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Study of DUX4 as a novel biomarker in Amyotrophic Lateral Sclerosis

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“Un segreto è

Fare tutto come se

Vedessi solo il sole.”

Summary

Abstract

Amyotrophic lateral sclerosis (ALS) is a degenerative neuromuscular disease of unknown etiology characterized by the impairment of both upper and lower motor neurons. The risk factors associated with ALS onset and progression remain unknown. The most accepted hypothesis on the etiopathogenesis of ALS foresees a concomitance between genetic and environmental factors that can cause or contribute to the development of familial and sporadic forms of ALS. Similar to other neurodegenerative diseases, ALS is characterized by an abnormal accumulation of insoluble proteins in the cytoplasm of degenerating motor neurons. In particular, in sporadic ALS, the aggregation of TDP-43 protein is thought to play a key role in the degeneration of motor neurons.

In this study, we investigated a novel possible biomarker for ALS: double homeobox 4 (DUX4). In fact, in a study by Homma and colleagues in 2015, it was shown that the overexpression of the DUX4 protein in myoblasts from patients with facioscapulohumeral muscular dystrophy (FSHD) can contribute to the accumulation and aggregation of TDP-43. Therefore, in the first part of our study, we evaluated the possible expression of DUX4 and its correlation with TDP-43 expression in peripheral blood mononuclear cells (PBMCs) from patients with ALS. Subsequently, we performed in neuroblastoma cell lines a DUX4 transfection and we evaluated the expression of TDP-43 before and after treatment. In the last part of this study, we assessed the possible effects of the two drugs approved by the FDA for the treatment of ALS, Edaravone and Riluzole, on DUX4 expression.

We analyzed peripheral blood mononuclear cells (PBMC) obtained from 46 patients with ALS and 26 controls for possible alterations in DUX4 expression. We found a significant (about threefold) increase in DUX4 mRNA and protein levels in PBMCs from patients

compared to controls. According to previous results from our group (Arosio et al., 2020), we subsequently confirmed an increase in TDP-43 protein levels in ALS PBMCs, as well as an increase in the truncated forms of TDP-35 and TDP-25, and we showed a positive correlation between DUX4 and TDP-43, TDP-35, and TDP-25 protein levels in ALS samples. In addition, our immunofluorescence analyses of ALS PBMCs showed DUX4-TDP-43 colocalization in the perinuclear region with a tendency to aggregate. Also experiments performed on fibroblasts ALS indicated an analogous increased DUX4 expression. Finally, immunohistochemistry studies were performed on cortex and spinal cord postmortem samples obtained by ALS patients. Upper motor neurons in ALS motor cortex samples showed a clearly detectable DUX-like immunoreactivity, while almost no signal was present in control samples. On the other hand, DUX4 was not expressed in spinal cord samples, regardless of the pathological status. Finally, we performed in a human neuroblastoma cell lines (SH-SY5Y) a DUX4 transfection and, showing that DUX4 upregulation after transfection induced an increase in the TDP-43 insoluble protein fraction.

To date, no therapy can cure ALS, although there are two drugs, Riluzole and Edaravone, have been approved by the Food and Drug Administration (FDA) for treatment. Thus, in the final part of this study, we investigated the possible effects of these two drugs on DUX4 mRNA and protein expression in neuroblastoma cell lines transfected with human SOD1 wild type or G93A mutation. Our results showed that Edaravone and Riluzole reduced DUX4 expression in basal conditions and following oxidative stress induced by H_2O_2 treatment.

Chapter 1:

Introduction

1. Neurodegenerative diseases

Neurodegenerative diseases are characterized by slow and progressive loss of various nerve functions. These disorders are becoming increasingly common and are related to age. There are various neurodegenerative diseases including amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD) (Gitler et al., 2017). Until recently, it was thought that there were no molecular mechanisms that accumulated in various pathological groups. To date, due to multiple studies, common characteristics have been observed: most neurodegenerative diseases, except for HD, can be both hereditary and sporadic, and familial forms are usually more severe and have an earlier onset than sporadic forms.

In most cases, the onset occurs in old age, around the fourth or fifth decade of life.

From a pathophysiological point of view, what distinguishes various pathologies is the production of abnormal proteins that can undergo a conformational change and acquire a toxic function or block their biological activity of origin (Soto et al 2001, Soto et al., 2003). These proteins tend to aggregate and accumulate, causing the production of fibrillar deposits. Therefore, based on this evidence these diseases are considered proteinopathies (Figure 1).

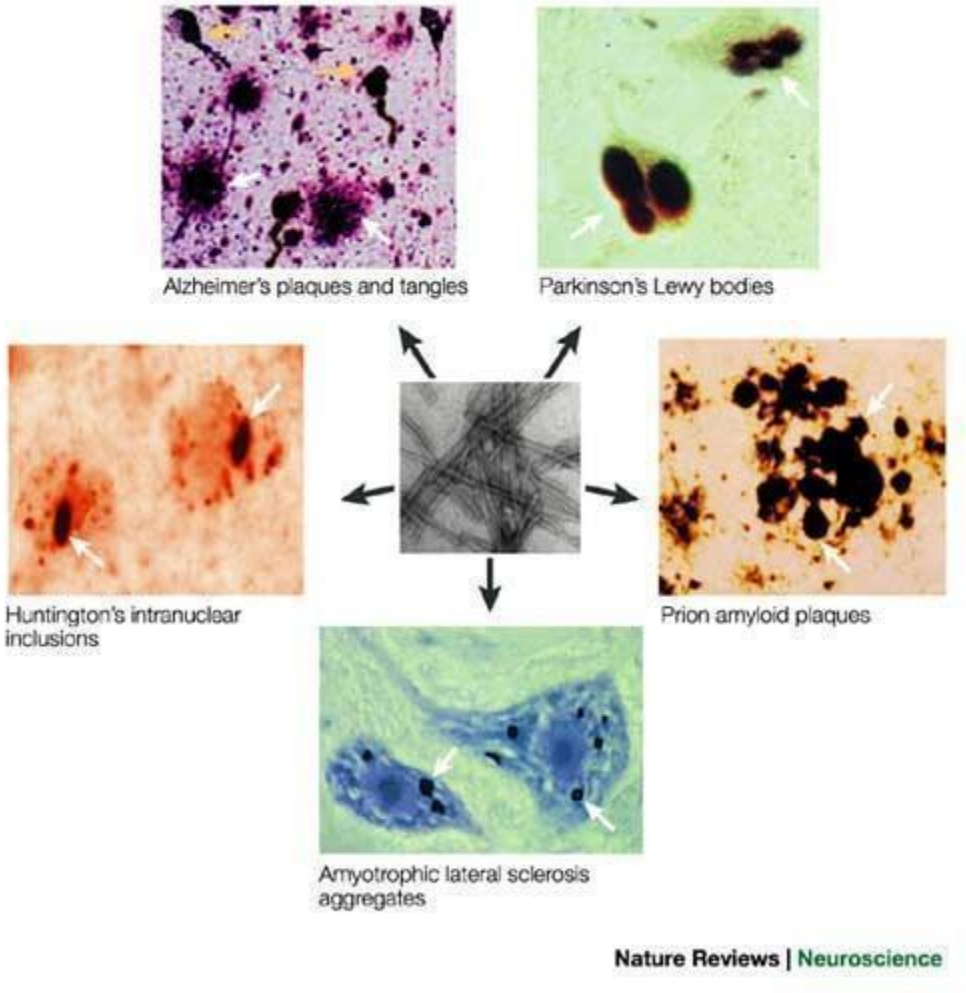


Figure 1: Brain aggregates in neurodegenerative diseases: extracellular amyloid plaques and intracytoplasmic neurofibrils characterize Alzheimer's disease. Intracytoplasmic aggregates in neurons of patients with Parkinson's and Amyotrophic Lateral Sclerosis. Intranuclear inclusions of Huntingtin in Huntington's disease and cerebral amyloidosis localized in various brain areas are observed in patients with transmissible spongiform encephalopathy. Despite the different protein compositions, the ultrastructures of these deposits seem to be similar to each other and composed of fibrillar polymers [source: Soto et al 2003].

2. Amyotrophic lateral sclerosis (ALS)

Amyotrophic lateral sclerosis (ALS), first described by Charcot in 1874, is a neurodegenerative disease of the first and second motor neurons (MNs), cells of the Central Nervous System (CNS) that control the voluntary muscles which involves a severe neurological disability. The degeneration progresses gradually causing weakening of the

muscles and loss of their function due to atrophy and spasticity. Neurodegeneration affects the motor cortex, brain stem, and spinal cord. The first motor neuron runs from the motor cortex to the spinal cord (called the upper motor neuron or first motor neuron); the second motor neuron or lower motor neuron goes from the brain stem or spinal cord to the muscle.

The name of this pathology derives from the characteristics that distinguish it:

- lateral sclerosis since the damage of the first motor neuron involves gliosis at the level of the lateral columns of the medulla where this motor neuron runs.
- amyotrophic because damage to the second motor neuron causes muscular amyotrophy, that is, the loss of mass, weakness, and fasciculations (Rowland et al 2001).

The disease is a proteinopathy, as following post-mortem histological examinations of the spinal cord of ALS patients, accumulations of protein aggregates were observed, which together with the degeneration of motor neurons, mitochondrial alterations, accumulations of neurofilaments in the surviving motor neurons the main causes of neurodegenerative damage (figure 2).

The rate of progression of the disease varies from one individual to another; it can be influenced by the site of onset but is usually rapid with an average survival of only 2-3 years from the onset of symptoms. The causes of the disease, in most cases, are not well defined; Clinical evaluations have allowed identifying the involvement of multiple cellular functions in the motor neurons of the patients, excessive increase in muscle excitatory tone, accumulation of misfolded proteins, impaired axonal transport, enhanced calcium metabolism (Cleveland et al 2001).

The disease can be classified according to various criteria:

- Environmental and lifestyle factors: Various studies have highlighted a higher frequency of ALS patients in groups of athletes than in the general population. Although it has not yet been confirmed, physical activity could represent a possible risk factor (Hardiman. et al 2017).
- Depending on the involvement of the first or second motor neuron causing a different clinical onset (bulbar or spinal).
- Genetic causes.

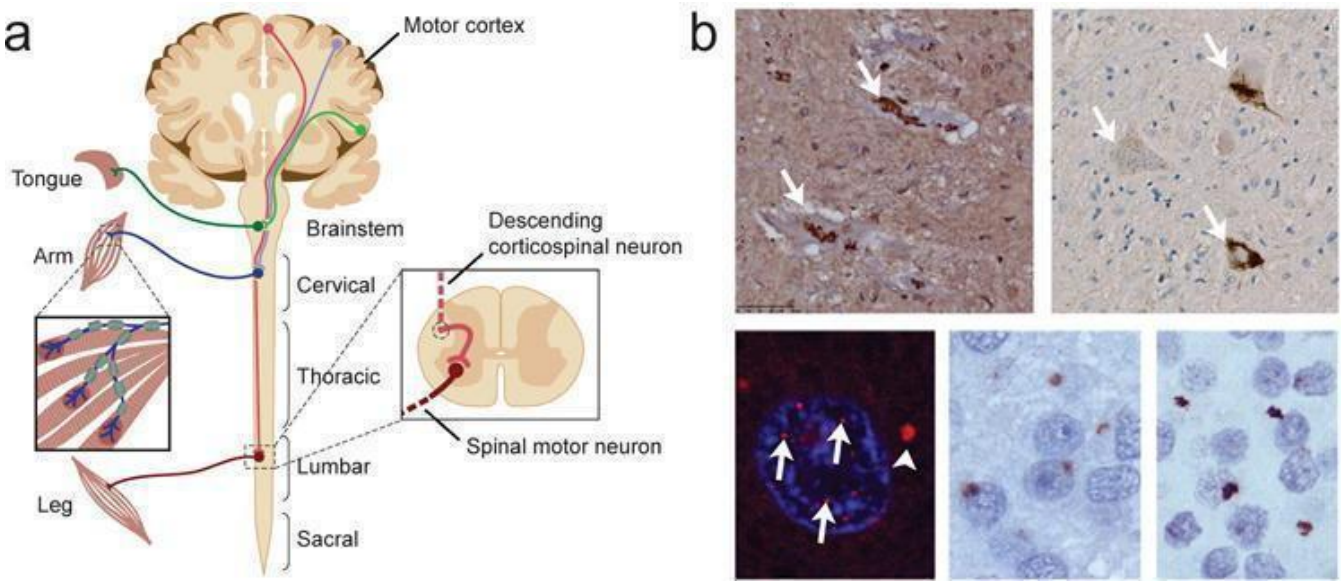


Figure 2. ALS primarily affects the upper motor neurons, which range from the motor cortex into the brain stem and spinal cord, and the lower motor neurons that arrive in the skeletal muscles. B ALS subtypes with typical pathological features: in the left panel aggregates of SOD1 in the spinal motor neuron in fALS patients, on the right cytoplasmic inclusions of TDP-43 in sALS patients [source: Taylor et al 2016].

ALS is the fourth largest neurodegenerative disease, after AD, PD, and Pick's disease, plus common with an incidence, worldwide, from 1 to 2.5 cases per 100,000 inhabitants and one prevalence from 4 to 8 per 100,000 (Logroscino et al 2005). This pathology, for 90% of patients, is sporadic (sALS) but thanks to the discovery of genes involved in the onset of the

latter, familial forms of ALS (fALS) have been studied which represent about 10% of total cases (Rowland 2001). The incidence rate of ALS patients in Europe ranges from 2 to 3 cases per 100,000 individuals (Hardiman et al. Et al 2017), worldwide has not yet been established with certainty. Men have a higher incidence rate (3 / 100.00 per year) than women (2 / 100.00 per year), although the incidence is similar in cases of familial ALS. ALS is a rare disease that mainly affects people between the ages of 50 and 60 for patients with sporadic ALS and between the ages of 40 and 50 with ALS family (Kiernan et al 2011). The onset and course of the disease may vary from one individual to another and there are several clinical subtypes of the disease.

a. Clinical heterogeneity of ALS

Familial and Sporadic ALS

The disease can present with a sporadic phenotype in most clinical cases (sALS) or with a familial phenotype (fALS) in about 10% of people with this disease (Byrne et al 2011). However, the clinical difference between fALS and sALS have not yet been understood and clarified, since different pathological and genetic links, suggest the hypothesis that these two forms have a link. From a clinical point of view, it is not possible to clearly distinguish sALS from fALS. The conditions have similar pathological patterns, such as the presence of similar inclusions, neurological conditions, and differences in patients with SOD1 and FUS mutations (Saberi et al 2015). In addition, Mendelian inheritance occurs in half of the patients with fALS, particularly autosomal dominant. In most cases of ALS, the genetic aspect is less clear and more complex, and this further enriches the characteristic of being a highly heterogeneous and complex pathology to study. In addition, there are cases where there is only one other family member with this condition. These cases are defined as

probable fALS when the sick family member is a close relative and possible fALS when the sick is distantly related. The most important link between sALS and fALS is the analysis of the genes involved in fALS whether they are mutated in patients with the uncertain sporadic form of the disease (Byrne et al 2011). ALS gene mutations were detected in about 15% of the sporadic forms (Lattante et al 2012, Renton et al 2014).

