J, Singleton A, Williams NM, Heutink P, Pickering-Brown S, Morris HR, Tienari PJ, Traynor BJ. A hexanucleotide repeat expansion in C9ORF72 is the cause of chromosome 9p21-linked ALS-FTD. Neuron. 2011; 72:257-68.

Riggs AD, Russo VEA, Martienssen RA. Epigenetic mechanisms of gene regulation, Plainview, N.Y, Cold Spring Harbor Laboratory Press, 1996.

Robberecht W, Philips T. The changing scene of amyotrophic lateral sclerosis. Nat Rev Neurosci. 2013; 14:248-64.

Rothstein JD, Martin LJ, Kuncl RW. Decreased glutamate transport by the brain and spinal cord in amyotrophic lateral sclerosis. N Engl J Med. 1992; 326:1464-8.

Rothstein JD. Current hypotheses for the underlying biology of amyotrophic lateral sclerosis. Ann Neurol 2009; 65(Suppl 1): S3-9.

Rowland LP, Shneider NA. Amyotrophic lateral sclerosis. N Engl J Med. 2001; 344:1688-1700.

Roy S, Benz F, Luedde T, Roderburg C. The role of miRNAs in the regulation of inflammatory processes during hepatofibrogenesis. Hepatobiliary Surg Nutr. 2015; 4:24-33.

Russell AP, Wada S, Vergani L, Hock MB, Lamon S, Léger B, Ushida T, Cartoni R, Wadley GD, Hespel P, Kralli A, Soraru G, Angelini C, Akimoto T. Disruption of skeletal muscle mitochondrial network genes and miRNAs in amyotrophic lateral sclerosis. Neurobiol Dis. 2013; 49:107-17.

Sareen D, O'Rourke JG, Meera P, Muhammad AK, Grant S, Simpkinson M, Bell S, Carmona S, Ornelas L, Sahabian A, Gendron T, Petrucelli L, Baughn M, Ravits J, Harms MB, Rigo F, Bennett CF, Otis TS, Svendsen CN, Baloh RH. Targeting RNA foci in iPSC-derived motor neurons from ALS patients with a C9ORF72 repeat expansion. Sci Transl Med. 2013; 5:208ra149.

Sasaki S, Maruyama S. Immunocytochemical and ultrastructural studies of the motor cortex in amyotrophic lateral sclerosis. Acta Neuropathol. 1994; 87:578-85.

Saxena S, Caroni P. Selective neuronal vulnerability in neurodegenerative diseases: from stressor thresholds to degeneration. Neuron. 2011; 71:35-48.

Schwarzenbach H, Nishida N, Calin GA, Pantel K. Clinical relevance of circulating cell-free microRNAs in cancer. Nat Rev Clin Oncol. 2014; 11:145-56.

Shaw PJ, Ince PG, Falkous G, Mantle D. Oxidative damage to protein in sporadic motor neuron disease spinal cord. Annals of Neurology. 1995; 38:691–95.

Shaw PJ. Molecular and cellular pathways of neurodegeneration in motor neurone disease. J Neurol Neurosurg Psychiatry. 2005; 76:1046-57.

Sheinerman KS, Tsivinsky VG, Abdullah L, Crawford F, Umansky SR. Plasma microRNA biomarkers for detection of mild cognitive impairment: biomarker validation study. Aging (Albany NY). 2013; 5:925-38.

Sheinerman KS, Umansky SR. Circulating cell-free microRNA as biomarkers for screening, diagnosis and monitoring of neurodegenerative diseases and other neurologic pathologies. Front Cell Neurosci. 2013; 7:150.

Shi L, Cheng Z, Zhang J, Li R, Zhao P, Fu Z, You Y. hsa-mir-181a and hsa-mir-181b function as tumor suppressors in human glioma cells. Brain Res. 2008; 1236:185-93.

Shioya M, Obayashi S, Tabunoki H, Arima K, Saito Y, Ishida T, Satoh J. Aberrant microRNA expression in the brains of neurodegenerative diseases: miR-29a decreased in Alzheimer disease brains targets neurone navigator 3. Neuropathol Appl Neurobiol. 2010; 36:320-30.

Simon NG, Turner MR, Vucic S, Al-Chalabi A, Shefner J, Lomen-Hoerth C, Kiernan MC. Quantifying disease progression in amyotrophic lateral sclerosis. Ann Neurol. 2014; 76:643-57.

Smith BN, Newhouse S, Shatunov A, Vance C, Topp S, Johnson L, Miller J, Lee Y, Troakes C, Scott KM, Jones A, Gray I, Wright J, Hortobágyi T, Al-Sarraj S, Rogelj B, Powell J, Lupton M, Lovestone S, Sapp PC, Weber M, Nestor PJ, Schelhaas HJ, Asbroek AA, Silani V, Gellera C, Taroni F, Ticozzi N, Van den Berg L, Veldink J, Van Damme P, Robberecht W, Shaw PJ, Kirby J, Pall H, Morrison KE, Morris A, de Belleroche J, Vianney de Jong JM, Baas F, Andersen PM, Landers J, Brown RH Jr, Weale ME, Al-Chalabi A, Shaw CE. The C9ORF72 expansion mutation is a common cause of ALS+/-FTD in Europe and has a single founder. Eur J Hum Genet. 2013; 21:102-8.

Steinacker P, Feneberg E, Weishaupt J, Brettschneider J, Tumani H, Andersen PM,

von Arnim CA, Böhm S, Kassubek J, Kubisch C, Lulé D, Müller HP, Muche R, Pinkhardt E, Oeckl P, Rosenbohm A, Anderl-Straub S, Volk AE, Weydt P, Ludolph AC, Otto M. Neurofilaments in the diagnosis of motoneuron diseases: a prospective study on 455 patients. J Neurol Neurosurg Psychiatry. 2016; 87:12-20.

Sun Z, Diaz Z, Fang X, Hart MP, Chesi A, Shorter J, Gitler AD. Molecular determinants and genetic modifiers of aggregation and toxicity for the ALS disease protein FUS/TLS. PLoS Biol. 2011; 9:e1000614.

Sutedja NA, Fischer K, Veldink JH, van der Heijden GJ, Kromhout H, Heederik D, Huisman MH, Wokke JJ, van den Berg LH. What we truly know about occupation as a risk factor for ALS: a critical and systematic review. Amyotroph Lateral Scler. 2009; 10:295-301.

Takahashi I, Hama Y, Matsushima M, Hirotani M, Kano T, Hohzen H, Yabe I, Utsumi J, Sasaki H. Identification of plasma microRNAs as a biomarker of sporadic Amyotrophic Lateral Sclerosis. Mol Brain. 2015; 8:67.

Tasca E, Pegoraro V, Merico A, Angelini C. Circulating microRNAs as biomarkers of muscle differentiation and atrophy in ALS. Clin Neuropathol. 2016; 35:22-30.

Teplyuk NM, Mollenhauer B, Gabriely G, Giese A, Kim E, Smolsky M, Kim RY, Saria MG, Pastorino S, Kesari S, Krichevsky AM. MicroRNAs in cerebrospinal fluid identify glioblastoma and metastatic brain cancers and reflect disease activity. Neuro Oncol. 2012; 14:689-700.

Teunissen CE, Tumani H, Engelborghs S, Mollenhauer B. Biobanking of CSF: international standardization to optimize biomarker development. Clin Biochem. 2014; 47:288-92.

Tohgi H, Abe T, Yamazaki K, Murata T, Ishizaki E, Isobe C. Increase in oxidized NO products and reduction in oxidized glutathione in cerebrospinal fluid from patients with sporadic form of amyotrophic lateral sclerosis. Neurosci Lett. 1999; 260:204-6.

Toivonen JM, Manzano R, Oliván S, Zaragoza P, García-Redondo A, Osta R. MicroRNA-206: a potential circulating biomarker candidate for amyotrophic lateral sclerosis. PLoS One. 2014; 9:e89065.

Tokumaru S, Suzuki M, Yamada H, Nagino M, Takahashi T. let-7 regulates Dicer expression and constitutes a negative feedback loop. Carcinogenesis. 2008; 29:2073-7.

Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol. 2007; 9:654-9.

Van Blitterswijk M, Landers JE. RNA processing pathways in amyotrophic

lateral sclerosis. Neurogenetics. 2010; 11:275-90.

Van Hoecke A, Schoonaert L, Lemmens R, Timmers M, Staats KA, Laird AS, Peeters E, Philips T, Goris A, Dubois B, Andersen PM, Al-Chalabi A, Thijs V, Turnley AM, van Vught PW, Veldink JH, Hardiman O, Van Den Bosch L, Gonzalez-Perez P, Van Damme P, Brown RH Jr, van den Berg LH, Robberecht W. EPHA4 is a disease modifier of amyotrophic lateral sclerosis in animal models and in humans. Nat Med. 2012; 18:1418-22.

Vance C, Rogelj B, Hortobágyi T, De Vos KJ, Nishimura AL, Sreedharan J, Hu X, Smith B, Ruddy D, Wright P, Ganesalingam J, Williams KL, Tripathi V, Al-Saraj S, Al-Chalabi A, Leigh PN, Blair IP, Nicholson G, de Belleroche J, Gallo JM, Miller CC, Shaw CE. Mutations in FUS, an RNA processing protein, cause familial amyotrophic lateral sclerosis type 6. Science. 2009; 323:1208-11.

Volonte C, Apolloni S, Parisi C. MicroRNAs: newcomers into the ALS picture. CNS Neurol Disord Drug Targets. 2015;14:194-207.

Weydt P, Oeckl P, Huss A, Müller K, Volk AE, Kuhle J, Knehr A, Andersen PM, Prudlo J, Steinacker P, Weishaupt JH, Ludolph AC, Otto M. Neurofilaments levels as biomarkers in asymptomatic and symptomatic familial ALS. Ann Neurol. 2015 Nov 3.

Wijesekera LC, Leigh PN. Amyotrophic lateral sclerosis. Orphanet J Rare Dis. 2009; 4:3.

Williams AH, Valdez G, Moresi V, Qi X, McAnally J, Elliott JL, Bassel-Duby R, Sanes JR, Olson EN. MicroRNA-206 delays ALS progression and promotes regeneration of neuromuscular synapses in mice. Science. 2009; 326:1549-54.

Wu Z, Sun L, Wang H, Yao J, Jiang C, Xu W, Yang Z. MiR-328 expression is decreased in high-grade gliomas and is associated with worse survival in primary glioblastoma. PLoS One. 2012; 7:e47270.

Xia J, Broadhurst DI, Wilson M, Wishart DS. Translational biomarker discovery in clinical metabolomics: an introductory tutorial. Metabolomics. 2013; 9:280-299.

Xie F, Xiao P, Chen D, Xu L, Zhang B. miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs. Plant Mol Biol. 2012. [Epub ahead of print].

Zhang L, Dong LY, Li YJ, Hong Z, Wei WS. miR-21 represses FasL in microglia and protects against microglia-mediated neuronal cell death following hypoxia/ischemia. Glia. 2012; 60:1888-95.

Zhang Z, Almeida S, Lu Y, Nishimura AL, Peng L, Sun D, Wu B, Karydas AM, Tartaglia MC, Fong JC, Miller BL, Farese RV Jr, Moore MJ, Shaw CE, Gao FB. Downregulation of microRNA-9 in iPSC-derived neurons of FTD/ALS patients with TDP-43 mutations. PLoS One. 2013; 8:e76055.

PART VII

ANNEXES

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HFE p.H63D polymorphism does not influence ALS phenotype and survival

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ABSTRACT

It has been recently reported that the p.His63Asp polymorphism of the *HFE* gene accelerates disease progression both in the SOD1 transgenic mouse and in amyotrophic lateral sclerosis (ALS) patients. We have evaluated the effect of *HFE* p.His63Asp polymorphism on the phenotype in 1351 Italian ALS patients (232 of Sardinian ancestry). Patients were genotyped for the *HFE* p.His63Asp polymorphism (CC, GC, and GG). All patients were also assessed for *C9ORF72*, *TARDBP*, *SOD1*, and *FUS* mutations. Of the 1351 ALS patients, 363 (29.2%) were heterozygous (GC) for the p.His63Asp polymorphism and 30 (2.2%) were homozygous for the minor allele (GG). Patients with CC, GC, and GG polymorphisms did not significantly differ by age at onset, site of onset of symptoms, and survival; however, in SOD1 patients with CG or GG polymorphism had a significantly longer survival than those with a CC polymorphism. Differently from what observed in the mouse model of ALS, the *HFE* p.His63Asp polymorphism has no effect on ALS phenotype in this large series of Italian ALS patients.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder of adult life characterized by a progressive loss of upper (cortical) and lower (bulbar and spinal) motor neurons. The disease has an invariably fatal course over a period of 3–5 years. No disease-modifying therapy is available, with the exception of riluzole, an antiexcytotoxic drug that prolongs patients' life by 3 months. The cause of ALS is still unknown. About 10% of ALS patients have a family history of ALS or frontotemporal dementia whereas in ~90% of cases the disease appears sporadically in the population. The most common genes related to ALS are *C9ORF72*, *SOD1*, *TARDBP*, and *FUS*, but at least 20 other rarer genes have been identified (Finsterer and Burgunder, 2014; Renton et al., 2014). In addition, some genes have been found to modify the phenotype or the survival of ALS.

Polymorphisms of Unc-13 homolog A (UNC13 A) (Chiò et al., 2013; Diekstra et al., 2012) of nonimprinted in Prader-Willi and Angelman syndrome 1 (*NIPA1*) (Blauw et al., 2012) genes and polyQ intermediate-length expansion of *ATXN2* (Chiò et al., 2014) have been associated with a shorter survival, whereas a locus on 1p34.128 has been associated with a younger age at onset (Ahmeti et al., 2013).

Recently, it has been reported that the p.His63Asp polymorphism of the *HFE* gene accelerates disease progression in the ALS *SOD1* transgenic mouse (Nandar et al., 2014). Conversely, in a small study on 35 ALS patients, it has been reported that patients carrying the p.H63D polymorphism of the *HFE* gene had a significantly longer survival than those with the wild-type gene (Su et al., 2013).

The aim of this study was to assess the influence of the p.H63D polymorphism of the *HFE* gene on the phenotype and survival of a large series of ALS patients of Italian and Sardinian ancestry.

2. Methods

2.1. Cases

ALS cases were collected through the Italian ALS Genetic (ITALSGEN) and the Sardinian ALS Genetic (SARDINIALS) consortia (Chiò et al., 2012, 2014). Cases were patients with definite, probable, probable-laboratory supported, and possible ALS diagnosed between 2006 and 2012. A total of 149 cases have been already reported (Restagno et al., 2007). All cases were also screened for most common ALS genes, that is, *C9ORF72*, *SOD1*, *TARDBP*, and *FUS*.

Table 1
Clinical characteristics of Italian and Sardinian ALS cases

	Italian ALS, $n = 1119$	Sardinian ALS, $n = 232$
Gender (female, %)	494 (44.1%)	92 (39.7%)
Mean age at onset (years)	62.2 (11.7)	60.5 (12.1)
Site of onset (Bulbar, %)	299 (26.7%)	48 (20.7%)
Genetic mutations		
Wild Type	1021 (91.2%)	139 (59.9%)
C9ORF72	50 (4.5%)	23 (9.9%)
SOD1	24 (2.1%)	2 (0.9%)
TARDBP	10 (0.9%)	68 (29.3%)
FUS	14 (1.3%)	0

Key: ALS, amyotrophic lateral sclerosis.

Table 2
Frequency of rs1799945 in Italian and Sardinian ALS patients and controls

Genotype	CC	GC	GG	Minor allele frequency	Total
Italians					
Cases	804 (71.8%)	293 (26.2%)	22 (2.0%)	0.151	1119
Controls	948 (72.8%)	322 (24.7%)	32 (2.5%)	0.148	1302
Sardinians					
Cases	154 (66.4%)	70 (30.2%)	8 (3.4%)	0.185	232
Controls	79 (65.3%)	38 (31.4%)	4 (3.3%)	0.190	121

Key: ALS, amyotrophic lateral sclerosis.

2.2. Controls

There were 1302 Italian and 121 Sardinian subjects without neurologic disorders, age- and gender-matched to cases. Of these, 162 Italian subjects have been previously reported (Restagno et al., 2007).

2.3. Genotyping

Cases and controls were genotyped using the Illumina NeuroX SNP array. The NeuroX platform consists of standard Illumina exome content of approximately 240,000 variants and over 24,000 custom content variants focusing on neurologic diseases (Nalls et al., 2015). Quality control parameters for genotype calling and filtering are as previously described (Nalls et al., 2015). Genotypes for rs1799945 (chr6:26091179, C > G, build 37) were extracted from the larger NeuroX dataset. SNP genotypes were not confirmed on another platform such as Sanger sequencing. However, the quality of genotyping has been assessed using Polar and Cartesian cluster plots for SNP rs1799945; the quality control metric of genotyping accuracy for this SNP was 0.835, indicating a high level of precision in assigning genotypes to samples (Supplementary Fig. 1).

2.4. Statistical methods

Comparisons between means were made with the Student's *t*-test or analysis of variance; comparison between categorical

Table 3
Mean age at onset according to *HFE* H63D genotype

	CC	GC	GG	*p* Value
Mean age at onset				
Italians	62.3 (11.2)	62.2 (11.7)	62.5 (11.2)	0.92
Sardinians	60.2 (12.8)	60.6 (10.5)	65.4 (10.3)	0.78

Table 4
Site of symptom onset according to *HFE H63D* genotype

Site of symptom onset	CC	GC	GG	*p* Value
Italians				
Bulbar	216	78	5	0.91
Spinal	588	215	17	
Sardinians				
Bulbar	31	15	2	0.93
Spinal	123	55	6	

variables was made with the χ^2 test; Levene's test was used to confirm the equality of variances. Survival was calculated from onset to death/tracheostomy or censoring date (October 31, 2014) using the Kaplan-Meier survival modeling, and differences in survival were measured by the log-rank test. No patients were lost to follow up. Multivariable analysis was performed with the Cox proportional hazards model (stepwise backward) with a retention criterion of $p < 0.1$. The significance level was set at $p < 0.05$. Data were processed using SPSS statistical package, version 22.0 (IBM Corporation, Chicago, IL, USA).

2.5. Ethical approval

The study has been approved by the ethical committees of the involved centers. All patients and controls signed a written informed consent. Databases were treated according to the Italian regulations for privacy.

3. Results

A total of 1119 Italian and 232 Sardinian ALS patients have been included in the study. Patients' clinical characteristics and genetic mutations are reported in Table 1.

3.1. HFE genotyping

The frequency of CC, GC, and GG genotypes in Italian and Sardinian ALS cases and controls is reported in Table 2. No significant differences were found in either population. Genotype frequencies are respected Hardy-Weinberg equilibrium in both cohorts (not shown).

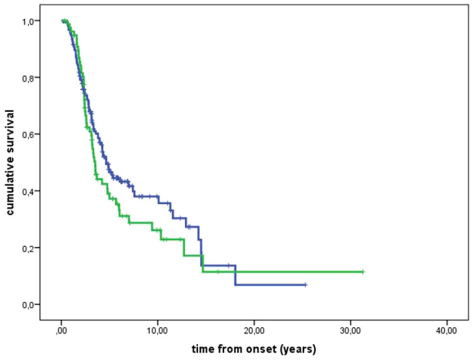

Fig. 2. Sardinian patients. Survival curves by *HFE* genotype. CC, median survival time 4.7 years (interquartile range 2.4–14.2); GC/GG, median survival time 3.5 years (interquartile range 2.3–10.3). p = n.s. CC, blue; GC/GG green.

3.2. Clinical characteristics of patients with different genotypes

Patients with CC, GC, and GG genotypes did not differ by age at onset and site of onset (Tables 3 and 4). No difference of survival was found considering both the CC/GC/GG phenotypes and the presence of a G allele in either cohorts of patients (Figs. 1 and 2). This finding has been confirmed in Cox multivariable analysis.

We also assessed the possible effect of HFE phenotypes in patients carrying genetic mutations. A list of identified genetic mutations is reported in Supplementary Table 1. No difference was found in the groups of patients carrying *C9ORF72*, *FUS*, and *TARDBP* mutations. In the 26 patients with *SOD1* mutations, we found an increased survival in patients with GC or GG compared with CC genotypes or in patients carrying the G allele (dominant assumption) ($p = 0.04$; Fig. 3). This finding is confirmed by the multivariable Cox model, where the G is retained as an independent prognostic factor ($p = 0.03$). A list of all SOD1 mutated cases with clinical details and HFE status is reported in Table 5.

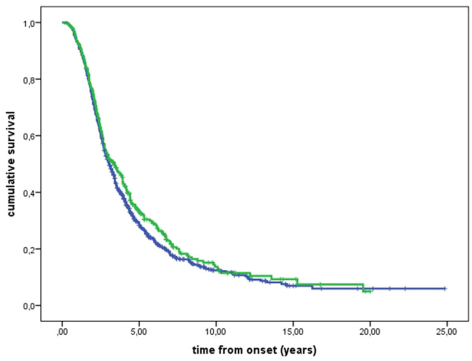

Fig. 1. Italian patients. Survival curves by *HFE* genotype. CC, median survival time 3.0 years (interquartile range 1.9–5.5); GC/GG, median survival time 3.4 years (interquartile range 2.0–6.7). p = n.s. CC, blue; GC/GG green.

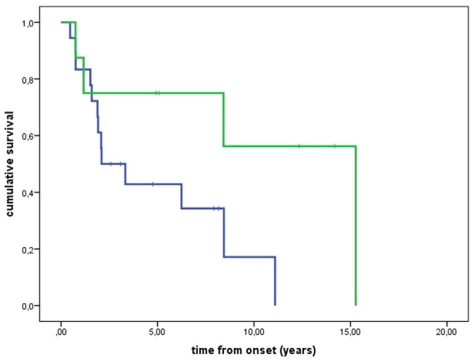

Fig. 3. Italian patients carrying *SOD1* mutations. CC, median survival time 2.1 years (interquartile range 2.6–8.4); GC/GG, median survival time 15.3 years (interquartile range 1.2–15.3). $p = 0.04$. CC, blue; GC/GG green.

Table 5
Clinical characteristics and HFE status in patients with *SOD1* mutation

Code	Sardinian	*SOD1* mutation	Age at onset	Type of onset	Gender	HFE alleles	Alive/dead	Duration (years)
P02007-295	N	A4V	82.74	S	F	CC	D	2.10
SLA2011-362	N	G93D	60.02	S	M	CC	D	0.75
SLA2012-313	N	S134N	73.47	S	F	CC	D	2.08
FALS-SI24	N	G41S	52.03	S	M	CC	D	0.76
SLA2010-240	N	G93D	45.00	S	F	CC	D	6.24
SLA2008-201	N	D101G	50.21	S	F	CC	D	1.92
SLA2010-292	N	L38V	46.74	S	F	CC	D	0.47
512-SN	N	N19S	28.14	S	F	CC	D	11.09
SLA2009-24	N	E132del	53.20	S	M	CC	D	8.44
SLA2010-495	N	D90A heterozygous	85.52	B	F	CC	D	1.52
SLA2009-217	Y	A4T	45.68	S	M	CC	D	3.33
SLA2009-28	N	G93D	57.95	S	F	CC	D	1.89
2543-SE	N	N19S	77.70	S	F	CC	D	1.59
SLA2010-489	N	D109Y	56.68	S	F	CC	A	7.92
SLA2011-455	N	N19S	57.04	S	F	CC	A	3.09
SLA2009-107	N	I150T	45.38	S	F	CC	A	8.17
SLA2013-60	N	G93D	18.00	S	F	CC	A	2.58
SLA2009-02	N	L144F	44.63	S	F	CC	A	4.75
SLA2008-187	N	D11Y	56.25	S	F	GC	D	8.42
SLA2008-37	N	D90A heterozygous	44.20	S	M	GC	D	15.27
SLA2010-146	N	D90N	70.70	S	F	GC	D	0.75
SLA2011-30	N	A4V	70.67	S	F	GC	D	1.17
SLA2012-141	N	D11Y	40.31	S	M	GC	A	14.18
SLA2009-178	Y	A95G	69.18	S	M	GC	A	12.34
SLA2011-197	N	D109Y	57.51	S	F	GC	A	5.09
SLA2009-299	N	L84F	27.97	S	M	GG	A	4.92

Sardinian: N, no; Y, yes; type of onset: S, spinal; B, bulbar; gender: F, female, M, male; alive/dead: A, alive, D, dead.

4. Discussion

In 2 cohorts of Italian and Sardinian patients, we have found that the p.H63D polymorphism of the *HFE* gene does not represent a risk factor for ALS. Moreover, we showed that the presence of the G allele does not modify the overall patients' clinical phenotype and survival. However, patients with *SOD1* mutations carrying the G allele had a better survival than other patients. In subjects with *C9ORF72*, *TARDBP*, and *FUS* mutations, the p.H63D polymorphism did not modify the phenotype and survival.

Several articles have suggested that the p.H63D polymorphism of *HFE* represents a risk factor for ALS (Goodall et al., 2005; Restagno et al., 2007; Sutedja et al., 2007), but others did not confirm this finding; 2 meta-analyses of literature arrived at opposite conclusions (Li et al., 2014; van Rheenen et al., 2013). This discrepancy may arise from several reasons: first, some articles are based on small, underpowered series; second, in some studies controls are not matched by ethnic origin to cases. A meta-analysis of worldwide HFE genotypes showed that the frequency of G polymorphism ranges from 30% in southern Europe, 20% in northern Europe, 15% in the Indian subcontinent, 5% in China, and 5% in sub-Saharan Africa (Hanson et al., 2001). Interestingly, the minor allele frequency observed for rs1799945 among the Italian samples analyzed in the present study (723 G alleles of 4842 alleles; minor allele frequency = 0.149) is comparable to its frequency reported in the ExAC browser (9128 G alleles of 66,738 alleles; minor allele frequency = 0.137; http://exac.broadinstitute.org/variant/6-26091179-C-G, accessed June 6, 2015).

In our 2 large cohorts of patients of Italian and Sardinian ancestry, compared with regionally matched controls, we have found that the p.H63D polymorphism of HFE does not increase the risk of developing ALS.

Recently, an article on a small series of ALS patients reported a significantly increased survival of subjects carrying the G allele compared with those who were homozygous for the C allele of the *HFE* gene (Su et al., 2013). The authors also found that the presence of the G allele was associated with a reduction of SOD1 activity in the muscle and that SOD1 protein expression was negatively associated with total disease duration in ALS patients. In a subsequent article, the same group reported that a double transgenic mouse line (SOD1/H67D) carrying the *H67D* HFE (homolog of human H63D) and *SOD1* (G93A) mutations have a shorter survival and an accelerated disease progression (Nandar et al., 2014); they therefore concluded that when HFE is combined with a mutation in an ALS gene the disease duration could be negatively impacted. The authors suggested that H63D *HFE* polymorphism can modify ALS pathophysiology via pathways involving oxidative stress, gliosis, and disruption of cellular functions.

Previously, the relationship between survival and HFE polymorphisms had been assessed only in a French series of ALS patients with negative results (Praline et al., 2012). However, in this article, no distinction between patients carrying or not-carrying *SOD1* mutations was made.

In our series, we found that in both populations the presence of a G allele or GG/GC phenotypes did not influence overall patients' survival. We also looked at the patients carrying mutations of major ALS genes. No effect of HFE status was found in patients with *C9ORF72*, *TARDBP*, and *FUS* mutations. Conversely, in patients with *SOD1* mutations the presence of a G allele was found to be significantly associated with a longer survival. This finding is in contrast with the reported shorter survival in the double transgenic mouse line (SOD1/H67D) (Nandar et al., 2014), highlighting the possibility that genetic interactions in mice compared with humans are biologically different. However, because of the small number of patients carrying a *SOD1* mutation in this series, our finding should be considered with caution, because of the possibility of a type 1 error.

In conclusion, we found that in 2 large cohorts of Italian and Sardinian patients, HFE p.H63D polymorphism is not a risk factor for ALS and does not modify the phenotype and survival of patients with ALS. However, we found a possible interaction between the presence of a *SOD1* genetic mutation and HFE genotype, with better survival in subjects carrying the G allele. Although based on a small cohort of patients, this interaction warrants further studies to better understand the genetic mechanisms underlying ALS.

Disclosure statement

The authors have no actual or potential conflicts of interest.

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Adriano Chiò had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. We thank the patient and her family for having collaborated to this study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neurobiolaging.2015.06.016.

References

Ahmeti, K.B., Ajroud-Driss, S., Al-Chalabi, A., Andersen, P.M., Armstrong, J., Birve, A., Blauw, H.M., Brown, R.H., Bruijn, L., Chen, W., Chiò, A., Comeau, M.C., Cronin, S., Diekstra, F.P., Soraya Gkazi, A., Glass, J.D., Grab, J.D., Groen, E.J., Haines, J.L., Hardiman, O., Heller, S., Huang, J., Hung, W.Y., ITALSGEN consortium, Jaworski, J.M., Jones, A., Khan, H., Landers, J.E., Langefeld, C.D., Leigh, P.N., Marion, M.C., McLaughlin, R.L., Meininger, V., Melki, J., Miller, J.W., Mora, G., Pericak-Vance, M.A., Rampersaud, E., Robberecht, W., Russell, L.P., Salachas, F., Saris, C.G., Shatunov, A., Shaw, C.E., Siddique, N., Siddique, T., Smith, B.N., Sufit, R., Topp, S., Traynor, B.J., Vance, C., van Damme, P., van den Berg, L.H., van Es, M.A., van Vught, P.W., Veldink, J.H., Yang, Y., Zheng, J.G., ALSGEN Consortium, 2013. Age of onset of amyotrophic lateral sclerosis is modulated by a locus on 1p34.1. Neurobiol. Aging 34, 357.e7–357.e19.

Blauw, H.M., van Rheenen, W., Koppers, M., an Damme, P., Waibel, S., Lemmens, R., van Vught, P.W., Meyer, T., Schulte, C., Gasser, T., Cuppen, E., Pasterkamp, R.J., Robberecht, W., Ludolp, A.C., Veldink, J.H., van den Berg, L.H., 2012. NIPA1 polyalanine repeat expansions are associated with amyotrophic lateral sclerosis. Hum. Mol. Genet. 21, 2497–2502.

Chiò, A., Borghero, G., Restagno, G., Mora, G., Drepper, C., Traynor, B.J., Sendtner, M., Brunetti, M., Ossola, I., Calvo, A., Pugliatti, M., Sotgiu, M.A., Murru, M.R., Marrosu, M.G., Marrosu, F., Marinou, K., Mandrioli, J., Sola, P., Caponnetto, C., Mancardi, G., Mandich, P., La Bella, V., Spataro, R., Conte, A., Monsurrò, M.R., Tedeschi, G., Pisano, F., Bartolomei, I., Salvi, F., Lauria Pinter, G., Simone, I., Logroscino, G., Gambardella, A., Quattrone, A., Lunetta, C., Volanti, P., Zollino, M., Penco, S., Battistini, S., ITALSGEN consortium, Renton, A.E., Majounie, E., Abramzon, Y., Conforti, F.L., Giannini, F., Corbo, M., Sabatelli, M., 2012. Clinical characteristics of patients with familial amyotrophic lateral sclerosis carrying the pathogenic GGGGCC hexanucleotide repeat expansion of C9ORF72. Brain 135, 784–793.

Chiò, A., Calvo, A., Moglia, C., Canosa, A., Brunetti, M., Barberis, M., Restagno, G., Conte, A., Bisogni, G., Marangi, G., Moncada, A., Lattante, S., Zollino, M., Sabatelli, M., Bagarotti, A., Corrado, L., Mora, G., Bersano, E., Mazzini, L., D'Alfonso, S., PARALS, 2014. ATXN2 polyQ intermediate repeats are a modifier of ALS survival. Neurology 84, 251–258.

Chiò, A., Mora, G., Restagno, G., Brunetti, M., Ossola, I., Barberis, M., Ferrucci, L., Canosa, A., Manera, U., Moglia, C., Fuda, G., Traynor, B.J., Calvo, A., 2013. *UNC13A* influences survival in Italian amyotrophic lateral sclerosis patients: a population-based study. Neurobiol. Aging 34, 357.e1–357.e5.

Diekstra, F.P., van Vught, P.W., van Rheenen, W., Koppers, M., Pasterkamp, R.J., van Es, M.A., Schelhaas, H.J., de Visser, M., Robberecht, W., Van Damme, P., Andersen, P.M., van den Berg, L.H., Veldink, J.H., 2012. *UNC13A* is a modifier of survival in amyotrophic lateral sclerosis. Neurobiol. Aging 33, 630.e3–630.e8.

Finsterer, J., Burgunder, J.M., 2014. Recent progress in the genetics of motor neuron disease. Eur. J. Med. Genet. 57, 103–112.

Goodall, E.F., Greenway, M.J., van Marion, I., Carroll, C.B., Hardiman, O., Morrison, K.E., 2005. Association of the H63D polymorphism in the *hemochromatosis* gene with sporadic ALS. Neurology 65, 934–937.

Hanson, E.H., Imperatore, G., Burke, W., 2001. HFE gene and hereditary hemochromatosis: a HuGE review. Am. J. Epidemiol. 154, 193–206.

Li, M., Wang, L., Wang, W., Qi, X.L., Tang, Z.Y., 2014. Mutations in the *HFE* gene and sporadic amyotrophic lateral sclerosis risk: a meta-analysis of observational studies. Braz. J. Med. Biol. Res. 47, 215–222.

Nalls, M.A., Bras, J., Hernandez, D.G., Keller, M.F., Majounie, E., Renton, A.E., Saad, M., Jansen, I., Guerreiro, R., Lubbe, S., Plagnol, V., Gibbs, J.R., Schulte, C., Pankratz, N., Sutherland, M., Bertram, L.M., Lill, C., DeStefano, A.L., Faroud, T., Eriksson, N., Tung, J.Y., Edsall, C., Nichols, N., Brooks, J., Arepalli, S., Pliner, H., Letson, C., Heutink, P., Martinez, M., Gasser, T., Traynor, B.J., Wood, N., Hardy, J., Singleton, A.B., International Parkinson's Disease Genomics Consortium (IPDGC) and the Parkinson's Disease meta-analysis consortium, 2015. NeuroX, a fast and efficient genotyping platform for investigation of neurodegenerative diseases. Neurobiol. Aging 36, 1605.e7–1605.e12.

Nandar, W., Neely, E.B., Simmons, Z., Connor, J.R., 2014. H63D *HFE* genotype accelerates disease progression in animal models of amyotrophic lateral sclerosis. Biochim. Biophys. Acta 1842, 2413–2426.

Praline, J., Blasco, H., Vourc'h, P., Rat, V., Gendrot, C., Camu, W., Andres, C.R., French ALS Study Group, 2012. Study of the HFE gene common polymorphisms in French patients with sporadic amyotrophic lateral sclerosis. J. Neurol. Sci. 15, 58–61.

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

Restagno, G., Lombardo, F., Ghiglione, P., Calvo, A., Cocco, E., Sbaiz, L., Mutani, R., Chiò, A., 2007. *HFE* H63D polymorphism is increased in patients with amyotrophic lateral sclerosis of Italian origin. J. Neurol. Neurosurg. Psychiatry 78, 327.

Su, X.W., Lee, S.Y., Mitchell, R.M., Stephens, H.E., Simmons, Z., Connor, J.R., 2013. H63D *HFE* polymorphisms are associated with increased disease duration and decreased muscle superoxide dismutase-1 expression in amyotrophic lateral sclerosis patients. Muscle Nerve 48, 242–246.

Sutedja, N.A., Sinke, R.J., Van Vught, P.W., Van der Linden, M.W., Wokke, J.H., van Duijn, C.M., Njajou, O.T., Van der Schouw, Y.T., Veldink, J.H., Van den Berg, L.H., 2007. The association between H63D mutations in *HFE* and amyotrophic lateral sclerosis in a Dutch population. Arch. Neurol. 64, 63–67.

van Rheenen, W., Diekstra, F.P., van Doormaal, P.T., Seelen, M., Kenna, K., McLaughlin, R., Shatunov, A., Czell, D., van Es, M.A., van Vught, P.W., van Damme, P., Smith, B.N., Waibel, S., Schelhaas, H.J., van der Kooi, A.J., de Visser, M., Weber, M., Robberecht, W., Hardiman, O., Shaw, P.J., Shaw, C.E., Morrison, K.E., Al-Chalabi, A., Andersen, P.M., Ludolph, A.C., Veldink, J.H., van den Berg, L.H., 2013. H63D polymorphism in HFE is not associated with amyotrophic lateral sclerosis. Neurobiol. Aging 34, 1517.e5–1517.e7.

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Research paper

Lack of relationship between the P413L chromogranin B variant and a SALS Italian cohort

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ABSTRACT

Chromogranins were reported to interact specifically with mutant forms of superoxide dismutase that are linked to amyotrophic lateral sclerosis (ALS). Particularly, a variation c.1238C>T (p.Pro413Leu) in the chromogranin B gene, CHGB, has been associated with an earlier age at onset in both familial and sporadic ALS in French/French–Canadian populations studied.

The aim of our study was to evaluate the P413L chromogranin variation in Italian patients with sporadic ALS. The study included 366 Italian patients with sporadic ALS and 382 control subjects. Genotyping of the polymorphism P413L in the CHGB gene was performed and the clinical characteristics of patients were analyzed in relation to their genotype. Our study on a cohort of Italian patients with SALS and controls failed to confirm an increased frequency of the 413L variant in SALS patients. Furthermore, we did not confirm the previous observation of a difference of age at onset between T-allele carriers and non-carriers (median age of onset 58.5 vs. 60.2 years of age, respectively). Our findings do not support the 413L variant as a risk factor for sporadic ALS in the Italian population.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the progressive loss of upper and lower motor neurons. Inheritance of the disease is seen in approximately 10% of ALS, with the remainder occurring as apparently sporadic cases (Wijesekera and Leigh, 2009). Around 25–35% of familial ALS (FALS) cases have been attributed to mutations in genes including SOD1, TARDBP, FUS and UBQLN2 (Rosen et al., 1993; Sreedharan et al., 2008; Vance et al., 2009; Deng et al., 2011). Rare mutations in OPTN, VCP and FIG4 are also thought to occur for a small proportion of cases (Andersen and Al-Chalabi, 2011). Recently, a pathogenic expansion of a non-coding hexanucleotide repeat sequence (GGGGCC) in the C9ORF72 gene was reported in familial and sporadic forms of ALS (DeJesus-Hernandez et al., 2011; Renton et al., 2011).

Chromogranins (CgA and CgB) are major constituents of secretory large dense-core vesicles in neurons (Taupenot et al., 2003) and may act as chaperone-like proteins promoting secretion of mutant SOD1 (Urushitani et al., 2006) that can activate microglia leading to neuronal death. Oxidized wild-type SOD1 appears to have similar binding properties to mutant SOD1 (Furukawa et al., 2006; Rakhit et al., 2004). Experiments on lysates of a neuroblastoma cell line treated with H_2O_2 showed that oxidized wt SOD1 coimmunoprecipitated with CgB (Ezzi et al., 2007). In addition, SOD1 and chromogranins have been viewed to colocalize in aggregates in motor neurons of SALS patients (Schrott-Fischer et al., 2009), suggesting a potential role for CgB in ALS pathogenesis.

Gros-Louis et al. (2009) performed a classical candidate gene case–control study on Chromogranin B (CHGB) variations in ALS patients of French, French–Canadian, and Scandinavian origins. They found a significant association between a missense variation c.1238C>T (rs742710) in exon 4, coding for a leucine in place of a proline at codon 413 (P413L), and ALS susceptibility. The presence of this variant conferred an ≈3.3-fold increased risk of ALS in the French/French–Canadian population studied. Furthermore, this c.1238C>T CHGB variant would also act as a modifier of disease onset by decreasing the median age at onset by 7 years in sporadic ALS patients and by as much as 11 years in familial ALS.

Abbreviations: ALS, amyotrophic lateral sclerosis; CHGB, chromogranin B; SLAS, sporadic amyotrophic lateral sclerosis; FALS, familial amyotrophic lateral sclerosis; PCR, polymerase chain reaction.

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On this trail, other two studies were performed by two different groups (Van Vught et al., 2010; Blasco et al., 2011) and did not support the 413L variant as a risk factor for sporadic ALS in French and Dutch populations.

Herein we present the screening of an Italian cohort of 366 SALS and 382 healthy individuals for the variant c.1238C>T of the CHGB gene.

2. Materials and methods

2.1. Patients

The study included 366 sporadic ALS patients and 382 control subjects of Caucasian origin, belonging to Italian ancestry and living in Northern and Central Italy.

ALS diagnosis was made accordingly to El Escorial Revisited criteria (Brooks et al., 2000). Only patients with diagnosis of definite, probable and probable laboratory supported ALS were included in the study. Briefly, sites of onset were recorded as spinal vs. bulbar. Age at onset was defined by the onset of the first symptoms. The survival endpoint was death or time of initiation of all forms of invasive ventilatory support. The mean duration of the disease was defined as the time occurring between onset and survival endpoint. Living cases were excluded from the calculation of the mean disease duration.

The control group consisted of age- and sex-matched individuals from the same ethnic background with no history of neurological diseases. Characteristics of ALS patients and controls are summarized in Table 1.

Written consent for genetic analysis was obtained from each individual. This study was approved by the local ethics committee in accordance with the ethical standards of the Declaration of Helsinki.

2.2. Genetic analysis

Genomic DNA from each ALS patient was extracted from peripheral blood leukocytes using standard procedures. Genotyping of the polymorphism c.1238C>T in the CHGB gene (NM_001819.2) was performed by PCR followed by restriction digest analysis using the enzyme MspI.

2.3. Statistical analysis

The estimation of the power of our sample to detect an association was performed by using the statistical program QUANTO version 1.2.4 (Gauderman, 2002).

Association analyses were carried out by using the software package SPSS v13.0. Interaction with single nucleotide polymorphisms (SNP) was tested by χ^2 analysis at genotypic and allelic levels. To evaluate the association of P413L variation with ALS clinical variables, patients were stratified in different groups for each variable. In particular, patients were stratified by gender (males/females), age of onset (<45 years/≥45 years, taking 45 years as arbitrary cut-off to discriminate early and late onset), and site of onset (spinal/bulbar). Association analyses were carried out by χ^2 analysis or Fischer's exact tests.

Genotype and allele associations with disease duration were estimated with univariate analysis according to the Kaplan–Meier method using the log-rank test to assess statistical differences between groups. Analysis was performed considering only deceased patients and considering both living and deceased individuals, using both censored and non-censored approaches. Analysis was performed stratifying patients in carriers and non-carriers of the P413L variation.

ALS onset probability based on age at symptom onset was also assessed by deriving Kaplan–Meier curves according to P413L polymorphism (carriers vs. non-carriers) and using the log-rank test. Unpaired *t* test (mean ± standard deviation) was used to calculate and compare the mean age of onset for each group.

Table 1
Characteristics of patients with ALS and controls.

	ALS patients	Controls
No. of subjects	366	382
Gender	194 M/172 F (1.13/1)	214 M/168 F (1.27/1)
Age at blood collection (years)	61.2 ± 15.1	56.4 ± 17.9
Age at onset	60.0 ± 14.4	
Bulbar onset	30.0%	
Disease duration (months)	35.8 ± 34.6	

3. Results

Our study had 80% power to detect an odds ratio of ≥1.80 given the known allele frequency and a significance cut-off of 0.05. The studied SNP was in Hardy–Weinberg equilibrium in both cases and controls ($p > 0.05$). Genotype and allele frequencies are shown in Table 2. Rare T-allele was only found in the heterozygous state both in patients and controls. No significant difference in distributions was observed in the two groups.

Association of genotypes and alleles with gender, site of disease onset (spinal versus bulbar) and age at disease onset was also evaluated. No significant associations were found with the ALS clinical variables examined. The results are summarized in Table 3. Survival analysis did not reveal any association of genotypes and alleles with the disease duration, using a censored approach (Table 3), a non-censored approach, and considering only deceased individuals (data not shown).

There was no association between age at onset and T-allele carrier status: ALS patients carrying the P413L variation had a median age of onset of 58.5 ± 15.4 years, compared to 60.2 ± 14.3 years for ALS patients without the variation ($p = 0.59$). Similarly, no significant difference was observed when ALS onset probability based on age at onset was evaluated by deriving Kaplan–Meier curves according to P413L polymorphism ($p = 0.768$) (Fig. 1).

4. Discussion

Chromogranin B belongs to the granin family and is a low affinity, high capacity calcium binding protein found in the hormone-storing organelles, the nucleus, and the endoplasmatic reticulum of excitable and non-excitable cells (Huh et al., 2005).

Evidence for a role of this protein in pathogenesis of ALS has been described (Rakhit et al., 2004; Urushitani et al., 2006; Furukawa et al., 2006; Ezzi et al., 2007; Schrott-Fischer et al., 2009). In particular, three studies tried to investigate the potential role of the polymorphism c.1238C>T in exon 4 of the CHGB gene in different ALS populations (Gros-Louis et al., 2009; Van Vught et al., 2010; Blasco et al., 2011). This SNP is located in the C-terminal region of CgB, crucial for inducing calcium release (Schmidt et al., 2011), but not within or in proximity of the known binding site to mutant SOD1, located in a Hsp-like domain (region 162–285) (Blasco et al., 2011). Moreover, the region containing the variation has not been conserved during evolution (Blasco et al., 2011), suggesting that the functional role for this region could be limited. None of the genome-wide association studies published to date showed an association of the region containing CHGB with ALS (Van Es et al., 2009). The first study, conducted by Gros-Louis, reported that rs742710 of the CHGB gene was associated with ALS susceptibility and age at onset in a population of 289 French (French or French–Canadian origins) ALS patients. The other two studies conducted on a large cohort

Table 2
Genotype and allelic distributions of P413L variation in sporadic ALS cases and controls.

	Cases (%)	Controls (%)	*p* value	OR	95% CI
P413L non-carriers (CC)	91.0	89.5	0.593	0.847	0.522–1.376
P413L carriers (TC)	9.0	10.5			
Allele C	95.5	94.8	0.604	0.858	0.511–1.442
Allele T	4.5	5.2			

Table 3
Genotype and allelic distributions of P413L variation for ALS clinical variables.

	Gender [a]	Age at onset <45 years/≥45 years[a]	Site of onset[a] Spinal/bulbar	Survival[b] Censored	ALS onset probability based on age[b]
Genotype *p* value	0.715	1.000	0.422	0.532	0.768
Allele *p* value	0.605	0.552	0.387	0.532	0.768

Key: ALS, amyotrophic lateral sclerosis.
[a] *p* values were calculated using χ^2 or Fischer's exact tests.
[b] Survival analysis and ALS onset probability based on age of onset were estimated using the Kaplan–Meier method and compared by the log-rank test. Regarding the alleles, patients were dichotomized in carriers and non-carriers of the risk allele (TC vs. CC).

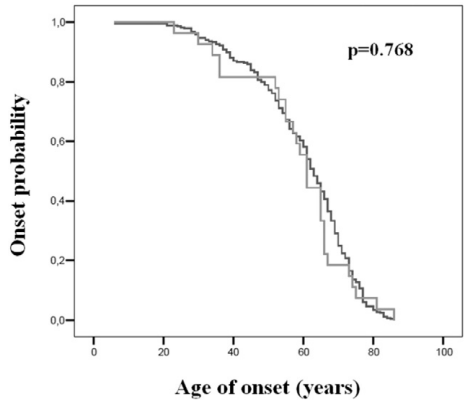

Fig. 1. Onset probability of ALS patients among carriers and non-carriers of the P413L variant. Gray curve corresponds to P413L variant carriers and black curve corresponds to non-carriers of P413L variant.

of 1082 Dutch patients and another French ALS population of 540 patients, respectively, did not find any association of the variation with an increased risk for developing ALS and with an earlier age at onset (Van Vught et al., 2010; Blasco et al., 2010); Table 4 reassumes the allele frequency in the different populations studied.

Therefore, we evaluated the potential role of the coding polymorphism c.1238C>T of the CHGB gene, in 366 patients with sporadic ALS and 382 healthy individuals belonging to Northern and Central Italy.

The frequency of the T-allele in our control group was 5.2%. This value was higher than those reported in French/French–Canadian (2.6%, n = 380), Swedish (1.8%, n = 303) (Gros-Louis et al., 2009) and Dutch (3.5%, n = 1812) (van Vught et al., 2010) control populations, but it was in line with the other French control populations (5.5%, n = 504) (Blasco et al., 2011) and with the results in the NCBI SNP database from European populations (http://www.ncbi.nlm.nih.gov/SNP/) that reports a frequency of the T-allele of 4.2%.

We are aware that French from France and French from Quebec are clearly distinct populations, indeed several studies highlight the great variety in types of relatedness present in the French Canadian founder population (Gauvin et al., 2014).

These data underline a distribution of T-allele quite uniform in Western–Southern Europe, but also a high variability among populations of different ethnic backgrounds.

Regarding the frequency of the mutated allele in SALS patients, we observed that the Italian and the French–Caucasian populations showed similar values (4.5% and 5.3% respectively) (Blasco et al., 2011). In the Dutch population, the frequency of the T-allele in SALS patients was lower (3.4%), however in these three populations there were no significant differences between patients and controls. On the contrary, Gros-Louis and colleagues reported a higher frequency of the T-allele in SALS patients in the French–Canadian population (8.3%), and a very low frequency in the Swedish population (2.6%). Anyway, in both cases there was an association trend between the presence of rare allele and the risk of ALS.

We also evaluated the influence of the c.1238C>T on the age at onset in the group of SALS patients. The T-allele was associated with an earlier age at onset by a decade in SALS French/French–Canadian and Swedish populations (53.3 years of age for the T-allele carriers versus 59.7 years of age for the non-carriers) (Gros-Louis et al., 2009). In our sample there was no relation between T-allele and age at onset (median age at onset: 58.5 vs. 60.2 years, respectively), in agreement with the data reported for French–Caucasian and Dutch populations (Blasco et al., 2011; van Vught et al., 2010).

Our work gives additional information in understanding the role of rs742710 of the CHGB gene in ALS. We can conclude that, in agreement with the data reported for French–Caucasian and Dutch populations, the rs742710 SNP does not confer an increased risk for developing ALS and an early age at onset on an Italian population.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Acknowledgments

All authors wish to thank the patients and their families.

Table 4
Allelic distributions of T allele (c.1238C>T) in different SALS populations.

SALS	Controls	Population	References
40/482 (8.3%)	20/760 (2.6%)	French/French Canadian	Gros-Louis et al. (2009)
16/630 (2.6%)	11/606 (1.8%)	Swedish	Gros-Louis et al. (2009)
70/2056 (3.4%)	127/3624 (3.5%)	Dutch	Van Vught et al. (2010)
57/1080 (5.3%)	55/1008 (5.4%)	French Caucasian	Blasco et al. (2011)

SALS, sporadic amyotrophic lateral sclerosis.

References

Andersen, P.M., Al-Chalabi, A., 2011. Clinical genetics of amyotrophic lateral sclerosis: what do we really know? Nat. Rev. Neurol. 7, 603–615.

Blasco, H., Corcia, P., Veyrat-Durebex, C., Coutadeur, C., Fournier, C., Camu, W., et al., 2011. The P413L chromogranin B variation in French patients with sporadic amyotrophic lateral sclerosis. Amyotroph. Lateral Scler. 12, 210–214.

Brooks, B.R., Miller, R.G., Swash, M., 2000. Munsat. World Federation of Neurology Research Group on Motor Neuron Diseases: El Escorial revisited: revised criteria for the diagnosis of amyotrophic lateral sclerosis. Amyotroph. Lateral Scler. Other Motor Neuron Disord. 1, 293–299.

DeJesus-Hernandez, M., Mackenzie, I.R., Boeve, B.F., Boxer, A.L., Baker, M., Rutherford, N.J., et al., 2011. Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS. Neuron 72, 245–456.

Deng, H.X., Chen, W., Hong, S.T., Boycott, K.M., Gorrie, G.H., Siddique, N., et al., 2011. Mutations in UBQLN2 cause dominant X-linked juvenile and adult-onset ALS and ALS/dementia. Nature 477, 211–215.

Ezzi, S.A., Urushitani, M., Julien, J.P., 2007. Wild-type superoxide dismutase acquires binding and toxic properties of ALS linked mutant forms through oxidation. J. Neurochem. 102, 170–178.

Furukawa, Y., Fu, R., Deng, H.X., Siddique, T., O'Halloran, T.V., 2006. Disulphide cross-linked protein represents a significant fraction of ALS-associated Cu/Zn superoxide dismutase aggregates in spinal cords of model mice. Proc. Natl. Acad. Sci. U. S. A. 103, 7148–7153.

Gauderman, W.J., 2002. Sample size requirements for association studies of gene–gene interaction. Am. J. Epidemiol. 155, 478–484.

Gauvin, H., Moreau, C., Lefebvre, J.F., Laprise, C., Vézina, H., Labuda, D., Gagnon, M.H., 2014. Genome-wide patterns of identity-by-descent sharing in the French Canadian founder population. Eur. J. Hum. Genet. 22, 814–821 (doi:10.1038).

Gros-Louis, F., Andersen, P.M., Dupre, N., Urushitani, M., Dion, P., Souchon, F., et al., 2009. Chromogranin B P413L variant as risk factor and modifier of disease onset for amyotrophic lateral sclerosis. Proc. Natl. Acad. Sci. U. S. A. 106, 21777–21782.

Huh, Y.H., Jeon, S.H., Yoo, J.A., Park, S.Y., Yoo, S.H., 2005. Effects of chromogranin expression on inositol 1,4,5-trisphosphate-induced intracellular Ca^{2+} mobilization. Biochemistry 44, 6122–6132.

Rakhit, R., Crow, J.P., Lepock, J.R., Kondejewski, L.H., Cashman, N.R., Chakrabartty, A., 2004. Monomeric Cu/Zn superoxide dismutase is a common misfolding intermediate in the oxidation models of sporadic and familial amyotrophic lateral sclerosis. J. Biol. Chem. 279, 15499–15504.

Renton, A.E., Majounie, E., Waite, A., Simón-Sánchez, J., Rollinson, S., Gibbs, J.R., et al., 2011. A hexanucleotide repeat expansion in C9ORF72 is the cause of chromosome 9p21-linked ALS-FTD. Neuron 72, 257–268.

Rosen, D.R., Siddique, T., Patterson, D., Figlewicz, D.A., Sapp, P., Hentati, A., et al., 1993. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature 362, 59–62.

Schmidt, S., Mo, M., Heidrich, F.M., Čelić, A., Ehrlich, B.E., 2011. C-terminal domain of chromogranin B regulates intracellular calcium signaling. J. Biol. Chem. 286, 44888–44896.

Schrott-Fischer, A., Bitsche, M., Humpel, C., Walcher, C., Maier, H., Jellinger, K., et al., 2009. Chromogranin peptides in amyotrophic lateral sclerosis. Regul. Pept. 152, 13–21.

Sreedharan, J., Blair, I.P., Tripathi, V.B., Hu, X., Vance, C., Rogelj, B., et al., 2008. TDP-43 mutations in familial and sporadic amyotrophic lateral sclerosis. Science 319, 1668–1672.

Taupenot, L., Harper, K.L., O'Connor, D.T., 2003. The chromogranin–secretogranin family. N. Engl. J. Med. 348, 1134–1149.

Urushitani, M., Sik, A., Sakurai, T., Nukina, N., Takahashi, R., Julien, J.P., 2006. Chromogranin-mediated secretion of mutant superoxide dismutase proteins linked to amyotrophic lateral sclerosis. Nat. Neurosci. 9, 108–118.

Van Es, M.A., Veldink, J.H., Saris, C.G., Blauw, H.M., van Vught, P.W., Birve, A., et al., 2009. Genome-wide association study identifies 19p13.3 (UNC13A) and 9p21.2 as susceptibility loci for sporadic amyotrophic lateral sclerosis. Nat. Genet. 41, 1083–1087.

Van Vught, P.W., Veldink, J.H., Van den Berg, L.H., 2010. P413L CHGB is not associated with ALS susceptibility or age at onset in a Dutch population. Proc. Natl. Acad. Sci. U. S. A. 107, E77.

Vance, C., Rogelj, B., Hortobgàyi, T., De Vos, K.J., Nishimura, A.L., Sreedharan, J., et al., 2009. Mutations in FUS, an RNA processing protein, cause familial amyotrophic lateral sclerosis type 6. Science 323, 1208–1211.

Wijesekera, L.C., Leigh, P.N., 2009. Amyotrophic lateral sclerosis. Orphanet J. Rare Dis. 4, 3. http://dx.doi.org/10.1186/1750-1172-4-3.

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RESEARCH

Genotyping of Macrophage Migration Inhibitory Factor (MIF) $CATT_{5–8}$ Repeat Polymorphism by Denaturing High-Performance Liquid Chromatography (DHPLC)

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Abstract Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine expressed in many different cell types and implicated in the pathogenesis of numerous acute and chronic inflammatory diseases. Variable Number of Tandem Repeat (VNTR) $CATT_{5–8}$ at position −794 in the promoter of the *MIF* gene has been associated with several human pathological conditions. Different methods for genotyping the CATT tetranucleotide repeats have been described. Here, we report, for the first time, the complete characterization of the $CATT_{5–8}$ repeat polymorphism using exclusively the denaturing high-performance liquid chromatography (DHPLC) technique under partially denaturing conditions. This approach, based on a step-by-step DHPLC protocol, allowed the accurate determination of all the homozygous and heterozygous genotypes in 350 DNA samples from control subjects. The results were validated by comparison to DNA sequencing, and the DHPLC approach was accurate, sensitive, and highly reproducible. Data from the current study demonstrate that this method of analysis by DHPLC may represent a powerful and sensitive alternative tool for a rapid and efficient genotyping of short tandem repeats presenting a limited number of alleles.

Keywords Macrophage migration inhibitory factor (MIF) · Denaturing high-performance liquid chromatography (DHPLC) · Genotyping · Polymorphisms · Variable number of tandem repeat (VNTR)

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Introduction

Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine which is implicated in the pathogenesis of many acute and chronic inflammatory diseases such as sepsis, rheumatoid arthritis, multiple sclerosis, and Alzheimer's disease [1–3].

MIF is expressed in many different cell types; in particular, it is produced by cells and tissues that are in direct contact with natural environment, such as the lung, the epithelial lining of the skin, and gastrointestinal and genitourinary tracts [1]. MIF is rapidly released by immune cells in response to microbial products, to proinflammatory cytokines, or during antigen-specific activation, and it has potent autocrine and paracrine effects that promote cell growth and survival [1]. Distinctive features of MIF include its capacity to counter-regulate the immunosuppressive effects of glucocorticoids on immune cells and sustain macrophage proinflammatory functions by inhibiting p53-dependent apoptosis [4, 5].

The human *MIF* gene spans less than 1 kilobase and is highly conserved. Two polymorphisms of *MIF* gene have been associated with human pathological conditions. Variable Number of Tandem Repeat (VNTR) CATT tetranucleotide sequence, repeated five to eight times at position −794 (rs5844572), correlates with disease severity in patients with rheumatoid arthritis [6], and G-to-C single-nucleotide polymorphism (SNP) at position −173 (rs755622) is associated with systemic-onset juvenile arthritis [7, 8]. CATT tetranucleotide is noted to influence *MIF* gene expression; in particular, the $CATT_5$ allele is typically referred as a "low-expression" allele, and the $CATT_6$, $CATT_7$, and (rare) $CATT_8$ alleles are considered "higher-expression" alleles [6]. The haplotype $CATT_7$/−173*C (contemporary presence of −173C allele and the CATT7 repeat in the same

chromosome) has been associated with susceptibility to inflammatory arthritis and atopy [8]. It is known that the distribution of MIF CATT allele frequencies is different in subjects from various populations [9].

Several methods have been previously described for genotyping the CATT tetranucleotide at position −794 in the *MIF* gene. These methods are based on PCR and single-strand conformation polymorphism (SSCP) [10], capillary electrophoresis on automated DNA sequencing system [6, 11, 12], or thin-film biosensor chips [13].

Here, we describe the use of the denaturing high-performance liquid chromatography (DHPLC) technique as a rapid, accurate, and cost-effective method to define the CATT tetranucleotide number variation in the *MIF* gene.

DHPLC is a technique commonly used to identify single-nucleotide substitutions, as well as small insertions and deletions, for mutation detection and genotyping [14]. At partially denaturing conditions, the separation chemistry is essentially based on sequence. After heating to 95 °C and slowly cooling, the PCR product of individuals, who are heterozygous in a single-nucleotide mutation or polymorphism, hybridizes and forms a mixture of hetero- and homoduplexes. Mutation detection analysis on the DHPLC system is performed at a temperature sufficient to partially denature (melt) the DNA heteroduplexes. The differential retention time (RT) on the cartridge allows for a rapid mutation or SNP detection. On the other hand, under non-denaturing conditions (50 °C), the sequence is not a factor in determining the elution behavior of the DNA, but the separation chemistry is based on size only. Thus, insertions and deletions are usually separated using non-denaturing conditions. Although the −794 CATT tetranucleotide polymorphism results in different sizes depending on the number of CATT repeats, in this work, the genotyping of the polymorphism has been performed exclusively by partially denaturing conditions. This analysis method has shown a better performance in genotyping than non-denaturing condition analysis and has provided high accuracy and precision in repeat determination.

Materials and Methods

DNA Samples

After informed consent, in accordance with local ethical committee guidelines, blood samples for DNA analysis were collected from 350 healthy subjects belonging to Italian ancestry. Genomic DNA was isolated from peripheral whole blood using standard procedures [15].

The whole sample series was previously genotyped for CATT tetranucleotide repeats by direct sequencing (ABI 310 Genetic Analyzer, Applied Biosystems).

PCR Analysis

The analysis of the CATT tetranucleotide repeated at position −794 in the regulatory region in MIF gene was carried out by PCR amplification using the following primers: Forward 5′-CTGCAGGAACCAATACCCAT-3′; Reverse 5′-GTCCCCGAGTTTACCATTAG-3′. The PCR reaction mixture (50 µl) contained AmpliTaq Gold PCR Master mix (Applied Biosystems), 0.2 µM each of the two oligonucleotide primers, and 20 ng of DNA. Cycling conditions consisted of initial denaturation and Taq polymerase activation at 95 °C for 18 min, followed by 30 cycles of 1 min at 94 °C, 1 min at 60 °C, 1 min at 72 °C, and a final extension of 10 min at 72 °C. The PCR products (345 bp) were visualized in a 2.5 % agarose gel containing ethidium bromide.

Denaturing High-Performance Liquid Chromatography (DHPLC)

PCR products were denatured for 10 min at 95 °C and then slowly reannealed by ramping the temperature down to 56 °C for 5 min. A 8 µl aliquot of each PCR product was injected into the DHPLC cartridge (Transgenomic Wave-MD Nucleic Acid Fragment Analysis System, Transgenomic Inc.) and eluted at a flow rate of 0.9 ml/min (run time: 7.5 min) with a mobile phase consisting of a mixture of buffer A (TEAA 0.1 M) and buffer B (TEAA 0.1 M and acetonitrile 25 %). The Navigator™ software (Vers. 1.6.4) was utilized to calculate both the specific temperature of analysis (57.3 °C) and the linear acetonitrile gradient buffer (buffer B was increased from 50.9 to 55.9 % in the first 30 s and then from 55.9 to 64.9 % in the following 5 min). Finally, the eluted DNA fragments were detected at 260 nm. The use of the Transgenomic Wave-MD system allows carrying out 96 chromatographic runs in a day.

In order to define the genotype of homoduplex samples, each homoduplex PCR product was mixed with an approximately equimolar volume of a control sample previously characterized as homozygous $CATT_{5/5}$. To distinguish between the different heteroduplex samples, each heteroduplex PCR product was mixed with an approximately equimolar volume of a control sample previously characterized as homozygous $CATT_{7/7}$. The mixtures were denatured by heating at 95 °C for 10 min and allowed to cool down to 56 °C for 5 min. 8 µl of each sample were then loaded on the DHPLC cartridge and analyzed as described above. In the case of two-step analysis, the time requested for the complete genotyping from the PCR

product includes 2 steps of denaturation (15 min/each step) and 2 DHPLC runs (7.5 min/each run), for a total of about 45 min.

Results

The first step of the DHPLC analysis was conducted in a subgroup of 50 samples, previously genotyped by automated sequencing, including all the six possible genotypes ($CATT_{5/5}$, $CATT_{6/6}$, $CATT_{7/7}$, $CATT_{5/6}$, $CATT_{5/7}$, and $CATT_{6/7}$). The $CATT_8$ allele was not present in our population, in agreement with previous studies failing to detect this allele or reporting a very low frequency in European populations [2, 16–18]. The homozygous genotypes $CATT_{5/5}$, $CATT_{6/6}$, and $CATT_{7/7}$ resulted in a chromatogram profile showing a single peak in DHPLC analysis, as depicted in Fig. 1a. PCR products heterozygous for $CATT_{5/6}$ and $CATT_{6/7}$ resulted in the same identical heteroduplex profile, characterized by two different peaks (defined "first heteroduplex profile," Fig. 2a). The PCR products heterozygous for $CATT_{5/7}$ showed a different characteristic heteroduplex profile displaying two clearly resolved peaks (defined "second heteroduplex profile," Fig. 2d) immediately identifiable.

Homozygote Characterization

The first DHPLC analysis was not able to discriminate among homozygous genotypes $CATT_{5/5}$, $CATT_{6/6}$, and $CATT_{7/7}$. For this reason, each sample showing a single elution peak was further analyzed by preparing an equimolar mixture with a PCR product obtained from a previously characterized homozygous $CATT_{5/5}$ control DNA to generate potential heteroduplex species and distinguish the homozygosity for $CATT_5$, $CATT_6$, and $CATT_7$ alleles. No alterations of chromatogram elution profile were detected for $CATT_{5/5}$ homozygote (Fig. 1b). Homozygous $CATT_{6/6}$ genotypes resulted in a characteristic heteroduplex chromatogram profile with two well-resolved peaks

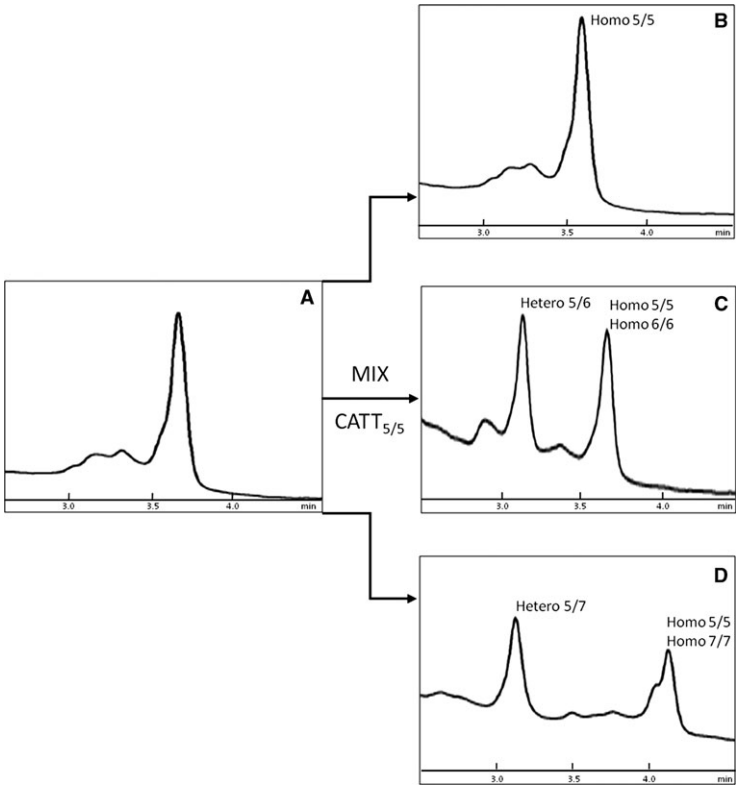

Fig. 1 Flowchart of DHPLC analysis for homozygote characterization. DHPLC elution profile of homozygous genotype is depicted in (**a**). After the first step, amplicons with homoduplex chromatogram profile were mixed in approximately equimolar proportions with a control sample previously characterized as homozygous $CATT_{5/5}$. This allowed differentiating the three homozygous genotypes. **b** Homoduplex profile of a sample with the same genotype as the added control sample (homozygous $CATT_5$). **c** Heteroduplex profile of a sample that differed from the control for one tetranucleotide repeat (homozygous $CATT_6$). **d** Heteroduplex profile of a sample that differed from the control for two repeats (homozygous $CATT_7$). On the top of each peak, the specific heteroduplex or homoduplex condition is indicated

(Fig. 1c). The second peak showed a typical retention time of 3.62 min ± 0.036. Homozygous $CATT_{7/7}$ genotypes resulted instead in a heteroduplex elution profile characterized by two less closely spaced peaks, with a typical RT for the second peak of 4.21 min ± 0.074. In addition, the second peak showed a distinctive weak shoulder in the left side (Fig. 1d).