Age of Onset

ALS affects people in adulthood, particularly between the ages of 60 and 80. From a recent publication by Longinetti et al, a prevalence of ALS between 4.1 and 8.4 per 100,000 has been reported on the incidence of the disease (Longinetti et al 2019). Clinical cases with early onset are very rare and are called "juvenile ALS". This form of ALS has the difference of being in most cases fALS, compared to cases of classic ALS, which usually has an autosomal recessive inheritance. Juvenile ALS has distinctive clinical features with predominant signs of UMN, mostly affecting men, and survival exceeding five years. However, it has not yet been understood and clarified whether the clinical features of juvenile ALS are linked to a pathological mechanism other than classical ALS. It is extremely difficult to diagnose classic sALS cases with an onset at 20 years. The vast majority of diagnosed cases have a de novo mutation in the FUS gene.

ALS forms: Classic ALS, LMN and UMN Form

This neurodegenerative disease is characterized by a combination of LMN and UMN clinical and physiological signs. However, the combination of UMN and LMN is highly variable, and the clinical manifestations of ALS are on a continuum whose extremes are represented both by patients who show LMN dysfunction and by patients with signs of UMN dysfunction. Charcot-type ALS is the most common form, it is represented by more than 70% of diagnoses, these cases are characterized by the presence of symptoms of LMN with pyramidal signs. ALS patients with no symptoms of UMN with no clinical signs present are

labeled as having progressive muscle atrophy (PMA) and represent approximately 5-10% of cases (Sabatelli et al 2011)

At least 5% of patients with motor neuron disease have a pyramidal shape with predominant spinal-bulbar onset, known as primary lateral sclerosis (PLS). The onset of PLS is similar to that of the classic form of ALS and the duration of the disease is longer. ALS patients with pyramidal signs consist mainly of spinal-bulbar insufficiency and have dominant amyotrophic lateral sclerosis of upper motor neurons (ALS UMN-D). These signs are also associated with mild signs of LMN. This form is more frequently found in young male patients and has a better prognosis than classical ALS (Sabatelli et al 2008, Sabatelli et al 2011, Swinnen et al 2014).

Site of Onset

ALS arises at a random site and progresses to involve other areas of the body or nearby parts of the body. The ALS bulbar onset is represented by about a quarter and is initially shown in the muscles innervated by motor neurons residing in the medulla. Spinal-onset ALS is represented by about one-third of patients who have symptoms in the upper limb muscles and one-third in the lower muscles whose motor neurons are found in the spinal cord. The area of onset can be an important tool for distinguishing the phenotypes of the disease. Bulbar onset is more common in female patients and has a worse prognosis than the spinal form.

Diagnosis

Even today, a specific examination is not available that allows to immediately ascertain the disease, but various medical investigations and clinical evaluations must be carried out to exclude other possible causes of the symptoms manifested, but to reach the definitive response it will be necessary to evaluate the progression of the disease.

To define the levels of diagnostic certainty, reference is made to the so-called El Escorial criteria which allow you to evaluate (Brooks et al. 2000):

- history of progressive weakness affecting various regions, such as the bulbar area (which affects speech and swallowing), cervical regions (which affects the upper limbs), and lumbar regions (which affects the lower limbs).
- involvement of the lower motor neuron (presence of denervation through electromyography) or upper (active tendon reflexes).

These criteria make it possible to define a clinically definitive or suspected diagnosis of ALS (Reniers et al 2017).

The standard diagnostic parameters do not include the assessment of the patient's cognitive and behavioral status, which is why screenings have been introduced, such as Cognitive and Behavioral ALS Screen (ECAS), which assess any cognitive and behavioral changes and are highly sensitive and low specificity (Pinto-Grau et al 2017).

To date, the most widely used evaluation scale in clinical practice is the ALS Functional Rating Scale (ALSFRS) by which it is possible to evaluate the patient's ability to carry out functional activities independently and therefore monitor the progression of disabilities (Cedarbaum 1999). Based on various areas of function, including bulbar, respiratory, and upper and lower limb functionality the patient is scored from 40 (functional integrity) to 0 (inability to perform any activity) (An et al. 2019).

ALS is a pathology with highly heterogeneous phenotypic manifestations, it is very important to have the availability of biomarkers to make a correct diagnosis and prognosis. Through the Magnetic Resonance Imaging (MRI) technique, it is possible to evaluate the degeneration at the corticospinal level with the involvement of the frontal and temporal regions in ALS patients. The use of electromyography (EMG) allows the evaluation of the

functionality of the nerves and peripheral muscles (Schuster et al 2017). Unfortunately, neither through imaging nor electromyography has it been possible, to date, to identify reliable biomarkers.

Etiopathogenesis

The pathogenetic processes underlying the disease are particularly complex and multifactorial, still little known today. It has now been clarified that the death of motor neurons is not due to a single event but is caused by the combination of various mechanisms that undergo alterations, including oxidative stress, mitochondrial dysfunction, excitotoxicity, formation of protein aggregates, and alterations in the metabolism of RNA (Strong et al 2001).

Oxidative stress

The damage caused by oxidative stress is highly implicated in various neurodegenerative diseases, including amyotrophic lateral sclerosis (Barber et al 2006). Free radicals cause an alteration in various cellular components, including proteins, lipids, and nucleic acids. Reactive oxygen species (ROS) are the product of aerobic metabolism, most of which derive from the mitochondrial respiratory chain due to a dispersion of electrons following an incomplete reduction of molecular oxygen, during oxidative phosphorylation, to produce hydrogen peroxide (H_2O_2) and the superoxide anionic radical (O_2) (Halliwell et al 1999). The latter alone is not highly reactive but can react with the nitric oxide radical (NO), produced by nitric oxide synthase, to produce peroxynitrite (ONOO−) (Pryor et al 1995). Hydrogen peroxide is also a poorly reactive oxidizing agent, but it slowly decomposes to form the hydroxyl radical (OH) which represents a highly reactive species (Halliwell et al 1999).

Both peroxynitrite and hydroxyl radicals cause an alteration of the protein conformation, destroy the active sites of enzymes, modify the physiological properties of cell membranes,

and induce mutations in the DNA. The cell activates mechanisms to counteract the production of ROS and various enzymatic species with antioxidant activity come into play, including superoxide dismutase (SOD) which catalyzes the removal of superoxide radicals (Fridovich 1995).

Motor neurons are particularly vulnerable to the toxic action of ROS and the discovery of mutations in SOD1 revealed a clear association between oxidative stress and the onset of the disease. Various mechanisms seem to be involved in the acquisition of the toxicity of SOD 1 based on the altered antioxidant activity:

- loss of its dismutation function, leading to an increase in the levels of superoxide radicals (Beckman et al 1993).
- the mutated SOD1 protein is not only inactive but inhibits the physiological activity of SOD1 expressed by the wild-type 17 allele.
- increased SOD1 activity causing levels of hydrogen peroxide and hydroxyl radicals (Rosen et al 1993).
- Oxidative stress is one of the mechanisms that come into play in cases of ALS linked to mutations in the gene that codes for SOD1 since this enzyme has the specific function of removing the reactive superoxide anion species formation of protein aggregates (Kaur et al 2016).

b. Genetic Characteristics of ALS

The high phenotypic heterogeneity of the clinical face of ALS pales in comparison to its biological heterogeneity. One of the first goals of ALS genetic research has been to identify variants in genes and to fit these genes into our current understanding of ALS biology. For almost 15 years, the only gene associated with fALS was the Cu-Zn superoxide dismutase

1 (SOD1) gene (Rosen et al. 1993), which accounts for 20% of fALS cases. The identification of SOD1 mutations in 1993 ushered in the molecular era of ALS research, and significant insight into ALS pathogenesis has been gained through the identification of pathways directly affected by the toxicity of mutant SOD1. Two main discoveries in mutant SOD1-mediated ALS in the models are the demonstration that the SOD1 protein aggregates produce a toxic gain of function that causes neuronal loss (Boillée et al., 2006; Yamanaka et al., 2008). A major shift in our understanding of ALS pathogenesis occurred in 2006 with the identification of a 43-kDa transactive response (TAR) DNA binding protein (TDP-43) as a key pathological substrate of cellular inclusions in sALS and non- SOD1 fALS, and frontotemporal lobar degeneration with ubiquitinated inclusions (FTLD-U) (Neumann et al., 2006).

It was soon followed in 2008 by the successful discovery of dominant mutations in the TDP-43 gene as a primary cause of ALS (Sreedharan et al., 2008), thus providing the proof of principle that aberrant TDP-43 can trigger neuronal degeneration and cause ALS. A total of 44 TDP-43 mutations (Da Cruz and Cleveland, 2011) have since been reported in ALS patients with or without an apparent family history (Da Cruz and Cleveland, 2011; Kabashi et al., 2008; Mackenzie et al., 2010; Sreedharan et al., 2008). The identification of TDP-43 mutations was followed in tandem by the discovery of mutations in another RNA/ DNA-binding protein, FUS/TLS (fused in sarcoma and translocated in liposarcoma) in 2009, as a primary cause of fALS (Kwiatkowski et al., 2009). Both in TDP-43- and FUS -linked ALS, the age and size of disease-onset and clinical phenotype are variable, as in sALS, and incomplete penetrance has been documented for several of these mutations (Da Cruz and Cleveland, 2011; Kabashi et al., 2008; Mackenzie et al., 2010; Sreedharan et al., 2008). Most patients with TDP-43 and FUS gene mutations develop the classical ALS phenotype, with characteristic pathological features of the disease.

There are other disease-related genes, including C9orf72, especially the expansion of the non-coding hexanucleotide GGGGCC, in healthy subjects <30 repeats are found while in ALS patients up to 1600 thus causing the production of an abnormal protein. C9orf72 was first investigated simultaneously in two studies (Renton et al 2011, DeJesus-Hernandez et al 2011) and was identified in families with multiple cases of ALS (Morita et al 2006). The first variant with an important influence on ALS genetic is C9orf72 HRE, explains about approximately 40% of fALS and 7% of sALS (Renton et al 2014). However, the frequency of this variant is strongly dependent by population (Zou et al 2017). To date, the physiological function of this protein remains to be clarified, in patients these nucleotide expansions form highly stable RNA G-quadruplexes that could influence the stability of telomeres, transcription, translation and transport of RNAs (Fratta et al 2012).

3. Pharmacological therapy

To date, there are no effective therapies in the treatment of ALS or capable of blocking its clinical progression, are mainly side-by-side palliative therapies such as ventilation non-invasive (NIV) (Bach et al 2002), tracheostomy and percutaneous endoscopic gastrostomy (PEG) since the disease progresses variably until complete degeneration motor neurons (Kaur et al 2016).

Despite the numerous clinical trials conducted in the last decade, the only drug approved by the Food and Drug Administration (FDA) for the treatment of this pathology is Riluzole (6-(trifluoromethoxy) benzothiazole-2-amine), capable of slowing down the progression of the disease and therefore lengthen the patient's median survival from 3 to 6 months. To date, there is no evidence that Riluzole exerts a therapeutic effect on the function of motor neurons, in the lungs, on the fasciculations, or in the muscles.

(https://www.ema.europa.eu/en/medicines/human/EPAR/rilutek).

It acts specifically by blocking the sodium channels by reducing the entry of calcium and the excessive stimulation of glutamate in the CNS (Reynolds et al 1996). It appears to have an antioxidant effect, i.e., capable of reducing the oxidative stress induced by various oxidizing agents (Storch et al 2000).

However, this drug is not able to improve the patient's quality of life or improve symptoms, which is why it arouses strong interest in researching more effective drugs.

In 2017, Radicava (Edaravone) was approved by the FDA for the treatment of ALS patients, given the many studies that highlight its potential in the therapeutic field (Maragakis et al 2017).

a. Edaravone

Edaravone (3-methyl-1-phenyl-2-pyrazoline-5-one) was first approved in Japan in 2001 for the improvement of neurological symptoms and associated functional disabilities of acute ischemic stroke (Müllges et al 2003). Properties chemicals of Edaravone and in vitro and in vivo studies suggest that it may have, primarily, an effective antioxidant. It also has specific chemical-physical properties thanks to which it is distributed both in hydrophilic conditions, for example, at the cytoplasmic level, but also in lipophilic conditions, for example at the cell membrane level. It is known to be a free radical scavenger, it acts mainly by reducing lipid peroxides, so efficient as known antioxidants such as vitamin E and ascorbic acid, but also hydroxyl radicals and peroxynitrites, other reactive oxygen species (Yamamoto et al 1996). Edaravone shows a protective effect at the neuronal, and glial levels (microglia, astrocytes, and oligodendrocytes) and endothelial vascular cells against oxidative stress. To date,

however, its exact mechanism of action for the treatment of ALS is still little known (Takei et al 2017).

Based on these findings, various clinical trials were conducted to evaluate the possible neuroprotective effect of Edaravone in this pathology.

A phase II trial was conducted to evaluate the safety and efficacy in ALS patients, observing a slowdown in motor neuron degeneration, and from a point of view, no adverse drug reactions occurred. In addition, the 3NT levels were analyzed and found to be reduced in the cerebrospinal fluid of almost all patients undergoing part of this trial, suggesting that a potential effect of Edaravone could be related to the protection of neuronal cells from nitrosative stress (Yoshino et al 2006). Over the years, various clinical trials have been conducted that show not only numerous failures but suggest that the effects of Edaravone are reliable and functionally significant (Maragakis et al 2017).

b. Riluzole

Riluzole (trade name: Rilutek) has been in use for the treatment of ALS since 1995 after the completion of a Phase III clinical trial indicated its effectiveness (Bensimon et al 1994). It has been shown that the drug not only increases survival time by approximately three months but also improves limb function in ALS patients (Miller et al 2007), although it appears to not affect overall muscle strength. This drug has been hypothesized to work through many different pathways, the best characterized of which is the reduction of glutamate excitotoxicity supposedly present in ALS through the blockage of N-Methyl-d-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) channels. Other potential mechanisms of action have also been proposed, including the selective blockage of voltage-gated calcium channels, sodium ion channels, the modification

of GABAergic systems, or a synergistic combination of all these effects, reducing neuronal damage and death (Cheah et al 2010).

Paradoxically, the purported benefits of Riluzole may not transfer effectively to animal models. A recent publication by Hogg et al. found that the drug did not increase lifespan or improve muscle function in three separate rodent models of ALS, indicating that our current rodent models are insufficiently recapitulating the pathophysiology of this disease (Hogg et al 2018).

4. Protein aggregation

Pathological lesions caused by protein aggregation are characteristic of numerous neurodegenerative diseases including ALS. The generation of these aberrant proteins can be attributed to numerous factors, including mutations, conformational alterations, and post-translational modifications that alter their native structure; deleterious environmental conditions such as altered pH, temperature, and oxidative state that influence protein folding; and deficiencies in the protein quality control system caused defections in the degradative systems or the chaperone protein system (Duranti and Villa 2023).

These anomalous proteins often have high hydrophobicity, which makes them prone to aggregation with a high propensity to organize themselves into complex structures such as oligomers, fibrils, and inclusions with a decreasing solubility (Kaganovich et al 2008). Many studies have identified oligomeric and proto-fibrillar intermediates as the species of aggregates most responsible for pathological processes, while it seems that insoluble and inert inclusions may have a protective function (Miller et al 2015, Baglioni et al 2006). This idea is supported by recent evidence showing that aberrant proteins are actively sequestered at specific storage sites during periods of proteotoxic stress so that they can

then be sorted into different degradation systems (Tyedmers et al 2010, Kaganovich et al 2008, Arrasate et al 2004). Therefore, these inclusions are protected if they are efficiently disposed of by the cell.

Amyotrophic lateral sclerosis is characterized by protein aggregates mainly of three proteins: a 43 kDa DNA-binding TAR protein (TDP-43) (Neumann et al 2006, Arai et al 2006), Cu / Zn superoxide dismutase (SOD1) (Bruijn et al 1998), or fused in sarcoma (FUS) (Kwiatkowski et al 2009, Vance et al. 2009, Duranti and Villa 2023). From a 2016 work, the identification of disease-associated mutations in many of the proteins identified in inclusions in ALS emerged from genetic screening (Taylor et al 2016). This evidence implies that the protein aggregation condition is not only a marker of disease but is also linked to the etiology and patho-mechanisms of neurodegeneration. Many of the proteins encoded by fALS-associated genes can also be found in neuronal protein aggregates of patients with sALS in their wild-type form (Blokhuis et al 2016); indicating that misfolding and aggregation are not entirely by mutation.

TDP-43 mislocalization and aggregation are observed in ~97% of all ALS cases, including all sALS cases, whereas SOD1 (2%) and FUS (1%) inclusions are associated with the remaining cases (Ling et al 2013). The most common genetic cause of ALS is the expansion of an intronic hexanucleotide repeat (GGGGCC) in chromosome 9 open reading frame 72 (C9orf72) (DeJesus-Hernandez et al 2011, Renton et al 2011). This expansion is present in 5–10% of sALS cases and 40–50% of fALS cases (Byrne et al 2012, Majounie et al 2012, Umoh et al 2016). In most ALS cases with C9orf72 mutations, TDP-43 aggregates are also present, and some evidence suggests that the deposition of DPRs initiates TDP-43 proteinopathy (Solomon et al 2018, Nonaka et al 2018, Khosravi et al 2017, Cooper-Knock Et al 2012). Although our understanding of the fundamental physiological mechanisms underlying ALS is incomplete, the key neuropathological hallmark of the disease is the

aggregation and accumulation of ubiquitinated proteinaceous inclusions in the cytoplasm of degenerating motor neurons and surrounding support cells (Saberi et al 2015). The anatomical regions of the CNS where these inclusions form include the motor cortex, brain stem, spinal cord, frontal cortex, temporal cortex, hippocampus, and cerebellum (Al-Chalabi, et al 2012). ALS is characterized by several distinct pathological protein inclusions (Saberi et al 2015). The type of inclusions present in a case varies depending on the ALS genotype or lack thereof. By assessing the levels and distribution of phosphorylated TDP-43 (pTDP-43) throughout the CNS, it was suggested that the progression of pTDP-43 pathology in ALS could be divided into four stages. Initially, pTDP-43 is found in the agranular neocortex, spinal motor neurons, and bulbar somatomotor neurons. Pathology then spreads sequentially through axonally connected areas throughout the CNS (Braak et al 2013). Both bulbar onset and spinal cases showed a similar pattern of TDP-43 pathology. Interestingly, there is reported to be a statistically significant correlation of pTDP-43 pathology with cell loss in several CNS regions, including beyond motor areas, as the disease progresses. Aggregates of pTDP-43 have also been observed in the interneurons of Clarke's column and the cerebellar dentate nucleus with significant loss of dopaminergic neurons of the substantia nigra (Brettschneider et al 2014). Neuronal lesions also correlate with similar lesions in grey and white matter oligodendroglial cells and astrocytes (Geser et al 2008). Whether these inclusions are a result, or a cause of toxicity remains to be conclusively demonstrated. Such diversity of inclusion characteristics indicates that different biochemical pathways govern their formation, yet the clinical phenotype of ALS is not specific to the observed pathological features.

While the deposition of TDP-43, SOD1, and FUS into aggregates is well established, how, and indeed whether the protein aggregates cause cell death remains under debate. There

are many possible mechanisms for how protein aggregation in ALS may result in cell toxicity (Figure 3).

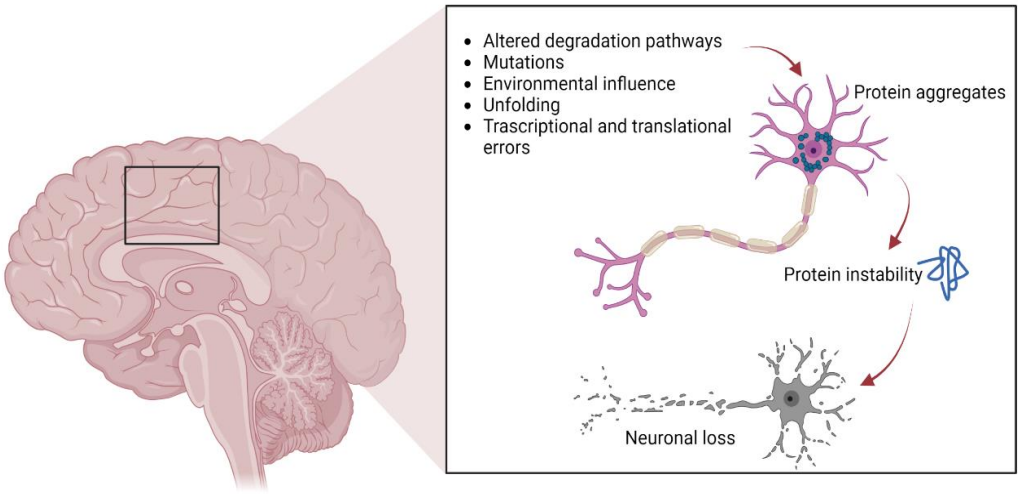


Figure 3: The pathways involved in protein aggregation in neuronal cells, leading to the neuronal death typically found in neurodegenerative diseases [source: Duranti and Villa 2023]

There is strong evidence linking many aggregating proteins, including SOD1, TDP-43, and FUS, to mitochondrial dysfunction and, indeed, complete mitochondrial degeneration (Nakaya et 2018, Tafuri et al 2015). The mechanism for this is yet to be elucidated, but possibilities include blocking mitochondrial transport pores, accumulating inside the intermembrane space, and/or activating the mitochondrial unfolded protein response. Protein aggregation is also known to disrupt the proteostasis network, including impairment of the key protein degradation pathways, the ubiquitin-proteasome system, and autophagy (Medinas et al 2017). The sequestration of other essential cellular molecules into protein aggregates would also be detrimental to cell health by inducing a loss of function in essential proteins (Yang et al 2016). Indeed, the expression of amyloidogenic proteins in cultured cells is found to sequester other proteins belonging to essential biochemical pathways such as proteostasis, cytoskeletal maintenance, chromatin organization, and RNA metabolism (Olzscha et al 2011) some pathways of which are found to disrupted in ALS (Taylor et al

2016, Ciryam et al 2017). Finally, evidence suggests protein aggregation in the cytoplasm inhibits nucleocytoplasmic transport, including the transport of mRNA, disrupting global RNA metabolism. Considering the diversity of downstream effects that protein aggregation has on cellular health, it remains difficult to identify specific interactions that would be viable therapeutic targets, other than the key aggregating proteins themselves (Woerner et al 2016).

a. Transactive response DNA binding 43-kDa (TDP-43)

TDP-43 was first isolated in 1995 when it was observed its ability to bind the Transactivation Response Region (TAR) of HIV DNA, hence the name TAR DNA binding protein (Ou et al., 1995) and was subsequently found in the human brain and several cell culture systems. In 2015, an interesting study showed for the first time that TDP-43 is expressed in muscle cells of patients with Facioscapulohumeral dystrophy (FSHD) (Homma et al, 2015).

TDP-43 has a ubiquitous expression in both humans and rodents, which is why it was possible to conduct multiple in vivo experiments on mutations, involving this protein, related to the pathology. It is a protein of 414 amino acids with a molecular weight of 43 kDa, encoded by the TARDBP gene located on chromosome 1, a member of a heterogeneous family of proteins that bind RNA, which is called hnRNP (heterogeneous ribonucleoprotein) (Lagier-Tourenne et al., 2010).

From a structural point of view, it consists of (Figure 4, Duranti and Villa 2023):

- Nuclear Localization Signal (NLS)
- Two regions for the recognition of RNA (RRM1 of about 106-175 amino acids and RRM2 of 191-162 amino acids) 64 which exhibit an export signal nuclear (NES)
- a N-terminal end.

- a C-terminal region that comprises an amino acid sequence rich in glycine (of about 274 413 amino acids) which mediates interaction with other proteins belonging to the hnRNP 65 families.

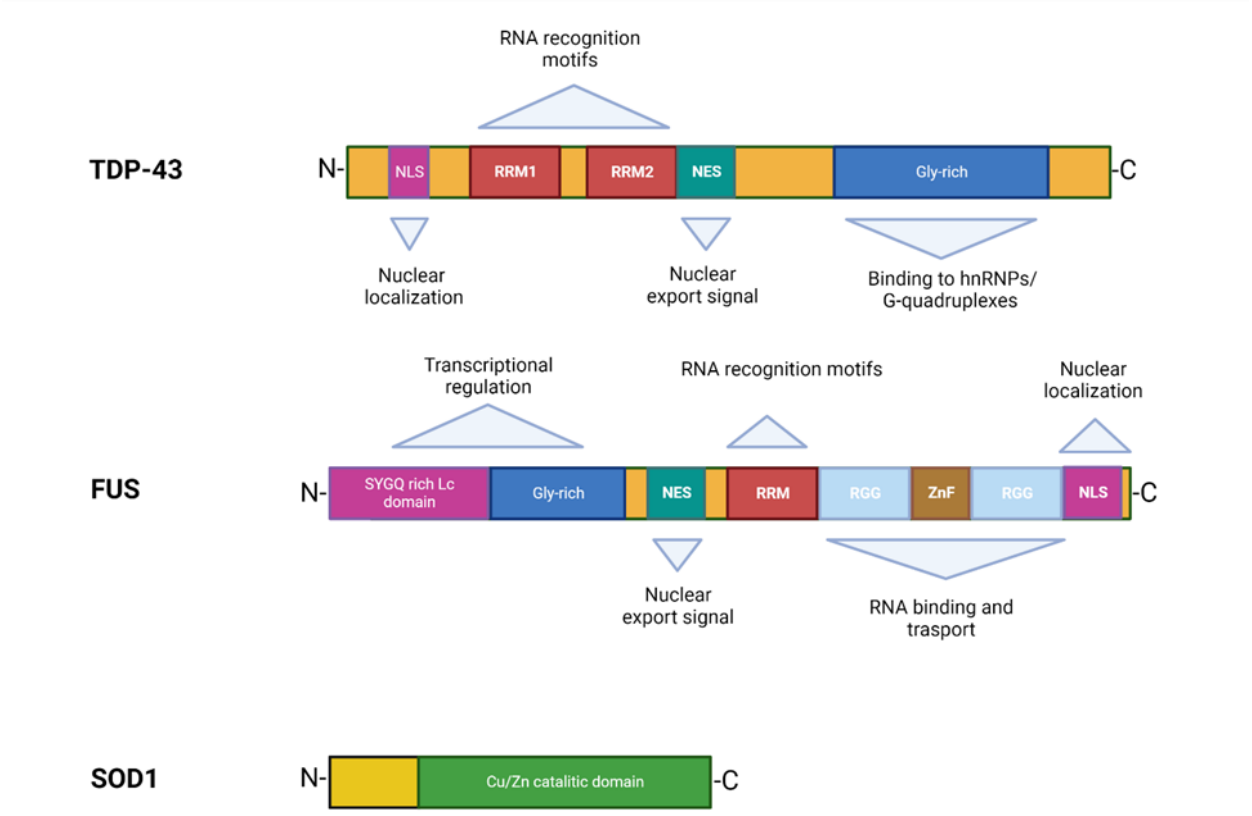


Figure 4: Structures and functional domains of TDP-43, FUS and SOD proteins [source: Duranti and Villa 2023].

The cellular function of TDP-43 is still unknown, but various studies have shown that this protein plays a fundamental role in various biological activities including regulation of gene transcription, control of splicing processes, and maintaining the stability of mRNA (Law et al 2006). It is a predominantly nuclear protein whose levels are strictly controlled through a self-regulation system, mainly involving the C-terminal region. Consequently, it is conceivable that mutations in this domain can interfere with the homeostatic control process by altering the recruitment of complexes required for self-regulation (Ayala et al 2011).

The TDP-43 protein is mainly involved in two neurodegenerative diseases: sporadic ALS and frontotemporal dementia (FTLD) (Arai et al 2006, Neumann et al 2006).

Furthermore, the gene coding for TDP-43 was investigated, revealing missense mutations in the C-terminal domain with dominant inheritance, these mutations are sufficient to cause the familial form, underlining its role in the pathogenesis (Gitcho et al 2008). Mutations affecting the TDP-43 protein represent about 5% of sporadic ALS and about 3% of familial ALS cases (Andersen et al 2011).

In pathological conditions, the protein tends to form ubiquitinated inclusions in the central nervous system, in the hippocampus, neocortex, and spinal cord. A further feature that differentiates it from the wild-type form is its localization that in the neurons of patients, of both pathologies, insoluble protein aggregates tend to form in the cytoplasm with a consequent reduction in the levels of the TDP-43 protein in the nucleus, unlike in the subjects healthy is expressed in the nucleus (Neumann et al 2006).

The altered localization of TDP-43 in the cytoplasm of neurons in ALS patients, regardless of the presence of genetic mutations, seems to be related to a pathogenetic mechanism associated with a loss of function of the nuclear protein in the regulation of mRNA transcription and splicing processes (Law et al 2006). The formation of cellular inclusions of TDP-43 induces toxicity to the cell, in this case, the protein acquires a toxic function (gain-of-function) (Mackenzie & Rademakers 2018).

The existence of a possible correlation between TDP-43 and the expression of the mutated SOD1 protein (Jeon et al 2018), previously believed to be two separate pathways, causative of neurodegeneration, has recently been highlighted.

Furthermore, the overexpression of this protein in neuronal cells and oligodendrocytes causes progressive degeneration of motor neurons in the SOD1 G93A (Lu et al 2016)

transgenic mouse models. In the study conducted by Jeon et al., they obtained confirmation that the increased expression of TDP-43 results consistently in ALS cell models expressing the human SOD1 G93A mutation and that the presence of the wild-type SOD1 protein has been associated with cytoplasmic accumulation of mutated TDP-43 in fALS patients and wild-type TDP-43 in sALS patients. Furthermore, the protein undergoes various modifications, such as phosphorylation, and translocates by passing into the cytoplasm of neurons, astrocytes, and microglial cells in SOD1 G93A transgenic mice (Jeon et al 2018). A further aspect is related to the accumulation of TDP-43 within ubiquitinated inclusions, which could lead to the altered regulation of genes or factors involved in the degradation processes of intracellular proteins, thus contributing to TDP-43 proteinopathy.