Heterozygote Characterization

In order to distinguish between the PCR products heterozygous for $CATT_{5/6}$ and $CATT_{6/7}$, we analyzed in DHPLC all heteroduplex samples showing the first heteroduplex profile (Fig. 2a) after mixing them with the PCR product from a previously characterized $CATT_{7/7}$ control DNA. No changes in the elution profiles were found for heterozygous $CATT_{6/7}$ samples (Fig. 2b). The heterozygous condition $CATT_{5/6}$ showed a DHPLC chromatogram profile characterized by a triple peak pattern, as depicted in Fig. 2c.

System Validation

In the second step of the analysis, the remaining 300 samples were examined by DHPLC in a blinded experiment. We first performed the DHPLC analysis and only after the examination of the chromatograms, the results were compared to those of the sequencing in order to validate the accuracy of the DHPLC technique. This comparison showed a 100 % match between the results obtained from the two methods.

The precision of the method, defined as the ability to obtain a reproducible chromatogram elution profile of PCR products from injection to injection using the DHPLC, was determined for a set of 100 samples analyzed in different days. All the included chromatogram profiles displayed distinguishable peaks (one, two, three peaks) and a minimal peak intensity of 2 mV. The retention time (RT) in heteroduplex species was considered and measured for both the first and the last peak in heteroduplex chromatograms. We subsequently determined the mean of Δ-Het (difference in RT between the two heteroduplex peaks) and Standard Deviation (SD) in both the first heteroduplex profile and the second heteroduplex profile. In particular, we revealed a Δ-Het of 0.617 ± 0.027 min for the first heteroduplex profile (Fig. 3a) and a Δ-Het of 0.985 ± 0.075 min for the second heteroduplex profile (Fig. 3b), indicating a good reproducibility of the method.

Discussion

In this study, we describe, for the first time to our knowledge, the use of the DHPLC technique for the complete characterization of CATT tetranucleotide at position −794 in the *MIF* gene.

The DHPLC analysis has been performed exclusively under partially denaturing conditions and has allowed the accurate determination of all the homozygous and heterozygous genotypes. Under the partially denaturing condition, the CATT tetranucleotide number variations may be detected based on their peak retention pattern, following a simple flowchart. The first DHPLC analysis allows defining homoduplex and heteroduplex profiles and thus distinguishing homozygous from heterozygous genotypes. The samples heterozygous for alleles that differ in two tetranucleotide repeats ($CATT_{5/7}$) are immediately identifiable. In this first step, however, it is not possible to define the exact genotype of the other heterozygous ($CATT_{5/6}$ and $CATT_{6/7}$) and homozygous ($CATT_{5/5}$, $CATT_{6/6}$, and $CATT_{7/7}$) samples. The second step of DHPLC analysis is then performed after mixing PCR samples with a PCR product obtained from a previously characterized homozygous control DNA: This allows discriminating all the possible homozygous and heterozygous genotypes. It is worth noting that the $CATT_8$ allele was not present in our population, either at a heterozygous or at a homozygous state. We can hypothesize that the approach described above could enable the identification of the $CATT_8$ allele using a mixture with an appropriate PCR product previously characterized, although this protocol needs to be validated before using in genotyping.

A similar DHPLC analysis has been formerly performed for the partial genotyping of the TA six and seven repeats at position −53 in the promoter of *UGT1A1* gene [19]. In this case, a two-step DHPLC analysis under partially denaturing conditions was used to identify $TA_{6/7}$ heterozygous samples at the first step and to distinguish between homozygous $TA_{6/6}$ and $TA_{7/7}$ genotypes at the second step by mixing PCR products with the PCR of a sample homozygous for $TA_{6/6}$ [19]. The DHPLC use is thus analogous to that typically used to identify point mutations in a homozygous state. Here, we propose a more complete approach, where DHPLC analysis allows the complete −794 CATT genotyping in *MIF* gene, including the accurate characterization of heteroduplex profiles corresponding to different heterozygous conditions.

The performance of the method has been evaluated and this approach has been found to be accurate and sensitive (with a 100 % match with sequencing results) and highly reproducible (with a SD of the Δ-Het between heteroduplex peaks less than 8 %). The interpretation of the chromatograms is particularly simple and rapid and the retention times (RT) in heteroduplex species are a constant characteristic making the interpretation of results easier.

The method presents several advantages in comparison to other techniques usually employed in this kind of analysis. The DHPLC analysis conducted under a partially

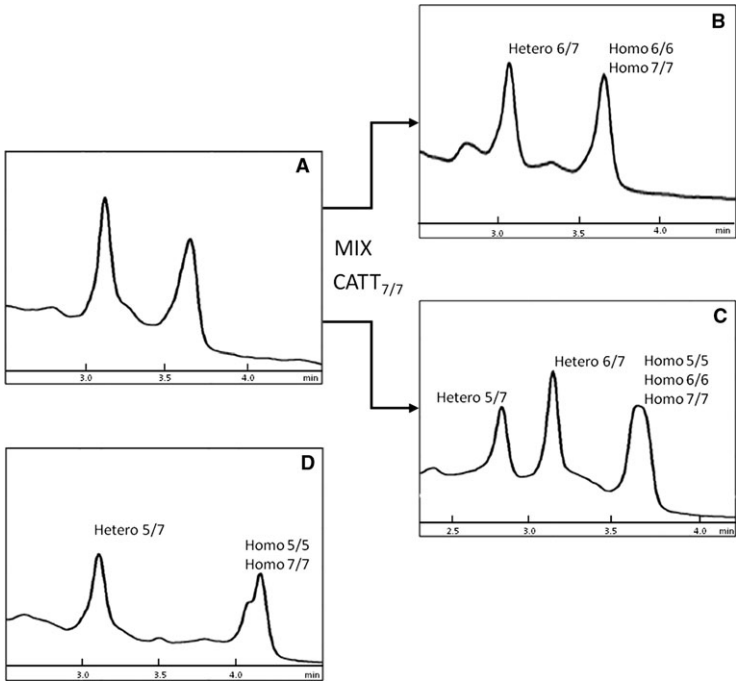

Fig. 2 Flowchart of DHPLC analysis for heterozygote characterization. DHPLC chromatogram of first heteroduplex profile is depicted in (**a**). The amplicons with this heteroduplex profile were mixed in approximately equimolar proportions with a control sample previously characterized as homozygous $CATT_{7/7}$. This allowed differentiating two heterozygote genotypes: **b** Elution profile without changes with respect to the first heteroduplex chromatogram, corresponding to a sample heterozygous $CATT_{6/7}$. **c** Elution profile with the characteristic triple peak, corresponding to heterozygous $CATT_{5/6}$. **d** DHPLC chromatogram of second heteroduplex profile, corresponding to heterozygous genotype $CATT_{5/7}$. On the top of each peak, the specific heteroduplex or homoduplex condition is indicated

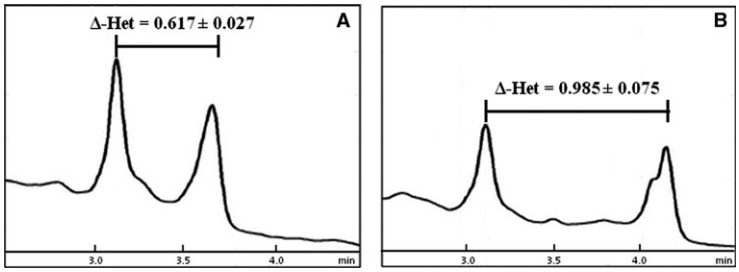

Fig. 3 Representation of the critical parameters of Δ-Het for system validation. The difference in retention time between the peaks (Δ-Het) in the first (**a**) and the second (**b**) heteroduplex profiles is indicated in minutes ± Standard Deviation (SD)

denaturing condition is more sensitive than the analysis under non-denaturing conditions, often used for determining the PCR product size. This analysis is rapid (about 20 min per sample), but in our experience it is not always very sensitive, in particular for PCR products which differ in only a few base pairs. In comparison to SSCP analysis, the DHPLC analysis applied to MIF −794 CATT genotyping is easier, more rapid, and more reproducible since it is an almost completely automated system that does not need to prepare and handle gels and reagents and strongly reduces the variability among different experiments and different operators [20, 21]. In addition, since the DHPLC is a technique usually available in the most of the genetic laboratories, its use is more accessible in comparison to the innovative methodology based on thin-film biosensor chips, which shows a high performance, but requires specific tools and technologies [22]. DHPLC analysis has a reliability similar to capillary electrophoresis on automated DNA analyzer; however, it is less expensive since it does not need specific labeled reagents: The cost per sample is about 1 € for one-step analysis and about 1.5 € for two-step analysis. The method shows an accuracy comparable with that of direct sequencing (100 % of matching), but it is more time effective and cost effective [23].

In conclusion, we provide evidence that the partially denaturing DHPLC analysis is an excellent alternative approach for MIF −794 CATT genotyping with respect to several techniques used in recent years. Since VNTR is involved in the pathogenesis of various diseases [24], we suggest that this DHPLC protocol could be used for the rapid and efficient screening of short tandem repeats identified in other genes.

References

1. Calandra, T., & Roger, T. (2003). Macrophage migration inhibitory factor: a regulator of innate immunity. *Nature Reviews Immunology, 3*, 791–800.
2. Donn, R. P., & Ray, D. W. (2004). Macrophage migration inhibitory factor: molecular, cellular and genetic aspects of a key neuroendocrine molecule. *Journal of Endocrinology, 182*, 1–9.
3. Popp, J., Bacher, M., Kölsch, H., Noelker, C., Deuster, O., Dodel, R., et al. (2009). Macrophage migration inhibitory factor in mild cognitive impairment and Alzheimer's disease. *Journal of Psychiatric Research, 43*, 749–753.
4. Donnelly, S. C., & Bucala, R. (1997). Macrophage migration inhibitory factor: a regulator of glucocorticoid activity with a critical role in inflammatory disease. *Molecular Medicine Today, 3*, 502–507.
5. Baugh, J. A., & Bucala, R. (2002). Macrophage migration inhibitory factor. *Critical Care Medicine, 30*, S27–S35.
6. Baugh, J. A., Chitnis, S., Donnelly, S. C., Monteiro, J., Lin, X., Plant, B. J., et al. (2002). A functional promoter polymorphism in the macrophage migration inhibitory factor (MIF) gene associated with disease severity in rheumatoid arthritis. *Genes and Immunity, 3*, 170–176.
7. Donn, R., Alourfi, Z., De Benedetti, F., Meazza, C., Zeggini, E., Lunt, M., et al. (2002). Mutation screening of the macrophage migration inhibitory factor gene: positive association of a functional polymorphism of macrophage migration inhibitory factor with juvenile idiopathic arthritis. *Arthritis and Rheumatism, 46*, 2402–2409.
8. Renner, P., Roger, T., & Calandra, T. (2005). Macrophage migration inhibitory factor: gene polymorphisms and susceptibility to inflammatory diseases. *Clinical Infectious Diseases, 7*, S513–S519.
9. Sreih, A., Ezzeddine, R., Leng, L., LaChance, A., Yu, G., Mizue, Y., et al. (2011). Dual effect of the macrophage migration inhibitory factor gene on the development and severity of human systemic lupus erythematosus. *Arthritis and Rheumatism, 63*, 3942–3951.
10. Shiroeda, H., Tahara, T., Nakamura, M., Shibata, T., Nomura, T., Yamada, H., et al. (2010). Association between functional promoter polymorphisms of macrophage migration inhibitory factor (MIF) gene and ulcerative colitis in Japan. *Cytokine, 51*, 173–177.
11. Gómez, L. M., Sánchez, E., Ruiz-Narvaez, E. A., López-Nevot, M. A., Anaya, J. M., & Martín, J. (2007). Macrophage migration inhibitory factor gene influences the risk of developing tuberculosis in north western Colombian population. *Tissue Antigens, 70*, 28–33.
12. Meyer-Siegler, K. L., Vera, P. L., Iczkowski, K. A., Bifulco, C., Lee, A., Gregersen, P. K., et al. (2007). Macrophage migration inhibitory factor (MIF) gene polymorphisms are associated with increased prostate cancer incidence. *Genes and Immunity, 8*, 646–652.
13. Zhong, X. B., Leng, L., Beitin, A., Chen, R., McDonald, C., Hsiao, B., et al. (2005). Simultaneous detection of microsatellite repeats and SNPs in the macrophage migration inhibitory factor (MIF) gene by thin-film biosensor chips and application to rural field studies. *Nucleic Acids Research, 33*, e121.
14. Marsh, D. J., & Howell, V. M. (2010). The use of denaturing high performance liquid chromatography (DHPLC) for mutation scanning of hereditary cancer genes. *Methods in Molecular Biology, 653*, 133–145.
15. Miller, S. A., Dykes, D. D., & Polesky, H. F. (1988). A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Research, 16*, 1215.
16. Lehmann, L. E., Book, M., Hartmann, W., Weber, S. U., Schewe, J. C., Klaschik, S., et al. (2009). A MIF haplotype is associated with the outcome of patients with severe sepsis: a case control study. *Journal of Translational Medicine, 7*, 100.
17. Makhija, R., Kingsnorth, A., & Demaine, A. (2007). Gene polymorphisms of the macrophage migration inhibitory factor and acute pancreatitis. *Journal of the Pancreas, 8*, 289–295.
18. Grigorenko, E. L., Han, S. S., Yrigollen, C. M., Leng, L., Mizue, Y., Anderson, G. M., et al. (2008). Macrophage migration inhibitory factor and autism spectrum disorders. *Pediatrics, 122*, e438–e445.
19. Pirulli, D., Giordano, M., Puzzer, D., Crovella, S., Rigato, I., Tiribelli, C., et al. (2000). Rapid method for detection of extra (TA) in the promoter of the bilirubin-UDP-glucuronosyl transferase 1 gene associated with Gilbert syndrome. *Clinical Chemistry, 46*, 129–131.
20. Bunn, C. F., Lintott, C. J., Scott, R. S., & George, P. M. (2002). Comparison of SSCP and DHPLC for the detection of LDLR mutations in a New Zealand cohort. *Human Mutation, 19*, 311.
21. Mlakar, S. J., & Ostanek, B. (2011). Development of a new DHPLC assay for genotyping UGT1A (TA)n polymorphism associated with Gilbert's syndrome. *Biochemia Medica (Zagreb), 21*, 167–173.
22. Frueh, F. W., & Noyer-Weidner, M. (2003). The use of denaturing high-performance liquid chromatography (DHPLC) for the analysis of genetic variations: impact for diagnostics and pharmacogenetics. *Clinical Chemistry and Laboratory Medicine, 41*, 452–461.
23. Mogensen, J., Bahl, A., Kubo, T., Elanko, N., Taylor, R., & McKenna, W. J. (2003). Comparison of fluorescent SSCP and denaturing HPLC analysis with direct sequencing for mutation screening in hypertrophic cardiomyopathy. *Journal of Medical Genetics, 40*, e59.
24. Bois, P., & Jeffreys, A. J. (1999). Minisatellite instability and germeline mutation. *Cellular and Molecular Life Sciences, 55*, 1636–1648.

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Lack of Mutations of the Telomerase RNA Component in Familial Papillary Thyroid Cancer with Short Telomeres

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Background: The occurrence of familial papillary thyroid cancer (FPTC) is well established but no susceptibility genes for this disease have been discovered. Our group has recently demonstrated that patients with FPTC have shorter telomeres, not associated with mutations in telomerase reverse transcriptase, gene than patients with sporadic papillary thyroid cancer (SPTC), healthy subjects (HS), and unaffected family members (UFMs). Several diseases, however, have short telomeres associated with mutations in the telomerase RNA component (*TERC*) gene or in the shelterin complex (*POT1*, *RAP1*, *TIN2*, *TPP1*, *TRF1*, and *TRF2*) genes. The objective of the present study was to verify whether short telomeres observed in FPTC patients were related to mutations in TERC or shelterin genes.
Methods: Sixty-six patients with FPTC, 46 UFMs, 111 patients with SPTC, and 153 HS were analyzed by polymerase chain reaction followed by denaturing high performance liquid chromatography analysis and direct sequencing for the presence of *TERC* or shelterin gene mutations. When present, single-nucleotide polymorphisms were tested by χ^2 analysis at the genotypic, allelic, and haplotypic levels.
Results: The entire sequence of the *TERC* gene was analyzed with particular attention to known mutations known to be associated with short telomeres. All samples appeared to be homozygous wild type for A-771G, C-99G, G305A, G322A, C323T, C408G, G450A, T467C, G508A, A514G, G623A, and C727G substitutions and for the 378Δ→3′ deletion in the *TERC* gene. In addition, upon analysis of all samples for shelterin proteins, we observed a significant decrease in POT1 and RAP1 protein expression in the blood of FPTC patients compared with SPTC subjects. However, no mutations or polymorphisms were found when in the coding sequences of both genes.
Conclusions: To our knowledge this is the first study of TERC mutations or alterations in the shelterin complex in relation to FPTC. Shorter telomeres observed in FPTC are not linked to mutations or polymorphisms in *TERC*, *POT1*, or *RAP1* genes.

Introduction

Differentiated thyroid cancer (DTC), although mostly sporadic, presents as familial occurrence (familial nonmedullary thyroid cancer [FNMTC]) with a prevalence of up to 10% (1,2). The risk of developing FNMTC in first-degree relatives of subjects with DTC is significantly higher (between 3.2 and 8.6) than in the general population (3,4). Several rare hereditary syndromes caused by germline mutations of known tumor suppressor genes are associated with the occurrence of DTC, mainly of the papillary histotype (familial papillary thyroid cancer [FPTC]), such as familial adenomatous polyposis, Cowden syndrome, Werner syndrome, and Carney complex (5–8). However, most FPTC patients have thyroid cancer as the only disease manifestation. At the moment, no candidate gene(s) has been discovered for FPTC and only in a minority of cases a locus of susceptibility has been identified, these being the locus TCO on 19p13.2 (9), the locus PRN1 on 1q21 (10), and the locus NMTC1 on 2q21 (11). Recent studies (12,13) carried out in our Unit provided clinical and molecular evidence that FPTC displays the features of clinical "anticipation" and that there are germline alterations in the telomere–telomerase complex. Patients with FPTC, compared with those with sporadic cancers, have significantly shorter telomeres and increased telomerase reverse transcriptase (TERT) activity.

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Short telomeres and inherited or acquired genetic defects in telomere maintenance have been associated with an increased risk of developing familial diseases such as dyskeratosis congenita (DC) syndrome (14) and familial forms of cancer such as head, neck, lung, breast, and renal cancers (15). Several mutations in telomerase RNA component (*TERC*) gene are associated with benign and malignant diseases such as DC (16), aplastic anemia (17,18), systemic sclerosis (19), myelodysplastic syndrome (18), and generally with a reduction in mean telomere length (20). Similarly, recent studies have highlighted an important role of the shelterin complex in cancer development (21). This complex is formed by six proteins that normally contribute to shape and safeguard human telomeres. In particular three shelterin subunits, TRF1, TRF2, and POT1, directly recognize the TTAGGG repeats. They are, moreover, interconnected by three additional shelterin proteins, TIN2, TPP1, and RAP1, forming a complex that allows cells to distinguish telomeres from extra-telomeric sites of DNA damage. Without the protective activity of shelterin, telomeres are no longer hidden from the DNA damage control mechanisms and chromosome ends are inappropriately processed by DNA repair pathways (22).

Based on these observations, the aim of the present work was to determine whether there are variations in *TERC* gene in the peripheral blood of patients with FPTC, perhaps explaining the telomere shortening observed in these patients. Shelterin proteins were also investigated to determine whether there were alterations in mRNA expression or the presence of gene mutations.

Patients and Methods

After informed consent in accordance with local ethical committee guidelines, blood samples for DNA analysis were collected from 66 patients with FNMTC (referred here as FPTC from the moment that all display the papillary histotype of the disease) (belonging to 38 kindreds), 46 unaffected family members (UFMs) (belonging to 23 kindreds), 111 sporadic papillary thyroid cancer (SPTC) patients, and 153 healthy subjects (HS). FPTC was defined as the presence of at least one first-degree relative with DTC. The HS were selected from volunteers of the "Blood donor centre" of the hospital of Siena (Italy). In these subjects, autoimmune diseases, cardiovascular diseases, and diabetes were excluded. Patients and HS with a history of radiation exposure and malignancies were excluded.

DNA extraction

Genomic DNA was extracted using salting out procedures. For each sample DNA concentration was assessed by spectrophotometry and stock solutions of 200 ng/50 μL were prepared and used for following experiments.

Search for TERC mutation

To determine the presence of *TERC* mutations, 25 μL/samples of AmpliTaq Gold PCR Master mix (Applied Biosystems) was been added to 1.5 mM $MgCl_2$, 400 μM dNTPs, and 300 nM of specific primers (primer sequences, annealing temperatures, and denaturing high performance liquid chromatography [DHPLC] conditions are available upon request) designed to sequence the entire gene in a final volume of 50 μL. Polymerase chain reaction (PCR) products were visualized with ethidium bromide in a 2% agarose gel. Samples were subsequently denatured (10 minutes at 95°C followed by 5 minutes at 56°C) and analyzed with the DHPLC (Transgenomic Wave Nucleic Acid Fragment Analysis System-Transgenomic Inc.) technique to confirm the presence/absence of mutations using specific temperatures and applying gradient conditions as calculated by the Navigator™ software (Vers. 1.6.4). Samples with different elution profile were subjected to direct pyrosequencing (99.5% accuracy) (Primm).

Shelterin protein expression

RNA was extracted from fresh blood of 51 FPTC and 82 SPTC patients using QIAamp RNA Blood Mini Kit (Qiagen). One microgram of each sample was retrotranscribed into complementary cDNA using iScript cDNA Synthesis Kit (Biorad) and 200 ng/μL was evaluated by real-time PCR using the MJ Mini Thermocycler (Biorad) in a mix containing 2× iQ™ Supermix (Biorad) and 20× TaqMan primer/probes (Applied Biosystems) in a final volume of 25 μL. Annealing temperature was 60°C for 35 cycles. Each sample was run in duplicate and for each run efficiency of real time PCR (RT-PCR) (E), slope values, and correlation coefficients (R^2) were determined. The expression level was calculated as ΔΔCt and reported as $2^{-\Delta\Delta Ct}$ against beta-actin and RPL13 chosen as reference genes by two different software programs for the selection of optimal control genes in qRT-PCR studies, *Normfinder* and *GenNorm*. The *GenNorm* provides a ranking of the tested genes, based on their expression stability, determining the two most stable reference genes or a combination of multiple stable genes for normalization. *NormFinder* identified the optimal normalization genes among a set of candidates according to their expression stability value in a given sample set and a given experimental design.

Search for POT1 and RAP1 point mutations

To determine the presence of *POT1* and *RAP1* point mutations, specific primers were designed using *Vector* NTI Software® to cover all the coding sequence of the genes (see Ensembl database). For each sample (66 FPTC, 111 PTC, 46 UFMs, and 153 HS), 200 ng of DNA was amplified in a final volume of 50 μL containing 2× AmpliTaq Gold PCR Master mix (Applied Biosystems) and 300 nM of specific primers (primers and PCR conditions are available upon request). Samples were subsequently denatured and analyzed with the DHPLC (Transgenomic Wave Nucleic Acid Fragment Analysis System-Transgenomic Inc.) technique to confirm the presence/absence of mutations. Samples with different elution profiles were subjected to pyrosequencing (Primm) (DHPLC conditions are available upon request).

Statistical analysis

Statistical analysis was performed using the software package SPSS version 13.0 (IBM Company). Interaction with single-nucleotide polymorphisms (SNPs) was tested by χ^2 analysis at genotypic, allelic, and haplotypic levels. $p < 0.05$ was considered statistically significant. Student's t test was used to calculate the difference in the expression levels of shelterin proteins using StatView for Windows, ver.5.00.1 (SAS Institute).

Table 1. Panel of Known Telomerase RNA Component Mutations (Reverse Strand) and Possible Associated Diseases

TERC variants	*Possible associated diseases*
A-771G	Myelodysplastic syndrome (MDS); aplastic anemia (AA)
C-99G	Aplastic anemia (AA); paroxysmal nocturnal hemoglobinuria (PNH)
821-bp deletion 3′-end 378Δ→3′	Autosomal dominant dyskeratosis congenita (DC)
G305A	Nonsevere aplastic anemia (NSAA)
G322A	Myelodysplastic syndrome (MDS)
C323T	Myelodysplastic syndrome (MDS)
C408G	Autosomal dominant dyskeratosis congenita (DC)
G450A	Severe aplastic anemia (SAA)
T467C	Aplastic anemia (AA)
G501A	Pathogenic significance unknown
G508A	Neural tube defects
A514G	Systemic sclerosis
G623A	Neural tube defects
C701T	Pathogenic significance unknown
C727G	Pathogenic significance unknown

TERC, telomerase RNA component.

Results

Search for TERC mutations

Samples were tested for known, generally disease-associated, *TERC* variations (Table 1) together with a complete sequencing of the entire gene. All of the subjects analyzed (FPTC patients, UFMs, SPTC patients, and HS) were homozygous wild type for A-771G, C-99G, G305A, G322A, C323T, C408G, G450A, T467C, G508A, A514G, G623A, and C727G substitutions and for the 378Δ→3′ deletion.

We found two polymorphisms indicated as polymorphism #1 (SNP rs2293607) and polymorphism #2 (SNP rs35073794)

Table 2. Frequency of Polymorphisms #1 and #2

	Polymorphism #1			*Polymorphism #2*		
Subjects		n	%		n	%
Healthy subjects (153)	TT	89	58.2	GG	148	96.7
	TC	48	31.4	GA	4	2.6
	CC	16	10.5	AA	1	0.7
FPTC patients (66)	TT	36	54.5	GG	62	93.9
	TC	26	39.4	GA	4	6.1
	CC	4	6.1	AA	0	0
Unaffected family members (46)	TT	23	50	GG	43	93.5
	TC	17	37	GA	3	6.5
	CC	6	13	AA	0	0
Sporadic PTC patients (111)	TT	67	60.4	GG	109	98.2
	TC	35	31.5	GA	2	1.8
	CC	9	8.1	AA	0	0

T = Wt allele and C = polymorphic allele for polymorphism #1; G = Wt allele and A = polymorphic allele for polymorphism #2.
FPTC, familial papillary thyroid cancer.

Table 3. Diplotype Distribution of the Two Polymorphisms Among Groups

Subjects	*Diplotype polymorphism #1/ polymorphism #2*	*Frequency (number of subjects)*	*Percent (%)*
Healthy subjects (153)	TT/GG	85	55.6
	TT/GA	3	2
	TT/AA	1	0.7
	TC/GG	47	30.7
	TC/GA	1	0.7
	CC/GG	16	10.5
FPTC patients (66)	TT/GG	32	48.5
	TT/GA	4	6.1
	TT/AA	0	0
	TC/GG	26	39.4
	TC/GA	0	0
	CC/GG	4	6.1
Unaffected family members (46)	TT/GG	20	43.5
	TT/GA	3	6.5
	TT/AA	0	0
	TC/GG	17	37
	TC/GA	0	0
	CC/GG	6	13
Sporadic PTC patients (111)	TT/GG	65	58.6
	TT/GA	2	1.8
	TT/AA	0	0
	TC/GG	35	31.5
	TC/GA	0	0
	CC/GG	9	8.1

of unknown pathogenic significance (Ensembl database). Polymorphism #1 consisted of n501 (T > C) substitution (forward strand), and was observed in the heterozygous form (TC) in 48/153 (31.4%) of HS, 26/66 (39.4%) of FPTC patients, 17/46 (37%) of UFMs, and 35/111 (31.5%) of SPTC patients (Table 2). The homozygous variant (CC) was detected in 16/153 (10.5%) of HS, 4/66 (6.1%) of FPTC patients, 6/46 (13%) of UFMs, and 9/111 (8.1%) of sporadic PTC patients (Table 2). The different distribution of this polymorphism in the four groups was not statistically significant (Pearson chi-square $p = 0.714$).

Polymorphism #2 was more rare and consisted of n701 (G > A) (forward strand) substitution. It was found as heterozygous (GA) in 4/153 (2.6%) of HS, 4/66 (6.1%) of FPTC patients, 3/46 (6.5%) of UFMs, and 2/111 (1.8%) of SPTC patients (Table 2). The homozygous variant (AA) was detected only in 1/153 (0.7%) of HS (Table 2). Also in this case, polymorphism frequency was not statistically different among groups (Pearson chi-square $p = 0.504$). In addition, the diplotype distribution obtained by considering the combination of polymorphisms #1 and #2 was not different in the various groups (Table 3) as well as the single allele frequency of both polymorphisms (Table 4, p value of 0.576 for polymorphism #1 and a p value of 0.417 for polymorphism #2, respectively) and the association between single alleles (Table 5, p value of 0.640).

When *TERC* copy number variations were analyzed, we found one sample belonging to the UFMs with two insertions and two deletions (Fig. 1) not reported before. These insertions concern nucleotides 1418 (1418insA) and 1437

Table 4. Single Allele Frequency

		Polymorphism #1			*Polymorphism #2*	
Subjects		n	%		n	%
Healthy subjects (153)	T	226	73.9	G	300	98
	C	80	26.1	A	6	2
FPTC patients (66)	T	98	74.2	G	128	97
	C	34	25.8	A	4	3
Unaffected family members (46)	T	63	68.5	G	89	96.7
	C	29	31.5	A	3	3.3
Sporadic PTC patients (111)	T	169	76.1	G	220	99.1
	C	53	23.9	A	2	0.9

Table 5. Association Between Single Alleles of Both Polymorphisms

	Allelic associations		
Subjects		n	%
Healthy subjects (153)	TG	221	72.2
	TA	5	1.6
	CG	79	25.8
	CA	1	0.3
FPTC patients (66)	TG	94	71.2
	TA	4	3.0
	CG	34	25.8
	CA	0	0
Unaffected family members (46)	TG	60	65.2
	TA	3	3.3
	CG	29	31.5
	CA	0	0
Sporadic PTC patients (111)	TG	167	75.2
	TA	2	0.9
	CG	53	23.9
	CA	0	0

(1437insT) while the deletions affected nucleotide 1440–1442 (1440_1442delCTG) and 1461–1467 (1461_1467delGGAAAAA). We did not find two other variations reported in genome databases (i.e., Ensembl) and indicated as CN_996453 and CN_996452. When the search was extended to the entire gene sequence, no mutations or polymorphisms were found.

Shelterin protein complex

We extended our research to the six proteins of the shelterin complex implicated in the regulation of telomere length. We previously analyzed their expression levels in blood of 51 patients with FPTC and 82 patients with SPTC and expressed the results as $2^{-\Delta\Delta Ct}$ with respect to two different reference genes (beta-actin and RPL13). Figure 2 reports the mean expression values for (Fig. 2A) TRF1 (SPTC: 9.39 ± 2.5, FPTC: 13.05 ± 3.2, $p = 0.9$), (Fig. 2B) TRF2 (SPTC: 3.04 ± 1, FPTC: 3.18 ± 0.8, $p = 0.1$), (Fig. 2C) TIN2 (SPTC: 6.16 ± 2.4, FPTC: 11.9 ± 2.7, $p = 0.05$), (Fig. 2D) TPP1 (SPTC: 5.18 ± 0.78, FPTC: 4.89 ± 1, $p = 0.4$), (Fig. 2E) POT1 (SPTC: 6.45 ± 1.2, FPTC: 2.8 ± 0.93, $p < 0.01$), and (Fig. 2F) RAP1 (SPTC: 11.5 ± 1.2, FPTC: 4.9 ± 1, $p = 0.03$). Only for POT1 and RAP1 did we observe a significant decrease in the expression level in patients with FPTC compared with patients with SPTC (Fig. 2). We then looked for point mutations in *POT1* and *RAP1* genes in the blood of 66 patients with FPTC, 111 patients with SPTC, 46 UFMs, and 153 HS. All samples were homozygous wild type in both genes. We found only the polymorphism rs4888444 in *RAP1*, with a frequency of 2.9%, in FPTC patients. This was considerably lower than that reported for normal population (20%) and, thus, not associated with the FPTC phenotype.

Discussion

Telomeres are structures of eukaryotic chromosomes consisting of 6 bp repeats (TTAGGG) that protect chromosome ends from degradation and from end-to-end fusions (23). Telomeres are maintained by telomerase complex composed by an enzymatic component (TERT) and an RNA component (TERC) (24), which acts as template for addition of telomeric repeats. In addition, accessory proteins such as dyskerin, NHP2, NOP110, pontin/reptin, and TCAB1 are found at telomere ends (24). Dyskerin, NHP2, and NOP10 are necessary for the stability and accumulation of TERC, whereas dyskerin collaborates with pontin/reptin to allow the assembly between TERC and TERT. Telomerase cooperates in telomere lengthening together with several proteins such as the shelterin family (TRF1, TRF2, RAP1, TIN2, POT1, and TPP1) and molecular chaperones such as HSP90 (25). The *TERC* gene maps on chromosome 3 and is characterized by one exon of 438-bp long (Ensembl database). As reported in aplastic anemia (AA) (26) and DC (14, 26), several mutations in *TERC* gene are associated with telomere shortening particularly those involving the pseudoknot domain (26). In DC, *TERC* mutations and short telomeres are responsible for the familial form (14, 27). In a previous study we have demonstrated that patients with FPTC have significantly shorter telomeres compared with patients with SPTC, UFM, and HS not related to mutations in *TERT* gene (12). In this report, we search for known/new *TERC* gene mutations as possible implicated factor for telomere shortening in FPTC. All samples analyzed, however, were homozygous wild type for

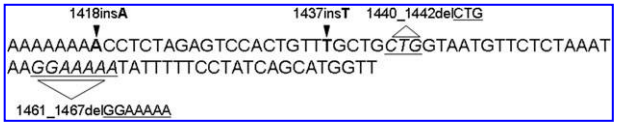

FIG. 1. Schematic representation of telomerase RNA component insertions and deletions found in one sample belonging to one unaffected family member.