The cytoplasmic aggregates of TDP-43 are constituted by the hyperphosphorylated form of the ubiquitinated protein that is cleaved generating C-terminal fragments (Ayala et al 2011, Neumann et al 2006). Caspase 3 acts mainly involve cleavage of TDP-43 into two fragments with a molecular weight of 25 kDa and 35 kDa (defined respectively as TDP-25 and TDP-35), which are highly prone to aggregate (Zhang et al 2007). These fragments, deriving from the entire wild-type TDP-43 protein chain, induce greater toxicity, contributing to the loss of function of TDP-43. Therefore, they must be efficiently cleared from cells to prevent their aggregation and the sequestration of other important neuronal components. The clearance of aberrant or misfolded proteins is mediated by the protein quality control system (PQC) (Cereda et al 2016). This system is composed of chaperone and co-chaperone proteins that recognize and bind damaged proteins and direct them towards degradation processes.

It is plausible that the cytoplasmic accumulation and aggregation of TDP-43 results in both a loss of function and a gain of toxic function, given the integral roles of nuclear TDP-43 in mRNA regulation and transport (Broeck et al 2014, Ederle et al 2017). The C-terminal low-complexity domain of TDP-43 controls its ability to undergo liquid-liquid phase separation

(Conicella et al 2016). Some evidence suggests that TDP-43 partitioning into stress granules is responsible for the formation of inclusions (Zhang et al 2019, Mackenzie et al 2017), whereas other evidence suggests that phase transitions independently of stress granules are responsible for pathological aggregation (Mann et al 2019, Gasset-Rosa et al 2019). Evidence also suggests that TDP-43 can aggregate through a ubiquitin-proteasome system-associated pathway (Farrawell et al 2015).

b. Cu/Zn superoxide dismutase (SOD1)

SOD1 mutations were the most frequently identified cause of fALS, before the discovery of C9orf72 expansion, accounting for ~20% of all patients. In 1993, Rosen et al. described eleven disease-associated mutations in the SOD1 gene, encoding for the Cu/Zn superoxide dismutase, a cytoplasmic enzyme responsible for the catabolism of superoxide radicals to hydrogen peroxide and molecular oxygen (McCord and Fridovich, 1969). SOD1 is ubiquitously expressed, highly conserved, and represents ~1% of all cytoplasmic proteins. Superoxide dismutase 1 (SOD-1) is a polypeptide of 153 amino acids with ubiquitous expression, composed of a binding site with a zinc atom and another for a copper atom (Figure 4). Predominantly present in the cytoplasm of cells, although it also appears to be present in the intramembrane space of the mitochondria and the nucleus (Liu et al 2004). The gene coding for this protein is located on chromosome 21(Wood-Allum et al 2006), a very small structural point of view composed of four exons separated by four introns. Its role is to convert superoxide anions, toxic to the cell, into peroxides of hydrogen and oxygen (Kaur et al 2016). SOD1 also performs a pro-oxidant activity including peroxidation with the production of hydroxyl radicals and nitration of tyrosines (Rowland et al 2001). This protein, as ubiquitinated, is widely expressed and constitutes about 0.5- 0.8% of soluble proteins in the human brain (Pardo 1995). To date, more than 140 SOD1 mutations have been identified (http://alsod.iop.kcl.ac.uk), most of them are missense or cause the replacement

of one amino acid with another while keeping the polypeptide chain intact, 11 are frameshift deletions and 5 insertions that cause premature termination of protein 42 expressions.

These mutations have been observed in approximately 20% of fALS patients and only 2% of sALS cases (Andersen et al 2006) and are characterized by inter-family and intra-family variability in phenotype concerning the age of onset and disease duration (Cudkowicz et al 1997). Common are G93A, A4V, H46R, D90A, which are inherited as a dominant trait, except for the D90A mutation, recessive inheritance in the Scandinavian population and dominant in the rest of the world (Andersen et al 1996).

The most studied mutation is G93A, which sees the substitution of glycine in position 93 with alanine, it is relatively rare, but it was one of the first to be studied in a G93A transgenic mouse model, and what concerns it is only one. Moderate alteration of its enzymatic activity that appears to be sufficient due to the neurodegenerative pathology in the mouse (Valentine & Hart 2003).

The two principal critical features of SOD1 mediated toxicity are toxic gain of function and the pathogenesis of the ubiquitously expressed mutant SOD1 is a non-cell autonomous process. This means that disease onset is driven by mutant protein synthesized inside motor neurons. Mutated SOD1 protein interacts specifically with neurofilament-light chain mRNA and the dynein/dynactin complex, thus inducing cytoskeletal defects or altering axonal transport. Furthermore, has an increased tendency to form aggregate-prone monomers, and the degree of instability correlates inversely with the length of survival; this suggests that increased propensity to aggregate may be the unifying common denominator for the 119 diverse SOD1 mutations (Andersen et al 2006). Some investigators (Bosco et al., 2010b) found misfolded SOD1 in motor neurons in a subset of patients with sALS without SOD1 mutations, thus suggesting a role for wild-type SOD1 in sALS, possibly after secondary (oxidative) modification (Robberecht and Philips 2013). Finally, aggregation and spreading

ability of mutant SOD1 has been demonstrated in cultured cells (Grad et al 2011) as well as seeding ability using spinal cord homogenate (Chia et al 2010).

To date there are no explanations that define how mutated SOD1 causes the disease, initially it was hypothesized that these mutations damage the enzymatic activity of the protein, causing an increase in the levels of reactive oxygen species (ROS) with consequent oxidative stress and death of neuronal cells (Deng et al 1993). Subsequent studies have shown that mutated forms of the protein kept their catalytic activity intact without correlating residual enzyme activity, clinical progression, and disease phenotype (Radunovic et al 1007). Recently, in vitro, and in vitro models Vivo that reproduce the pathology have highlighted that the dismutation reaction mediated by SOD1 appears to be normal or increased. This aspect has allowed us to advance a further hypothesis on the activity of the mutated SOD1 related to the acquisition of a new function (gain-of-function), that is, it induces toxicity to motor neurons.

The mutated SOD1 protein accumulates in the oligomeric form and subsequently forms cytoplasmic aggregates, which could induce neuronal cell death by sequestering other cytoplasmic proteins essential for neuronal survival, blocking the ubiquitin/proteasome system with consequent loss of chaperone proteins, destruction of mitochondria, blockade of cytoskeletal or axonal transport (Ticozzi et al 2011).

The mutated SOD1 seems to compromise the two main degradation processes of the cell: the proteasome pathway and autophagy (Bendotti et al 2012).

c. Fused-in-Sarcoma (FUS)

FUS is a protein encoded by 15 exons; the gene contains an N-terminal domain enriched QGSY region, an RRM, an arginine-rich region, an arginine and glycine-rich (RG rich) region, and a C-terminal zinc finger motif (Figure 4) (Sreedharan et al., 2008; Lanson and

Pandey, 2012). As with the TDP-43 gene, most pathogenic FUS mutations are clustered in the glycine-rich region, and these mutations are found in about 5% of fALS cases and 1% of sALS cases (Da Cruz and Cleveland, 2011; Lanson and Pandey, 2012). FUS has a nuclear distribution in a lot of cell types and the analysis of the tissue's principal involved in ALS patients with FUS mutations has shown abnormal FUS cytoplasmic aggregates in neurons and glial cells (Da Cruz and Cleveland, 2011; Kwiatkowski et al., 2009; Lagier-Tourenne et al., 2010). These mislocalized FUS protein inclusions are not correlated with TDP-43, suggesting that this neurodegenerative pathway is independent of TDP-43.

The FUS protein appears to have a similar role to that of TDP-43 and is involved in the most cellular process: genomic stability, transcriptional regulation, splicing, transport, and maturation of mRNAs (Law et al., 2006). Furthermore, in the central nervous system, the protein is involved in regulating mRNA transport towards the dendrites and synaptic plasticity upon the activation of glutamate receptors (Fujii et al., 2005).

Clinically, ALS represents the outcome with juvenile and adult-onset, ALS, and DFT (Boylan et al 2015).

5. Double Homeobox 4 (DUX4)

DUX4 (double homeobox 4) is a retrogene whose coding sequence is highly conserved in primates (Clapp et al 2007), but whose physiological role has not yet been well defined. This gene encodes a transcription factor of which two isoforms are known: DUX4-FL, whole, and DUX4-S, shorter; the latter originates from alternative splicing and lacks the C terminal domain, its physiological function is still to be clarified e. when over-expressed, it does not induce cell apoptosis (Sacconi et al 2015, Geng et al 2012). The other isoform, DUX4-FL, is of pathogenetic interest and the one to which we will implicitly refer from now on. It is

normally produced in the early stages of development, in stem cells and germ lines, especially in the testis. While it is repressed, through a repetition-mediated epigenetic silencing (methylation) mechanism (Tawil et al 2014), during cell differentiation (Sacconi et al 2015) and in most adult somatic tissues, including muscle (Zernov et al 2019, Snider et al 2010), except for thymus (Das et al 2016) and keratinocytes (Hamel et al 2018). This expression pattern, similar to that of other homeodomain-containing proteins, suggests a possible role of DUX4 in promoting transcription at the embryonic level (De Iaco et al 2017) and in reproductive biology (Geng et al 2012). Among the targets regulated by DUX4, in addition to the genes normally expressed in germ cells and the testis, there are also genes involved in the processing of RNA and proteins, coding for example for splicing factors and ubiquitin ligase, components of the Polymerase II complex, pathway of ubiquitin-mediated protein degradation, repetitive elements and non-coding RNA, each of which could have pathophysiological implications (Tawil et al 2014, Geng et al 2012, Young et al 2013). The upregulation of many factors involved in RNA transcription and processing suggests that DUX4 may be a key component of a complex gene regulatory network (Geng et al 2012). Contrary to the hundreds of up-regulated genes, there appear to be very few downs regulated by DUX4, most of which are involved in innate immunity (Yao et al 2014).

As for the location within the genome, this gene is found in the subtelomeric region of chromosome 4q35, where a highly polymorphic microsatellite array is present. Each of the repeating units in this array is named D4Z4 and consists of 3,300 base pairs (3.3 kb), while the number of repeats within the array is normally between 11 and 100 (Zernov et al 2019). Each repetition has within it a complex content of DNA, which is always part of, in addition to heterochromatin including a series of non-coding RNAs (IncRNAs, miRNAs, siRNAs), also a copy of the DUX4 gene. Only the most telomeric repetition can produce functional transcripts since it is the only one to contain a polyadenylation site necessary for their

stabilization (Tawil et al 2014). Furthermore, at this same level, chromosome 4 can present itself in two main variants, the haplotypes 4qA and 4qB, which have approximately the same frequency of recurrence (Lemmers et al 2002, Lemmers et al 2010). The 4qA haplotype has functional polymorphic polyadenylation (PAS) signal and therefore allows the ectopic production of the protein (with pathological significance). For this reason, it is defined as "permissive" (Hamel et al 2018, Lemmers et al 2007); as opposed to 4qB, which contains single nucleotide polymorphisms (SNPs) which break down the PAS and render it non-functional (Tawil et al 2014). On the other hand, mRNA transcribed by both permissive and non-permissive alleles has been identified in the testes (which we recall are a normal site of DUX4 production); in fact, here other polyadenylation sites are active, distal to the PAS sequence, which always allows the expression of DUX4, even in the haplotype 4qB (Sacconi et al 2015, Snider et al 2010).

The entire D4Z4 region is normally hypermethylated in somatic tissues and consequently, DUX4, as already mentioned, is silenced and does not allow the production of any protein. Changes at this level that lead to inefficient epigenetic repression of the gene and therefore its expression, in skeletal muscle (and other tissues), are involved in the pathogenesis of facioscapulohumeral dystrophy (FSHD), the main pathological drift of the gene under consideration (Sacconi et al 2015, Tawil et al 2014). It is shared opinion that the expression of DUX4 is a necessary condition to be able to observe the disease, playing a leading role in its pathogenesis, although other genes are also involved (Yao et al 2014). FSHD is the third most common muscular dystrophy after Duchenne muscular dystrophy and myotonic dystrophy (Hamel et al 2018, Geng et al 2012), with a prevalence that varies, in different populations, between 1: 14,000 and 1: 20,000 (Zernov et al 2019). It is a heterogeneous disorder that includes genetic and epigenetic alterations and of which two types are currently described, FSHDI and FSHD2. They have different genetic causes but are united by

hypomethylation of the D4Z4 units and the consequent relaxation of chromatin, which, in the presence of a permissive 4qA haplotype, allows transcription, and ectopic expression of DUX4, toxic to skeletal muscle (Zernov et al 2019, Hamel et al 2018).

The presence of the DUX4 protein within skeletal muscle damages and destroys muscle cells, leading to progressive muscle weakness and atrophy. However, the precise mechanism by which this gene induces dystrophic changes and the changes themselves induced at the cellular level, are still controversial and the subject of research (Zernov et al 2019, Hamel et al 2018). There are numerous hypotheses in the field: ectopic activation of the expression of stem cell genes, suppression of the innate immune response (Geng et al 2012), and RNA degradation pathways (leading to aberrant RNA accumulation) (Feng et al 2015, Shadle et al 2017), PITXI cytotoxicity -mediated (Dixit et al 2007), induction of cell death through apoptotic pathways (such as p-53, caspase-3 and caspase-7 mediated), inhibition of myogenesis and muscle regeneration (Wallace et al 2011, Kowaljow et al 2007), etc. Despite numerous supporting evidence, in the past, the primary role of DUX4 in FSHD has been questioned due to the extremely low amount of its messenger RNA found in muscle cells and biopsies of affected patients, although it is still much higher compared to healthy controls (Homma et al 2015). These poor levels reflect a varied pattern of expression, with a small number of nuclei expressing the gene in relatively large quantities at any given time (Snider et al 2010). It has been shown that these short bursts of DUX4 mRNA production occurring over time in different muscle cell nuclei are sufficient to activate its gene expression program downstream (Geng et al 2012).

In recent years, the aberrant expression of DUX4 has also been reported to be associated in several forms of solid cancer. Recent studies showed DUX4 overexpression in Sarcoma of Ewing, in cancers of the bladder, esophagus, breast, cervix, ovary, endometrium, kidney, lung, stomach, soft tissues, and frequently expressed in testicular germ cell cancers, and

thymomas (Chew et al 2019). In this context, DUX4 drives an aberrant program of embryonic gene expression and delivers a distinct advantage for cancer cells by allowing suppression of antigen presentation of class I-dependent MHC and immune evasion, thus reducing the likelihood of a possible positive response to immunotherapy as DUX4 would prevent immune cells from recognizing cancer cells (Chew et al 2019).

Recent molecular studies in Acute Lymphoblastic Leukemia have identified new mutated genes associated with oncogenic subtypes including in some clinical cases of acute lymphoblastic leukemia have found DUX4 rearrangements. In leukemia cells, DUX4 induces expression of ERG dominant negative isoforms, resulting in the loss of function of wild-type ERG, which is essential for the correct leukemogenesis pathway (Karpukhina et al 2021, Dib et al 2019).