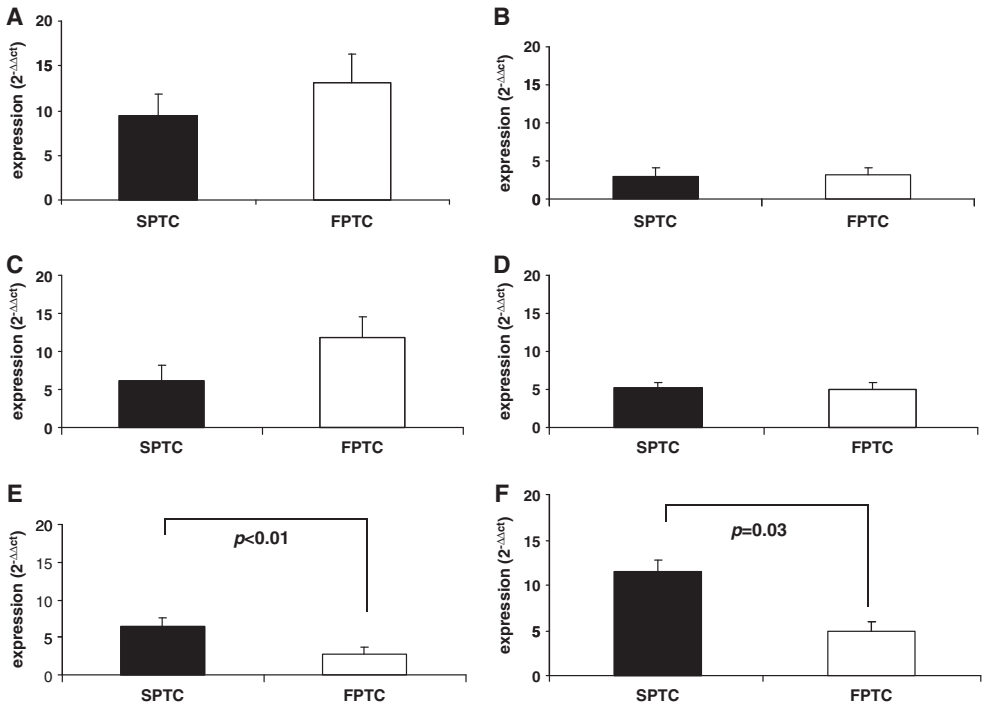

FIG. 2. Mean expression levels of the six shelterin proteins expressed as $2^{-\Delta\Delta Ct}$ and calculated in comparison to two different reference genes (beta-actin and RPL13). **(A)** TRF1, **(B)** TRF2, **(C)** TIN2, **(D)** TPP1, **(E)** POT1, and **(F)** RAP1. SPTC, sporadic papillary thyroid cancer; FPTC, familial papillary thyroid cancer.

TERC mutations with the exception of two SNPs (T501C and G701A) of unknown pathogenetic significance. Statistical analysis of the distribution of the two single polymorphisms, of the combination of both polymorphisms, and of the association between single alleles of polymorphisms #1 and #2 among FPTC patients, SPTC patients, and HS yielded no statistically significant difference. We then extended our research to accessory proteins involved in telomere maintenance. We excluded from this analysis proteins such as dyskerin that has been clearly associated with DC development (28) or hoyeraal-hreidarsson syndrome (29) completely absent in our patients (Genecards database) and focused our attention on shelterin complex. We found a significant reduction in mRNA expression of POT1 and RAP1, not linked to known mutations in both genes.

To our knowledge this is the first report in which mutations in *TERC*, *POT1*, and *RAP1* have been searched in FPTC and our results exclude that variations of these genes are responsible for telomere shortening observed in familial form of PTC. However, a possible implication of shelterin proteins needs to be further investigated (i.e., miRNA regulation) from the moment that FPTC patients display a decrease in POT1 and RAP1 mRNA expression.

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Disclosure Statement

The authors declare that no conflict of interest exists.

References

1. Stoffer SS, Van Dyke DL, Bach JV 1986 Familial papillary carcinoma of the thyroid. Am J Med Genet **25:**775–782.
2. Loh KC 1997 Familial non-medullary thyroid carcinoma: a meta-review of case series. Thyroid **7:**107–113.
3. Goldgar DE, Easton DF, Cannon-Albright LA, Skolnick MH 1994 Systematic population-based assessment of cancer risk in first-degree relatives of cancer probands. J Nat Cancer Inst **86:**1600–1608.
4. Hemminki K, Eng C, Chen B 2005 Familial risks for non-medullary thyroid cancer. J Clin Endocrinol Metab **90:**5747–5753.
5. Giardiello FM, Offerhaus GJA, Lee DH, Krush AJ, Tersmette AC, Booker SV, Kelley NC, Hamilton SR 1993 Increased risk

of thyroid and pancreatic carcinoma in familial adenomatous polyposis. Gut **34:**1394–1396.
6. Liaw D, Marsh DJ, Li J, Dahia PLM, Wang SI, Zheng Z, Bose S, Call KM, Tsou HC, Peacocke M, Eng C, Parsons R 1997 Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. Nat Genet **16:**64–67.
7. Goto M, Miller RW, Ishikawa Y, Sugano H 1996 Excess of rare cancers in Werner syndrome (adult progeria). Cancer Epidemiol Biomarkers Prev **5:**239–246.
8. Stratakis CA, Courcoutsakies NA, Abati A, Filie A, Doppman JL, Carney A, Shawker T 1997 Thyroid gland abnormalities in patients with the syndrome of spotty skin pigmentation, myxomas, endocrine overactivity, and schwannomas (Carney Complex). J Clin Endocrinol Metab **82:**2037–2043.
9. Canzian F, Amati P, Harach HR, Kraimps JL, Lesueur F, Barbier J, Levillain P, Romeo G, Bonneau D 1998 A gene predisposing to familial thyroid tumors with cell oxyphilia maps to chromosome 19p13.2. Am J Human Genet **63:**1743–1748.
10. Malchoff CD, Sarfarazi M, Tendler B, Forouhar F, Whalen G, Joshi V, Arnold A, Malchoff DM 2000 Papillary thyroid carcinoma associated with papillary renal neoplasia: genetic linkage analysis of a distinct heritable tumor syndrome. J Clin Endocrinol Metab **85:**1758–1764.
11. McKay JD, Lesueur F, Jonard L, Pastore A, Williamson J, Hoffman L, Burgess J, Duffield A, Papotti M, Stark M, Sobol H, Maes B, Murat A, Kaariainen H, Bertholon-Gregoire M, Zini M, Rossing MA, Toubert ME, Bonichon F, Cavarec M, Bernard AM, Boneu A, Leprat F, Haas O, Lasset C, Schlumberger M, Canzian F, Goldgar DE, Romeo G 2001 Localization of a susceptibility gene for familial nonmedullary thyroid carcinoma to chromosome 2q21. Am J Hum Genet **69:**440–446.
12. Capezzone M, Cantara S, Marchisotta S, Filetti S, De Santi MM, Rossi B, Ronga G, Durante C, Pacini F 2008 Short telomeres, telomerase reverse transcriptase gene amplification, and increased telomerase activity in the blood of familial papillary thyroid cancer patients. J Clin Endocrinol Metab **93:**3950–3957.
13. Capezzone M, Marchisotta S, Cantara S, Busonero G, Brilli L, Pazaitou-Panayiotou K, Carli AF, Caruso G, Toti P, Capitani S, Pammolli A, Pacini F 2008 Familial non-medullary thyroid carcinoma displays the features of clinical anticipation suggestive of a distinct biological entity. Endocr Relat Cancer **15:**1075–1081.
14. Vulliamy T, Marrone A, Szydlo R, Walne A, Mason PJ, Dokal I 2004 Disease anticipation is associated with progressive telomere shortening in families with dyskeratosis congenita due to mutations in TERC. Nat Gen **36:**447–449.
15. Gilson E, Londono-Vallejo A 2007 Telomere length profile in humans. Cell Cycle **6:**1–9.
16. Vulliamy T, Marrone A, Goldman F, Dearlove A, Bessler M, Mason PJ, Dokal I 2001 The RNA component of telomerase is mutated in autosomal dominant dyskeratosis congenita. Nature **413:**432–435.
17. Vulliamy T, Marrone A, Dokal I, Mason PJ 2002 Association between aplastic anaemia and mutations in telomerase RNA. Lancet **359:**2168–2170.
18. Yamaguchi H, Baerlocher GM, Lansdorp PM, Chanock SJ, Nunez O, Sloand E, Young NS 2003 Mutations of the human telomerase RNA gene (*TERC*) in aplastic anaemia and myelodysplastic syndrome. Blood **102:**916–918.
19. Ohtsuka T, Yamakage AY, Yamazaki SY 2002 The polymorphism of telomerase RNA component gene in patients with systemic sclerosis. Br J Dermatol **147:**250–254.
20. Codd V, Mangino M, van der Harst P, Braund PS, Kaiser M, Beveridge AJ, Rafelt S, Moore J, Nelson C, Soranzo N, Zhai G, Valdes AM, Blackburn H, Mateo Leach I, de Boer RA; Wellcome Trust Case Control Consortium, Goodall AH, Ouwehand W, van Veldhuisen DJ, van Gilst WH, Navis G, Burton PR, Tobin MD, Hall AS, Thompson JR, Spector T, Samani NJ 2010 Common variants near TERC are associated with mean telomere length. Nat Genet **42:**197–199.
21. Martinez P, Blasko MA 2010 Role of shelterin in cancer and aging. Aging Cell **9:**653–666.
22. de Lange T 2005 Shelterin: the protein complex that shapes and safeguards human telomeres. Genes Dev **19:**2100–2110.
23. Marrone A, Dokal I 2004 Dyskeratosis congenital: molecular insights into telomerase function, ageing and cancer. Expert Rev Mol Med **6:**1–23.
24. Wyatt HDM, Stephen CW, Beattie TL 2010 InTERTpreting telomerase structure and function. Nucleic Acids Res **38:**5609–5622.
25. Fu D Collins K 2007 Purification of human telomerase complexes identifies factors involved in telomerase biogenesis and telomere length regulation. Mol Cell **28:**773–785.
26. Fogarty PF, Yamaguchi H, Wiestner A, Baerlocher GM, Sloand E, Zeng WS, Read EJ, Lansdorp PM, Young NS 2003 Late presentation of dyskeratosis congenita as apparently acquired aplastic anaemia due to mutations in telomerase RNA. Lancet **362:**1628–1630.
27. Marrone A, Sokhal P, Walne A, Beswick R, Kirwan M, Killick S, Williams M, Marsh J, Vulliamy T, Dokal I 2007 Functional characterization of novel telomerase RNA (TERC) mutations in patients with diverse clinical and pathological presentations. Haematologica **92:**1013–1020.
28. Heiss NS, Girod A, Salowsky R, Wiemann S, Pepperkok R, Poustka A 1999 Dyskerin localizes to the nucleolus and its mislocalization is unlikely to play a role in the pathogenesis of dyskeratosis congenita. Hum Mol Genet **8:**2515–2524.
29. Knight SW, Heiss NS, Vulliamy TJ, Aalfs CM, McMahon C, Richmond P, Jones A, Hennekam RC, Poustka A, Mason PJ, Dokal I 1999 Unexplained aplastic anaemia, immunodeficiency, and cerebellar hypoplasia (Hoyeraal-Hreidarsson syndrome) due to mutations in the dyskeratosis congenita gene, DKC1. Br J Haematol **107:**335–339.

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Negative results

No association of *MTHFR* c.677C>T variant with sporadic ALS in an Italian population

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Abstract

The c.677C>T polymorphism in the 5,10-methylenetetrahydrofolate reductase gene (*MTHFR*) has been recently associated with susceptibility to sporadic amyotrophic lateral sclerosis (ALS). We have investigated this association in 450 ALS patients and 700 control subjects from Italy. No significant association was observed at the genotype and allelic level, either for the c.677C>T variant alone or in combination with *PON1* polymorphisms. Our negative results suggest that the *MTHFR* c.677C>T polymorphism is not a risk factor for ALS in the Italian population.

Keywords: Amyotrophic lateral sclerosis; *MTHFR* and *PON* genes; Association study

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive and fatal disease characterized by the progressive loss of motor neurons in the cerebral cortex, brain stem, and spinal cord. About 90% of all the ALS cases are sporadic. To date, the etiology of the sporadic form is poorly understood and several genetic risk factors have been implicated in its pathogenesis. Among these, the c.677C>T polymorphism (rs1801133) in the 5,10-methylenetetrahydrofolate reductase gene (*MTHFR*) has been recently reported to be significantly associated with ALS in a German/Swiss ALS population, in particular with bulbar onset ALS (Kuhnlein et al., 2010). The c.677C>T variant encodes a thermolabile form of the enzyme, which reduces enzyme activity and results in increased blood homocysteine levels. In the present study, we tested the reported association of the *MTHFR* c.677C>T variant in an Italian ALS population. Because paraoxonase 1 (PON1) is an esterase which protects against protein homocysteinylation, we also examined the association of this variant in combination with *PON1* polymorphisms that have been related to increased homocysteine levels in the blood.

2. Methods

The study included 450 sporadic ALS patients and 700 age- and sex-matched control subjects from Italy (northern Italy: 235 patients and 353 controls; central Italy: 215 patients and 347 controls). The sample series from northern Italy partially overlapped with that previously studied by Penco et al. (2008). The whole sample series included the samples formerly analyzed for *PON1* and *PON2* polymorphisms (Ricci et al., 2011). Characteristics of ALS patients and controls are summarized in Supplementary Table 1. Genotyping of *MTHFR* c.677C>T variant was performed

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Table 1
Genotype and allelic distributions of *MTHFR* c.677C>T polymorphism in sporadic ALS cases and control subjects

	CC	CT	TT	*p* value	C	T	*p* value	OR	95% CI
Case	0.292	0.508	0.200	0.256	0.546	0.454	0.102	0.869	0.734–1.029
Controls	0.256	0.511	0.233		0.511	0.489			

Key: ALS, amyotrophic lateral sclerosis; CI, confidence interval; OR, odds ratio.

by restriction digest analysis using the enzyme HinfI. Frequencies were compared using χ^2 statistics. Survival analysis was performed using the Kaplan-Meier method. For details see Supplementary data.

3. Results

Our sample had > 95% power to detect the reported association, assuming an allele frequency of 0.44 (Botto and Yang, 2000) and an arbitrary odds ratio of 1.5, at a significance level of $\alpha = 0.05$. The polymorphism was in Hardy-Weinberg equilibrium in both cases and controls ($p = 0.604$ and $p = 0.536$, respectively). No significant difference in *MTHFR* c.677C>T distributions was observed in patients compared with controls (Table 1). Statistical analysis failed to find any association of c.677C>T polymorphism with bulbar ALS in our population (in bulbar patients: CC/CT/TT 0.266/0.527/0.207, T allele 0.470; in controls: 0.256/0.511/0.233, T allele 0.489; $p = 0.548$, odds ratio [OR], 0.93; confidence interval [CI], 0.733–1.179). *MTHFR* c.677C>T variant was not associated with ALS clinical variables (gender, site and age at onset, disease duration; Supplementary Table 3). Finally, no significant differences were observed when the risk allele (T) for *MTHFR* c.677C>T was present in combination with risk allele for *PON1* L55M (M) and for *PON1* Q192R (Q), respectively (Supplementary Table 4).

4. Discussion

We failed to find any association between the *MTHFR* c.677C>T polymorphism and the risk of ALS in an Italian population. The c.677C>T polymorphism was not associated with ALS clinical variables, either at the genotype or allelic level. No association was found when *MTHFR* c.677C>T variant was considered in combination with the L55M and Q192R polymorphisms in *PON1* gene. A recent study on *MTHFR* c.677C>T polymorphism has shown a positive association in a German/Swiss ALS population (Kuhnlein et al., 2010). In this population the frequency of T allele was quite low (28%), with a homozygosity frequency of 6%. On the contrary, it is known that in Italy the mean T allele frequency is considerably higher (about 44%), with a mean homozygosity frequency of about 18%. In Europe the prevalence of the TT genotype increases from low values in the north (4%–7%), to higher values in southern Europe (12%–15%), peaking in southern-central Italy (20%–30%) (Wilcken et al., 2003). The mechanisms generating this gradient seem to involve gene-nutrient interactions. Because the TT genotype encodes for a thermolabile MTHFR variant that reduces the enzyme activity in presence of low folate intake, dietary folate may be one factor that has influenced the prevalence of T allele in Europe. The highest T allele frequency is reported in the geographic regions with the highest folate intake and the consequent highest plasmatic folate levels. In these areas the influence of the TT genotype on homocysteine (Hcy) plasma levels is the lowest, whereas it is the most evident in the regions with low frequency of T allele and low dietary intake of folate. Thus, *MTHFR* c.677C>T polymorphism is a risk factor for defects associated with high levels of Hcy (e.g., neural tube defects) in northern Europe, but it is neutral in western and southwestern Europe (Guéant-Rodriguez et al., 2006). A similar scenery may be hypothesized for the risk of ALS in the Italian population. Here, the c.677C>T variant is not associated with ALS and it is unlikely that it is involved in ALS pathogenesis, at least in presence of a sufficient dietary intake of folate. Whether Hcy plasma levels are a risk factor for ALS, other genetic and/or environmental factors, causing an alteration in homocysteine metabolism, could be responsible for the motor neuron damage observed in ALS in the Italian population.

Disclosure statement

The authors disclose no conflicts.

References

See Supplementary data.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neurobiolaging.2011.07.010.

Telomere Length in Neoplastic and Nonneoplastic Tissues of Patients with Familial and Sporadic Papillary Thyroid Cancer

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Introduction: Many studies have found an association between altered telomere length (TL), both attrition or elongation, and cancer phenotype. Recently, we have reported that patients with the familial form of papillary thyroid cancer (FPTC) have short telomeres in blood leucocytes.

Aim: To evaluate relative TL (RTL) at somatic level in neoplastic and nonneoplastic tissues of patients with FPTC (n = 30) and sporadic PTC (n = 46).

Methods: RTL was measured by quantitative PCR in neoplastic thyroid tissues, in the corresponding nontumor thyroid tissues (normal contralateral thyroid), and in other extrathyroidal tissues (lymph nodes, muscles, or buccal mucosa). RTL was also measured in adenomas and hyperplastic nodules. In a subset of samples, telomerase expression was measured by quantitative PCR.

Results: Mean ± SD RTL of FPTC patients was short in neoplastic thyroid tissues (0.87 ± 0.2) with no difference from the normal contralateral thyroid tissues (0.85 ± 0.11) and extrathyroidal tissues (0.85 ± 0.31). On the contrary, in patients with sporadic PTC, the mean ± SD RTL in the neoplastic tissues (1.73 ± 0.63) was significantly shorter than that found in normal contralateral tissues (2.58 ± 0.89) and extrathyroidal tissues (2.5 ± 0.86). For all tissue samples (cancer, normal thyroid, and nonthyroidal tissues) the mean ± SD RTL of familial cases was shorter ($P < 0.0001$) than that found in tissues from sporadic PTC. RTL of FPTC was also lower ($P < 0.0001$) than that of 23 follicular adenomas (1.6 ± 0.7) and 24 hyperplastic nodules (2.2 ± 0.9).

Conclusions: Our results demonstrate that short telomeres are a consistent feature of PTC, which in familial cases, is not restricted to the tumor tissue. This finding suggests that FPTC has a distinct, heritable, genetic background. **(*J Clin Endocrinol Metab* 96: E1852–E1856, 2011)**

Telomeres are repetitive structures located at the chromosome ends that progressively shorten with each cell replication due to incomplete lagging DNA strand synthesis and oxidative damage (1). Previous studies (2, 3) have indicated that human malignant cells in general have shorter telomeres than normal cells, and a relationship between telomere shortening and risk of cancer has been advocated. Many studies (4–6) have found an association

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Abbreviations: FPTC, Familial recurrence of papillary thyroid cancer; RTL, relative telomere length.

between altered relative telomere length (RTL), both attrition and elongation, and cancer phenotype. The few studies that have analyzed RTL in thyroid cancer tissues have found shorter RTL in primary tissues compared with normal peritumoral tissues or benign adenomas (7–9). Recently, our group reported that patients with the familial recurrence of papillary thyroid cancer (FPTC) display short telomeres in the peripheral blood compared with sporadic PTC patients, suggesting that this alteration may be implicated in the inherited predisposition to FPTC (10). To further extend this observation, the present report investigated RTL at the somatic level, analyzing primary PTC tissues, normal contralateral thyroid tissues, and nonthyroidal tissues (muscles, normal lymph nodes, and buccal mucosa) in patients with familial and sporadic PTC, adenomas, and hyperplastic nodules.

Patients and Methods

Patients

We studied a total of 76 patients (60 females) with PTC. The mean ± SD age at diagnosis was 49.2 ± 16.6 yr (range 17–83 yr). Sixty-six of these patients were treated in the Section of Endocrinology of the University of Siena, and 10 were provided by the Department of Endocrinology of the University of Thessaloniki (Greece). Thirty patients (25 females) had the familial recurrence of the disease defined as the presence of at least one first-degree relative with PTC. All patients had been treated with total thyroidectomy.

No clinical or epidemiological differences were present between familial and sporadic cases (sex, age at diagnosis, primary tumor diameter, tumor extension, lymph node metastases, and presence of *BRAF* mutation) with the exception of more frequent multifocality in the familial cases. We also analyzed 23 patients with follicular adenomas (16 females) with mean ± SD age at diagnosis of 60.9 ± 13.7 yr (range, 32–85 yr) and 24 patients with hyperplastic nodules (19 females), with mean ± SD age of 59.4 ± 14.1 yr (range, 32–83 yr).

Tissue samples

For molecular analysis, a total of 252 tissue specimens were obtained from these patients after signed informed consent according to a protocol approved by the local ethical committee. Samples of the primary tumor and their normal contralateral tissues were available in all 76 patients with cancer (30 familial and 46 sporadic PTC). Nonthyroidal tissues were available in 53 cases (18 with FPTC and 35 with sporadic PTC) and consisted in muscles (n = 13), reactive lymph nodes (n = 11), and buccal mucosa obtained by scraping (n = 29). Twenty-three samples were from patients with follicular adenoma, and 24 were from patients with hyperplastic nodules.

DNA extraction

Fresh tissues were collected at surgery into a 1.5-ml microcentrifuge tube containing Allprotect Tissue reagent (QIAGEN, Milan, Italy). After 24 h at 4 C, the solution was removed and tissues were frozen at −80 C. Genomic DNA was extracted using the QIAamp DNA Micro Kit (QIAGEN) following kit instructions. For paraffin-embedded tissues, approximately 25 mg tissue was incubated overnight at 56 C in lysis buffer (provided by the kit) in the presence of proteinase K (20 μl). Samples were then incubated for 15 min at 95 C to allow paraffin melting. After centrifugation for 3 min at 14,000 rpm, DNA was extracted following QIAamp DNA Micro Kit instructions (QIAGEN).

Measurement of RTL

RTL was assessed with two different techniques.

Quantitative PCR

A quantitative PCR assay was carried out on 30 ng/μl genomic DNA using an MJ mini personal thermal cycler (Bio-Rad, Milan, Italy) as already described (11). Briefly, telomere length quantification involved determining the relative ratio of telomere repeat copy number to a single-copy gene copy number in experimental samples using standard curves. This ratio is proportional to the average telomere length. The *36B4* gene, encoding acidic ribosomal phosphoprotein P0, has been used as the single-copy gene. Primers and conditions are detailed in Ref. 11.

Southern blot

The TeloTAGGG telomere length assay (Roche, Milan, Italy) kit was used to perform the Southern blot experiments. Briefly, 1.5 μg purified genomic DNA was digested by an optimized mixture of restriction enzymes provided by the kit. After digestion, the DNA fragments were separated by gel electrophoresis (voltage 0.5/cm) for 4 h and transferred overnight to a nylon membrane. Hybridization was carried out as described by the kit and telomere length determine by chemiluminescence. Telomere length was calculated as mean telomere restriction fragment using the following formula: $\Sigma(OD_i)/\Sigma(OD_i/L_i)$, where OD_i is the chemiluminescent signal and L_i is the length of telomere restriction fragment at position i.

hTERT mRNA expression

hTERT mRNA expression was evaluated by quantitative RT-PCR from 24 FPTC cancer tissues and 14 normal contralateral tissues, 12 sporadic PTC cancer specimens and six normal contralateral tissues, 11 follicular adenoma, and seven hyperplastic nodules. RNA was extracted using the RNeasy Mini Kit (QIAGEN) and retrotranscribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Approximately 100 ng/sample was analyzed using the MJ mini thermocycler (Bio-Rad) in a mix containing 2× SsoFast EvaGreenSupermix (Bio-Rad) and 300 nM specific primers (Beacon designer software version 7.7; Bio-Rad) (*hTERT* forward, 5′-ACGGCGACATGGAGAACAA-3′, and *hTERT* reverse, 5′-CACTGTCTTCCGCAAGTTCAC-3′) in a final volume of 20 μl. To exclude the presence of nonspecific binding between EvaGreen and primers, a melting curve was added at the end of all PCR amplification reactions. The PCR protocol was as follows: step 1, 3 min at 95 C; step 2 (40 repetitions), 15 sec at 95 C and 30 sec at 60 C, followed by a melting curve of step 1, 1 min at 95 C; step 2, 30 sec at 55 C, and step 3, 80 cycles of 0.5 C increments (10 sec each) from 55–95 C.

Each sample was run in duplicate, and for each run, efficiency of RT-PCR, slope values, and correlation coefficients (R^2) were determined. The expression level was calculated as ΔΔCt and reported as $2^{-\Delta\Delta Ct}$ against *GAPDH*, which was chosen as the

optimal reference gene by two well-validated software programs, Normfinder and GenNorm.

Statistical analysis

Statistical analysis was conducted with StatView for Windows, version 5.00.1 (SAS Institute, Cary, NC). All data are presented as mean ±SD. To calculate differences in RTL among different groups, the one way ANOVA test with Dunn's multiple comparisons was used. For the analysis of RTL in tumoral thyroid tissues, normal thyroid tissues and extrathyroidal tissues in the same patient, the paired *t* test was used. A *p*-value <0.05 was considered significant. ANOVA multiple comparison was also used to determine the difference in hTERT mRNA expression levels among groups.

Results

As shown in Fig. 1, mean ± SD RTL in primary tumor samples of FPTC patients was 0.87 ± 0.2 (range: 0.62–1.23), not different from the mean ± SD RTL of their corresponding normal thyroid tissues (0.85 ± 0.11; range, 0.66–1.2) or extrathyroidal tissues (0.85 ± 0.31; range, 0.16–1.48).

On the contrary, in sporadic cases, the mean ± SD RTL of primary cancer tissues was significantly ($P < 0.0001$) shorter (1.73 ± 0.63; range, 0.18–3.2) compared with the mean ± SD RTL found in normal contralateral tissues (2.58 ± 0.89; range, 0.78–6.5) or extrathyroidal tissues (2.5 ± 0.86; range, 1–4.8). For all tissue samples (cancer, normal thyroid, and nonthyroidal tissues) the mean ± SD RTL of familial cases was significantly shorter ($P < 0.0001$) than that found in the tissues from sporadic PTC. Similarly, RTL of FPTC (of all tissues) was significantly lower ($P < 0.0001$) than that of 23 follicular adenomas (1.6 ± 0.7; range, 0.32–3.1) and 24 hyperplastic nodules (2.2 ± 0.9; range, 0.64–3.8). The difference between RTL of cancer tissues, normal thyroid tissues, or extrathyroidal tissues of sporadic cases is even more apparent when the data were analyzed within each individual patient by paired *t* test (Fig. 2). RTL was shorter in cancer tissues compared with normal thyroid tissues and extrathyroidal tissues in the majority of cases (80%).

Measurement of RTL by Southern blot technique confirmed the results obtained by quantitative PCR. In terms of kilobases, we found an average telomere length of 2.3 ± 0.03 and 2.68 ± 0.1 for primary tumors and contralateral tissues of FPTC, respectively, and 3 ± 0.04 and 10.04 ± 0.21 for primary tumors or contralateral tissues of sporadic PTC, respectively (data not shown).

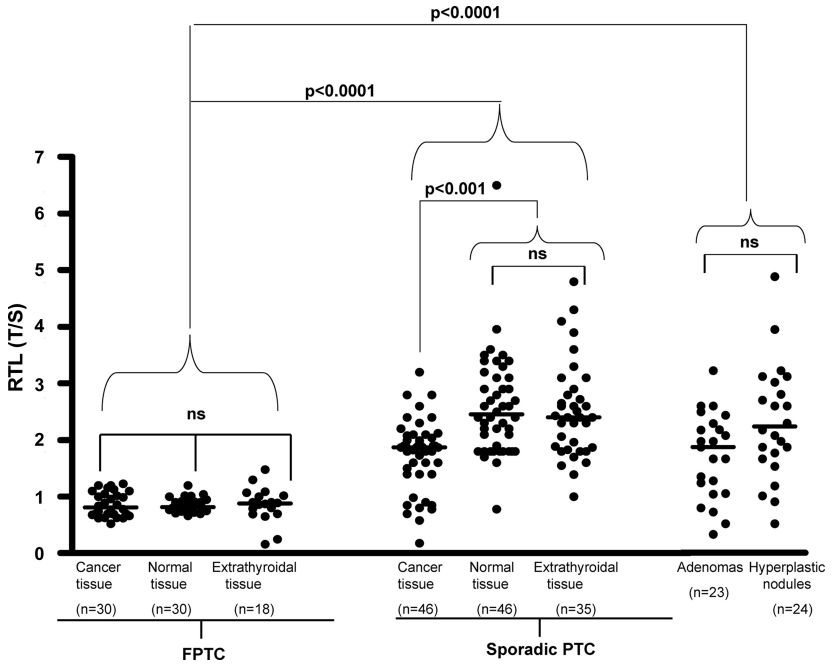

FIG. 1. RTL measured by quantitative PCR and expressed as ratio of telomere (T) repeat copy number to a single-copy gene (S) copy number (T/S ratio) in cancer tissues, normal thyroid tissues, and extrathyroidal tissues of FPTC and sporadic PTC, adenomas, and hyperplastic nodules. ns, Not significant.

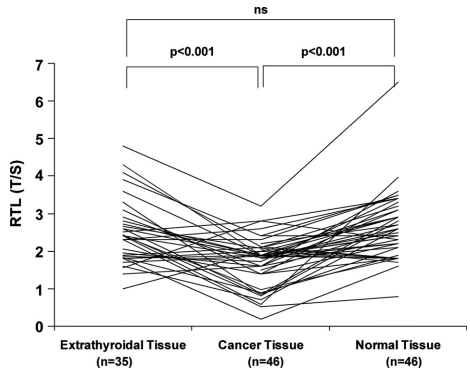

FIG. 2. RTL in tumor thyroid tissues, normal thyroid tissues, and extrathyroidal tissues of individual patients with sporadic PTC. T/S ratio is the ratio of telomere (T) repeat copy number to a single-copy gene (S) copy number.

Both in familial and sporadic cases, no correlation was found between RTL values and any clinical or pathological parameter (gender, age, primary tumor diameter, bilaterality, tumor extension, lymph node metastases, and *BRAF* status).

To address whether a change in telomerase expression is involved in telomere length, we evaluated *hTERT* mRNA expression (expressed as $2^{-\Delta\Delta Ct}$) in 24 primary cancer tissues and 14 contralateral tissues of FPTC, 12 primary tumors and six contralateral tissues of sporadic PTC, 11 adenomas, and seven hyperplastic nodules.

hTERT mRNA expression was found in 20 of 24 FPTC cancer tissues (mean ± SD value of 3.2 ± 0.5) and in 11 of 14 contralateral tissues (mean ± SD value of 2.77 ± 0.3). In sporadic cases, we found *hTERT* mRNA expression only in primary tumors (seven of 12, mean ± SD value of 4.3 ± 0.7) but never in contralateral tissues. Among benign lesions, eight of 11 adenomas had *hTERT* mRNA expression (mean ± SD value of 0.55 ± 0.24), whereas it was completely absent in all the hyperplastic lesions. The *hTERT* mRNA expression found in FPTC and in sporadic primary tumors was significantly higher ($P = 0.04$ and 0.018, respectively) compared with adenomas, hyperplastic nodules, and sporadic contralateral tissues. No significant correlation between *hTERT* activity and RTL was found.

Discussion

Our study confirms that short telomeres are present in tissue samples from papillary and follicular thyroid cancer compared with normal thyroid tissues and benign thyroid nodules (7–9). Follicular adenomas behave in an intermediate fashion between malignant and hyperplastic nodules, probably reflecting their tumoral (although biologically benign) nature. In addition, our study has the novelty of analyzing tissue from familial cases of PTC. In these familial cases, we previously demonstrated short telomere length in blood leukocytes compared with patients with sporadic PTC and postulated that this alteration may be heritable (10). In the present study, we have extended this observation by measuring the telomere length at the somatic level. Although the RTL was reduced in both sporadic tumors and, even more, in familial tumors, the RTL of normal thyroid tissues and extrathyroidal tissues differed in the two cohorts of patients. In familial cases, RTL was similarly short in any tissue examined, whereas in sporadic cases, normal thyroid tissues and extrathyroidal tissues had longer telomeres compared with the primary tumor.

This finding demonstrates that in familial patients, the presence of short telomeres is a peculiar feature of all the cells of the body, likely inherited from the parents. This concept is in agreement with a recent report by Chiang *et al.* (12), who elegantly showed in a shortened-telomerase mouse model that the set-point of telomere length of offspring is determined by the telomere length of their parents.

Short telomeres in the genome have been associated with chromosome instability and predisposition to several benign and malignant diseases, particularly of the familial type (4, 6, 13–16). The contribution of short telomeres in our FPTC patients to develop thyroid cancer is uncertain. However, recent studies in normal individuals prospectively followed for many years have shown that incident cancers were significantly associated with those subjects who had short telomere length at baseline, independently of standard cancer risk factors (17). In addition, the same study found a significant inverse correlation between telomere length and cancer mortality. This finding might support our previous report (18) showing that kindred with the FPTC display the phenomenon of genetic anticipation and have, in general, a more aggressive course compared with sporadic cases. The shorter telomere length in the tumor tissue of FPTC found in the present report compared with the tissue from sporadic cases may be somehow linked with the higher aggressiveness of familial tumors.

The telomerase experiments have shown that this enzyme is expressed in most malignant tissues, both familial and sporadic, and in some adenomas (at lower levels) but never in hyperplastic nodules or normal tissue of sporadic patients. Interestingly, telomerase was expressed also in normal tissue of familial patients, strengthening the concept that the alterations of the telomere-telomerase complex are peculiar and possibly constitutive features of FPTC.

In conclusion, our results confirm that short telomeres are a constant feature of PTC, both sporadic and familial. However, in familial cases, we have demonstrated that short telomeres are not restricted to the tumor tissue but are consistently present in other tissues, suggesting that FPTC has a distinct, heritable, genetic background.

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References

1. **Ju Z, Rudolph L** 2008 Telomere dysfunction and stem cell ageing. Biochimie (Paris) 90:24–32
2. **Wu X, Amos CI, Zhu Y, Zhao H, Grossman BH, Shay JW, Luo S, Hong WK, Spitz MR** 2003 Telomere dysfunction: a potential cancer predisposition factor. J Nat Cancer Inst 95:1211–1218
3. **Hiraga S, Ohnishi T, Izumoto S, Miyahara E, Kanemura Y, Matsumura H, Arita N** 1998 Telomerase activity and alterations in telomere length in human brain tumors. Cancer Res 58:2117–2125
4. **Broberg K, Björk J, Paulsson K, Höglund M, Albin M** 2005 Constitutional short telomeres are strong genetic susceptibility markers for bladder cancer. Carcinogenesis 26:1263–1271
5. **Meeker AK, Hicks JL, Iacobuzio-Donahue CA, Montgomery EA, Westra WH, Chan TY, Ronnett BM, De Marzo AM** 2004 Telomere length abnormalities occur early in the initiation of epithelial carcinogenesis. Clin Cancer Res 10:3317–3326
6. **Jang JS, Choi YY, Lee WK, Choi JE, Cha SI, Kim YJ, Kim CH, Kam S, Jung TH, Park JY** 2008 Telomere length and the risk of lung cancer. Cancer Sci 99:1385–1389
7. **Kammori M, Takubo K, Nakamura K, Furugouri E, Endo H, Kanauchi H, Mimura Y, Kaminishi M** 2000 Telomerase activity and telomere length in benign and malignant human thyroid tissues. Cancer Lett 159:175–181
8. **Jones CJ, Soley A, Skinner JW, Gupta J, Haughton MF, Wyllie FS, Schlumberger M, Bacchetti S, Wynford-Thomas D** 1998 Dissociation of telomere dynamics from telomerase activity in human thyroid cancer cells. Exp Cell Res 240:333–339
9. **Achille M, Boukheris H, Caillou B, Talbot M, de Vathaire F, Sabatier L, Desmaze C, Schlumberger M, Soria JC** 2009 Expression of cell cycle biomarkers and telomere length in papillary thyroid carcinoma: a comparative study between radiation-associated and spontaneous cancers. Am J Clin Oncol 32:1–8
10. **Capezzone M, Cantara S, Marchisotta S, Filetti S, De Santi MM, Rossi B, Ronga G, Durante C, Pacini F** 2008 Short telomeres, telomerase reverse transcriptase gene amplification, and increased telomerase activity in the blood of familial papillary thyroid cancer patients, J Clin Endocrinol Metab 93:3950–3957
11. **Cawthon RM** 2002 Telomere measurement by quantitative PCR. Nucleic Acids Research 30:e47
12. **Chiang YJ, Calado RT, Hathcock KS, Lansdorp PM, Young NS, Hodes RJ** 2010 Telomere length is inherited with resetting of the telomere set-point. Proc Natl Acad Sci USA 107:10148–10153
13. **Armanios MY, Chen JJ, Cogan JD, Alder JK, Ingersoll RG, Markin C, Lawson WE, Xie M, Vulto I, Phillips 3rd JA, Lansdorp PM, Greider CW, Loyd JE** 2007 Telomerase mutations in families with idiopathic pulmonary fibrosis. N Engl J Med 356:1317–1326
14. **Vulliamy T, Marrone A, Szydlo R, Walne A, Mason PJ, Dokal I** 2004 Disease anticipation is associated with progressive telomere shortening in families with dyskeratosis congenita due to mutations in TERC. Nat Genet 36:447–449
15. **Shen J, Terry MB, Gurvich I, Liao Y, Senie RT, Santella RM** 2007 Short telomere length and breast cancer risk: a study in sister sets. Cancer Res 67:5538–5544
16. **Hills M, Lansdorp PM** 2009 Short telomeres resulting from heritable mutations in the telomerase reverse transcriptase gene predispose for a variety of malignancies. Ann NY Acad Sci 1176:178–190
17. **Willeit P, Willeit J, Mayr A, Weger S, Oberhollenzer F, Brandstätter A, Kronenberg F, Kiechl S** 2010 Telomere length and risk of incident cancer and cancer mortality. JAMA 304:69–75
18. **Capezzone M, Marchisotta S, Cantara S, Busonero G, Brilli L, Pazaitou-Panayiotou K, Carli AF, Caruso G, Toti P, Capitani S, Pammolli A, Pacini F** 2008 Familial non-medullary thyroid carcinoma displays the features of clinical anticipation suggestive of a distinct biological entity. Endocr Relat Cancer 15:1075–1081

EUROPEAN NEUROLOGICAL JOURNAL REVIEW ARTICLE

SOD1 Mutations in Amyotrophic Lateral Sclerosis

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a progressive and fatal disorder characterized by degeneration of motor neurons in the cerebral cortex, brain stem, and spinal cord. Most cases of ALS appear sporadically but about 1–13.5% of patients have a family history of ALS. Although the precise cause for the majority of cases is still unknown, mutations in the gene encoding for copper–zinc superoxide dismutase (*SOD1*) have been found in 12–23% of familial cases of the disease. Currently, more than 150 different *SOD1* gene mutations have been identified in ALS patients most of which with autosomal dominant transmission. Occasionally, specific mutations are associated with a particular phenotype. Some *SOD1* mutations occur as recurrent or founder mutations with a different geographic distribution. The discovery of mutations in the *SOD1* gene has marked a change in ALS research and enabled the development of novel experimental rodent models to investigate the pathogenesis of familial ALS. However, the mechanism by which mutant SOD1 causes neural death remains elusive. Several lines of evidence suggest that ALS is a protein-folding disease and the increased propensity of mutant SOD1 to form aggregates may confer toxicity in motor neurons. Despite the apparent selectivity for motor neurons, recent data indicate that non-neuronal cell types contribute to pathogenesis and disease progression.

Keywords: Amyotrophic lateral sclerosis, familial and sporadicals, superoxide dismutase 1 (SOD1) Gene, mutation, founder effect, animal models, protein aggregation

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS), the most common adultonset motor neuron disease, is a progressive and fatal disorder characterized by neurodegeneration of motor neurons in the motor cortex, brain stem, and spinal cord [1]. Although motor neurons are selectively affected by degeneration and death, increasing evidence indicates that non-neuronal neighboring cell types contribute to pathogenesis and disease progression [2, 3]. Motor neuron degeneration results in progressive weakness of bulbar, thoracic, abdominal, and limb muscles. Dysfunction of upper motor neurons (UMN) in the motor cortex leads to hyperreflexia, extensor plantar response, and increased muscle tone, whereas dysfunction of lower motor neuron (LMN) in the brainstem and spinal cord triggers generalized weakness, hyporeflexia, muscle atrophy, muscle cramps, and fasciculations [1]. Symptoms present in early disease may vary. Affected individuals most often present with asymmetrical distal onset in a limb with both UMN and LMN signs from the onset (classical Charcot ALS). Patients with bulbar-onset ALS typically have slurred speech and difficulty swallowing, and the condition is designated progressive bulbar palsy (PBP). Limbs symptoms in the majority of cases will occur within 1–2 years. During the course of the disease, most cases become generalized with a combination of both LMN and UMN signs affecting spinal and brainstem regions [4].

At presentation, limb involvement occurs more often than bulbar involvement, which accounts for about 25% of ALS cases. Other brain functions, as well as oculomotor and sphincter functions, are rarely involved. Mild cognitive impairment is described in 20–50% of cases, and a frontotemporal dementia (FTLD) is reported in 3–5% [5]. Cognitive abnormalities may precede or occur after the onset of motor symptoms. Death, mainly because of respiratory failure, occurs 2–4 years after onset; however, a small group of patients may have a disease duration of 10 years or even more. There is no objective test capable of providing the diagnosis of ALS. It remains essentially a clinical diagnosis based on clinical features, electrodiagnostic testing, and exclusion of conditions that can mimic ALS. The clinical diagnosis of ALS may be categorized into various levels of certainty by clinical and laboratory assessment based on El Escorial criteria [6].

Epidemiology

The incidence (1–2/100 000 person-year) and the prevalence (4–13/100 000) of the disease are relatively uniform in European and North American populations, although several high-incidence foci occur in the Western pacific [4, 7]. Four European population-based registers showed an increase of the incidence of ALS after the age of 40 years, with a peak in the late 60s or early 70s, followed by a rapid decline [8]. The lifetime risk for sporadic ALS by the age of 70 has been estimated at 1 in 1000 [4]. Several non-population based

studies consistently report that males are more likely to be affected than females with a M:F ratio of about 1.5:1. However, more recent data from European population-based registers report a change in the gender ratio, which is approaching equality [4]. A better ascertainment among women and/or a change in prevalence of risk factors across genders as a consequence of socioeconomic changes have been suggested as possible explanations for the increase of ALS incidence among women [8].

Over the years, a multitude of environmental exposure and lifestyle risk factors have been proposed as possible contributors to the cause of ALS including manual work, exposure to lead/solvents, pesticides, cigarette smoking, and intense physical activity. At present, however, no conclusive data are available, and further studies are needed to define exogenous risk factors of ALS [8, 9].

Negative prognostic indicators, arising from population-based studies, include site of disease onset, higher age of onset, and progression rate of respiratory, bulbar, and lower-limb symptoms [10].

Most cases (90%) are classified as sporadic ALS (SALS), as they are not associated with a documented family history. In retrospective epidemiological studies, in 1–13% of patients the disease is reported to be inherited and referred to as familial ALS (FALS), most commonly with a Mendelian dominant mode of inheritance and high penetrance, although pedigrees with incomplete penetrance or recessive inheritance have been reported [11]. The mean age of onset for SALS is 56 years and for FALS is 46 years. Age of onset in FALS shows a Gaussian distribution, whereas SALS is characterized by an age-dependant incidence [4]. The term ''juvenile ALS'' is used for patients with onset of disease prior to age 25 [12].

Apart from a mean age of onset for SALS that is about a decade later than for FALS, sporadic and familial forms are clinically indistinguishable suggesting a common pathogenesis. The precise cause of the selective death of motor neuron in the disease at present remains elusive, and progress in understanding the mechanisms underlying familial ALS may shed light on both forms of the disease [13].

Genetic Factors

Eight ALS genes have currently been identified: Cu/Zn superoxide dismutase (*SOD1*) [14], alsin (*ALS2*) [15, 6], senataxin (*SETX*) [17], fused in sarcoma/translated in liposarcoma (*FUS/TLS*) [18, 19] vesicle-associated membrane protein (*VAPB*) [20], angiogenin (*ANG*) [21, 22], Tar DNA-binding protein 43 (*TARDBP*) [23–25], and dynactin (*DCTN1*) [26, 27] (**Table 1**). Among these genes, ALS2 encodes a protein that may act as a GTPase regulator; SETX, FUS/TLS, ANG, and TARDBP encode proteins involved in the RNA metabolism; VABP and DCTN1 presumably regulate axonal transport. Therefore, familial ALS genes control different cellular mechanisms, suggesting that the pathogenesis of ALS may be related to several different processes finally leading to motor neuron degeneration.

COPPER–ZINC SUPEROXIDE DISMUTASE (*SOD1*) GENE

The most important contribution toward an understanding of ALS thus far has come from the discovery in 1993 of missense mutations in the gene encoding for copper–zinc superoxide dismutase (*SOD1*) on chromosome 21q22.1 associated with an adult-onset autosomal dominant form of the disease (ALS1) [14]. The human *SOD1* gene is a small gene comprising five exons, separated by four introns, encoding for a metalloenzyme of 153 highly conserved amino acids [14]. The *SOD1* protein is a homodimeric Cu/Zn-binding enzyme, composed of eight antiparallel β strands and two metal atoms, that cat alyzes the conversion of the toxic superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). SOD1 is ubiquitously expressed and account for about 0.5–0.8% of the soluble protein in the human brain [28]. It is found in the cytosol and nucleus, and in the intermembrane space of the mitochondria [29].

Currently, more than 150 different mutations in the *SOD1* gene (**Figure 1**) have been described worldwide in ALS patients, and an updated list can be found at the ALS Online Genetic Database (ALSOD: http://alsod.iop.kcl.ac.uk/) [30]. The majority of mutations in *SOD1* gene are missense, with a small number of deletion and insertion mutations resulting in truncated *SOD1* polypeptides. Missense mutations that cause ALS phenotype have been documented at 80 codons in *SOD1* gene, with multiple amino acid substitutions involving a given position (up to six different substitutions at position G93) [31].

In studies from different populations, the frequency of *SOD1* gene mutations ranges from 12% to 23% in patients diagnosed with FALS and from 0% to 7% in patients diagnosed with SALS [11, 32, 33], with an overall frequency, including all the published studies, of 20.7% for FALS and 2.2% for SALS (**Table 2**). In the Italian population, our early-referred study found a frequency of *SOD1* gene mutations in FALS cases (17.9%) similar to figures reported in studies from different countries [11]. However, a significant difference was found between the frequency of *SOD1* mutations in SALS cases (0%) [34] and that previously reported in the literature [11]. Since our study was published, a total of 390 SALS cases were screened and a low mutation frequency (0.8%) was confirmed in our series. It is interesting to note that our data are in agreement with a recent population-based study from Italy that found a figure of 0.7% in SALS, and suggested that the frequency of *SOD1* mutations in sporadic cases observed in case series could be overestimated because of referral bias [35]. A low *SOD1* mutation frequency was recently found also in the Dutch population both in familial (1.8%) and sporadic (0.4%) cases [36]. These findings suggest heterogeneity in the genetic background of ALS within different populations.

Recurrent Mutations and Genotype–Phenotype Correlation

Among the known *SOD1* gene mutations a geographic distribution is beginning to emerge [11]. The D90A mutation

Table 1. ALS-Causing Genes

ALS type	Gene	Protein	Locus	Inheritance	Clinicai features
ALS1	SOD1	Cu/Zn superoxide dismutase	21q22.1	AD/AR	Adultonset, Classical
ALS2	ALS2	ALsin	2q33	AR	Juvenile onset, UMN
ALS4	SETX	Senataxin	9q34	AD	Juvenile onset, slow
ALS6	FUS/TLS	Fused in sarcoma/translated in liposarcoma	16p11.2	AD	Adultonset, Classical
ALS8	VAPB	VAMP-associated protein	20q13.3	AD	Adult onset, Atypical features
ALS9	ANG	Angiogenin	14q11.2	AD	Adultonset, Classical
ALS10	TARDBP	Tar DNA-binding protein 43 (TDP-43)	1q36	AD	Adultonset, Classical
LMND	DCTN1	Dynactin 1	2PI3	AD	LMND

AD, autosomal dominant; AR, autosomal recessive; UMN, upper motor neuron; LMND, lower motor neuron disease.

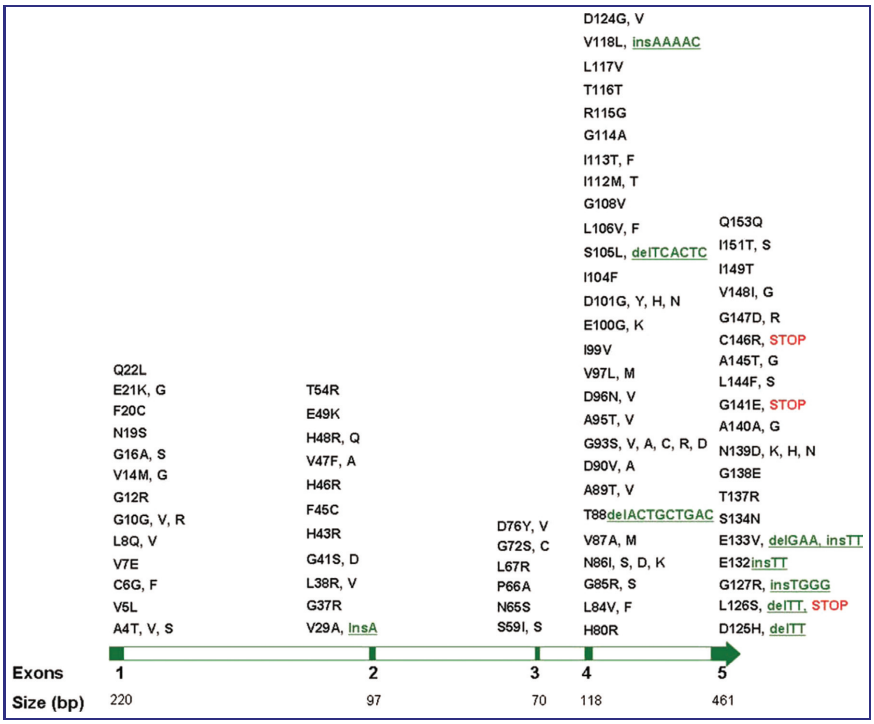

Figure 1. Mutation in the *SOD1* gene of ALS patients. Exons 1–5 of the *SOD1* gene are shown as green boxes; for each exon the size in base pairs is indicated. Different mutations are indicated in different colors (black: missense and silent mutation; green: deletion (del) and insertion (ins); red: nonsense mutations).

is the most common mutation globally and the most prevalent one in Europe, accounting for 50% of overall ALS cases in some areas of Sweden and Finland. The A4V mutation is the most prevalent mutation in the United States, accounting for about 50% of all SOD1 mutated patients, and the I113T mutation is by far the most common mutation in the United Kingdom. In the Balkans the most prevalent mutation seems to be the L144F [52]; in Germany, the R115G [41]; and in Italy, the G41S [53].

All SOD1 mutations are autosomal dominantly inherited with some exceptions. Although a single familial case homozygous for the L84F mutation has been reported [38], only the D90A substitution has been clearly shown to be inherited as both a dominant and a recessive trait [39]. In addition, compound heterozygous D90A and D96N patients have been described in a recessive French family [54]. In Scandinavia, the D90A allele exists as a polymorphism because it is found in 0.5–5% of the population [55] In the

Table 2. Frequency of *SOD1* Mutations in FALS and SALS in Different Populations

	FALS		SALS		
Country	**n° Studied**	**n° SOD1 positive**	**n° Studied**	**n° SOD1 positive**	**References**
Belgium	23	7 (30.4%)	69	2 (2.9%)	Aguirre et al. [37]
Canada	117	20 (17.1%) Range: 14.3–21.3%	159	3 (1.9%)	Eisen et al. [33], Boukaftane et al. [38]
Denmark	2	2 (100%)	25	1 (4.0%)	Andersen et al. [39]
Finland	21	9 (42.9%)	80	9 (11.3%)	Andersen et al. [39]
France	–	(15.0%)	–	–	Jafari-Schluep et al. [40]
Germany	75	9 (12.0%)	–	–	Niemann et al. [4i]
Ireland	8	1 (12.5%)	90	2 (2.2%)	Alexander et al. [42]
Italy	99	15 (15.2%) Range: 13.6–17.9%	675	10 (1.5%) Range: *0.7–4.5%	Gellera et al. [43], Battistinietal. [34]*, Corrado et al. [44], Chiò et al. [35]
Japan	7	5 (71.4%)	–	–	Abe et al. [45]
Norway	4	0 (0.0%)	37	0 (0.0%)	Andersen et al. [39]
Scotland	10	5 (50.0%)	57	4 (7.0%)	Jones et al. [46]
Spain (Catalonia)	30	5 (16.7%)	94	4 (4.3%)	Gamez et al. [47]
Sweden	45	16 (35.6)	213	4 (1.9)	Andersen et al. [39]
The Netherlands	55	1 (1.8%)	451	2 (0.4%)	van Es et al. [36]
UK	123	25 (20.3%) Range: 20.0–21.0%	330	9 (2.7%) Range: 2.6–2.9%	Jackson et al. [48], Shaw et al. [49], Orrell et al. [50]
USA	290	68 (23.4%)	–	–	Cudkowicz et al. [51]
Overall frequency		**20.7%**		**2.2%**	

*The frequency of *SOD1* mutation in SALS cases previously reported by the authors as 0%[34]. has been updated to 0.8% (3 out of 390 cases) (unpublished data).

majority of cases the D90A mutation causes ALS as a recessive trait. In all reported D90A homozygous patients, the disease shows a homogeneous phenotype with an initial pre-paretic phase followed by a paretic phase and a mean disease duration of about 14 years. In the first phase, patients have sensory complaints such as lower back, hip, or knee pain or heat sensations usually remitting at the onset of the second, slowly ascending phase. Atypical features have been reported, including bladder disturbance, intermitted ataxia, and aching pain [11]. A few D90-Aheterozygous patients have been described, predominantly as sporadic cases but also in dominant pedigrees, in Sweden and Finland [39], Belgium [56], Russia [57], the United Kingdom [48], United States [55], the United States of German ancestry [58], France [59], Spain [47], and Italy [60–62], and recently in The Netherlands [36]. The D90A-heterozygous patients have been divided in two groups: the first group resembles the D90A-homozygous condition, whereas the second one shows a more variable and aggressive phenotype, with spinal or bulbar onset, short or intermediate survival, and a lack of atypical features [11].

Phenotypic heterogeneity in terms of age at onset, disease duration, penetrance, and clinical manifestations is not uncommon among patients with different *SOD1* mutations and also in members of the same family. Clinical presentation can occasionally be correlated with *SOD1* mutations. For example, the phenotype of the A4V mutation is characterized by a sudden symptom onset and relatively rapid disease progression, with a mean survival of usually 1–2 years. Muscle weakness can start in the limbs or in the bulbar muscles. LMN signs usually dominate the clinical presentation [51]. A uniform phenotype has been described in six Italian FALS with the G41S mutation [53], confirming previous observations that this mutation is consistently associated with a dramatic and fast-progressing phenotype [14, 35, 51, 63]. The clinical picture of these G41S mutated FALS patients was characterized by spinal onset with early UMN and LMN involvement, appearance of bulbar signs usually within 1 year, and death a few months later. The patients displayed a rapidly progressive disease course, with a mean age at onset of 49.3 ± 11.3 years (range 25–66 years) and an illness duration of 0.9 ± 0.3 years (range 0.2–1.2 years) [53]. On the opposite end of the spectrum, an extreme phenotypic variability has been reported for patients with the I113T mutation with a range of disease duration of 2–20 years, spinal or bulbar onset, and variable penetrance even among members of the same family [33, 51, 64].

Haplotype Studies

Though some *SOD1* mutations occur as recurrent, few data have been reported in the literature regarding haplotype studies. A founder effect has been demonstrated for the I113T mutation in six Scottish SALS and FALS cases [65], for the D90A in recessive and dominant European and North American families [55], for the R115G in four German cases of FALS, [41]. for the A4V in many North American [66–68] and European families [68], and for the G41S in six Italian FALS [53]. Interestingly, the age of the mutation has been estimated for a limited number of mutations. In the case of the D90A mutation, individuals from dominant and recessive D90A pedigrees have been shown to share a common founder existing around 895 generations ago in Eurasia, with recessive families arising around 63 generations ago in the founding populations of Finland [55, 69]. It was initially proposed by the authors that some modifying factors in the coding or regulatory regions of *SOD1* gene could be able to slow the disease progression in recessive cases [55]. However, subsequent studies failed to confirm the presence of such neuroprotective factors in the genomic region around the *SOD1* gene [70, 71]. In addition, two different founders have been identified for the North American A4V mutation. One founder, responsible for 18% of North American A4V patients, had a genetic background similar to that of European A4V patients. The other founder, accounting for the remaining 82%, was genetically similar to Native Americans, who reached the Americas from Asia. The authors have estimated that the mutation has been introduced into the white population about 400–500 years ago [68]. Another study on A4V North American ALS patients identified a conserved minimal haplotype more similar to Asian than European population, confirming that this mutation occurred in an Asian population who migrated into the North America through the Bering Strait, and arose in this population about 540 generations ago [66]. These findings may explain why this mutation is rare in Europe. Finally, founder analysis showed that the G41S mutation, identified so far only in Italian ALS families, may have originated in Italy approximately 45 generations ago from a common founder from Northwest Tuscany region [53]. These reports may contribute to a better understanding of the evolutionary history of the disease and how ALS originated and spread within populations.

ANIMAL MODELS OF *SOD1*-LINKED ALS

The discovery of mutations in the *SOD1* gene in 1993 [14] has marked a change for the scientific research of ALS and prompted many investigators to develop experimental rodent models to investigate the mechanism of neuronal death associated with *SOD1* gene defects. Several transgenic mouse strains expressing the human *SOD1* gene with different mutations, including missense mutations and C-terminally truncated variants, have been generated to date. The most extensively used strains for studying the disease pathogenesis are [72] $SOD1^{G93A}$ [73], $SOD1^{A4V}$ [74], $SOD1^{G37R}$ [75] $SOD1^{G85R}$ [76], $SOD1^{G86R}$ [77], $SOD1^{D90A}$ [78], $SOD1^{L84V}$ [79], $SOD1^{I113T}$ [80], $SOD1^{H46R/H48Q}$ [81], and $SOD1^{H46R/H48Q/H63G/H120G}$ [82] for the missense mutations, and $SOD1^{L126X}$ [74, 83], $SOD1^{L126delTT}$ [84], and $SOD1^{G127X}$ [85] as C-terminally truncated variants. In addition to the mice, there are two examples of transgenic rats carrying two different human SOD1 mutations, the $SOD1^{H46R}$ rats[86] and the $SOD1^{G93R}$ rats [86, 87]. Mutant SOD1 transgenic mice and rats develop muscle wasting and progressive paralysis, which clinically resemble human ALS. They also express histopathological features that reflect several characteristics of the human disease, in particular, selective degeneration of spinal motor neurons [88] aggregation of ubiquitinated proteins in motor neuron, decrease of constitutive proteasome levels with a concomitant increase of immunoproteasome [89], and, finally, microglial activation in the degeneration area [90]. The various transgenic mouse strains, however, show some genetic and phenotypic differences consisting in the transgene copy number, expression levels of mutant SOD1 protein, disease onset, and disease duration. Overall, it is widely accepted that transgenic rodents are a good model of human SOD1-linked FALS. However, the main limitation of the use of rodent models is that the level of expression needed in transgenic rodents to induce the pathological phenotype is much higher than that in patients with heterozygous *SOD1* mutation, so these differences between mice and men should be taken into account when using mouse models [91].

In addition to transgenic models, a first spontaneously occurring animal model of ALS has recently been reported. Indeed, a mutation in the *SOD1* gene (E40K), recessively inherited, has been identified in canine degenerative myelopathy (DM), a disease characterized by symptoms and histopathologic and immune-histophalogical lesions similar to those present in ALS patients [92]. Dogs with DM, compared with the transgenic rodents, are more similar to humans with ALS in terms of structure and complexity of the nervous system, disease duration, and mutant SOD1 expression levels, and may be a faithful model to investigate the processes underlying the motor neuron degeneration in ALS.

STRUCTURAL PROPERTIES OF SOD1 PROTEIN AND DISEASE MECHANISM

The role of SOD1 in detoxifying superoxide anion, together with its high expression levels in neural tissue, suggested the initial hypothesis that the *SOD1* mutations resulted in an enzyme unable to neutralize reactive oxygen species. This loss of enzymatic activity could lead to accumulation of toxic radicals, and oxidative damage and death of neural cells. However, SOD1 knockout mice do not develop a murine motor neuron disease [93]. Moreover, transgenic mice carrying human mutant SOD1, in addition to their own endogenous SOD1, develop symptoms similar to those observed in human patients, whereas transgenic mice expressing human wild-type SOD1, in addition to their own endogenous SOD1, do not [73, 76]. These collective observations converge to indicate that the mutant SOD1 protein acts through the gain of a toxic property and not through a loss of function. The current hypotheses for the mechanism of toxicity include oxidative

stress, mitochondrial dysfunction, impairment of axonal transport, aberrant RNA metabolism, glial cell pathology, and glutamate excitotoxicity [2]. Although the mechanism by which mutant SOD1 causes neural death remains elusive, several lines of evidence suggest that ALS is a protein-folding disease analogous to other neurodegenerative disorders like Alzheimer's, Parkinson's, and Creutzfeldt–Jakob's disease [31, 94, 95].

Structure of the SOD1 Protein

SOD1 is a 32-kDa homodimeric enzyme in which each subunit contains an 8-stranded Greek key β barrel and several loops (**Figure 2**A). Each subunit can form an intramolecular disulfide bond and coordinate one copper ion and one zinc ion in its active site [96]. Two loop elements, termed the ''zinc loop'' (loop IV, residues 50–83) and the ''electrostatic loop'' (loop VII, residues 121–142) project from the β barrel and are important in metal ion binding and formation of the active site. In the mature enzyme, the C57 is covalently linked to the C146 through a disulfide bond [97]. The fully metallated SOD1 protein is a highly stable dimeric molecule that remains active under a broad range of denaturing conditions. This conformational stability seems to be closely linked to copper and zinc ion coordination, which leads to the formation of the intramolecular disulfide bond; in addition, metallation and disulfide bond promote the homodimerization of SOD1 subunits [98–100].

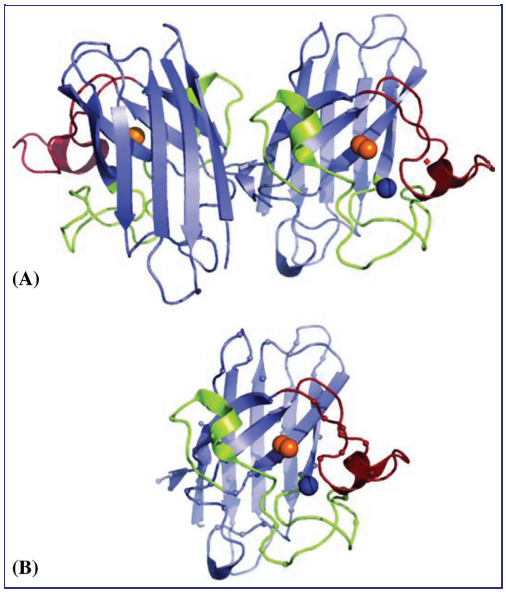

Figure 2. A) Structure of human SOD1 protein. The β barrel is shown in blue, and the zinc loop and the electrostatic loop are shown in green and red, respectively. The copper and zinc ions are represented as orange and blue spheres, respectively. B) ALS-associated mutations in the SOD1 protein. The position of the *SOD1* mutations are represented as small spheres within the SOD1 monomer.

SOD1 Mutants and Their Effects on Enzyme Structure

The pathogenic SOD1 mutations are classified into two groups based on their position in the structure (**Figure 2**B). The β-barrel mutants are characterized by a metal content comparable to that of the wild-type SOD1 and their three-dimensional structures are all similar to that of the wild-type protein except for a perturbation near the site of mutation (eg, A4V, G93A, I113T). In contrast, the metal-binding mutants are generally deficient in copper and/or zinc, and are characterized by conformational disorder of the electrostatic and zinc loop elements (eg, H46R, G85R, D125H) [101]. The β-barrel mutations can result in local perturbations that are able to alter the protein structure by affecting selectively the monomer stability, or weakening exclusively the dimer interface, or doing both at the same time [102]. The metal-binding mutations can diminish the metal coordination and lead to altered SOD1–SOD1 interactions. In both cases, the mutations cause a destabilization of SOD1 structure and could promote oligomerization and aggregation. In addition, several ALS-associated SOD1 mutations decrease the net negative charge of the SOD1 protein, and this reduction could promote aggregation. Most proteins possess a net surface charge at physiological pH, and the prevalence of net charge has been supposed as a general biological mechanism that prevents aggregation. Thus, some ALS-associated SOD1 mutations, such as the D101N and D76V, that are unlikely to cause SOD1 aggregation by protein destabilization or inhibition of metal binding, might promote the aggregate formation by decreasing the net negative charge of the SOD1 protein [103]. Based on these assumptions, the potential effect of a specific SOD1 mutation on protein stability can be predicted using computational bioinformatics tools (eg, the ''Panther software'' and the ''SOD1 Database—Motor Neuron Disease Mutations,'' directly available from the ALSOD database, and the ''Swiss model server,'' accessible via the ExPASy web server) [30, 104, 105]. For example, the modeling of the novel G10R mutation showed a relevant increase in energy around the site of mutation causing a strong destabilization of the protein that could influence the strength of the dimer interface [106].

It has also been suggested that mutant SOD1 proteins have a reduced ability to interact properly with the copper chaperone for SOD1 (CCS), a polypeptide that confers two critical stabilizing posttranslational modifications on the newly synthesized SOD1: the insertion of the copper ion [107], and the oxidation of the disulfide bond within each SOD1 subunit [108]. The reduced interaction with this chaperone might result in an increased destabilization and aggregation propensity [101].

SOD1 Aggregation and Motor Neuron Degeneration

The accumulation of detergent-insoluble aggregates of mutant SOD1 has been observed to coincide with the manifestation of disease symptoms in all mouse models

[31, 97]. Similar evidence that mutant SOD1 aggregation is a pathologic feature has been reported in human SOD1-associated ALS [109–111]. So, the presence of detergent-insoluble aggregated forms of mutant SOD1 in the spinal cord seems to strictly correlate with the disease. Whether the toxic action in SOD1-associated ALS is caused by the misfolded pathogenic SOD1 monomers, soluble oligomers, or insoluble aggregates remains unclear. Data from cell models suggest that the formation of large SOD1 aggregates could be the main mechanism of toxicity [112]. However, it remains possible that it is the soluble precursors of these large SOD1 aggregates (rather than the aggregates themselves) that are toxic. Indeed, in mouse models of SOD1-associated ALS, the most significant accumulation of mutant SOD1 aggregates occurs late in the disease [31], after the appearance of multiple pathologic abnormalities [113]. Thus, it has been supposed that a toxic form of mutant SOD1, different from the larger protein aggregates, is responsible for initiating the disease [31].

Recent studies have used several biophysical data to calculate the aggregation rates for ALS mutants, suggesting that the aggregation of mutant protein might be a key element in disease progression [102, 114]. A study analyzed the aggregation propensity in about 30% of all known SOD1 mutants in a cell culture model, providing definitive evidence that increased aggregation propensity is highly likely to be a universal feature of mutant SOD1 [31]. However, it failed to identify a specific biochemical or biophysical property that adequately explains the variability in the propensity of different mutant proteins to aggregate, even in cases in which multiple mutations target a single amino acid position. Interestingly, no obvious relationship between aggregation propensity and age of onset was found. However, SOD1 mutations with a high aggregation propensity, such as the A4V and the G41S, are generally correlated with a more rapidly progressing disease and a shorter duration [31]. For the G41 position it has been demonstrated that the G41S mutation showed an *in vitro* aggregation propensity higher than the G41D substitution, which is associated with a less aggressive phenotype and a longer survival (<1 year vs. 17 years). The association with the disease duration appears to be a distinctive feature of ALS, because in other examples of neurodegenerative disorders associated with aggregation, aggregation propensity is best correlated with disease onset [115, 116].