Chapter 2:

Aims

Amyotrophic lateral sclerosis (ALS) is a rare and deadly disease that causes motor neuron degeneration. Today, ALS is considered to be a proteinopathy, a disease characterized by the formation of protein inclusions in the cytosol of degenerative motor neurons; in sporadic forms of ALS, the protein that is most present in these aggregates is TDP-43 misfolded, and TDP-35 and 25 C-terminal fragments from the cleavage of TDP-43, which enhance cellular toxicity and are highly likely to aggregate (Zhang et al. 2007).

DUX4: possible involvement in ALS?

An interesting 2015 study (Homma et al. 2014) aimed at better understanding the cellular mechanisms of damage induced by DUX4, and more generally how the cell responds to the expression of this gene, associated this protein to TDP-43. In particular, the Authors decided to analyze two factors, ubiquitinated proteins, and TDP-43, for multiple reasons. First of all, the fact that the altered functioning of the turnover systems, which deal with the processing of ubiquitinated proteins, is often associated with two events: increased cell death, also at the muscle level (Sassone et al. 2006) as observed in FSHD, and protein aggregation, with the formation of cytoplasmic inclusions of ubiquitinated proteins, as found in at least two myopathies, oculopharyngeal muscular dystrophy and inclusion body myopathy, in which aggregates of TDP-43 have already been reported (Askanas et al. 1998; Kusters et al. 2009). Furthermore, in the present study and others (Snider et al. 2010; Tassin et al. 2013), the immunodetection of DUX4 appeared sometimes punctuated, suggesting that it too may form aggregates within the nucleus, perhaps precisely due to the malfunctioning of the turnover systems. Finally, in the past, DUX4 expression has been correlated with increases in some ubiquitin-related proteins, such as ubiquitin ligases (Geng et al. 2012; Vanderplanck et al. 2011). The evaluation of these two targets was done by analyzing both the endogenous and exogenous expression of DUX4, using different cell types: differentiated cells

expressing myosin, myoblasts, and myotubes. The results obtained are largely overlapping: the expression of DUX4 seemed to lead to accumulation and altered intracellular distribution of both ubiquitinated proteins, with relatively large, but reduced aggregates, inside the nucleus and increased signaling at the cytoplasmic level, both of TDP-43, which no longer appears uniformly distributed in the nucleus, as is common to observe in many cell types, but rather with a much more punctuated nuclear pattern which suggests aggregation. Subsequently, the authors of the study also demonstrated that pharmacological inhibition of the ubiquitin-proteasome system (by MG132) produced cellular changes similar to those described, which could therefore represent the result of a functional impairment, induced by DUX4, of this system, perhaps particularly sensitive to gene expression (Von Mikecz 2006). These cellular changes are also potential mechanisms involved in the pathogenesis of FSHD and there are several hypotheses regarding how DUX4 would implement them, although further studies are needed to clarify the picture. The alteration of protein homeostasis and the formation of TDP-43 aggregates have been linked to several other diseases, neurological and muscular (Ling et al. 2013). For example, nuclear, cytoplasmic, and neuritic inclusions of TDP-43 are found in the disease object of this work, amyotrophic lateral sclerosis, but also in frontotemporal lobar degeneration with positive inclusions for ubiquitin (FTLD-U) and myopathy: from inclusion bodies (Greenberg 2011; Cohen et al. 2011); however, in these cases, the protein is found ubiquitinated in cytoplasmic aggregates, while following the expression of DUX4 it appears in nuclear aggregates and not ubiquitinated (Arai et al. 2006). Further studies are also needed to understand whether DUX4 expression leads to impaired TDP-43 functionality, possibly contributing to the disease through a loss-of-function mechanism (Yang et al. 2014). We have already mentioned in FSHD, the attempt to develop gene therapy strategies that have DUX4 as a target; according to the above, the blocking of its function, if effective in patients, could lead to the normalization of the function of the proteasome and of the aggregation of TDP-43,

with potential (and desirable) therapeutic implications also for other pathologies, including ALS, in which there is an alteration of these mechanisms.

Aims of this PhD Thesis: unraveling a potential role for DUX4 in ALS

Considering these premises, our first objective was to verify whether DUX4 is expressed in peripheral cells obtained from ALS patients, considering a possible involvement in TDP-43 aggregation (see Chapter 3). This hypothesis is completely innovative as to date there are no studies in the literature that link the expression of DUX4 to ALS.

The next part of our study (Chapter 4) focused on studying DUX4 expression also in the central nervous system of ALS patients, in order to further theorize on a possible pathogenic role.

The final part of this study (Chapter 5) focused on studying the modulation of DUX4 expression in vitro, in cellular models associated to ALS and compare it with TDP-43, to verify the existence of a possible interaction between the two proteins and, therefore, of new molecular mechanisms and intracellular pathways involved in the accumulation and aggregation of TDP-43 in ALS. In this section, we also evaluated the potential protective effects of Riluzole and Edaravone, the only two available ALS-approved drugs, in counteracting a toxic insult, verifying, at the same time, for potential modification in DUX4 expression.

Chapter 3*:*

Increased DUX4 expression and correlation with TDP-43 in peripheral cells from ALS patients.

An interesting previous study showed human FSHD myotubes with endogenous DUX4 expression are characterized by altered nuclear and cytoplasmic distributions of TAR DNA binding protein-43 (TDP-43) in FSHD myoblasts (Homma S. et al 2015). DUX4-FL expression also inhibited protein turnover and increased the amounts of insoluble ubiquitinated proteins and insoluble TDP-43 in DUX4-transfected myoblasts. These findings suggest the idea that DUX4 up-regulation in human FSHD myotubes can alter TDP-43 expression leading to its accumulation with consequent impairment of protein homeostasis.

In this study, we decided to verify if DUX4 is expressed in peripheral cells of ALS patients and its possible involvement in TDP-43 aggregation. Protein and gene expression of DUX4 was assessed in peripheral blood mononuclear cells (PBMCs) obtained from 46 ALS patients and 26 healthy controls. Moreover, we confirmed the DUX4 overexpression in ALS patients with the analysis of expression levels in fibroblast.

1. Subjects and Methods

Recruited subjects

After informed consent, n=46 patients affected by sporadic ALS (sALS) diagnosed according to El Escorial revised criteria (Brook 2000) were recruited at the NEMO center (Milano, Italy) (sex: 32M/14F; age: 67.2 ± 11.1 [42-82] years-old, mean ± SD [range]). ALS patients negative for SOD1, TARDBP and FUS gene mutations and patients with C9orf72 hexanucleotide repeats <14 were considered eligible for the study. ALSFRS-R scores were recorded at the time of recruitment: 26.1 ± 12.1 (4-45). N=26 age- and sex- comparable healthy subjects (sex: 18M/8F; age: 65.6 ± 12.1 [42-77] years-old) were also recruited among patient spouses and caregivers. Exclusion criteria for all the recruited subjects were considered cancer, autoimmune and inflammatory diseases. Moreover, healthy controls

were not affected by any neurological or psychiatric condition, nor were taking psychoactive drugs.

PBMCs isolation

Peripheral blood samples (20 ml) were drawn from each subject in tubes containing K2-EDTA after overnight fasting (Sala G et al. 2012). Briefly, after dilution with the same amount of saline solution, whole blood samples were layered on Ficoll-Histopaque (GE Healthcare) and centrifuged (490 g, 30 min, room temperature). PBMCs were collected from the interface between plasma and Ficoll-Histopaque, washed with saline solution, aliquoted, and stored at -80°C.

Real-time qPCR

Total RNA was extracted from PBMCs using the RNeasy Mini kit (Qiagen), according to the manufacturer's instructions. RNA concentration was determined spectrophotometrically at 260 nm. cDNA was synthesized using the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) at the following conditions: 10 min at 25°C and 60 min at 42°C. The reaction was terminated at 85 °C for 5 min and cDNAs were stored at -20°C.

For the analysis of the DUX4 mRNA level, cDNAs obtained from RNA were amplified in triplicate using the HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) (Solis BioDyne) at the following conditions: 50°C for 2 min, 95°C for 15 min, 40 cycles of 95°C for 15 sec, 60°C for 20 sec and 72°C for 30 sec. The sequences of the primers used: DUX4: Forward GAGCTCCTGGCGAGCCCGGAGTTTCTG, Reverse CTAAAGCTCCTCCAGCAGAGCCCGGTATTCTTCCTC, RPLP0: Forward ATGTGGGCTTTGTGTTCACC, Reverse TCCAGTCTTGATCAGCTGCA. For the relative quantification of each target vs. RPLP0 mRNA, the comparative Ct method was used as previously described. Analysis of the Ct values from two replications for a single sample.

Western Blotting

The pellets were lysed in cell extraction buffer (Invitrogen) with 1 mM PMSF (Sigma-Aldrich) and protease inhibitor cocktail (Sigma-Aldrich) and protein concentrations were determined by Bradford's method. Based on the previous assay, we load 30 µg of proteins to analyze DUX4 and TDP-43 expression. Sample lysates were diluted in Laemmli's loading buffer pH 6.8, denaturated at 95°C for 2 min, separated by SDS PAGE in 4-12% tris glycine gels (Invitrogen), and transferred into nitrocellulose. Nitrocellulose membrane was blocked for 1 h, incubated overnight at 4 °C with primary antibodies: mouse monoclonal anti-TDP-43 (Santa Cruz; dilution 1:500); mouse monoclonal anti-DUX4 (MerkMillipore; dilution 1:250). GAPDH (MerckMillipore; 1:20,000 dilution) was used as an internal standard. Signals were revealed by chemiluminescence and quantified using the software ImageJ. The protein expression of each sample was calculated as the ratio between the optical densities of the target protein and internal standard and expressed as a percentage versus the mean value of a control.

Immunofluorescence

PBMCs were fixed with 4% paraformaldehyde for 15 min and permeabilized using 0.2% Triton X-100 for 15 min at RT. Cells were incubated overnight at 4°C with the primary antibody (1:100, mouse anti-TDP-43, Santa Cruz. 1:250, mouse anti-DUX4, MerkMillipore) and, after washing, with the fluorochrome-conjugated secondary antibody (1:200, Alexa Fluor 546 goat anti-mouse, Thermo Fisher Scientific) for 1 h. After staining with DAPI for 2 min (1:50,000, Sigma-Aldrich), glass slides were mounted with polyvinyl alcohol (Polyvinyl Alcohol mounting medium with DABCO® Antifading pH 8.7, Sigma-Aldrich). Immunofluorescence analysis was performed with laser confocal microscope (Radiance 2100; Biorad Laboratories) and analyzed with ImageJ software.

Statistical analysis

Statistical analysis was performed using Prism 7.04 (GraphPad Software). Data are expressed throughout the paper as mean ± standard deviation (SD). Two-tailed unpaired Student's *t*-test. A correlation was computed with the two-tailed Pearson's *r*-test. Values that differ by two standard deviations from the mean value of each group were excluded from the analysis.

2. Results

DUX4 is detectable in PBMC and increased in ALS patients

At first, we investigate the possible expression of DUX4 in PBMCs from sALS patients, we started to analyze the presence of DUX4 protein, we performed a western blot assay and our results revealed a significant increase of DUX4 levels in PBMCs of sporadic ALS patients concerning controls in which found low protein level (Figure 1 A-B). DUX4 protein levels of sporadic patients were more than twofold higher (****p<0.0001) as compared to healthy controls.

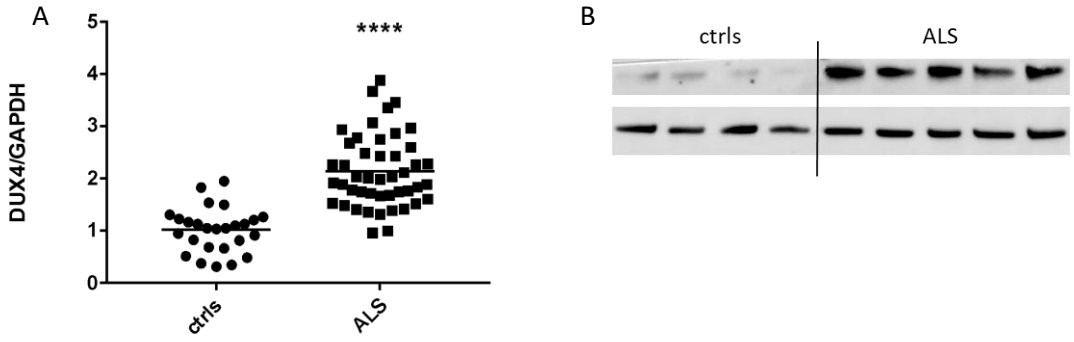


Figure. 1 A and B: DUX4 protein expression in PBMCs of patients and healthy controls. Increased DUX4 protein levels in PBMCs of sALS patients compared to healthy controls. Relative quantification of DUX4 protein levels is calculated as a ratio to GAPDH. Unpaired Student's t-test (****p<0.0001, SD: C: 1,017 ± 0,08326, n=26, ALS: 2,138 ± 0,102, n=46)

To clarify the nature of this increase, we subsequently quantified the gene expression by real-time qPCR of DUX4 mRNA levels. We found similar results that we previously found for protein levels, which were obtained for DUX4 mRNA levels that were significantly up regulated (****p<0,0001) in sporadic ALS patients when compared to healthy controls (Figure 2).

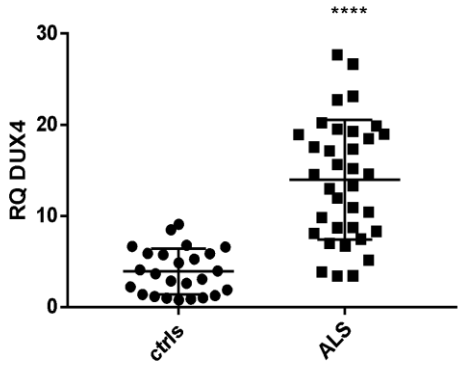


Figure. 2: DUX4 mRNA expression in PBMCs of patients and healthy controls. Increased DUX4 mRNA levels in PBMCs of sALS patients to healthy controls. Relative quantification of DUX4 mRNA levels is calculated as a ratio to GAPDH. Two-tailed Student's t-test (****p<0.0001, SD: C: 3,906 ± 0,5033, n=25, ALS: 13,96 ± 1,11, n=35)

DUX-4 and TDP-43 are consensually increased in ALS PBMC

To understand if DUX4 may be implicated in TDP-43 aggregation, we also analyzed in the same patients the TDP-43 protein expression and also of the truncated forms. Our results showed an important increase of protein levels in both TDP-43 (+44%, **p<0.005), TDP-35 (+77%, ****p<0.0001) and TDP-25 (+84%, ****p<0.0001), in PBMCs of sporadic ALS patients compared with controls (Figure 3 A-B-C-D). In addition, we analyzed for possible correlations with DUX4, we found a positive correlation between protein levels of DUX4 and TDP-43, and also for TDP-35 and TDP-25 was observed. TDP-43 (r=0.61, p<0.0001), TDP-35 (r=0.48, p<0.001) or TDP-25 (r=0.46, p<0.005) (Figure 4).