Several hypotheses have been proposed regarding the toxicity mechanism of SOD1 aggregates, including the perturbation of mitochondrial function, the alteration of axonal transport, the aberrant binding of apoptosis regulators, and the glutamate exocitotoxicity [103]. Various lines of evidence suggest that the presence of SOD1 aggregates affects the capability of the cell to preserve the protein homeostasis. In the cell, chaperones aid partially folded or unfolded polypeptides to revert to their functional conformation, preventing their aggregation and their interaction with inappropriate partners [117]. If proteins cannot be refolded, they are escorted to the proteasome system for degradation [118]. In the case of SOD1 mutations, the chaperones might be engaged in the unproductive effort to remove protein aggregates, and might become not available for productive functions in protein folding. At the same time, the mutant proteins may overburden the proteasome system, resulting in an impairment of the degradation activity. A general dysfunction in protein-folding and metabolism, caused by the alteration of these crucial protein homeostatic processes, could be responsible for the rapid progression of the disease.

Mutant SOD1 Toxicity in Non-neuronal Cells

The collective evidence is that, although ALS is characterized by motor neuron degeneration and death, toxicity of SOD1 mutants is produced by damage developed not only within motor neurons, but also by other non-neuronal neighbors [3]. SOD1 aggregates occur not exclusively in neurons, but also in the glial cells surrounding the motor neuron [76, 119, 120]. The role of glial cells in SOD1-linked ALS pathogenesis has been initially demonstrated in mice chimeric for a human SOD1 mutation [121]. In these mice, degeneration of motor neurons expressing mutant SOD1 was delayed or prevented when they were surrounded by wild-type non-neuronal cells, whereas wild-type motor neurons developed degenerative changes when surrounded by non-neuronal cells expressing mutant SOD1 [121]. It is now accepted that damage within motor neurons is a primary determinant of disease onset. During this initial phase, mutant SOD1 primarily acts directly within motor neurons, where aggregation of misfolded SOD1 damages cellular machinery and alters normal cellular functions. SOD1 mutant injury is amplified by the action within other cell types, especially the microglia, which respond to the initial damage and lead to a more rapid disease progression [3]. Progression is indeed characterized by a massive activation of microglia and astrocytes, in addition to continuing damage within motor neurons themselves. Misfolded SOD1 mutant within microglial cells and astrocytes, together with their activation in response to neuronal damage, can cause inflammation and provoke an increased release of toxic factors. These molecules in turn exert a toxic effect on neighboring cells and cause acceleration of the disease progression [122]. In addition, recent findings have shown that protein components of neurosecretory vesicles in neurons and neuroendocrine system, termed chromogranins, can interact with mutant SOD1, but not with the wild-type protein [123]. Chromogranins may act as chaperone-like proteins to promote the secretion of SOD1 mutants by motor neuron and astrocytes. Extracellular mutant SOD1 in turn can activate microgliosis and cause neuronal cell death. This model emphases the likely crosstalk between motor neurons, microglial cells, and potentially other nonneuronal cells that may cooperate to drive disease progression. In the light of these assumptions, the selective sensitivity of motor neurons to toxicity from ubiquitously expressed SOD1 mutants can be explained by the accidental convergence of the peculiar properties of motor neurons and the combination of injuries sustained by those cells and their multiple cellular neighbors.

SUMMARY

ALS is a progressive and fatal disorder characterized by degeneration of motor neurons in the cerebral cortex, brain stem, and spinal cord. Mutations in the *SOD1* gene have been found in 12–23% of patients with a diagnosis of FALS. To date, more than 150 mutations located in all five exons of the *SOD1* gene have been described worldwide in ALS patients, with autosomal dominant transmission or, rarely, recessive transmission. A genotype–phenotype correlation has been defined for only a few mutations. In particular, the A4V mutation is mostly associated with a rapidly progressive form of LMN ALS with a survival time usually of 1–2 years. A uniform phenotype with spinal onset, bulbar involvement at the end of the disease, and a short survival time of about 1 year has been described for the G41S mutation. On the opposite end of the spectrum, an extreme phenotypic variability has been reported for patients with the I113T mutation with a range of disease duration of 2–20 years, spinal or bulbar onset, and variable penetrance even among members of the same family. Some *SOD1* mutations occur as recurrent or founder mutations, and a geographic distribution is beginning to emerge. The D90A mutation is the most common mutation globally and the most prevalent one in Europe, the A4V mutation is the most prevalent mutation in the United States, accounting for about 50% of all *SOD1* mutated patients, and the I113T mutation is by far the most common mutation in the United Kingdom.

Although the mechanism by which mutant SOD1 causes neural death remains elusive, several lines of evidence suggest that ALS is a protein-folding disease and that the toxic action in SOD1-associated ALS is caused by the misfolded pathogenic SOD1 monomers, soluble oligomers, or insoluble aggregates. Mutant SOD1 aggregation is indeed a common pathologic feature reported in human SOD1-associated ALS. SOD1 is a homodimeric enzyme in which each subunit forms an intramolecular disulfide bond and coordinates one copper ion and one zinc ion in its active site. The SOD1 mutations are classified into β-barrel mutations, which result in local perturbations that are able to affect the monomer stability, the dimer interface, or both at the same time, and metal binding mutations, which diminish the metal coordination and lead to altered SOD1–SOD1 interactions. In both cases, the mutations cause a destabilization of SOD1 structure and may promote oligomerization and aggregation. Experimental evidence has shown that the increased aggregation propensity is highly likely to be a universal feature of mutant SOD1, although it has not been identified as a specific biochemical or biophysical property able to adequately explain the variability in propensity of the different mutant proteins to aggregate. No obvious relationship between aggregation propensity and age of onset has been found; however, the aggregation propensity appears to be involved in the progression and inversely associated with the disease duration. Among the hypotheses advanced to explain the toxicity mechanism of misfolded SOD1, it has been proposed that SOD1 aggregates play a direct role in altering the cellular protein homeostasis, particularly affecting the chaperone activity and the proteasome system.

In addition, it has been demonstrated that mutant SOD1 may be secreted in the extracellular environment by motor neurons and astrocytes and in turn can activate microgliosis and cause neuronal cell death. This model for the pathogenic mechanism mediated by SOD1 mutants is consistent with the concept that ALS is a non-cell autonomous disease, and that motor neuron degeneration requires the expression of mutant SOD1 also in non-neuronal cell types.

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REFERENCES

1. Rowland LP, Shneider NA. Amyotrophic lateral sclerosis. *N Engl J Med*. 2001;344:1688–1700.
2. Rothstein JD. Current hypotheses for the underlying biology of amyotrophic lateral sclerosis. *Ann Neurol*. 2009;65:S3–S9.
3. Ilieva H, Polymenidou M, Cleveland DW. Non-cell autonomous toxicity in neurodegenerative disorders: ALS and beyond. *J Cell Biol*. 2009;187:761–772.
4. Wijesekera LC, Leigh PN. Amyotrophic lateral sclerosis. *Orphanet J Rare Dis*. 2009;4:3.
5. Phukan J, Pender NP, Hardiman O. Cognitive impairment in amyotrophic lateral sclerosis. *Lancet Neurol*. 2007;6:994–1003.
6. Brooks BR, Miller RG, Swash M, *et al*. El Escorial revisited: revised criteria for the diagnosis of amyotrophic lateral sclerosis. *Amyotroph Lateral Scler Other Motor Neuron Disord*. 2000;1:293–299.
7. Abhinav K, Stanton B, Johnston C, *et al*. Amyotrophic lateral sclerosis in South-East England: a population-based study. The South-East England register for amyotrophic lateral sclerosis (SEALS Registry). *Neuroepidemiology*. 2007;29:44–48.
8. Logroscino G, Traynor BJ, Hardiman O, *et al*. Descriptive epidemiology of amyotrophic lateral sclerosis: new evidence and unsolved issues. *J Neurol Neurosurg Psychiatry*. 2008;79:6–11.
9. Sutedja NA, Fischer K, Veldink JH, *et al*. What we truly know about occupation as a risk factor for ALS: a critical and systematic review. *Amyotroph Lateral Scler*. 2009;10:295–301.
10. Chiò A, Logroscino G, Hardiman O, *et al*. Prognostic factors in ALS: a critical review. *Amyotroph Lateral Scler*. 2009;10:310–323.
11. Andersen PM. Amyotrophic lateral sclerosis associated with mutations in the CuZn superoxide dismutase gene. *Curr Neurol Neurosci Rep*. 2006;6:37–46.
12. Ben Hamida M, Hentati F, Ben Hamida C. Hereditary motor system diseases (chronic juvenile amyotrophic lateral sclerosis). Conditions combining a bilateral pyramidal syndrome with limb and bulbar amyotrophy. *Brain*. 1990;113:347–363.
13. Bruijn LI, Miller TM, Cleveland DW. Unraveling the mechanisms involved in motor neuron degeneration in ALS. *Annu Rev Neurosci*. 2004;27:723–749.
14. Rosen DR, Siddique T, Patterson D, *et al*. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature*. 1993;362:59–62.
15. Hadano S, Hand CK, Osuga H, *et al*. A gene encoding a putative GTPase regulator is mutated in familial amyotrophic lateral sclerosis 2. *Nat Genet*. 2001;29:166–173.
16. Yang Y, Hentati A, Deng HX, *et al*. The gene encoding alsin, a protein with three guanine-nucleotide exchange factor domains, is mutated in a form of recessive amyotrophic lateral sclerosis. *Nat Genet*. 2001;29:160–165.

17. Chen YZ, Bennett CL, Huynh HM, *et al*. DNA/RNA helicase gene mutations in a form of juvenile amyotrophic lateral sclerosis (ALS4). *Am J Hum Genet*. 2004;74:1128–1135.
18. Kwiatkowski TJ Jr, Bosco DA, Leclerc AL, *et al*. Mutations in the FUS/TLS gene on chromosome 16 cause familial amyotrophic lateral sclerosis. *Science*. 2009;323:1205–1208.
19. Vance C, Rogelj B, Hortobágyi T, *et al*. Mutations in FUS, an RNA processing protein, cause familial amyotrophic lateral sclerosis type 6. *Science*. 2009;323:1208–1211.
20. Nishimura AL, Mitne-Neto M, Silva HC, *et al*. A mutation in the vesicletrafficking protein VAPB causes late-onset spinal muscular atrophy and amyotrophic lateral sclerosis. *Am J Hum Genet*. 2004;75:822–831.
21. Greenway MJ, Alexander MD, Ennis S, *et al*. A novel candidate region for ALS on chromosome 14q11.2. *Neurology*. 2004;63:1936–1938.
22. Greenway MJ, Andersen PM, Russ C, *et al*. ANG mutations segregate with familial and 'sporadic' amyotrophic lateral sclerosis. *Nat Genet*. 2006;38:411–413.
23. Yokoseki A, Shiga A, Tan CF, *et al*. TDP-43 mutation in familial amyotrophic lateral sclerosis. *Ann Neurol*. 2008;63:538–542.
24. Sreedharan J, Blair IP, Tripathi VB, *et al*. TDP-43 mutations in familial and sporadic amyotrophic lateral sclerosis. *Science*. 2008;319:1668–1672.
25. Kabashi E, Valdmanis PN, Dion P, *et al*. TARDBP mutations in individuals with sporadic and familial amyotrophic lateral sclerosis. *Nat Genet*. 2008;40:572–574.
26. Puls I, Jonnakuty C, LaMonte BH, *et al*. Mutant dynactin in motor neuron disease. *Nat Genet*. 2003;33:455–456.
27. Münch C, Sedlmeier R, Meyer T, *et al*. Point mutations of the p150 subunit of dynactin (DCTN1) gene in ALS. *Neurology*. 2004;63:724–726.
28. Pardo CA, Xu Z, Borchelt DR, *et al*. Superoxide dismutase is an abundant component in cell bodies, dendrites, and axons of motor neurons and in a subset of other neurons. *Proc Natl Acad Sci USA*. 1995;92:954–958.
29. Okado-Matsumoto A, Fridovich I. Subcellular distribution of superoxide dismutases (SOD) in rat liver: Cu, Zn-SOD in mitochondria. *J Biol Chem*. 2001;276:38388–38393.
30. Wroe R, Wai-Ling Butler A, Andersen PM, *et al*. ALSOD: the Amyotrophic Lateral Sclerosis Online Database. *Amyotroph Lateral Scler*. 2008;9:249–250.
31. Prudencio M, Hart PJ, Borchelt DR, *et al*. Variation in aggregation propensities among ALS-associated variants of SOD1: correlation to human disease. *Hum Mol Genet*. 2009;18:3217–3226.
32. Andersen PM, Borasio GD, Dengler R, *et al*. Good practice in the management of amyotrophic lateral sclerosis: clinical guidelines. An evidence-based review with good practice points. EALSC Working Group. *Amyotroph Lateral Scler*. 2007;8:195–213.
33. Eisen A, Mezei MM, Stewart HG, *et al*. SOD1 gene mutations in ALS patients from British Columbia, Canada: clinical features, neurophysiology and ethical issues in management. *Amyotroph Lateral Scler*. 2008;9:108–119.
34. Battistini S, Giannini F, Greco G, *et al*. SOD1 mutations in amyotrophic lateral sclerosis. Results from a multicenter Italian study. *J Neurol*. 2005;252:782–788.
35. Chiò A, Traynor BJ, Lombardo F, *et al*. Prevalence of SOD1 mutations in the Italian ALS population. *Neurology*. 2008;70:533–537.
36. van Es MA, Dahlberg C, Birve A, *et al*. Large scale SOD1 mutation screening provides evidence for genetic heterogeneity in amyotrophic lateral sclerosis. *J Neurol Neurosurg Psychiatry*. 2010;81:562–566.
37. Aguirre T, Matthijs G, Robberecht W, *et al*. Mutational analysis of the Cu/Zn superoxide dismutase gene in 23 familial and 69 sporadic cases of amyotrophic lateral sclerosis in Belgium. *Eur J Hum Genet*. 1999;7:599–602.
38. Boukaftane Y, Khoris J, Moulard B, *et al*. Identification of six novel SOD1 gene mutation in familial amyotrophic lateral sclerosis. *Can J Neurol Sci*. 1998;25:192–196.
39. Andersen PM, Nilsson P, Keranen ML, *et al*. Phenotypic heterogeneity in motor neuron disease patients with CuZn-superoxide dismutase mutations in Scandinavia. *Brain*. 1997;120:1723–1737.
40. Jafari-Schluep HF, Khoris J, Mayeux-Portas V, *et al*. Superoxide dismutase 1 gene abnormalities in familial amyotrophic lateral sclerosis: phenotype/genotype correlations. The French experience and review of the literature. *Rev Neurol (Paris)*. 2004;160:44–50. French.
41. Niemann S, Joos H, Meyer T, *et al*. Familial ALS in Germany: origin of the R115G SOD1 mutation by a founder effect. *J Neurol Neurosurg Psychiatry*. 2004;75:1186–1188.
42. Alexander MD, Traynor BJ, Coor B, *et al*. SOD1 Mutation analysis in the Irish ALS population—a preliminary report. *Amyotroph Lateral Scler Other Motor Neuron Disord*. 2000;1:99 (Abstract).
43. Gellera C, Castellotti B, Riggio MC, *et al*. Superoxide dismutase gene mutations in Italian patients with familial and sporadic amyotrophic lateral sclerosis: identification of three novel missense mutations. *Neuromuscul Disord*. 2001;11:404–410.
44. Corrado L, D'Alfonso S, Bergamaschi L, *et al*. SOD1 gene mutations in Italian patients with Sporadic Amyotrophic Lateral Sclerosis (ALS). *Neuromuscul Disord*. 2006;16:800–804.
45. Abe K, Aoki M, Ikeda M, *et al*. Clinical characteristics of familial amyotrophic lateral sclerosis with Cu/Zn superoxide dismutase gene mutations. *J Neurol Sci*. 1996;136:108–116.
46. Jones CT, Swingler RJ, Simpson SA, *et al*. Superoxide dismutase mutations in an unselected cohort of Scottish amyotrophic lateral sclerosis patients. *J Med Genet*. 1995;32:290–292.
47. Gamez J, Corbera-Bellalta M, Nogales G, *et al*. Mutational analysis of the Cu/Zn superoxide dismutase gene in a Catalan ALS population: should all sporadic ALS cases also be screened for SOD1? *J Neurol Sci*. 2006;247:21–28.
48. Jackson M, Al-Chalabi A, Enayat ZE, *et al*. Copper/Zinc superoxide dismutase 1 and sporadic amyotrophic lateral sclerosis: analysis of 155 cases and identification of a novel insertion mutation. *Ann Neurol*. 1997;42:803–807.
49. Shaw CE, Enayat ZE, Chioza BA, *et al*. Mutations in all five exons of SOD-1 may cause ALS. *Ann Neurol*. 1998;43:390–394.
50. Orrell RW, Habgood JJ, Malaspina A, *et al*. Clinical characteristics of SOD1 gene mutations in UK families with ALS. *J Neurol Sci*. 1999;169:56–60.
51. Cudkowicz ME, McKenna-Yasek D, Sapp PE, *et al*. Epidemiology of mutations in superoxide dismutase in amyotrophic lateral sclerosis. *Ann Neurol*. 1997;41:210–221.
52. Ferrera L, Caponnetto C, Marini V, *et al*. An Italian dominant FALS Leu144Phe SOD1 mutation: genotype-phenotype correlation. *Amyotroph Lateral Scler Other Motor Neuron Disord*. 2003;4:167–170.
53. Battistini S, Ricci C, Giannini F, *et al*. G41S SOD1 mutation: a common ancestor for six ALS Italian families with an aggressive phenotype. *Amyotroph Lateral Scler*. 2010;11:210–215.
54. Hand CK, Mayeux-Portas V, Khoris J, *et al*. Compound heterozygous D90A and D96N SOD1 mutations in a recessive amyotrophic lateral sclerosis family. *Ann Neurol*. 2001;49:267–271.
55. Parton MJ, Broom W, Andersen PM, *et al*. D90A-SOD1 mediated amyotrophic lateral sclerosis: a single founder for all cases with evidence for a Cis-acting disease modifier in the recessive haplotype. *Hum Mutat*. 2002;20:473–480.
56. Robberecht W, Aguirre T, Van den Bosch L, *et al*. D90A heterozygosity in the SOD1 gene is associated with familial and apparently sporadic amyotrophic lateral sclerosis. *Neurology*. 1996;47:1336–1339.
57. Skvortsova VI, Limborska SA, Slominsky PA, *et al*. Sporadic ALS associated with the D90A Cu, Zn superoxide dismutase mutation in Russia. *Eur J Neurol*. 2001;8:167–172.
58. Andersen PM, Sims KB, Xin WW, *et al*. Sixteen novel mutations in the Cu/Zn superoxide dismutase gene in amyotrophic lateral sclerosis: a decade of discoveries, defects and disputes. *Amyotroph Lateral Scler Other Motor Neuron Disord*. 2003;4:62–73.
59. Corcia P, Jafari-Schluep HF, Camu W. Reply to ''Disease penetrance in Amyotrophic Lateral Sclerosis associated with mutations in the SOD1 gene''. *Ann Neurol*. 2004;55:299.
60. Giannini F, Battistini S, Mancuso M, *et al*. D90A-SOD1 mutation in ALS: the first report of heterozygous Italian patients and unusual findings. *Amyotroph Lateral Scler*. 2010;11:216–219.
61. Origone P, Caponnetto C, Mascolo M, *et al*. Heterozygous D90A-SOD1 mutation in an Italian ALS patient with atypical presentation. *Amyotroph Lateral Scler*. 2009;10:492.

62. Luigetti M, Conte A, Madia F, *et al*. Heterozygous SOD1 D90A mutation presenting as slowly progressive predominant upper motor neuron amyotrophic lateral sclerosis. *Neurol Sci*. 2009;30:517–520.
63. Rainero I, Pinessi L, Tsuda T, *et al*. SOD1 missense mutation in an Italian family with ALS. *Neurology*. 1994;44:347–349.
64. Lopate G, Baloh RH, Al-Lozi MT, *et al*. Familial ALS with extreme phenotypic variability due to the I113T SOD1 mutation. *Amyotroph Lateral Scler*. 2010;11:232–236.
65. Hayward C, Swingler RJ, Simpson SA, *et al*. A specific superoxide dismutase mutation is on the same genetic background in sporadic and familial cases of amyotrophic lateral sclerosis. *Am J Hum Genet*. 1996;59:1165–1167.
66. Broom WJ, Johnson DV, Auwarter KE, *et al*. SOD1A4V-mediated ALS: absence of a closely linked modifier gene and origination in Asia. *Neurosci Lett*. 2008;430:241–245.
67. Rosen DR. A shared chromosome-21 haplotype among amyotrophic lateral sclerosis families with the A4V SOD1 mutation. *Clin Genet*. 2004;66:247–250.
68. Saeed M, Yang Y, Deng HX, *et al*. Age and founder effect of SOD1 A4V mutation causing ALS. *Neurology*. 2009;72:1634–1639.
69. Al-Chalabi A, Andersen PM, Chioza B, *et al*. Recessive amyotrophic lateral sclerosis families with the D90A SOD1 mutation share a common founder: evidence for a linked protective factor. *Hum Mol Genet*. 1998;7:2045–2050.
70. Broom WJ, Russ C, Sapp PC, *et al*. Variants in candidate ALS modifier genes linked to Cu/Zn superoxide dismutase do not explain divergent survival phenotypes. *Neurosci Lett*. 2006;392:52–57.
71. Broom WJ, Johnson DV, Garber M, *et al*. DNA sequence analysis of the conserved region around the SOD1 gene locus in recessively inherited ALS. *Neurosci Lett*. 2009;463:64–69.
72. Turner BJ, Talbot K. Transgenics, toxicity and therapeutics in rodent models of mutant SOD1-mediated familial ALS. *Prog Neurobiol*. 2008;85:94–134.
73. Gurney ME, Pu H, Chiu AY, *et al*. Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. *Science*. 1994;264:1772–1775.
74. Deng HX, Shi Y, Furukawa Y, *et al*. Conversion to the amyotrophic lateral sclerosis phenotype is associated with intermolecular linked insoluble aggregates of SOD1 in mitochondria. *Proc Natl Acad Sci USA*. 2006;103:7142–7147.
75. Wong PC, Pardo CA, Borchelt DR, *et al*. An adverse property of a familial ALS-linked SOD1 mutation causes motor neuron disease characterized by vacuolar degeneration of mitochondria. *Neuron*. 1995;14:1105–1116.
76. Bruijn LI, Becher MW, Lee MK, *et al*. ALS-linked SOD1 mutant G85R mediates damage to astrocytes and promotes rapidly progressive disease with SOD1-containing inclusions. *Neuron*. 1997;18:327–338.
77. Ripps ME, Huntley GW, Hof PR, *et al*. Transgenic mice expressing an altered murine superoxide dismutase gene provide an animal model of amyotrophic lateral sclerosis. *Proc Natl Acad Sci USA*. 1995;92:689–693.
78. Jonsson PA, Graffmo KS, Brännström T, *et al*. Motor neuron disease in mice expressing the wild type-like D90A mutant superoxide dismutase-1. *J Neuropathol Exp Neurol*. 2006;65:1126–1136.
79. Tobisawa S, Hozumi Y, Arawaka S, *et al*. Mutant SOD1 linked to familial amyotrophic lateral sclerosis, but not wild-type SOD1, induces ER stress in COS7 cells and transgenic mice. *Biochem Biophys Res Commun*. 2003;303:496–503.
80. Shibata N. Transgenic mouse model for familial amyotrophic lateral sclerosis with superoxide dismutase-1 mutation. *Neuropathology*. 2001;2:82–92.
81. Wang J, Xu G, Gonzales V, *et al*. Fibrillar inclusions and motor neuron degeneration in transgenic mice expressing superoxide dismutase 1 with a disrupted copper-binding site. *Neurobiol Dis*. 2002;10:128–138.
82. Wang J, Slunt H, Gonzales V, *et al*. Copper-binding-site-null SOD1 causes ALS in transgenic mice: aggregates of non-native SOD1 delineate a common feature. *Hum Mol Genet*. 2003;12:2753–2764.
83. Wang J, Xu G, Li H, *et al*. Somatodendritic accumulation of misfolded SOD1-L126Z in motor neurons mediates degeneration: alphaB-crystallin modulates aggregation. *Hum Mol Genet*. 2005;14:2335–2347.
84. Watanabe Y, Yasui K, Nakano T, *et al*. Mouse motor neuron disease caused by truncated SOD1 with or without C-terminal modification. *Brain Res Mol Brain Res*. 2005;135:12–20.
85. Jonsson PA, Ernhill K, Andersen PM, *et al*. Minute quantities of misfolded mutant superoxide dismutase-1 cause amyotrophic lateral sclerosis. *Brain*. 2004;127:73–88.
86. Nagai M, Aoki M, Miyoshi I, *et al*. Rats expressing human cytosolic copper-zinc superoxide dismutase transgenes with amyotrophic lateral sclerosis: associated mutations develop motor neuron disease. *J Neurosci*. 2001;21:9246–9254.
87. Howland DS, Liu J, She Y, *et al*. Focal loss of the glutamate transporter EAAT2 in a transgenic rat model of SOD1 mutant-mediated amyotrophic lateral sclerosis (ALS). *Proc Natl Acad Sci USA*. 2002;99:1604–1609.
88. Martin LJ. Transgenic mice with human mutant genes causing Parkinson's disease and amyotrophic lateral sclerosis provide common insight into mechanisms of motor neuron selective vulnerability to degeneration. *Rev Neurosci*. 2007;18:115–136.
89. Cheroni C, Peviani M, Cascio P, *et al*. Accumulation of human SOD1 and ubiquitinated deposits in the spinal cord of SOD1G93A mice during motor neuron disease progression correlates with a decrease of proteasome. *Neurobiol Dis*. 2005;18:509–522.
90. Lee J, Kannagi M, Ferrante RJ, *et al*. Activation of Ets-2 by oxidative stress induces Bcl-xL expression and accounts for glial survival in amyotrophic lateral sclerosis. *FASEB J*. 2009;23:1739–1749.
91. Bendotti C, Carrì MT. Lessons from models of SOD1-linked familial ALS. *Trends Mol Med*. 2004;10:393–400.
92. Awano T, Johnson GS, Wade CM, *et al*. Genome-wide association analysis reveals a SOD1 mutation in canine degenerative myelopathy that resembles amyotrophic lateral sclerosis. *Proc Natl Acad Sci USA*. 2009;106:2794–2799.
93. Reaume AG, Elliott JL, Hoffman EK, *et al*. Motor neurons in Cu/Zn superoxide dismutase-deficient mice develop normally but exhibit enhanced cell death after axonal injury. *Nat Genet*. 1996;13:43–47.
94. Nordlund A, Oliveberg M. SOD1-associated ALS: a promising system for elucidating the origin of protein-misfolding disease. *HFSP J*. 2008;2:354–364.
95. Stefani M, Dobson CM. Protein aggregation and aggregate toxicity: new insights into protein folding, misfolding diseases and biological evolution. *J Mol Med*. 2003;81:678–699.
96. Strange RW, Antonyuk SV, Hough MA, *et al*. Variable metallation of human superoxide dismutase: atomic resolution crystal structures of Cu-Zn, Zn-Zn and as-isolated wild-type enzymes. *J Mol Biol*. 2006;356:1152–1162.
97. Hart PJ. Pathogenic superoxide dismutase structure, folding, aggregation and turnover. *Curr Opin Chem Biol*. 2006;10:131–138.
98. Arnesano F, Banci L, Bertini I, *et al*. The unusually stable quaternary structure of human Cu,Zn-superoxide dismutase 1 is controlled by both metal occupancy and disulfide status. *J Biol Chem*. 2004;279:47998–48003.
99. Lindberg MJ, Normark J, Holmgren A, *et al*. Folding of human superoxide dismutase: disulfide reduction prevents dimerization and produces marginally stable monomers. *Proc Natl Acad Sci USA*. 2004;101:15893–15898.
100. Doucette PA, Whitson LJ, Cao X, *et al*. Dissociation of human copper-zinc superoxide dismutase dimers using chaotrope and reductant. Insights into the molecular basis for dimer stability. *J Biol Chem*. 2004;279:54558–54566.
101. Seetharaman SV, Prudencio M, Karch C, *et al*. Immature copper-zinc superoxide dismutase and familial amyotrophic lateral sclerosis. *Exp Biol Med (Maywood)*. 2009;234:1140–1154.
102. Lindberg MJ, Byström R, Boknäs N, *et al*. Systematically perturbed folding patterns of amyotrophic lateral sclerosis (ALS)-associated SOD1 mutants. *Proc Natl Acad Sci USA*. 2005;102:9754–9759.
103. Shaw BF, Valentine JS. How do ALS-associated mutations in superoxide dismutase 1 promote aggregation of the protein? *Trends Biochem Sci*. 2007;32:78–85.
104. Thomas PD, Campbell MJ, Kejariwal A, *et al*. PANTHER: a library of protein families and subfamilies indexed by function. *Genome Res*. 2003;13:2129–2141.

105. Arnold K, Bordoli L, Kopp J, *et al*. The SWISS-MODEL Workspace: a web-based environment for protein structure homology modelling. *Bioinformatics*. 2006;22:195–201.
106. Ricci C, Benigni M, Battistini S, et al. A novel exon 1 mutation (G10R) in the SOD1 gene in a patient with familial ALS. *Amyotroph Lateral Scler*. 2010 Mar 24 [Epub ahead of print].
107. Culotta VC, Klomp LW, Strain J, *et al*. The copper chaperone for superoxide dismutase. *J Biol Chem*. 1997;272:23469–23472.
108. Brown NM, Torres AS, Doan PE, *et al*. Oxygen and the copper chaperone CCS regulate posttranslational activation of Cu, Zn superoxide dismutase. *Proc Natl Acad Sci USA*. 2004;101:5518–5523.
109. Shibata N, Asayama K, Hirano A, *et al*. Immunohistochemical study on superoxide dismutases in spinal cords from autopsied patients with amyotrophic lateral sclerosis. *Dev Neurosci*. 1996;18:492–498.
110. Watanabe M, Dykes-Hoberg M, Culotta VC, *et al*. Histological evidence of protein aggregation in mutant SOD1 transgenic mice and in amyotrophic lateral sclerosis neural tissues. *Neurobiol Dis*. 2001;8:933–941.
111. Wood JD, Beaujeux TP, Shaw PJ. Protein aggregation in motor neurone disorders. *Neuropathol Appl Neurobiol*. 2003;29:529–545.
112. Matsumoto G, Stojanovic A, Holmberg CI, *et al*. Structural properties and neuronal toxicity of amyotrophic lateral sclerosis-associated Cu/Zn superoxide dismutase 1 aggregates. *J Cell Biol*. 2005;171:75–85.
113. Karch CM, Prudencio M, Winkler DD, *et al*. Role of mutant SOD1 disulfide oxidation and aggregation in the pathogenesis of familial ALS. *Proc Natl Acad Sci USA*. 2009;106:7774–7779.
114. Wang Q, Johnson JL, Agar NY, *et al*. Protein aggregation and protein instability govern familial amyotrophic lateral sclerosis patient survival. *PLoS Biol*. 2008;6:e170.
115. Gusella JF, Macdonald ME. Huntington's disease: seeing the pathogenic process through a genetic lens. *Trends Biochem Sci*. 2006;31:533–540.
116. Atsuta N, Watanabe H, Ito M, *et al*. Natural history of spinal and bulbar muscular atrophy (SBMA): a study of 223 Japanese patients. *Brain*. 2006;129:1446–1455.
117. Höhfeld J, Cyr DM, Patterson C. From the cradle to the grave: molecular chaperones that may choose between folding and degradation. *EMBO Rep*. 2001;2:885–890.
118. Kabashi E, Durham HD. Failure of protein quality control in amyotrophic lateral sclerosis. *Biochim Biophys Acta*. 2006;1762:1038–1050.
119. Kato S, Hayashi H, Nakashima K, *et al*. Pathological characterization of astrocytic hyaline inclusions in familial amyotrophic lateral sclerosis. *Am J Pathol*. 1997;151:611–620.
120. Vlug AS, Teuling E, Haasdijk ED, *et al*. ATF3 expression precedes death of spinal motoneurons in amyotrophic lateral sclerosis-SOD1 transgenic mice and correlates with c-Jun phosphorylation, CHOP expression, somato-dendritic ubiquitination and Golgi fragmentation. *Eur J Neurosci*. 2005;22:1881–1894.
121. Clement AM, Nguyen MD, Roberts EA, et al. Wild-type nonneuronal cells extend survival of SOD1 mutant motor neurons in ALS mice. *Science*. 2003;302:113–117. Erratum in: *Science*. 2003;302:568.
122. Boillée S, Vande Velde C, Cleveland DW. ALS: a disease of motor neurons and their nonneuronal neighbors. *Neuron*. 2006;52:39–59.
123. Urushitani M, Sik A, Sakurai T, *et al*. Chromogranin-mediated secretion of mutant superoxide dismutase proteins linked to amyotrophic lateral sclerosis. *Nat Neurosci*. 2006;9:108–118.

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Short communication

Severe familial ALS with a novel exon 4 mutation (L106F) in the *SOD1* gene

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ABSTRACT

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease associated with a positive familial history in 5–10% of ALS cases. Mutations in the superoxide dismutase-1 (*SOD1*) gene have been found in 12%–23% of patients diagnosed with familial ALS. Here we report a novel mutation in exon 4 of *SOD1* gene in a 55-year-old ALS patient belonging to a large Italian family with ALS first clinically described in 1968. In the family the clinical presentation was characterized by relatively early age of onset, spinal onset with proximal distribution weakness, bulbar involvement and a rapid disease course. Molecular analysis showed a heterozygous mutation at codon 106 resulting in a substitution of phenylalanine for leucine in the SOD1 protein (L106F). In analogy with the previously reported L106V mutation, we propose that the L106F causes a relevant destabilization of the protein chain around the mutation site, able to affect the SOD1 monomer and dimer structures suggesting a pathogenic role for this novel mutation.

1. Introduction

Amyotrophic Lateral Sclerosis (ALS) is an adult-onset, progressive and fatal neurodegenerative disease with unknown pathogenesis, characterized by degeneration of motor neurons although increasing evidence indicates that non-neuronal neighboring cell types contribute to pathogenesis and disease progression [1,2]. The annual incidence of the disease is about 1–3/100,000, increasing with age. Approximately 90% of cases present as sporadic form of ALS (SALS), whereas 5%–10% are described as familial form of ALS (FALS). Mutations in the copper/zinc superoxide dismutase-1 (*SOD1*) gene are responsible for 12%–23% of all FALS cases [3]. More than 150 *SOD1* mutations have been identified (ALS Online Genetic Database, ALSOD: http://www.alsod.iop.kcl.ac.uk/) [4], with autosomal dominant transmission or, rarely, recessive transmission. We here report a novel missense mutation (L106F) in exon 4 of *SOD1* gene in an Italian ALS patient belonging to a large family with ALS. We describe the clinical features of the nine affected family members and evaluate the potential effect of this novel mutation on SOD1 protein structure and stability.

2. Materials and methods

2.1. Case report

A 55-year-old man (individual IV-9, Fig. 1,) came to our observation because of a three-month history of left leg weakness and gait impairment. Neurological examination showed left leg atrophy and weakness affecting proximal muscles (Medical Research Council, MRC 3/5) more than distal muscles (MRC 4/5), diffuse fasciculations in the lower limbs with hypotonia and normal deep tendon reflexes. Within few months the patient's conditions worsened with progressive weakness accompanied by wasting of the right upper and lower extremity. He could walk only with support and couldn't stand up. Moreover the patient developed right facial nerve palsy, dysarthria, nasal voice and severe dysphagia with loss of 5 kg in weight. Routine blood examination was unremarkable apart from mildly elevated serum creatine kinase level (600 UI/L; n.v.: 25–195 UI/L). Magnetic resonance imaging (MRI) of the brain was normal. Spinal cord MRI showed anterior displacement of the spinal cord at the T4 level with an enlargement of the dorsal subarachnoid space suggestive for idiopathic spinal cord herniation or arachnoid cyst. Motor evoked potentials (MEP) by transcranial magnetic stimulation, recorded from the abductor pollicis brevis (APB) and the tibialis anterior (TA) muscles, revealed normal central conduction time (APB: left, 5.4 ms; right, 5.6 ms; n.v.<7.4 ms. TA: left, 13.5 ms; right, 10.7 ms; n.v.<15.7). Somatosensory evoked potentials (SEP)

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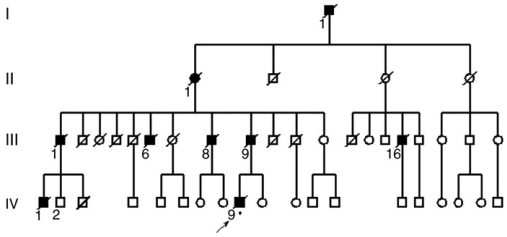

Fig. 1. Pedigree of the family with the L106F mutation. circle = female; square = male; filled symbol = ALS-affected individual; open symbol = unaffected individual; arrow = proband; diagonal line = individual deceased; and asterisk = mutation.

from tibial nerve stimulation showed slowing of conduction in the dorsal columns with prolonged bilateral cortical P37 latency (left and right, 48.0 ms; n.v.<41 ms). These findings caused initial diagnostic uncertainty and a myelopathy was hypothesized. Electromyography (EMG) showed neurogenic pattern with denervation activity at rest in the cervical, thoracic, and lumbo-sacral regions. ALS Functional Rating Scale (ALS-FRS), at the first observation, was 35 and forced vital capacity as percent predicted (FVC%) was 103%. At the last follow-up, three months later, ALS-FRS was 27 and FVC% decreased of 15% in upright position and of 50% sitting and non-invasive nocturnal ventilatory support was initiated. His condition rapidly deteriorated. The patient declined invasive ventilatory treatment and expired of respiratory insufficiency 9 months after onset of the first symptom.

Our patient belonged to a large kindred with a multigenerational history of FALS first clinically described by Avanzini et al. in 1968 [5]. At the time of the original description, he was still unaffected. The pedigree is shown in Fig. 1. In the study by Avanzini et al. a total number of 8 affected family members distributed across four generations were identified: three affected family members (individuals III-1, III-8, III-9) were directly examined and others five affected family members (individuals I-1, II-1, III-6, III-16 and IV-1) were identified on the basis of anamnestic data and medical records. There were no skipped generations and there was evidence of an autosomal dominant inheritance. The main clinical features of these nine affected family members (including our case) are summarized in Table 1. The clinical picture of our patient and of the other affected family members previously reported [5] was quite uniform, characterized by spinal onset with proximal distribution weakness, upper (UMN) and lower (LMN) motor neuron involvement and appearance of bulbar signs at the end of the disease. All patients displayed a rapidly progressive disease course, with a mean age at onset of 46.9 ± 4.8 years (range 38–55 years) and an illness duration of 1.9 ± 1.1 years (range 0.8–4.0 years).

Table 1
Summary of the clinical features of the affected family members.

Patient	Sex	Age at onset (years)	Site of onset	Clinical presentation	Bulbar signs	Age at death (years)	Disease Duration (years)
I-1	M	43	NA	NA	NA	45	2
II-1	F	45	LL	NA	+	47	2
III-1	M	46	Left LL, proximal	UMN + LMN	+	47	0.9
III-6	M	50	LL + UL	NA	+	52	2
III-8	M	47	LL, proximal	UMN + LMN	+	51	4
III-9	M	48	LL, proximal	UMN + LMN	+	50	1.5
III-16	M	50	Left LL	UMN + LMN	NA	NA	NA
IV-1	M	38	Left LL	UMN + LMN	NA	NA	NA
IV-9	M	55	Left LL, proximal	UMN + LMN	+	56	0.8

Abbreviations: LMN, lower motor neuron; UMN, upper motor neuron; LL, lower limb; UL, upper limb; and NA, data not available.

2.2. Molecular analysis

After obtaining written informed consent, genomic DNA from the proband was extracted from peripheral blood using standard procedures [6]. Exons 1 to 5 and 3′ untranslated regions of *SOD1* gene were amplified from genomic DNA by polymerase chain reaction (PCR). All the amplicons were screened for sequence variations by direct sequencing using the Big-Dye Terminator v3.1 sequencing kit (Applied Biosystems, Milan, Italy) and ABI 310 Genetic Analyzer (Applied Biosystems, Milan, Italy).

2.3. Bioinformatics analysis and Modelling of the SOD1 L106F mutation

The effect of the newly detected *SOD1* missense mutation on protein structure or function was analyzed with the prediction programs PredictProtein (http://www.predictprotein.org) [7] and PolyPhen (http://www.genetics.bwh.harvard.edu/pph/) [8]. The likelihood of the mutation to cause a functional impact on the protein, based on alignment of evolutionarily related proteins, was calculated using the pathogenicity predictor Panther software, directly available from the ALSOD database (http://www.pantherdb.org/tools/csnpScoreForm.jsp) [9].

Modelling of the L106F mutation was performed using the Swiss model server (http://www.swissmodel.expasy.org/) [10,11] and the molecular visualization system Pymol (http://www.pymol.sourceforge.net/) from the crystal structure of the normal SOD1 protein (PDB2c9vA). The atomic empirical mean force potential atomic non-local environment assessment (ANOLEA) [12] was used to assess the packing quality of the models. The program performs energy calculations on a protein chain evaluating the "non-local environment" (NLE) of each heavy atom in the molecule. In addition, the impact of the mutation on the protein structure was evaluated using the "*SOD1 Database-Motor Neuron Disease Mutations*" link (http://www.bioinf.org.uk/mndb/), accessible from the ALSOD database.

3. Results

3.1. Molecular analysis

DNA analysis of the proband showed a heterozygous mutation c.319C>T in the *SOD1* gene (Fig. 2). The mutation of *CTC* to *TTC* at codon 106 in exon 4 determined a substitution of leucine to phenylalanine in the SOD1 protein (L106F). No other family members were available for *SOD1* screening and therefore it was not possible to confirm the co-segregation of this mutation with the disease. The mutation was absent in 400 chromosomes from healthy controls.

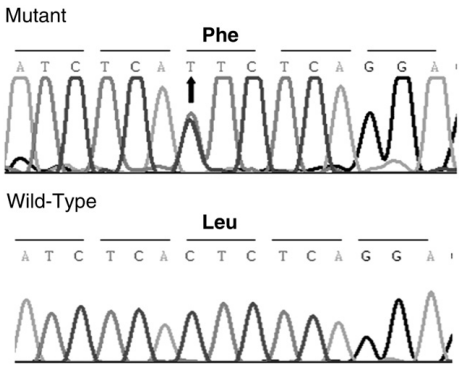

Fig. 2. Automated sequence analysis of the *SOD1* gene. Automated sequence analysis of exon 4 from the *SOD1* gene showing the heterozygous mutation c.319C>T (arrow).

Table 2
Protein alignment of the residues conserved across different species.

Species	106
Human	ADVSIEDSVISL**L**SGDHCIIGR
Rat	ANVSIEDRVISL**L**SGEHSIIGR
Mouse	ANVSIEDRVISL**L**SGEHSIIGR
Chiken	AEVEIEDSVISL**L**TGPHCIIGR
Rana Catesbeiana	ADINIKDKLISL**L**SKGEHSIIGR
Drosophila	LNVTITDSVLSL**L**TGKHIIIGR
Cristaria Plicata	CNVNITDSVISL**L**TGERSIIGR
S. Pombe	ADVSIEDSVISL**L**SGDHCIIGR

Multi-species comparison of the SOD1 protein in the region surrounding leucine in position 106 (grey box).

Multi-species comparisons showed that the leucine at codon 106 is a highly conserved residue among various species (Table 2). The pathogenicity predictor Panther software evidenced a score of −3.478, suggesting a highly harmful mutation.

3.2. Modelling of the SOD1 L106F mutation

The PredictProtein server showed that L106F mutation could have a non-neutral effect with an accuracy of 93%. This prediction was confirmed using PolyPhen. The calculation showed that PSIC score differences (Position-Specific Independent Counts) was in the interval 1.5–2.0 and this value predicts a possibly damaging effect resulting in a cavity creation.

The model of the mutant SOD1 showed a destabilization of the secondary structure around the phenylalanine in position 106. The substitution of leucine with the aromatic amino acid phenylalanine would cause an alteration of the protein secondary structure, due to the increased steric hindrance of the benzene ring in a region where it interacts with another phenylalanine in position 20 located in the β barrel (Fig. 3). Moreover, the energy calculation of the protein chain showed a relevant increase in energy, corresponding to an unfavourable energy environment, around the phenylalanine in the mutant SOD1 compared with the wild-type protein (Fig. 3).

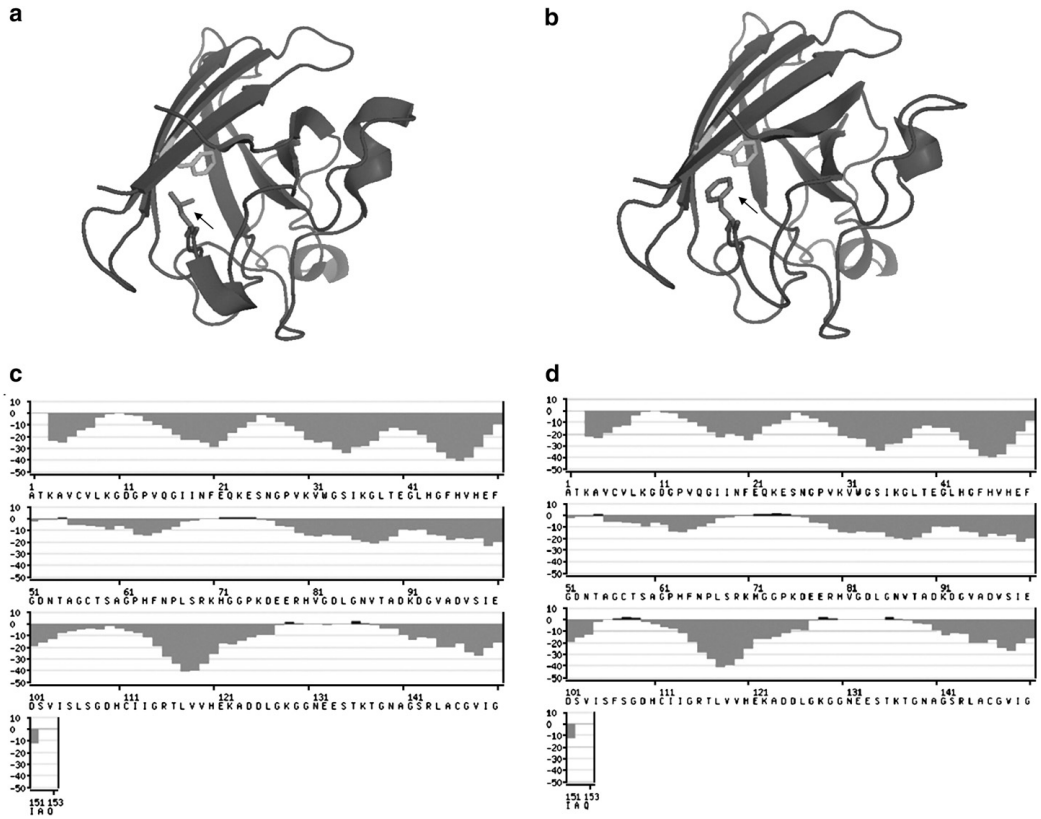

Fig. 3. Three-dimensional structure of both wild-type human SOD1 protein and a model of the SOD1 L106F variant, and their specific atomic non-local environment assessment (ANOLEA) plots. a. Three-dimensional x-ray diffraction structure of wild-type human SOD1 protein (PDB2c9vA). b. Model of the superoxide dismutase L106F mutation. The leucine and phenylalanine in position 106 and the phenylalanine in position 20 are represented with sticks and are indicated by an arrow. c. ANOLEA plot with the atomic empirical mean force potential of the normal SOD1 protein. d. ANOLEA plot of the SOD1 L106F mutation. The y-axis of the plots represents the energy for each amino acid in the protein chain. Negative energy values (grey) represent a favourable energy environment, whereas positive values (black) represent an unfavourable energy environment for a given amino acid.

4. Discussion

Here we report a novel exon 4 missense mutation (L106F) in the *SOD1* gene in an ALS patient belonging to a large Italian family with ALS first described in 1968 [5]. In this family there was evidence of an autosomal dominant inheritance. The clinical presentation of the nine affected family members was characterized by relatively early age of onset, spinal onset with proximal distribution weakness, bulbar involvement and a rapid disease course of about two years.

In the same codon, two different missense mutations have been previously described. A mutation resulting in the substitution of leucine to valine (L106V) has been identified in a Bulgarian ALS family [6,13] and in a Japanese family [14], with a quite similar disease course, characterized by early age of onset and rapid disease progression [15]. Another mutation, the L106P, has been found in an Italian patient who, analogously with our cases, presented with spinal onset with weakness mainly in proximal areas, a rather uncommon feature in ALS. However, this patient differed from our cases since, 30 months after disease onset, the pattern of weakness remained restricted to the upper limbs without pyramidal tract dysfunction, and it was consistent with brachial amyotrophic diplegia (BAD), a relatively slowly progressive variant of motor neuron disease [16,17].

The L106 amino acid residue is highly conserved in different species (Table 2) suggesting an important role for the proper structure and function of the SOD1 protein. The leucine 106 is localized in a completely buried position in the connecting loop between the β-6 strand and the α-helix 2. In the case of the L106V mutation, it has been previously observed that the introduction of an oversized chain alters the folded structure through steric clashes and destabilizes both the monomer and dimer interface [18]. Regarding the L106F mutation reported in our study, the presence of phenylalanine could cause an even stronger alteration, due to the increased steric hindrance of the benzene ring. In support of this explanation, a similar obstructive effect has been reported for the substitution of phenylalanine for isoleucine at position 104 (I104F) [18]. In addition, the modelling of L106F mutation has shown a relevant destabilization of the protein chain around the mutation site, able to affect the SOD1 monomer and dimer structures (Fig. 3), suggesting a pathogenic role for this novel mutation.

In conclusion, molecular genetic analysis showed that the Italian ALS family clinically described in 1968 [5] harboured a novel *SOD1* gene mutation. Further studies of this and other families with the L106F mutation will help to confirm its pathogenicity and the correlation with a severe ALS phenotype.

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References

[1] Rothstein JD. Current hypotheses for the underlying biology of amyotrophic lateral sclerosis. Ann Neurol 2009;65:S3–9.
[2] Ilieva H, Polymenidou M, Cleveland DW. Non-cell autonomous toxicity in neurodegenerative disorders: ALS and beyond. J Cell Biol 2009;187:761–72.
[3] Andersen PM. Amyotrophic lateral sclerosis associated with mutations in the CuZn Superoxide dismutase gene. Curr Neurol Neurosci Rep 2006;6:37–46.
[4] Wroe R, Wai-Ling Butler A, Andersen PM Powell JF, Al-Chalabi A. ALSOD: the amyotrophic lateral sclerosis. Online database. Amyotroph Lateral Scler 2008;9: 249–50.
[5] Avanzini G, Lechi A, Mancia D. Familial amyotrophic lateral sclerosis. Apropos of a clinical case. Sist Nerv 1968;20:311–9 Italian.
[6] Rosen DR, Siddique T, Patterson D, Figlewicz DA, Sapp P, Hentati A, et al. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature 1993;362:59–62.
[7] Rost B, Yachdav G, Liu J. The predictProtein server. Nucleic Acids Res 2004;32: W321–6.
[8] Ramensky V, Bork P, Sunyaev S. Human non-synonymous SNPs: server and survey. Nucleic Acids Res 2002;30:3894–900.
[9] Thomas PD, Campbell MJ, Kejariwal A, Mi H, Karlak B, Daverman R, et al. PANTHER: a library of protein families and subfamilies indexed by function. Genome Res 2003;13:2129–214.
[10] Arnold K, Bordoli L, Kopp J, Schwede T. The SWISS-MODEL Workspace: a web-based environment for protein structure homology modelling. Bioinformatics 2006;22:195–201.
[11] Schwede T, Kopp J, Guex N, Peitsch MC. SWISS-MODEL: an automated protein homology-modeling server. Nucleic Acids Res 2003;31:3381–5.
[12] Melo F, Feytmans E. Assessing protein structures with a non-local atomic interaction energy. J Mol Biol 1998;277:1141–52.
[13] Deng HX, Hentati A, Tainer JA, Iqbal Z, Cayabyab A, Hung WY, et al. Amyotrophic lateral sclerosis and structural defects in Cu, Zn superoxide dismutase. Science 1993;261:1047–51.
[14] Kawamata J, Hasegawa H, Shimohama S, Kimura J, Tanaka S, Ueda K. Leu106–>Val (CTC–>GTC) mutation of superoxide dismutase-1 gene in patient with familial amyotrophic lateral sclerosis in Japan. Lancet 1994;343:1501.
[15] Cudkowicz ME, McKenna-Yasek D, Sapp PE, Chin W, Geller B, Hayden DL, et al. Epidemiology of mutations in superoxide dismutase in amyotrophic lateral sclerosis. Ann Neurol 1997;41:210–21.
[16] Valentino P, Conforti FL, Pirritano D, Nisticò R, Mazzei R, Patitucci A, et al. Brachial amyotrophic diplegia associated with a novel SOD1 mutation (L106P). Neurology 2005;64:1477–8.
[17] Wijesekera LC, Mathers S, Talman P, Galtrey C, Parkinson MH, Ganesalingam J, et al. Natural history and clinical features of the flail arm and flail leg ALS variants. Neurology 2009;72:1087–94.
[18] Lindberg MJ, Byström R, Boknäs N, Andersen PM, Oliveberg M. Systematically perturbed folding patterns of amyotrophic lateral sclerosis (ALS)-associated SOD1 mutants. Lindberg. Proc Natl Acad Sci U S A 2005;102:9754–9.

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Lack of association of PON polymorphisms with sporadic ALS in an Italian population

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Abstract

Paraoxonase (*PON*) gene polymorphisms have been associated with susceptibility to sporadic amyotrophic lateral sclerosis (ALS). We have investigated the role of the previously associated single nucleotide polymorphisms rs854560, rs662, and rs6954345 in 350 ALS patients and 376 matched controls from Italy. No significant association was observed at genotype and haplotype level. Our data suggest that *PON* polymorphisms are not involved in ALS pathogenesis in an Italian population.

Keywords: Amyotrophic lateral sclerosis; Paraoxonase (*PON*) genes; Single nucleotide polymorphisms

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive and fatal disease characterized by the degeneration of motor neurons of the cerebral cortex, brain stem, and spinal cord. Sporadic ALS (SALS) accounts for about 90% of all ALS cases and is thought to result from the interaction of several susceptibility genes with environmental factors. Among the genetic factors, special attention has been reserved for the paraoxonase (*PON*) genes (*PON1*, *PON2*, *PON3*), which express detoxifying enzymes involved in the metabolism of a large number of substrates. Some single nucleotide polymorphisms (SNPs) have been identified in the *PON* genes. Several studies in different populations have investigated the association between *PON* genetic variants and the risk for sporadic ALS, and reported conflicting results (Cronin et al., 2007; Landers et al., 2008; Morahan et al., 2007; Saeed et al., 2006; Slowik et al., 2006; Valdmanis et al., 2008; Wills et al., 2009). In the light of the discrepancy in results, replication in independent populations can be of substantial importance to better understand the role of paraoxonase genes in sporadic ALS.

2. Methods

The study included 350 sporadic ALS patients and 376 control subjects from Italy. ALS diagnosis was made according to El Escorial Revisited criteria (Brooks et al.,

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Table 1
Genotype and allelic distributions of *PON* gene polymorphisms in sporadic ALS cases and controls

SNP	Cases (%)			Controls (%)			*p* value	OR	95% CI
PON1-L55M	LL	LM	MM	LL	LM	MM			
Genotype	41.8	44.7	13.5	36.9	46.3	16.8	0.292		
Allele	L 64.1		M 35.9	L 60.0		M 40.0	0.225	0.840	0.678–1.041
PON1-Q192R	QQ	QR	RR	QQ	QR	RR			
Genotype	50.1	40.0	9.9	53.6	36.4	10.0	0.596		
Allele	Q 70.1		R 29.9	Q 71.8		R 28.2	0.958	1.085	0.864–1.364
PON2-S311C	SS	SC	CC	SS	SC	CC			
Genotype	60.0	34.6	5.4	61.2	35.6	3.2	0.337		
Allele	S 77.3		C 22.7	S 79.0		C 21.0	0.098	1.106	0.862–1.420

Key: ALS, amyotrophic lateral sclerosis; CI, confidence interval; OR, odds ratio; SNP, single nucleotide polymorphism.

2000). Control group consisted of age- and sex-matched individuals from the same ethnic background with no history of neurological diseases. Characteristics of ALS patients and controls are summarized in Table 1S in the Supplementary material. Genotypes for the SNPs rs854560 (L55M) and rs662 (Q192R) in the *PON1* gene and rs6954345 (S311C) in the *PON2* gene were determined using restriction enzyme digestion and/or direct sequencing. Frequencies were compared using χ^2 statistics. Survival analysis was performed using the Kaplan-Meier method. For details see Supplementary material.

3. Results

Based on frequency and odds ratio data previously reported for the examined SNPs (Cronin et al., 2007; Slowik et al., 2006; Wills et al., 2009), our sample had >85% power to detect the risk allele at a significance level of $\alpha = 0.05$. All the studied SNPs were in Hardy-Weinberg equilibrium in both cases and controls ($p > 0.05$). No significant difference in rs854560 (L55M), rs662 (Q192R), and rs6954345 (S311C) distributions was observed in patients compared with controls, assuming additive, dominant, and recessive models (see Table 1 and Table 2S). Haplotype analysis did not reveal a significant association for any haplotypes (Table 3S). None of the genotypes/haplotypes were associated with ALS clinical variables (i.e., gender, location of disease onset, age at the onset, and disease duration; see Table 4S).

4. Discussion

The present study investigated the association between *PON* gene polymorphisms and susceptibility to ALS in an Italian population. To our knowledge, no studies on *PON* genes and ALS had been previously performed in patients from Italy. No significant association was observed between the examined SNPs and the risk of ALS, either at genotype or haplotype level. Previous studies involving *PON* genes have reported positive associations in several ALS populations. However, each study found different SNPs and/or haplotypes to be associated with ALS susceptibility. This may reflect heterogeneity among different populations at the genetic level, but it should not be overlooked that conflicting results can originate from the large range of *PON* SNPs chosen for the analysis in each report. Here, we have examined some of the most frequently assayed *PON* polymorphisms (Wills et al., 2009), analyzed in the first association study (Slowik et al., 2006), which are able to influence/modulate paraoxonase activity *in vivo* (Li et al., 2003). Our findings in the Italian population agree with the results of a recent meta-analysis of *PON* gene polymorphisms in sporadic ALS that failed to confirm any positive association in a comprehensive study on more than 8000 patients and controls, including the SNPs assayed in the present report (Wills et al., 2009). Lack of association of SNPs with ALS in the Italian population may indicate that *PON* genes play a minor role, and other gene-environment interactions are involved in ALS pathogenesis.

Disclosure statement

Competing interests: none.

References

Brooks, B.R., Miller, R.G., Swash, M., Munsat, T.L., World Federation of Neurology Research Group on Motor Neuron Diseases, 2000. El Escorial revisited: revised criteria for the diagnosis of amyotrophic lateral sclerosis. Amyotroph. Lateral Scler. Other Motor Neuron. Disord. 1, 293–9.

Cronin, S., Greenway, M.J., Prehn, J.H., Hardiman, O., 2007. Paraoxonase promoter and intronic variants modify risk of sporadic amyotrophic lateral sclerosis. J. Neurol. Neurosurg. Psychiatry 78, 984–986 [DOI: 10.1136/jnnp.2006.112581] [PubMed: 17702780].

Landers, J.E., Shi, L., Cho, T.J., Glass, J.D., Shaw, C.E., Leigh, P.N., Diekstra, F., Polak, M., Rodriguez-Leyva, I., Niemann, S., Traynor, B.J., McKenna-Yasek, D., Sapp, P.C., Al-Chalabi, A., Wills, A.M., Brown, R.H.J., 2008. A common haplotype within the PON1 promoter region is associated with sporadic ALS. Amyotroph. Lateral Scler. 9, 306–314 [DOI: 10.1080/17482960802233177] [PubMed: 18618303].

Li, H.L., Liu, D.P., Liang, C.C., 2003. Paraoxonase gene polymorphisms, oxidative stress, and diseases. J. Mol. Med. 81, 766–779 [DOI: 10.1007/s00109-003-0481-4] [PubMed: 14551701].

Morahan, J.M., Yu, B., Trent, R.J., Pamphlett, R., 2007. A gene-environment study of the paraoxonase 1 gene and pesticides in amyotrophic lateral sclerosis. Neurotoxicology 28, 532–540 [DOI: 10.1016/j.neuro.2006.11.007] [PubMed: 17204329].

Saeed, M., Siddique, N., Hung, W.Y., Usacheva, E., Liu, E., Sulfit, R.L., Heller, S.L., Haines, J.L., Pericak-Vance, M., Siddique, T., 2006. Paraoxonase cluster polymorphisms are associated with sporadic ALS. Neurology 67, 771–776.

Slowik, A., Tomik, B., Wolkow, P.P., Partyka, D., Turaj, W., Malecki, M.T., Pera, J., Dziedzic, T., Szczudlik, A., Figlewicz, D.A., 2006. Paraoxonase gene polymorphisms and sporadic ALS. Neurology 67, 766–770 [DOI: 10.1212/01.wnl.0000219565.32247.11] [PubMed: 16822965].

Valdmanis, P.N., Kabashi, E., Dyck, A., Hince, P., Lee, J., Dion, P., D'Amour, M., Souchon, F., Bouchard, J.P., Salachas, F., Meininger, V., Andersen, P.M., Camu, W., Dupré, N., Rouleau, G.A., 2008. Association of paraoxonase gene cluster polymorphisms with ALS in France, Quebec, and Sweden. Neurology 71, 514–520 [DOI: 10.1212/01.wnl.0000324997.21272.0c].

Wills, A.M., Cronin, S., Slowik, A., Kasperaviciute, D., Van Es, M.A., Morahan, J.M., Valdmanis, P.N., Meininger, V., Melki, J., Shaw, C.E., Rouleau, G.A., Fisher, E.M., Shaw, P.J., Morrison, K.E., Pamphlett, R., Van den Berg, L.H., Figlewicz, D.A., Andersen, P.M., Al-Chalabi, A., Hardiman, O., Purcell, S., Landers, J.E., Brown, R.H.J., 2009. A large-scale international meta-analysis of paraoxonase gene polymorphisms in sporadic ALS. Neurology 73, 16–24 [DOI: 10.1212/WNL.0b013e3181a18674] [PubMed: 19321847].

Supplementary material

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a relatively rare (incidence: 1–3/100,000/year), progressive, and fatal disease characterized by the neurodegeneration of motor neurons of the cerebral cortex, brain stem, and spinal cord. Familial ALS (FALS) accounts for about 10% of the cases, and is usually inherited as an autosomal dominant trait. Currently eight ALS genes and several additional chromosomal loci have been identified (Beleza-Mereiles and Al-Chalabi, 2009; Kwiatkowski et al., 2009; Valdmanis and Rouleau, 2008; Vance et al., 2009); approximately 20% of familial ALS cases are attributed to mutations in *SOD1* gene (Andersen, 2006). In contrast, 90% of all the ALS cases are sporadic (SALS). To date, the etiology of the sporadic form is undisclosed and little is known about the factors contributing to the development of the disease. SALS is thought to result from the interaction of several genes with environmental factors, including exposure to adverse environmental agents, thus depicting sporadic ALS as a typical multifactorial disease (Schymick et al., 2007; Simpson and Al-Chalabi, 2006).

Among the genetic factors, special attention has been reserved to the paraoxonase (*PONs*) genes, that express detoxifying enzymes involved in the metabolism of a large number of substrates. The genetic locus encoding *PON* is localized on chromosome 7q21.3 and contains 3 genes (ordered *PON1*, *PON3* and *PON2*). PON1 is synthesized in the liver and associated with high-density lipoprotein (HDL) in the circulation; it exhibits antioxidative properties, preventing low-density lipoprotein (LDL) from peroxidation and inactivating oxidized lipids in LDL (Li et al., 2003). PON1 also detoxifies organophosphate insecticides, pesticides and nerve gases (Li et al., 2003). PON2 is a ubiquitously expressed intracellular protein able to protect the cells against the oxidative damage (Ng et al., 2001). PON3 displays properties similar to PON1, but differs from it in regulation pathway and substrate specificity (Reddy et al., 2001).

Some single nucleotide polymorphisms (SNPs) have been identified in the *PON* cluster. In particular, L55M (rs854560) and Q192R (rs662) SNPs have been detected in the coding region of the *PON1* gene and related to the enzyme activity. The 192RR and 55LL genotypes are associated with the greatest hydrolytic activity against paraoxon, whereas the 192QQ and 55-MM genotypes possess the highest protective capacity against LDL oxidation (Humbert et al., 1993). In addition, individuals with the M allele have lower levels of *PON1* mRNA and plasma concentration of the enzyme, thus showing a 30% reduction in the serum PON activity (Garin et al., 1997). In the coding region of *PON2*, the S311C (rs6954345) polymorphism has been associated with variations in lipoprotein metabolism and plasma lipoprotein concentration (Li et al., 2003).

Several studies have investigated the association between *PON* genetic variants and the risk for sporadic ALS. The first study reported a significant association of the R allele at Q192R in *PON1* and the C allele at S311C in *PON2* with SALS in a Polish population (Slowik et al., 2006). Subsequently, 5 additional case-control association studies were performed in Australian (Morahan et al., 2007), Irish (Cronin et al., 2007), North-American (Saeed et al., 2006; Landers et al., 2008), French, Quebec, and Swedish (Valdmanis et al., 2008) populations. These studies assayed a heterogeneity of SNPs across the *PON* locus and reported conflicting results. Recently, a meta-analysis of all these data, also including genome-wide association studies (GWAS), did not confirm the previous results and failed to find a significant association between the most common variants in *PON* genes and the risk for sporadic ALS (Wills et al., 2009).

In the light of this discrepancy in results, replication in independent populations could be of substantial importance to better understand the role of paraoxonase genes in sporadic ALS.

2. Methods

2.1. Patients

The study included 350 sporadic ALS patients and 376 control subjects of Caucasian origin, belonging to Italian ancestry and living in Northern and Central Italy. ALS diagnosis was made accordingly to El Escorial Revisited criteria (Brooks et al., 2000). Only patients diagnosed to have Definite, Probable and Probable laboratory supported ALS, who gave their informed consent, were included in the study. Briefly, sites of onset were recorded as spinal versus bulbar. Age at onset was defined by the onset of first symptoms. The survival endpoint was death or time of initiation of all forms of invasive ventilatory support. The mean duration of the disease was defined as the time occurring between onset and survival endpoint. Living cases were excluded from the calculation of the mean duration.

Control group consisted of age- and sex-matched individuals from the same ethnic background with no history of neurological diseases. Characteristics of ALS patients and controls are summarized in Table 1S. This study was approved by the local ethics committee.

2.2. Genotyping

Genomic DNA was obtained from peripheral blood samples by using standard procedure. The analysis of the L55M and Q192R polymorphisms within the *PON1* gene and the S311C within *PON2* gene was carried out by PCR amplification using the following primers:

L55M: forward 5′-GCTCTAGTCCATCAATTTAAAACAAA-3′, reverse 5′-TGGGTATACAGAAAGCCTAAGTGA-3′; Q192R: forward 5′-AGACAGTGAGGAATGCCAGTT-3′, reverse 5′-CAGAGAGTTCACATACTTGCCATC-3′; S311C: forward 5′-TTCAACAGCATGTCCCCTTA-3′, reverse 5′-AGTGCCTATGAGCAGCTTCC-3′.

PCR products for *PON1*-L55M and *PON2*-S311C were analyzed by restriction enzyme digestion, by using NlaIII and DdeI, respectively; Denaturing High Performance Liquid Chromatography (DHPLC) screening and subsequent sequence analysis of the samples with aberrant elution profiles were performed for *PON1*-Q192R.