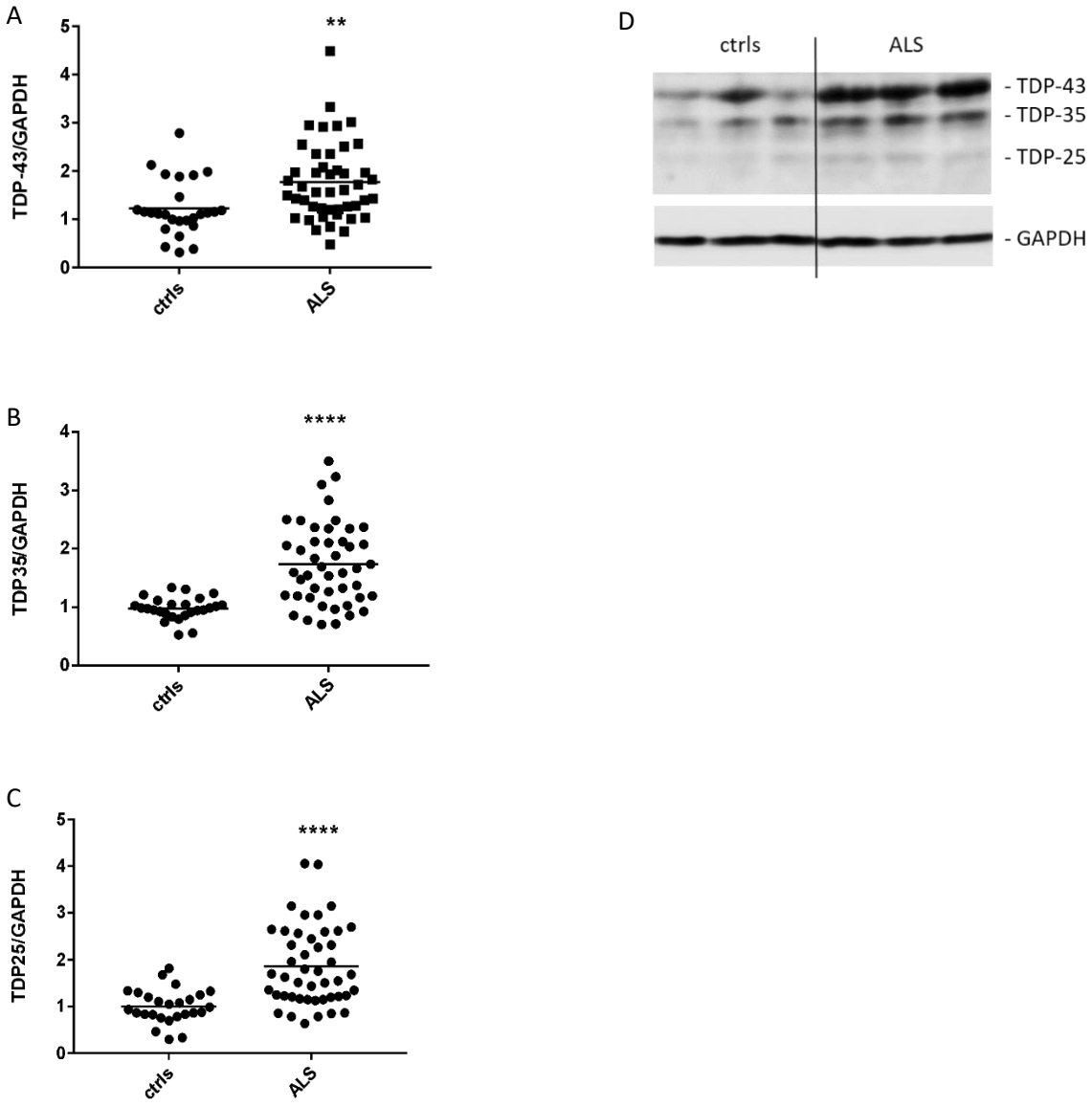


Figure.3: A-B-C-D TDP-43 and truncated protein expression in PBMCs of patients and healthy controls. Increased DUX4 protein levels in PBMCs of sALS patients with respect to healthy controls. Relative quantification of DUX4 protein levels is calculated as a ratio to GAPDH. Two-tailed Student's t-test (TDP-43 **p<0.0032, SD: C: 1,227 ± 0,1138, n=26, ALS: 1,769 ± 0,1172, n=46. TDP-35 ****p<0.0001, SD: C: 0,98 ± 0,03822, n=26 ALS: 1,737 ± 0,1027, n=46. TDP-25 ****p<0.0001, DS: C: 1,008 ± 0,07184, n=26 ALS: 1,859 ± 0,1248, n=46)

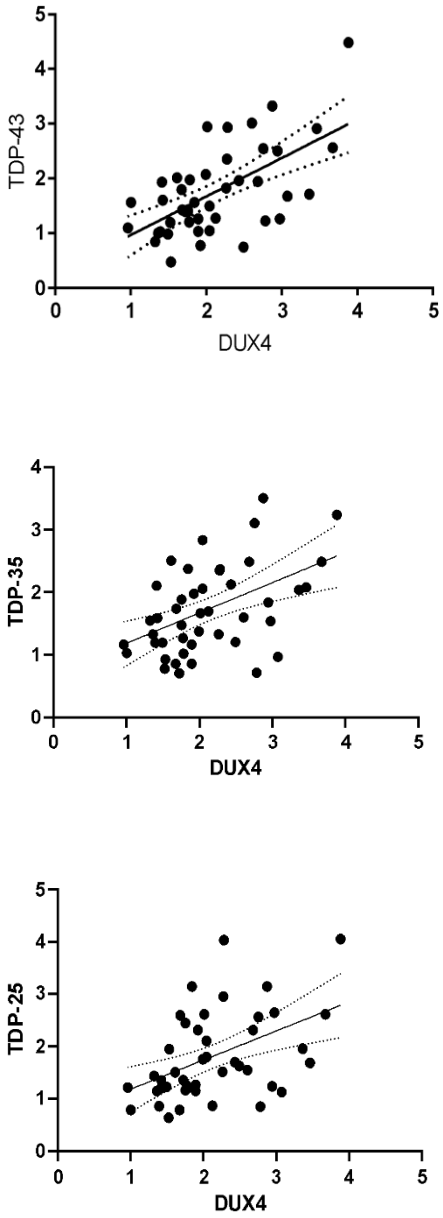


Figure 4: Correlation between protein levels of different TDP-43 isoforms with DUX4. Protein levels showed a positive correlation in PBMCs of healthy controls (DUX4 vs TDP-43: r= 0.61 $p<0.0001$, 95% Confidence Intervals: 0,4284 to 0,9805. DUX4 vs TDP-35: r= 0.48 $p<0.001$, 95% Confidence Intervals: 0,2159 to 0,7524. DUX4 vs TDP-25: r= 0.46 $p<0.005$, 95% Confidence Intervals: 0,2287 to 0,8838

DUX4 expression in ALS and a mis-localization in the perinuclear and cytoplasmic areas

Moreover, to investigate the intracellular distribution of DUX4 protein, an immunofluorescence assay was performed on fixed PBMCs derived from sALS patients and controls. We found Low levels of DUX4 at the nuclear level in controls, while in ALS patients we found an increment of DUX4 immunoreactive signal in perinuclear and cytoplasmatic areas (Figure 5 C-D). Similar results were found for the TDP-43 immunoreactive signal (Figure 5 F-G) and our results showed that DUX4 co-localized with TDP-43 in the perinuclear region and showed a tendency to aggregate.

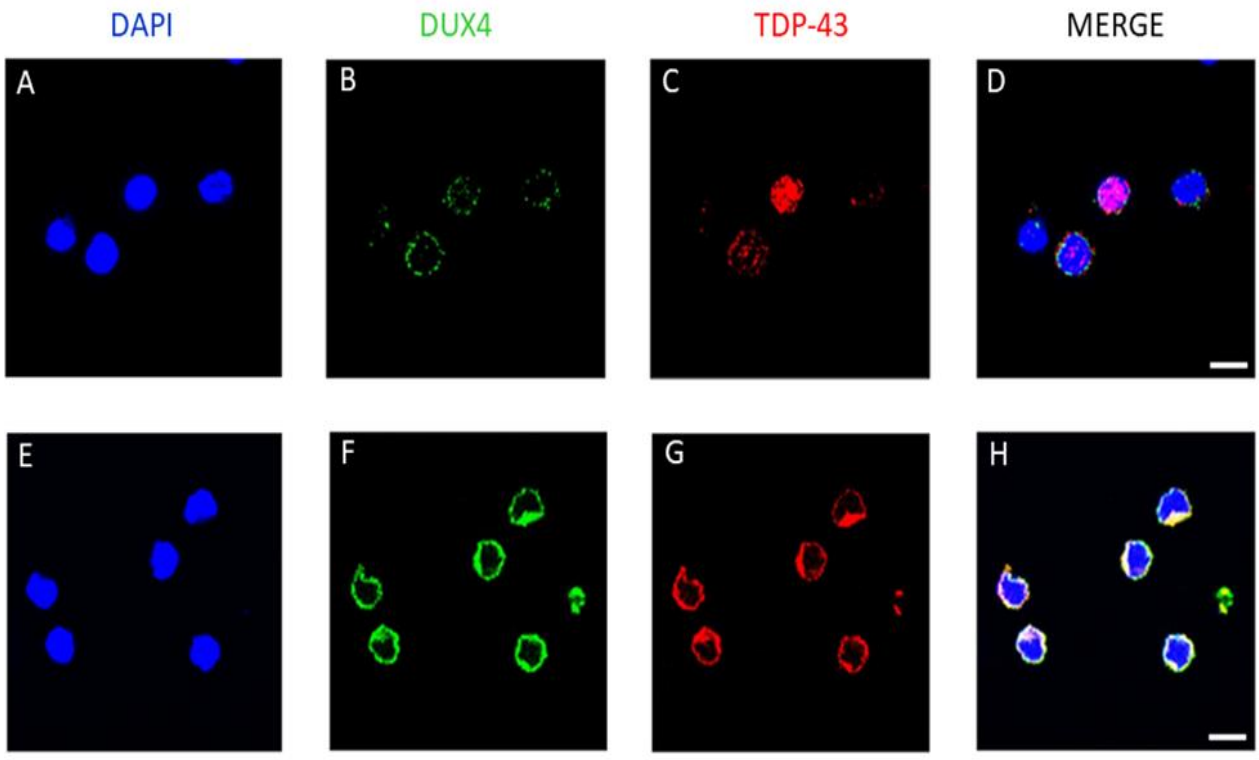


Figure 5: Localization of TDP-43 and DUX4 in PBMCs from sALS patients (E-H) and controls (A-D). Representative immunofluorescence assay showing DUX4 (green. B and F) and TDP-43 (red. C and G) intracellular expression. Nuclei are stained in blue (DAPI).63x

DUX4 expression is increased in ALS skin fibroblasts.

Fibroblasts obtained from ALS patients with respect to those obtained from CTRL displayed an increase in DUX4 protein (about threefold, 2.92 ± 0.13, n=4 vs. 0.94 ± 0.14, n=5, ALS vs. CTRL respectively, p<0.0001), and mRNA (about fourfold, 3.14 ± 0.72, n=4 vs. 0.80 ± 0.12, n=5, ALS vs. CTRL respectively, p=0.008) (Figure. 6 A-B-C).

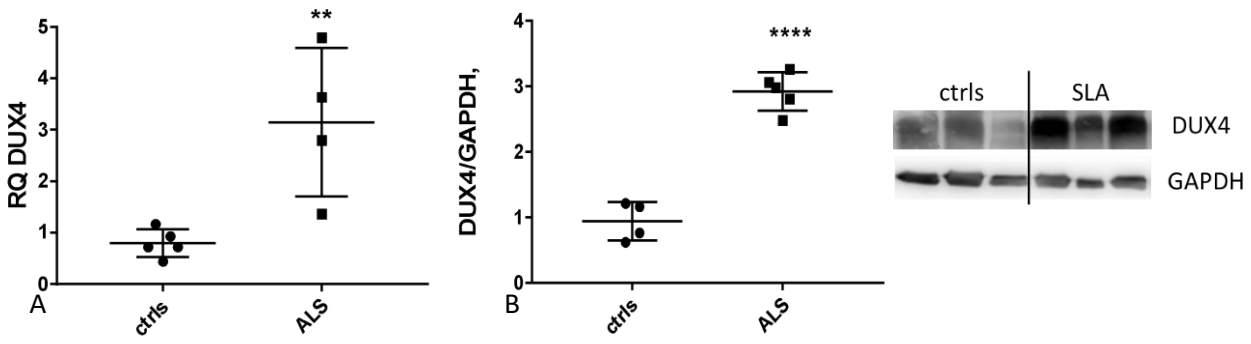


Figure. 6: A DUX4 mRNA expression in fibroblasts of patients and healthy controls. B DUX4 protein expression in fibroblasts of patients and healthy controls. Increased DUX4 mRNA and protein levels in fibroblasts of sALS patients to healthy controls. Relative quantification of DUX4 protein levels is calculated as a ratio to GAPDH.

Clinical correlations

Finally, we investigated possible correlations between the levels of mRNA and protein of DUX4 and the different clinical-demographic data available. At the moment there were no correlations, suggesting that DUX4-increase in ALS should be considered as a trait marker of the disease.

3. Discussion

DUX4 is a double-homeobox transcription factor that regulates the expression of genes associated with stem cells and germline development (Gabriels et al. 1999, Geng et al. 2012). Aberrant expression of this protein in skeletal muscle induces cell death and formation of atrophic myotubes, suggesting DUX4 as the more interesting gene causative of Facioscapulohumeral dystrophy (FSHD) (Lemmers et al. 2010, Daxinger et al. 2015). Previous studies by Homma et al. showed human FSHD myotubes with endogenous DUX4 expression are characterized by altered nuclear and cytoplasmic distributions of TAR DNA binding protein-43 TDP-43 (Homma et al. 2015). Furthermore, FSHD myotubes exhibited an increased amount of ubiquitinated proteins and an abnormal TDP-43 aggregation.

In this study, we investigated the DUX4 expression in peripheral blood mononuclear cells (PBMCs) of healthy controls and ALS patients. Besides their accessibility, PBMCs were selected since they have been repeatedly shown to recapitulate ALS pathogenic changes expressed by motor neurons, *i.e.*, TDP-43 aggregates (Arosio et al. 2019, De Marco et al. 2011).

We report here a strong overexpression of DUX4 in mRNA and protein levels in ALS samples when compared to control ones. This overexpression was confirmed by the images of immunofluorescence, which show that the protein is poorly present in healthy PBMCs and much more expressed in PBMCs from ALS patients. Furthermore, from DUX4 intracellular localization we also observed a different pattern of distribution in immunofluorescence signals performed in a small subgroup of sALS. In particular, we found a nuclear localization in control, and a high expression in perinuclear and cytoplasmic areas in sALS PBMCs.

Moreover, to verify the possible involvement of DUX4 in TDP-43 aggregation, we assessed TDP-43 expression in ALS and control samples. We found overexpression of TDP-43

protein levels in sALS patients, confirming our previous observations (Arosio et al. 2019). Furthermore, we evaluated the TDP-43 truncated forms: TDP-35 and TDP-25, and we obtained similar results. Finally, we found possible correlations between the levels of mRNA and protein of DUX4 and TDP forms. We also analyzed in the same sub-group of controls and sALS patients a possible co-localization of DUX4 and TDP-43, to verify whether the increase of TDP-43 is associated or not with an altered intracellular distribution. The images obtained have a diffuse nuclear presence of TDP-43 in control PBMCs and perinuclear accumulation, with the tendency to aggregation, in ALS patients. This data confirms the altered intracellular localization typically observed in motor neurons of ALS patients, where the protein appears localized in the cytoplasm, but already reported also in the PBMCs of affected subjects within which the protein assumes a perinuclear pattern, due to the poor representation of cytoplasm in these cells (Arosio et al. 2019, De Marco et al. 2011). Interestingly, the results by immunofluorescence of DUX4 and TDP-43, in PMBCs showed a direct colocalization between DUX4 and TDP-43 and a tendency to aggregate. These results confirm the observations of previous studies (Homma et al. 2015, Snider et al. 2010, Tassin et al. 2013), in which the nuclear immunodetection of DUX4 is described as in some cases with the tendency to aggregate, assuming a cause of malfunctioning of the turnover that the protein itself would induce.

Finally, DUX4 expression was found to be increased also in ALS skin fibroblast, further suggesting that ALS patients have an abnormal and aberrant expression of DUX4.

In conclusion, our research emerged for the first time the overexpression of DUX4 in ALS patients, in particular in PBMCs and fibroblasts. Our data suggest that DUX4 is increased in ALS patients and abnormal expression of DUX4 may be implicated in TDP-43 aggregation. In fact, from our study, emerged an important positive correlation between DUX4 and TDP-

43 and truncated forms in ALS patients. Clearly, the exact mechanisms governing the interaction between these two proteins will remains to be elucidated.

The lack of clinical or demographic correlations at the moment, suggests that DUX4 is a trait marker of ALS and fails to be a state marker. However, we are confirming this observation in a follow-up study that is recruiting a larger number of patients, carefully phenotyped for disease characteristics.

To conclude, DUX4 overexpression has never been previously reported in ALS. The next question to be urgently answered was then: is it overexpressed into the central nervous system of these patients as well or is this phenomenon confined just to peripheral tissues?

Chapter 4:

Is the peripheral increase in DUX4 expression in ALS a phenomenon involving the CNS as well?

The title question is extremely relevant in order to acquire further information on this novel phenomenon (DUX4 overexpression in ALS), which is still largely unclear. Therefore, our next step consisted in searching for a collaboration with the brain bank of the San Raffaele University (Milano, Italy), asking for the possibility of running immunohistochemistry experiments aimed at determining DUX expression in central nervous system (CNS) samples. Both motor cortex and spinal cord samples obtained from ALS patients and controls were then challenged for DUX4 immunoreactivity.

1. Material and Methods

For immunoperoxidase studies in human CNS tissues, paraffin-embedded sections of motor and occipital cortex were obtained from non-neurological controls and ALS patients (n=3) from the Target ALS Human Postmortem Tissue Core and stored at the INSPE Tissue Bank (San Raffaele Scientific Institute). Sections were dewaxed in xylene, passed through alcohol graded series, and rehydrated. Heat-induced epitope retrieval in a Tris-EDTA pH 9 in a water bath at 97°C for 30 min was required for the DUX4 antibody. For immunohistochemistry, endogenous peroxidase activities were blocked with 3% hydrogen peroxide for 10 min. Sections were washed in TBS for 5 min and nonspecific background blocked with Super block (ScyTek, AAA500) for 5 min at RT. Anti-DUX4 rabbit polyclonal (1:25, R&D system, MAB9535) antibody was applied on sections for 1 h at RT. After 5 min rinsing in TBS, CRF Anti-Polyvalent HRP Polymer (ScyTek, ABZ015) was applied for 30 min and signal amplified. Peroxidase reaction was developed for 5 min using DAB Chromogen concentrate (ScyTek, ACB030). Counterstain with hematoxylin was performed. After dehydration through ethanol grading scales followed by 20 min in xylene, coverslips were applied with Eukitt mounting medium and analyzed by light microscopy (Olympus BX51).

We acknowledge The Target ALS Human Postmortem Tissue Core for providing ALS postmortem tissue slides and Lyle W. Ostrow, K Wilsbach and K Gallo for their support.

2. Results

Immunohistochemistry studies on motor cortex and spinal cord sections postmortem obtained from ALS patients were performed. The representative image in Figure 1 shows that the immunoreactivity for DUX4 was not spread throughout the entire section, but peculiar of specific neuronal cell types. Immunostaining showed a nuclear DUX4 signal in upper motor neurons (UMN) of patient with ALS; DUX4 immunoreactivity was absent in control motor cortex. What is intriguing is that DUX4 is not expressed in lower motor neurons (LMN) in spinal cord in ALS (Figure 2).

A human testis section was used as positive control, showing as expected from the literature, a robust and diffuse expression of DUX4 (data not shown; courtesy of Prof. Giorgio Cattoretti, UNIMIB, Italy).

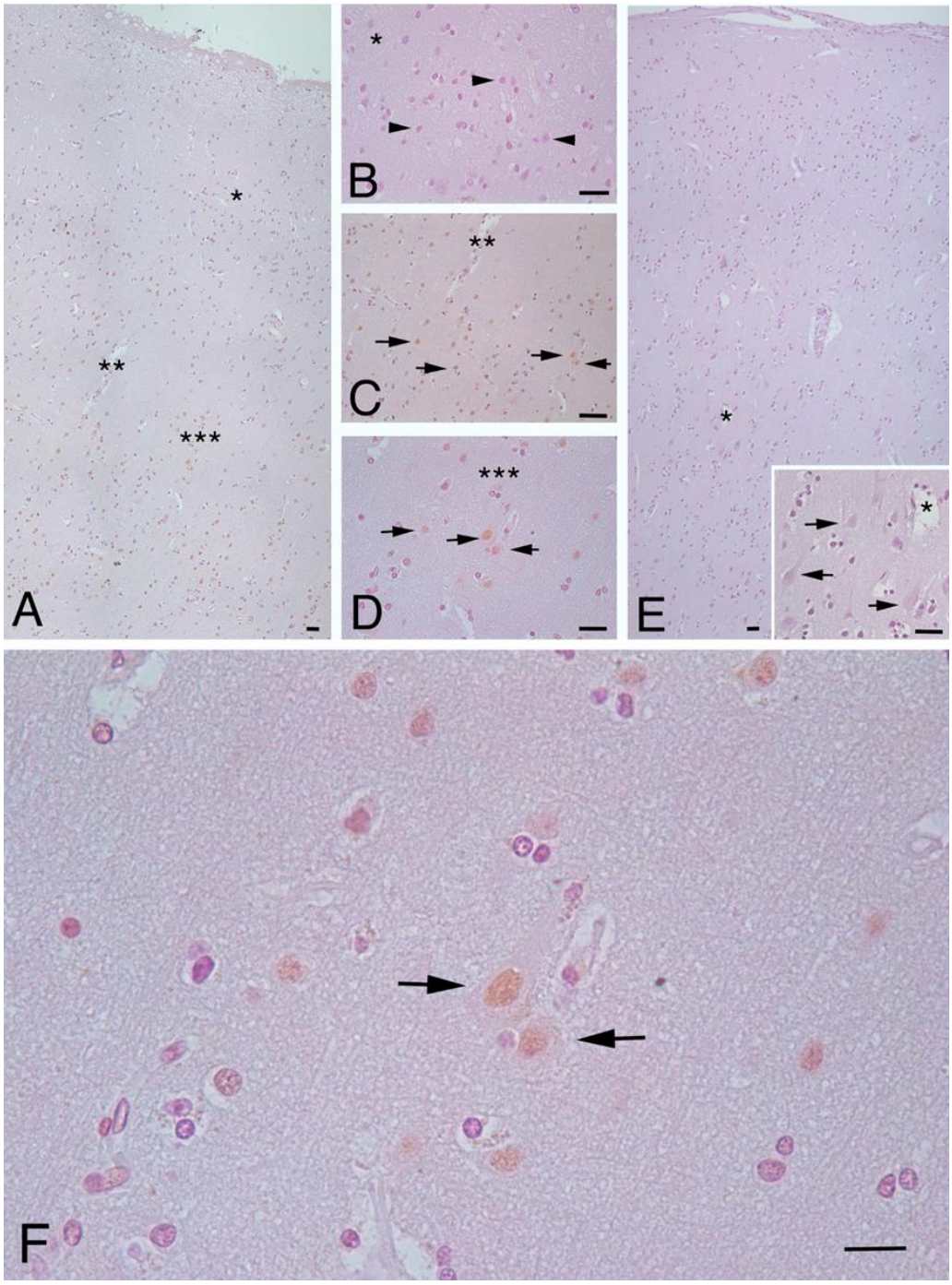


Figure 1. IHC of motor cortex coronal sections, showing DUX-positive UMN in the motor cortex of ALS patient (A-D and F, arrows). DUX is expressed predominantly between layers 4 and 6; arrowhead in B mark negative DUX immunoreactivity (B – D). (E) Representative negative immunoreactivity in the motor cortex of control, including UMN (arrows). Bar: A - E 200µm; F 100µm

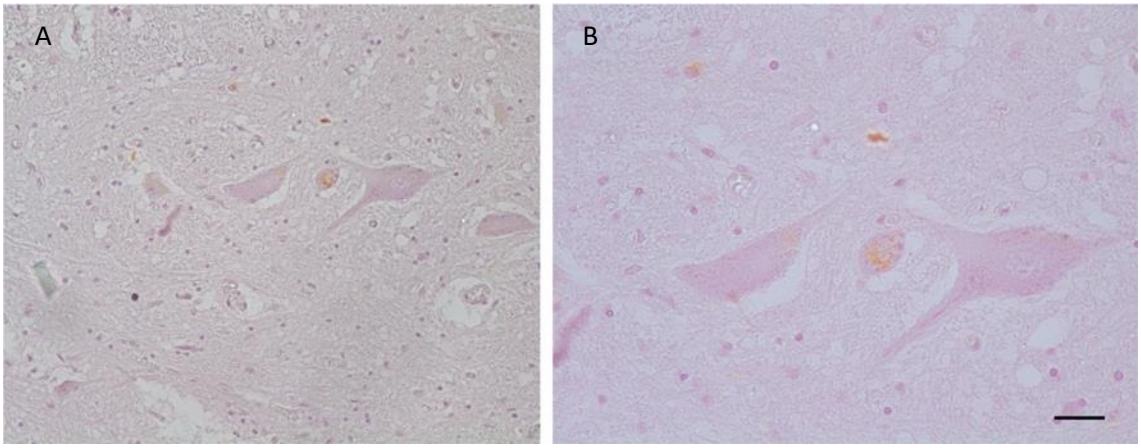


Figure 2: motor neurons anterior horns negative. Bar: A = 100µm, B = 50µm.

3. Discussion

Postmortem study on brain and spinal cord samples showed that DUX4 is indeed overexpressed in UMN of ALS patients. Intriguingly, control samples did not display an immunoreactivity for this target, suggesting that DUX4 belongs for all intents and purposes to the pathogenic milieu characteristic of ALS. What is more, DUX4 pathology appeared to be UMN-confined, since LMN failed to stain with two different antibodies. This could perhaps sustain the literature's fascinating hypothesis that cortical dysfunction may be an early event in ALS, preceding the onset of lower motor neuron dysfunction (Geevasinga et al. 2016). Given these observations, we concluded that further data should have been collected in order to clarify the (causal?) relationship existing between DUX4 and TDP-43, the protein considered the major culprit in ALS motor neuron.

Chapter 5:

Effect of DUX4 overexpression and analysis of DUX4 expression and pharmacological modulation in ALS cellular models.

We performed a series of experiments in a human neuroblastoma cell line (SH-SY5Y) with the aim of assessing the effect of DUX4 overexpression on TDP-43 protein expression and intracellular localization.

Furthermore, we verified the possible effect of Riluzole and Edaravone, two drugs approved by the Food and Drug Administration (FDA) for the treatment of ALS, on DUX4 expression in parental cells and in cells overexpressing the G93A SOD1 mutation, associated with a familial form of ALS, or overexpressing wild-type SOD1.

1. Materials and methods

Cells culture

Human SH-SY5Y neuroblastoma cells were cultured in medium DMEM-F12, 10% fetal bovin serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine at 37°C, 5% CO2, 90-95% humidity.

In addition, a set of experiments was also done in:

- SH-SY5Y overexpressing wild-type human SOD1 (WT)

- SH-SY5Y overexpressing G93A SOD1 mutation (G93A)

These two lines, kindly provided by the Center of Experimental Neurobiology 'Mondino-Tor Vergata-S.Lucia' of Rome, were obtained from the parental cell line SH-SY5Y, coming from the "European Collection of Cell Culture", by transfection with the cDNA encoding the gene SOD1 wild-type and with the mutation G93A (replacement of alanine with glycine in position 93) associated with a severe form of familial ALS. Cell lines were grown in Dulbecco's MEM-F12 culture medium supplemented with 15% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin, at a temperature of 37° and in a modified 5% CO2 atmosphere.

The WT and G93A lines were maintained in selection by adding 16 µg/ml of geneticin to the culture medium, which was removed 24 hours (h) before the experiments.

A set of experiments was also done in differentiated SH-SY5Y cells.

For differentiation, 48h after the seeding of SH-SY5Y cells, the % of serum in the culture medium was reduced to 3%, and retinoic acid (10 µM) was added for 7 days; at the end of the differentiation, cells were exposed to different treatments.

Cell transfection

Lipofectamine 2000 (Invitrogen) with plasmid vector RC238145 DUX4 (NM_001293798) Human tagged ORF Clone (OriGene) was used for cell transfection. Lipofectamine and vector were diluted (3:1) in Opti-MEM (Invitrogen), incubated for 5 min and added to cells for 48h.

Extraction of soluble and insoluble protein fractions

RIPA-soluble and RIPA-insoluble/urea-soluble protein cell fractions were obtained from the total protein lysates after centrifugation at 12,000xg for 20 min at 4°C. The supernatant corresponds to soluble protein fraction, and the obtained pellet, resuspended in the same volume of lysis buffer as that in the supernatant, represents the insoluble protein fraction.

MTT

A cell viability test was performed on SH-SY5Y cells to verify the toxicity of the treatment. For this analysis, the cells were sown in a 96-well culture plate with a flat bottom at a density of about 150,000 cells/ml, allowed to grow for 24h, and then exposed to stimuli for an additional 24h. At the end of the treatment period, the culture medium was removed and 500 µl of 0,5 mg/ml MTT medium was added to each well. MTT is converted by mitochondrial dehydrogenase of viable cells into formazan, which precipitates to form blue-violet crystals.

After incubation at 37° for 45 minutes, the MTT solution was aspirated and the formazan crystals were dissolved in 100 µl of DMSO. Finally, the staining intensity of each well, directly proportional to the number of viable cells, was quantified by a spectrophotometer, which measures the absorbance of each well at 570 nm.