2.3. Statistical analysis

The estimation of the power of our sample to detect an association was performed by using the statistical program QUANTO version 1.2.4 (Gauderman, 2002) the parameters used for the calculation were outcome: disease; design: unmatched case-control (1:1.1); hypothesis: gene only; mode of inheritance: additive; significance: 0.05, 2-sided. For each SNP, frequencies and odds ratios (OR), previously reported in studies showing a positive association with ALS, were used. In particular: for *PON1*-L55M OR = 1.5 and frequency = 0.37 (Cronin et al., 2007), for *PON1*-Q192R OR = 1.4 and frequency = 0.33, for *PON2*-S311C OR = 1.4 and frequency = 0.30 (Slowik et al., 2006).

Haplotype frequencies and association statistics for the polymorphisms were constructed using PHASE version 2 software (Stephens et al., 2001; Stephens and Donnelly, 2003).

Association analyses were carried out by using the software package SPSS v13.0 (IBM Company, Chicago, Illinois). Interaction with SNPs was tested by χ^2 analysis at genotypic, allelic, and haplotypic levels. In addition to basic tests, the association of genotype with ALS was evaluated assuming dominant and recessive models.

To evaluate the association between *PON* polymorphisms and ALS clinical variables, patients were stratified in different groups for each variable. In particular, patients were stratified by gender (males/females), age of onset (<45 years/≥45 years, taking 45 years as arbitrary cut-off to discriminate early and late onset), and site of onset (spinal/bulbar). Association analyses were carried out by χ^2 analysis using the software package SPSS v13.0.

Genotype and allele associations with disease duration were estimated with univariate analysis according to the Kaplan-Meier method using the log-rank test to assess statistical differences between groups. Analysis was performed considering: (1) deceased patients; and (2) both living and deceased individuals, using both censored and noncensored approaches.

3. Results

Based on frequency and odds ratio previously reported for the examined SNPs (Slowik et al., 2006; Cronin et al., 2007; Wills et al., 2009), our sample had >85% power to detect the risk allele at a significance level of $\alpha = 0.05$. All the studied SNPs were in Hardy-Weinberg equilibrium in both cases and controls ($p > 0.05$). Genotype and allele frequencies for each of the analyzed polymorphisms are shown in Table 1. No significant difference in *PON1*-L55M, *PON1*-Q192R and *PON2*-S311C distributions was observed in patients compared with controls. Statistical analysis failed to find an association between genotypes and ALS also assuming the dominant and recessive models (Table 2S).

Haplotype analysis did not reveal a significant difference in distribution in cases and controls. Results are summarized in Table 3S.

Association of genotypes and haplotypes with gender, site of disease onset (spinal vs. bulbar) and age at disease

Table 1S
Characteristics of patients with ALS and control subjects

	ALS patients	Controls
n	350	376
Gender, n	186 M/164 F (1.13/1)	205 M/171 F (1.2/1)
Age at blood collection, years	60.6 ± 13.1	59.5 ± 15.3
Age at onset, years	59.8 ± 12.7	
Bulbar onset	27.4%	
Disease duration, months	34.7 ± 28.1	

Data are given as mean ± SD, except where noted.
Key: ALS, amyotrophic lateral sclerosis; F, female; M, male.

Table 2S
Association of polymorphisms in paraoxonase genes in sporadic ALS, assuming recessive and dominant models

SNP	Cases (%)	Controls (%)	*p* value
PON1-L55M recessive			
MM	13.6	16.8	0.225
LL+LM	86.4	83.2	
PON1-L55M dominant			
MM+LM	58.2	63.1	0.177
LL	41.8	36.9	
PON1-Q192R recessive			
RR	9.9	10.0	1.000
QQ+QR	90.1	90.0	
PON1-Q192R dominant			
RR+QR	49.9	46.4	0.369
QQ	50.1	53.6	
PON2-S311C recessive			
CC	5.4	3.2	0.140
SS+SC	94.6	96.8	
PON2-S311C dominant			
CC+SC	40.0	38.8	0.735
SS	60.0	61.2	

Key: ALS, amyotrophic lateral sclerosis.

Table 3S
Haplotype distribution in patients with sporadic ALS and controls

Haplotype	Cases (%)	Controls (%)	*p* value
L-Q-S	22.0	19.2	0.198
L-Q-C	13.2	13.0	0.929
L-R-S	22.8	22.5	0.867
L-R-C	6.2	5.3	0.446
M-Q-S	32.2	36.6	0.077
M-Q-C	3.2	2.6	0.528
M-R–S	0.3	0.5	0.688
M-R-C	0.1	0.3	1.000

Key: ALS, amyotrophic lateral sclerosis.

Table 4S
Genotype and allelic distributions of *PON* gene polymorphisms for ALS clinical variables

SNP	Gender		Age at onset (years), ≥45/<45		Site of onset, spinal/bulbar		Survival	
	Genotype *p* value[a]	Allele *p* value[a]	Genotype *p* value[a]	Allele *p* value[a]	Genotype *p* value[a]	Allele *p* value[a]	Genotype *p* value[b]	Allele *p* value[b]
PON1-L55M	0.421	0.578	0.446	0.724	0.781	0.749	0.541	0.562
PON1-Q192R	0.446	0.702	0.441	0.203	0.304	0.339	0.338	0.146
PON2-S311C	0.305	0.741	0.608	0.681	0.641	0.383	0.108	0.907

Regarding the alleles, patients were dichotomized in carriers and noncarriers of the risk allele: PON1-L55M: LL/(LM + MM); PON1-Q192R: QQ/(QR + RR); and PON2-S311C: SS/(SC + CC).

[a] *p* values were calculated using χ^2 or Fischer's exact tests.

[b] Survival analysis was estimated using the Kaplan-Meier method and compared by the log-rank test.

Key: ALS, amyotrophic lateral sclerosis, SNP, single nucleotide polymorphisms.

onset was also evaluated. None of the genotypes and haplotypes were significantly associated with ALS clinical variables examined. The results are summarized in Table 4S. Survival analysis did not reveal any association of genotypes and alleles with the disease duration, using a censored approach (Table 4S), a noncensored approach, and considering only deceased individuals (data not shown).

4. Discussion

The present study investigated the association between paraoxonase gene polymorphisms and susceptibility to ALS in an Italian population. To our knowledge, no studies on *PON* genes and ALS have been previously performed in patients from Italy.

Genotype and allelic frequencies in the examined population were quite similar in cases and controls. We did not find any positive association between the examined SNPs (L55M and Q192R in *PON1* gene and S311C in *PON2* gene) and the risk of ALS, either at the genotype or haplotype level.

Association studies performed on *PON* genes have previously reported positive associations in several ALS populations (Slowik et al., 2006; Morahan et al., 2007; Cronin et al., 2007; Saeed et al., 2006; Landers et al., 2008; Valdmanis et al., 2008). However, each study found different SNPs and/or haplotypes to be associated with ALS susceptibility. This can reflect the heterogeneity among different populations on a genetic level. It is known that the genetic frequencies for SNPs, as well the level of association with a specific phenotype, can largely diverge among different populations (Goldstein and Cavalleri, 2005; International HapMap Consortium, 2005). By contrast, it should not be overlooked that these conflicting results can originate from the large range of paraoxonase SNPs chosen for the analysis in each report.

In the present report, we have examined the 3 single nucleotide polymorphisms analyzed in the first association study (Slowik et al., 2006). These SNPs are some of the most frequently assayed *PON* polymorphisms and are able to influence/modulate paraoxonase activity *in vivo* (Li et al., 2003). Our data agree with the recent results of the meta-analysis of *PON* gene polymorphisms in sporadic ALS (Wills et al., 2009). This analysis failed to confirm any positive association, in a comprehensive study on more than 4000 ALS cases and 4000 controls, including the SNPs assayed in the present report. Lack of association of such SNPs with ALS can indicate that in the Italian population, as well as already observed in the Swedish population (Valdmanis et al., 2008), *PON* genes play a minor role, and other gene-environment interactions are involved in ALS pathogenesis.

References

Andersen, P.M., 2006. Amyotrophic lateral sclerosis associated with mutations in the CuZn Superoxide Dismutase gene. Curr. Neurol. Neurosci. Rep. 6, 37–46.

Beleza-Meireles, A., Al-Chalabi, A., 2009. Genetic studies of amyotrophic lateral sclerosis: Controversies and perspectives. Amyotroph. Lateral Scler. 10, 1–14 [DOI: 10.1080/17482960802585469] [PubMed: 19110986].

Brooks, B.R., Miller, R.G., Swash, M., Munsat, T.L., World Federation. of Neurology Research Group on Motor Neuron Diseases, 2000. El Escorial revisited: revised criteria for the diagnosis of amyotrophic lateral sclerosis. Amyotroph. Lateral Scler. Other Motor Neuron. Disord. 1, 293–9.

Cronin, S., Greenway, M.J., Prehn, J.H., Hardiman, O., 2007. Paraoxonase promoter and intronic variants modify risk of sporadic amyotrophic lateral sclerosis. J. Neurol. Neurosurg. Psychiatry 78, 984–986 [DOI: 10.1136/jnnp.2006.112581] [PubMed: 17702780].

Garin, M.C., James, R.W., Dussoix, P., Blanché, H., Passa, P., Froguel, P., Ruiz, J., 1997. Paraoxonase polymorphism Met-Leu54 is associated with modified serum concentration of the enzyme. A possible link between the paraoxonase gene and increased risk of cardiovascular disease in diabetes. J. Clin. Invest. 99, 62–69 [DOI: 10.1172/JCI119134].

Gauderman, W.J., 2002. Sample Size Requirements for Association Studies of Gene-Gene Interaction. Am. J. Epidemiol. 155, 478–484 [DOI: 10.1093/aje/155.5.478] [PubMed: 11867360].

Goldstein, D.B., Cavalleri, G.L., 2005. Genomics: understanding human diversity. Nature 437, 1241–1242 [DOI: 10.1038/4371241a] [PubMed: 16251937].

Humbert, R., Adler, D.A., Disteche, C.M., Hassett, C., Orniecinski, C.J., Furlong, C.E., 1993. The molecular basis of the human serum paraoxonase activity polymorphism. Nat. Genet. 3, 73–76.

International HapMap Consortium, 2005. A haplotype map of the human genome. Nature 437, 1299–1320.

Kwiatkowski, T.J., Jr, Bosco, D.A., Leclerc, A.L., Tamrazian, E., Vanderburg, C.R., Russ, C., Davis, A., Gilchrist, J., Kasarskis, E.J., Munsat, T., Valdmanis, P., Rouleau, G.A., Hosler, B.A., Cortelli, P., de Jong, P.J., Yoshinaga, Y., Haines, J.L., Pericak-Vance, M.A., Yan, J., Ticozzi, N., Siddique, T., McKenna-Yasek, D., Sapp, P.C., Horvitz, H.R., Landers, J.E., Brown, R.H., Jr, 2009. Mutations in the FUS/TLS gene on chromosome 16 cause familial amyotrophic lateral sclerosis. Science 323, 1205–1208 [DOI: 10.1126/science.1166066] [PubMed: 19251627].

Landers, J.E., Shi, L., Cho, T.J., Glass, J.D., Shaw, C.E., Leigh, P.N., Diekstra, F., Polak, M., Rodriguez-Leyva, I., Niemann, S., Traynor, B.J., McKenna-Yasek, D., Sapp, P.C., Al-Chalabi, A., Wills, A.M., Brown, R.H.J., 2008. A common haplotype within the PON1 promoter region is associated with sporadic ALS. Amyotroph. Lateral Scler. 9, 306–314 [DOI: 10.1080/17482960802233177] [PubMed: 18618303].

Li, H.L., Liu, D.P., Liang, C.C., 2003. Paraoxonase gene polymorphisms, oxidative stress, and diseases. J. Mol. Med. 81, 766–779 [DOI: 10.1007/s00109-003-0481-4] [PubMed: 14551701].

Morahan, J.M., Yu, B., Trent, R.J., Pamphlett, R., 2007. A gene-environment study of the paraoxonase 1 gene and pesticides in amyotrophic lateral sclerosis. Neurotoxicology 28, 532–540 [DOI: 10.1016/j.neuro.2006.11.007] [PubMed: 17204329].

Ng, C.J., Wadleigh, D.J., Gangopadhyay, A., Hama, S., Grijalva, V.R., Navab, M., Fogelman, A.M., Reddy, S.T., 2001. Paraoxonase-2 is a ubiquitously expressed protein with antioxidant properties and is capable of preventing cell-mediated oxidative modification of low density lipoprotein. J. Biol. Chem. 276, 44444–44449 [DOI: 10.1074/jbc.M105660200] [PubMed: 11579088].

Reddy, S.T., Wadleigh, D.J., Grijalva, V., Ng, C., Hama, S., Gangopadhyay, A., Shih, D.M., Lusis, A.J., Navab, M., Fogelman, A.M., 2001. Human paraoxonase-3 is an HDL-associated enzyme with biological activity similar to paraoxonase-1 protein but is not regulated by oxidized lipids. Arterioscler. Thromb. Vasc. Biol. 21, 542–547 [PubMed: 11304470].

Saeed, M., Siddique, N., Hung, W.Y., Usacheva, E., Liu, E., Sulfit, R.L., Heller, S.L., Haines, J.L., Pericak-Vance, M., Siddique, T., 2006. Paraoxonase cluster polymorphisms are associated with sporadic ALS. Neurology 67, 771–776.

Schymick, J.C., Talbot, K., Traynor, B.J., 2007. Genetics of sporadic amyotrophic lateral sclerosis. Hum. Mol. Genet. 16, R233–R242 [DOI: 10.1093/hmg/ddm215] [PubMed: 17911166].

Simpson, C.L., Al-Chalabi, A., 2006. Amyotrophic lateral sclerosis as a complex genetic disease. Biochim. Biophys. Acta 1762, 973–985 [PubMed: 16973338].

Slowik, A., Tomik, B., Wolkow, P.P., Partyka, D., Turaj, W., Malecki, M.T., Pera, J., Dziedzic, T., Szczudlik, A., Figlewicz, D.A., 2006. Paraoxonase gene polymorphisms and sporadic ALS. Neurology 67, 766–770 [DOI: 10.1212/01.wnl.0000219565.32247.11] [PubMed: 16822965].

Stephens, M., Smith, N.J., Donnelly, P., 2001. A new statistical method for haplotype reconstruction from population data. Am. J. Hum. Genet. 68, 978–989 [DOI: 10.1086/319501] [PubMed: 11254454].

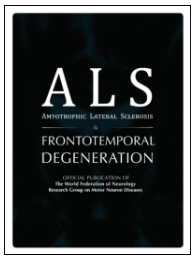
Stephens, M., Donnelly, P., 2003. A comparison of bayesian methods for haplotype reconstruction from population genotype data. Am. J. Hum. Genet. 73, 1162–1169 [DOI: 10.1086/379378] [PubMed: 14574645].

Valdmanis, P.N., Rouleau, G.A., 2008. Genetics of familial amyotrophic lateral sclerosis. Neurology 70, 144–152 [DOI: 10.1212/01.wnl.0000296811.19811.db] [PubMed: 18180444].

Valdmanis, P.N., Kabashi, E., Dyck, A., Hince, P., Lee, J., Dion, P., D'Amour, M., Souchon, F., Bouchard, J.P., Salachas, F., Meininger, V., Andersen, P.M., Camu, W., Dupré, N., Rouleau, G.A., 2008. Association of paraoxonase gene cluster polymorphisms with ALS in France, Quebec, and Sweden. Neurology 71, 514–520 [DOI: 10.1212/01.wnl.0000324997.21272.0c].

Vance, C., Rogelj, B., Hortobágyi, T., De Vos, K.J., Nishimura, A.L., Sreedharan, J., Smith, B., Ruddy, D., Wright, P., Ganesalingam, J., Williams, K.L., Tripathi, V., Al-Saraj, S., Al-Chalabi, A., Leigh, P.N., Blair, I.P., Nicholson, G., de Belleroche, J., Gallo, J.M., Miller, C.C., Shaw, C.E., 2009. Mutations in FUS, an RNA processing protein, cause familial amyotrophic lateral sclerosis type 6. Science 323, 1208–1211 [DOI: 10.1126/science.1165942].

Wills, A.M., Cronin, S., Slowik, A., Kasperaviciute, D., Van Es, M.A., Morahan, J.M., Valdmanis, P.N., Meininger, V., Melki, J., Shaw, C.E., Rouleau, G.A., Fisher, E.M., Shaw, P.J., Morrison, K.E., Pamphlett, R., Van den Berg, L.H., Figlewicz, D.A., Andersen, P.M., Al-Chalabi, A., Hardiman, O., Purcell, S., Landers, J.E., Brown, R.H.J., 2009. A large-scale international meta-analysis of paraoxonase gene polymorphisms in sporadic ALS. Neurology 73, 16–24 [DOI: 10.1212/WNL.0b013e3181a18674] [PubMed: 19321847].



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SHORT REPORT

A novel exon 1 mutation (G10R) in the *SOD1* gene in a patient with familial ALS

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Abstract

Mutations in the superoxide dismutase-1 (*SOD1*) gene have been found in 12–23% of patients with a diagnosis of ALS. Although the mechanism by which mutant *SOD1* causes neural death remains elusive, several lines of evidence suggest that ALS is a protein-folding disease. Here we report a novel missense mutation in exon 1 of the *SOD1* gene in a 68-year-old female with familial ALS characterized by spinal onset with upper and lower motor neuron signs and early neuroimaging evidence of corticospinal tract involvement. Molecular analysis identified a heterozygous mutation in codon 10, with substitution of a highly conserved glycine with arginine (G10R). Modelling of the mutant *SOD1* showed a strong destabilization of the protein secondary structure that could influence the strength of the dimer interface. This property can result in a failure of the protein to fold and generation of toxic intracellular aggregates, suggesting a pathogenic role for the mutation.

Key words: *Amyotrophic lateral sclerosis, SOD1 mutation, protein structural modelling*

Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive and fatal disorder characterized by selective degeneration of motor neurons in the cerebral cortex, brainstem, and spinal cord. About 1–13.5% of patients have a family history of ALS (FALS). A mutation in the *SOD1* gene has been found in 12–23% of patients with a diagnosis of familial ALS (1). The *SOD1* protein is a homodimeric Cu/Zn binding enzyme, composed of eight anti-parallel β-strands and two metal atoms, that catalyses the conversion of the toxic superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). More than 150 different *SOD1* gene mutations have been identified in ALS patients (ALS Online Genetic Database, ALSOD: http://alsod.iop.kcl.ac.uk/) (2), mostly with autosomal dominant transmission. Although the mechanism by which mutant *SOD1* causes neural death remains elusive, several lines of evidence suggest that ALS is a protein-folding disease analogous to other neurodegenerative disorders such as Alzheimer's, Parkinson's, and Creutzfeldt-Jakob's disease (3–5). The propensity of mutant *SOD1* protein to misfold and form toxic intracellular aggregates is thought to be a common mechanism in ALS caused by *SOD1* variations (4,6). Most *SOD1* mutations, when mapped onto the crystallographic structure of the *SOD1* protein, localize near the dimer interface and at the beginning of the active loop (7). *SOD1* mutations are proposed to perturb the protein stability by destabilizing the precursor monomer, weakening the dimer interface, or both (8). Here we report a novel missense mutation (G10R) in exon 1 of the *SOD1* gene in a FALS patient. The mutation was mapped onto the three-dimensional structure of *SOD1* in order to predict how this amino acid substitution affects the structure of the *SOD1* protein, and to evaluate its potential role in protein stability and aggregation.

Case report

The proband, a 68-year-old female, presented with a nine-month history of difficulty in climbing stairs, followed by progressive weakness in the lower limbs. Neurological examination showed reduced strength in proximal and distal sites of the right lower limb and

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in the distal site of the left lower limb. Deep tendon reflexes were brisk in the upper limbs and at the knees. Achilles reflexes were absent. Hoffmann and Babinski signs were present on the right side. No autonomic or cognitive impairments were present. An EMG study showed active neurogenic changes in the cervical, thoracic, and lumbosacral regions. Motor evoked potential (MEP) by transcranial magnetic stimulation 10 months after symptom onset showed normal latency and amplitude in all four limbs (Table I). MRI Flair images disclosed bilateral hyperintensity of the centrum semiovale and the corticospinal pathways in the brainstem. Twenty-one months after the onset of symptoms, the patient showed severe weakness and muscular atrophy in the lower limbs and mild weakness in the proximal site of the right upper limb. The patient was able to stand only with bilateral aid. At the last follow-up, three months later, the patient showed further worsening of strength in the lower limbs and she was wheelchair-bound. At this time MEP showed no changes and normal values in upper limbs while responses in the lower limbs were lost, also by recording from the tibialis anterior muscles, probably due to severe peripheral denervation (Table I). Bulbar and respiratory functions were normal. Her father developed bulbar onset ALS at the age of 55 years and died seven months after the onset of symptoms. The patient had two daughters, aged 45 and 39 years, who were clinically unaffected at the time of our examination. Due to ethical concerns, *SOD1* screening was not performed in these subjects. No other family members were available for clinical examination and genetic testing.

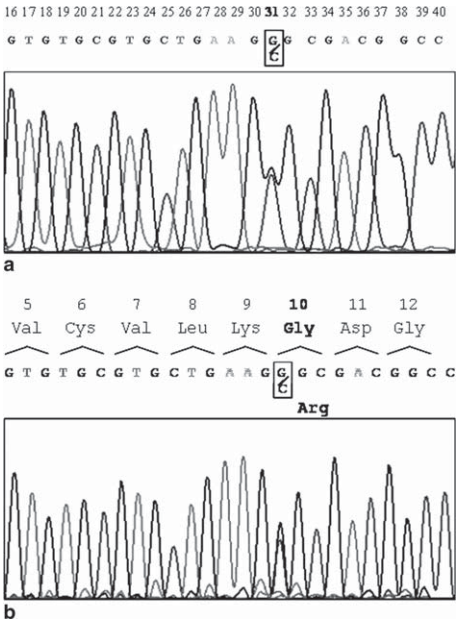

Figure 1. Automated sequence analysis of both the *SOD1* gene and cDNA. a. Automated sequence analysis of exon 1 from the *SOD1* gene showing the heterozygous mutation c.31G > C. b. Automated sequence analysis of *SOD1* cDNA with the variation *GGC* to *CGC* at codon 10 resulting in the substitution of arginine for glycine in the *SOD1* protein (G10R).

Molecular analysis

After obtaining written informed consent, genomic DNA was extracted from peripheral blood using standard procedures. Exons 1 to 5 of the *SOD1* gene were amplified from genomic DNA by polymerase chain reaction (PCR), as previously described (9). cDNA was obtained by reverse transcription of total RNA extracted from the patient's white blood cells using standard procedures, and amplified by PCR to confirm the presence of the mutation and evaluate *SOD1* expression. All PCR products were sequenced by an automated sequencing system (ABI 310 Genetic Analyzer, Applied Biosystems). The presence and zygosity of the G10R mutation was also confirmed by restriction digestion (*Cfo*I from Roche Diagnostic).

Modelling of the SOD1 G10R mutation

The pathogenicity of the mutation was evaluated using Panther software (www.pantherdb.org/tools/csnpScore Form.jsp), directly available from the ALSOD database (2). Modelling of the G10R mutation was performed using the Swiss model server (http://swissmodel.expasy.org/) (10,11) and the molecular visualization system Pymol (http://pymolsourceforge.net/) from the crystal structure of the normal *SOD1* protein (PDB2c9vA).

Table I. MEP values by transcranial magnetic stimulation.

	Amplitude (% of M-wave)			Latency (contracted) (msec)			CMCT (root stimulation) (msec)		
Muscle	I	II	NL	I	II	NL	I	II	NL
R thenar	60	54	≥20	21.1	20.6	<24.1	5.3	4.8	<7.4
L thenar	65	64		20.2	20.2		5.2	5.2	
R abductor hallucis	60	NR	≥15	40.4	NR	<48.0	13.1	NR	<15.7
L abductor hallucis	59	NR		39.9	NR		13.5	NR	

Table I. I: 10 months after disease onset; II: 24 months after disease onset; CMCT: central motor conduction time; NL: normal limit; R: right; L: left; NR: no response.

The atomic empirical mean force potential, atomic non-local environment assessment (ANOLEA) (12) was used to assess the packing quality of the models. The program performs energy calculations on a protein chain evaluating the 'non-local environment' (NLE) of each heavy atom in the molecule. In addition, the effect of the mutation on the protein structure was evaluated using the link to the '*SOD1* Database-Motor Neuron Disease Mutations' (http://www.bioinf.org.uk/mndb/), accessible from the ALSOD database (2).

Results

Molecular analysis

DNA analysis showed a heterozygous mutation c.31G > C in the *SOD1* gene in the proband (Figure 1a). The mutation of *GGC* to *CGC* at codon 10 in exon 1 determined a substitution of arginine for glycine in the *SOD1* protein (G10R) (Figure 1b). RT-PCR revealed the presence of both wild-type and G10R mutant *SOD1* mRNA, confirming that the mutation was present in a heterozygous state and that both alleles were transcribed into mRNA. No additional mRNA alterations were observed. Since no other family members were available for genetic testing, it was not possible to confirm the cosegregation of this mutation with the disease.

Multi-species comparisons showed that the glycine at codon 10 is a highly conserved residue among various species (Figure 3). The pathogenicity predictor software revealed a score of –3.109, which suggests a highly harmful mutation. This mutation was absent in 400 chromosomes from healthy controls.

Modelling the SOD1 G10R mutation

The model of the mutant *SOD1* showed destabilization of the secondary structure around the arginine in position 10 involving the structure of the surrounding β-barrel, particularly in the β8-strand (Figure 2a, b). The energy calculation of the protein chain showed a relevant increase in energy, corre-

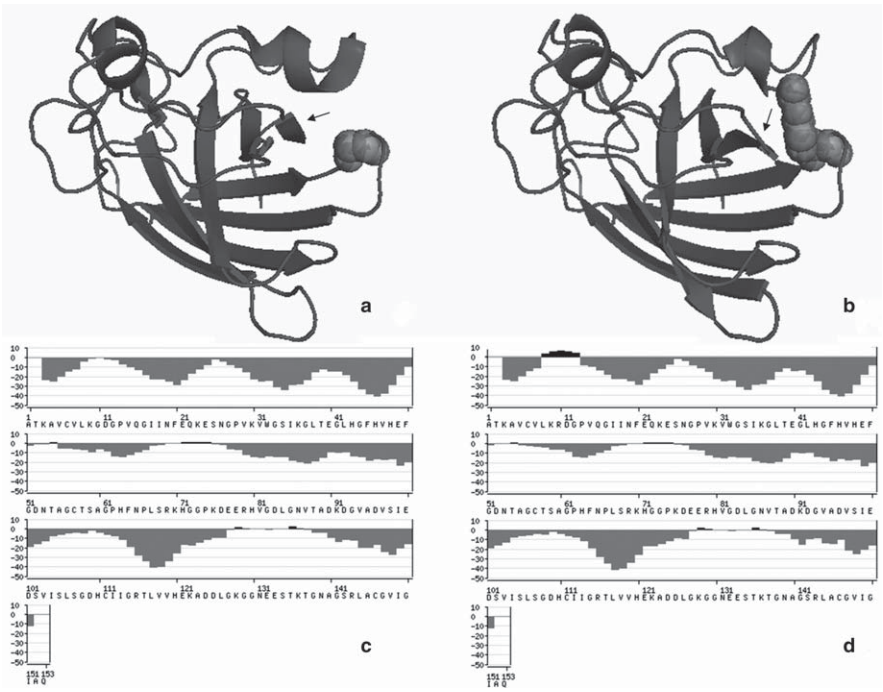

Figure 2. Three-dimensional structure of both wild-type human *SOD1* protein and a model of the *SOD1* G10R variant, and their specific atomic non-local environment assessment (ANOLEA) plots. a. Three-dimensional X-ray diffraction structure of wild-type human *SOD1* protein. b. Model of the superoxide dismutase G10R mutation. The steric hindrance of glycine and arginine is represented with spheres and the backbone distortion caused by the amino acid substitution is indicated by arrows. c. ANOLEA plot with the atomic empirical mean force potential of the normal *SOD1* protein. d. ANOLEA plot of the *SOD1* G10R mutation. The y-axis of the plots represents the energy for each amino acid in the protein chain. Negative energy values (grey) represent a favourable energy environment, whereas positive values (black) represent an unfavourable energy environment for a given amino acid.

Human	MAT- KAVCVLK **G** DGPVQG
Rat	MAM-KAVCVLK **G** DGPVQG
Mouse	MAM-KAVCVLK **G** DGV- QG
Chicken	MATLKAVCVMK **G** DAPVEG
Xenopus	MV- - KAVCVL - **G** SGDVKG
Drosophila	MVV -KAVCVI N **G** DAK - -G
Nematode	MSNR -AVAVLR **G** DPGVTG
S. Cerevisiae	MVQ- -AVAVLR- **G** DSK VSG

Figure 3. Protein alignment of the residues conserved across different species. Multi-species comparison of the *SOD1* protein in the region surrounding glycine in position 10 (grey box).

sponding to an unfavourable energy environment, around the arginine in the mutant *SOD1* compared with the wild-type protein (Figure 2c, d). Moreover, the substitution of glycine with the positively charged arginine would cause, under physiological conditions, a decrease in the magnitude of the net negative charge compared with the normal *SOD1*.

Discussion

In this study, we report a novel exon 1 missense mutation (G10R) in the *SOD1* gene in an Italian patient with familial ALS. The proband's clinical picture was characterized by spinal onset with upper and lower motor neuron signs and early neuroimaging evidence of corticospinal tract involvement. Although not specific, this conventional MRI finding has been reported in up to 17% of early-phase ALS patients (13). Bulbar and respiratory functions were normal 24 months after onset. In contrast, her father developed ALS with bulbar onset at the age of 55 years and showed a very rapid course of the disease (<1 year). Heterogeneity in the age of onset, disease duration, and disease severity is not uncommon between members of the same family (1,14), suggesting that the phenotype is modified by elements other than the mutation, such as different genetic or environmental factors.

In the present study it was not possible to demonstrate whether this novel mutation segregates with the disease in the family. However, some evidence supports this mutation as causative. First, the G10R mutation was not present in 400 control chromosomes. In addition, in the same codon, as well as a synonymous mutation found in a sporadic ALS case, a different missense mutation, *GGC* to *GTT* resulting in the substitution of glycine to valine (G10V), has been identified previously in a Korean ALS family associated with a quite uniform phenotype, characterized by relatively early age of onset and rapid disease progression (15).

Furthermore, the G10R mutation involves an amino acid residue that is highly conserved in different species and appears to be important for the proper structure and function of the protein (Figure 2). The G10R substitution is located at the beginning of the first loop connecting the β1 and β2 strands, which form the dimer interface (7). It is known that glycine is a critical amino acid residue for protein secondary structure formation, since, when localized at the beginning of a loop, it can interrupt the regularity of the β strand and α-helix conformations. The presence of an amino acid other than glycine in position 10 is hypothesized to alter the correct folding of *SOD1* protein. As shown in Figure 3, the steric hindrance of arginine leads to a relevant backbone distortion that can be propagated over a considerable distance in the protein structure, significantly altering the stability of the free monomer and influencing the strength of the dimer interface. Moreover, since the G10R substitution introduces an extra positive charge, this decreases the magnitude of the net negative charge and could consequently alter the aggregation propensity of the protein, in agreement with previous findings (3,4). These properties can result in a failure of proper protein folding and/or genesis of toxic intracellular aggregates, suggesting a pathogenic role for the G10R *SOD1* mutation in ALS.

This report of a novel *SOD1* mutation with the corresponding clinical data and the hypothetical protein structural rearrangement expands the number of ALS associated *SOD1* gene mutations stored in the ALSOD online database (2). Further studies on ALS patients carrying the G10R mutation may validate its pathogenicity, and reveal a possible genotype-phenotype correlation.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

1. Andersen PM. Amyotrophic lateral sclerosis associated with mutations in the Cu/Zn superoxide dismutase gene. Curr Neurol Neurosci Rep. 2006;6:37–46.
2. Wroe R, Wai-Ling Butler A, Andersen PM, Powell JF, Al-Chalabi A. ALSOD: the Amyotrophic Lateral Sclerosis Online Database. Amyotroph Lateral Scler. 2008;9:249–50.
3. Nordlund A, Oliveberg M. *SOD1* associated ALS: a promising system for elucidating the origin of protein-misfolding disease. HFSP J. 2008;2:354–64.
4. Prudencio M, Hart PJ, Borchelt DR, Andersen PM. Variation in aggregation propensities among ALS associated variants of *SOD1*: correlation to human disease. Hum Mol Genet. 2009;18:3217–26.
5. Stefani M, Dobson CM. Protein aggregation and aggregate toxicity: new insights into protein folding, misfolding diseases and biological evolution. J Mol Med. 2003;81: 678–99.
6. Valentine JS, Hart PJ. Misfolded Cu/Zn SOD and amyotrophic lateral sclerosis. Proc Natl Acad Sci USA. 2003;100: 3617–22.
7. Deng HX, Hentati A, Tainer JA, Iqbal Z, Cayabyab A, Hung WY, et al. Amyotrophic lateral sclerosis and structural defects in Cu/Zn superoxide dismutase. Science. 1993;261: 1047–51.
8. Lindberg MJ, Byström R, Boknäs N, Andersen PM, Oliveberg M. Systematically perturbed folding patterns of

amyotrophic lateral sclerosis (ALS) associated *SOD1* mutants. Proc Natl Acad Sci U S A. 2005;102:9754–9.
9. Rosen DR, Siddique T, Patterson D, Figlewicz DA, Sapp P, Hentati A, et al. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature. 1993;362:59–62.
10. Arnold K, Bordoli L, Kopp J, Schwede T. The SWISS-MODEL Workspace: a web-based environment for protein structure homology modelling. Bioinformatics. 2006;22: 195–201.
11. Schwede T, Kopp J, Guex N, Peitsch MC. SWISS-MODEL: an automated protein homology-modelling server. Nucleic Acids Res. 2003;31:3381–5.
12. Melo F, Feytmans E. Assessing protein structures with a non-local atomic interaction energy. J Mol Biol. 1998;277:1141–52.
13. Charil A, Corbo M, Filippi M, Kesavadas C, Agosta F, Munerati E, et al. Structural and metabolic changes in the brain of patients with upper motor neuron disorders: a multiparametric MRI study. Amyotroph Lateral Scler. 2009;26:1–11.
14. Chiò A, Traynor BJ, Lombardo F, Fimognari M, Calvo A, Ghiglione P, et al. Prevalence of *SOD1* mutations in the Italian ALS population. Neurology. 2008;70:533–7.
15. Kim NH, Kim HJ, Kim M, Lee KW. A novel *SOD1* gene mutation in a Korean family with amyotrophic lateral sclerosis. J Neurol Sci. 2003;206:65–9.