Real-time qPCR

Total RNA was extracted from PBMCs using the RNeasy Mini kit (Qiagen), according to the manufacturer's instructions. RNA concentration was determined spectrophotometrically at 260 nm. cDNA was synthesized using the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) at the following conditions: 10 min at 25°C and 60 min at 42°C. The reaction was terminated at 85 °C for 5 min and cDNAs were stored at -20°C.

For the analysis of the DUX4 mRNA level, cDNAs obtained from RNA were amplified in triplicate using the HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) (Solis BioDyne) at the following conditions: 50°C for 2 min, 95°C for 15 min, 40 cycles of 95°C for 15 sec, 60°C for 20 sec and 72°C for 30 sec. The sequences of the primers used: DUX4: Forward GAGCTCCTGGCGAGCCCGGAGTTTCTG, Reverse CTAAAGCTCCTCCAGCAGAGCCCGGTATTCTTCCTC, TDP-43: Forward TGTTTTGCAGCCCTGAATGC, Reverse CAGGAGGACAAAGCCCATTC and RPLP0: Forward ATGTGGGCTTTGTGTTCACC, Reverse TCCAGTCTTGATCAGCTGCA. For the relative quantification of each target vs. RPLP0 mRNA, the comparative Ct method was used as previously described. Analysis of the Ct values from two replications for a single sample.

Western Blotting

The pellets were lysed in cell extraction buffer (Invitrogen) with 1 mM PMSF (Sigma-Aldrich) and protease inhibitor cocktail (Sigma-Aldrich) and protein concentrations were determined

by Bradford's method. Based on the previous assay, we load 30 µg of proteins to analyze DUX4 and TDP-43 expression. Sample lysates were diluted in Laemmli's loading buffer pH 6.8, denaturated at 95°C for 2 min, separated by SDS PAGE in 4-12% tris glycine gels (Invitrogen), and transferred into nitrocellulose. Nitrocellulose membrane was blocked for 1 h, incubated overnight at 4 °C with primary antibody: mouse monoclonal mouse monoclonal anti-DUX4 (Biotechne, 1:2000 diluition). GAPDH (MerckMillipore; 1:20,000 dilution) was used as an internal standard. Signals were revealed by chemiluminescence and quantified using the software ImageJ. The protein expression of each sample was calculated as the ratio between the optical densities of the target protein and internal standard and expressed as a percentage versus the mean value of a control.

Immunofluorescence

PBMCs were fixed with 4% paraformaldehyde for 15 min and permeabilized using 0.2% Triton X-100 for 15 min at RT. Cells were incubated overnight at 4°C with the primary antibody (1:100, mouse anti-TDP-43, Santa Cruz. 1:250, mouse anti-DUX4, MerkMillipore) and, after washing, with the fluorochrome-conjugated secondary antibody (1:200, Alexa Fluor 546 goat anti-mouse, Thermo Fisher Scientific) for 1 h. After staining with DAPI for 2 min (1:50,000, Sigma-Aldrich), glass slides were mounted with polyvinyl alcohol (Polyvinyl Alcohol mounting medium with DABCO® Antifading pH 8.7, Sigma-Aldrich). Immunofluorescence analysis was performed with laser confocal microscope (Radiance 2100; Biorad Laboratories) and analyzed with ImageJ software.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism 4.0 software. All data are expressed as mean ± standard deviation (SD). Differences between groups were tested by

Anova test, followed by the Tuckey test (to compare all groups) or Dunnett test (to compare all groups vs. basal).

2. Results

Upregulation of DUX4 induces TDP-43 insoluble fraction.

DUX4 was transiently transfected in human neuroblastoma cells SH-SY5Y with a plasmid vector to induce overexpression. DUX4 overexpression was verified by analyzing DUX4 mRNA (data not shown) and protein levels (Figure 1B, upper panel), which are both marked increased in transfected cells. Subsequently, RIPA-soluble and -insoluble fractions were analyzed and a significant increase in TDP-43 was found in the RIPA-insoluble fraction in DUX4-transfected cells with respect to the control ones (Figure 1).

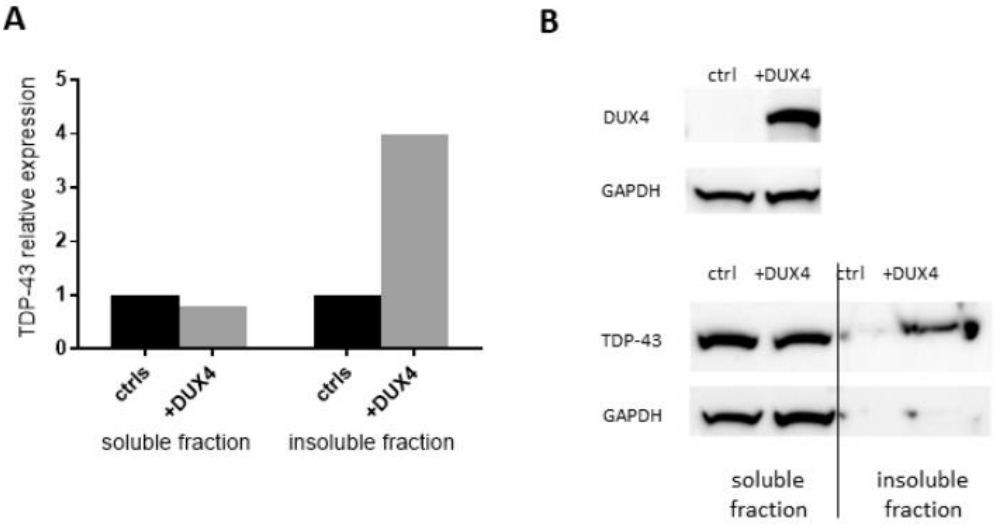


Figure 1: Effect of DUX4 overexpression on TDP-43 protein expression. A) Expression of TDP-43 in soluble and insoluble protein fractions in DUX4-transfected (grey) human neuroblastoma SH-SY5Y cells and vehicle (black). B) Representative Western blot image showing the increased DUX4 protein level (upper panel) and TDP-43 protein expression (lower panel) in DUX4-transfected cells and vehicle. GAPDH was used as internal standard for total and soluble protein fractions.

We also investigated the intracellular localization of TDP-43 and DUX4 in DUX4 overexpressing cells.

As shown in figure 2, DUX4 is expressed at very low level in non-transfected cells (Fig. 2B) and TDP-43 immunoreactivity, as previously described, appears diffused in the nucleus (Fig. 2C). In transfected cells, DUX4 is clearly expressed and localized at the nuclear and perinuclear level (Fig. 2F), while TDP-43 is strongly accumulated in the nucleus, without delocalization in the cytoplasm, in large clusters similar to the inclusions reported in damaged ALS motor neurons (Fig. 2G).

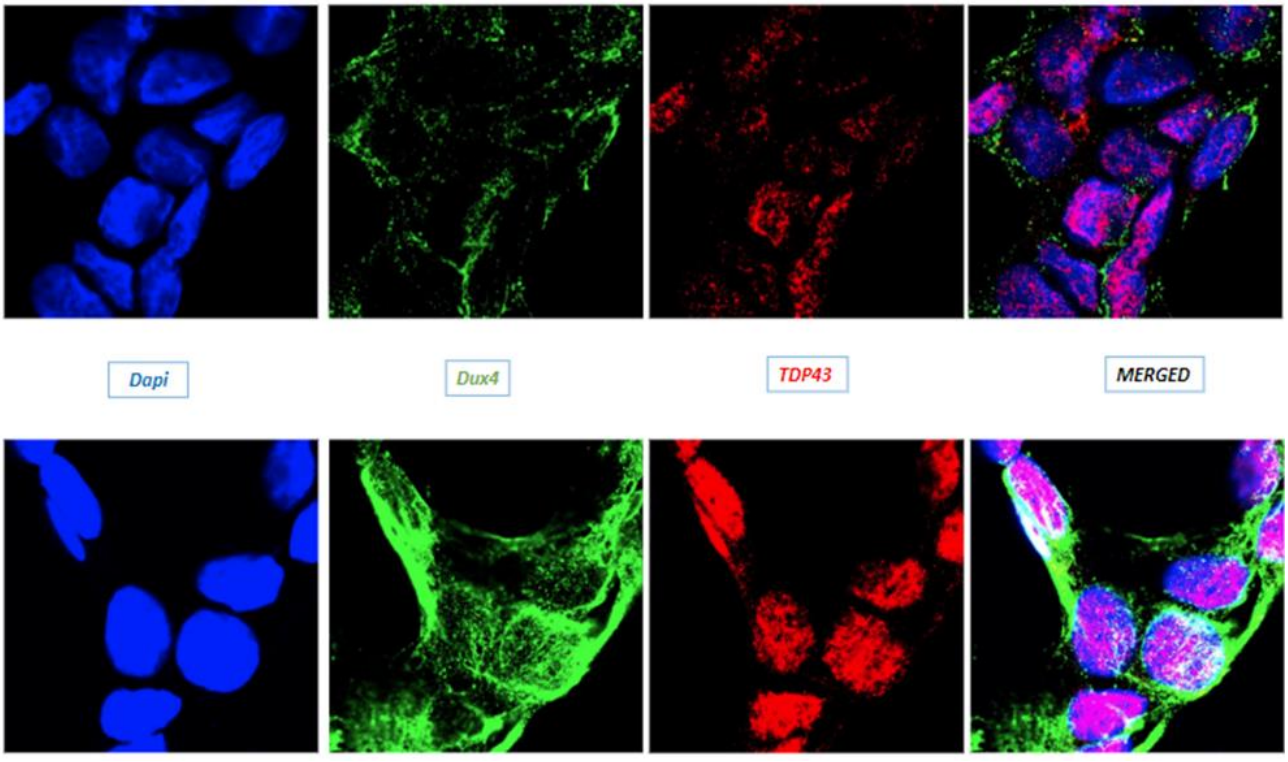


Figure 2: Intracellular localization of DUX4 (green) and TDP-43 (red) in vehicle- (A-D) and DUX4-transfected (E-H) human neuroblastoma SH-SY5Y cells. Nuclei are stained in blue (DAPI).63x

Effects of Edaravone and Riluzole on DUX4 expression

To date, there are no effective therapies in the treatment of ALS or capable of blocking its clinical progression, they are mainly palliative therapies side by sides such as non-invasive

ventilation (NIV) (Bach, J. R. 2002), tracheostomy and percutaneous endoscopic gastrostomy (PEG) since the disease progresses variably until the complete degeneration of motor neurons (Kaur, S. J., et al 2016). Despite the numerous clinical trials conducted in the last decade, to date the only drugs approved by the Food and Drug Administration (FDA) for the treatment of this disease are Riluzole and Edaravone. Recently, in September 2022, a new compound RELYVRIO (AMX0035), combination of the two drugs sodium phenylbutyrate and taurursodiol, has been approved based on its efficacy in preventing nerve cell death by blocking stress signals in cells.

The first drug approved by FDA for the treatment of ALS was Riluzole (6- (trifluoromethoxy) benzothiazole-2-amine), capable of slowing down the progression of the disease and therefore lengthen the patient's median survival from 3 to 6 months. To date, there is no evidence that Riluzole exerts a therapeutic effect on the function of motor neurons, at the pulmonary level, on the fasciculations, or at the muscular level (https://www.ema.europa.eu/en/medicines/human/EPAR/rilutek). It acts specifically by blocking the sodium channels by reducing the entry of calcium and the excessive stimulation of glutamate in the CNS (Reynolds, D. J. M. 1996). It appears to have an antioxidant effect, i.e. capable of reducing the oxidative stress induced by various oxidizing agents (Storch, A., et al 2000). However, this drug is not able to improve the patient's quality of life or improve symptoms, which is why it arouses strong interest in researching more effective drugs.

In 2017, Radicava (Edaravone) was approved by the FDA for the treatment of ALS patients, given the many studies that highlight its potential in the therapeutic field (Maragakis, N. J. 2017). Edaravone (3-methyl-1-phenyl-2-pyrazoline-5-one) was first approved in Japan in 2001 for the improvement of neurological symptoms and associated functional disabilities of acute ischemic stroke (Müllges W. 2003). Chemical properties of Edaravone and in vitro and in vivo studies suggest that it may have, mainly, an antioxidant effect. It is known to be

a free radical scavenger, it acts mainly by reducing lipid peroxides, efficiently as antioxidants such as vitamin E and ascorbic acid do, but also hydroxyl radicals and peroxynitrites, other reactive oxygen species (Yamamoto, Y et al 1996). Edaravone shows a protective effect on neuronal, glial (microglia, astrocytes, and oligodendrocytes), and endothelial vascular cells against oxidative stress.

As from literature data on facioscapulohumeral muscular dystrophy (FSHD) emerged a possible role for DUX4 in inducing oxidative stress and in the regulation of the altered expression of the gene sensitive to this cellular damage, like Pitx-1 (Bosnakovski D. et al 2014, Dmtriev P et al 2016, Karpukhina A et al 2021). We decided to investigate if DUX4 could be involved in oxidative damage also in ALS cellular models and if the two drugs approved for ALS and endowed with antioxidant properties, Riluzole and Edaravone, could affect DUX4 expression.

Effect of Edaravone and Riluzole on cell viability

In the therapeutic field, Riluzole has been the first drug used for the treatment of ALS because various studies have shown its ability to block sodium channels by reducing the entry of calcium and excessive stimulation of the glutamate receptor at the level of the central nervous system (Reynolds, D. J. M.1996).

It has also been hypothesized that this drug had an anti-oxidant action in the presence of various toxic agents, which induce oxidative stress and can counteract nitrosative stress (Storch et al 2000)**.**

Figure 3 illustrates the results obtained in SH-SY5Y cells after exposure to H_2O_2 200 μM for 24 h, which significantly reduces cell viability (-50%, $p<0.01$). Co-treatment with Riluzole prevents the cell death induced by the toxic insult, the maximum effect is observed at Riluzole concentrations ranging from 1 to 10 μM (Sala G et al 2019).

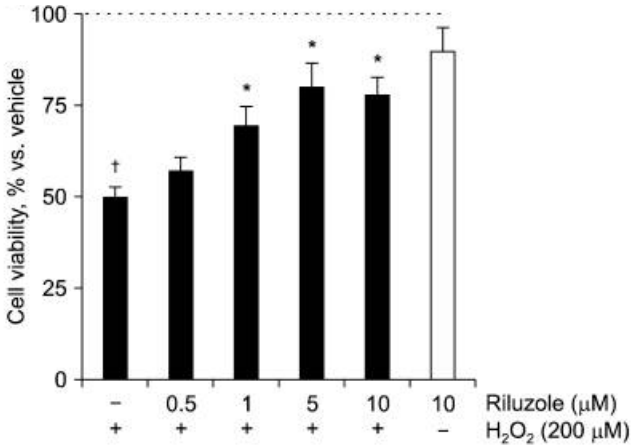


Figure 3. Effect of Riluzole (0,5-10 μM) on cell viability in SH-SY5Y cells exposed to H2O2 200 uM. *p<0.01 vs cells treated with H2O2., p<0.001 vs vehicle. [Source: Sala et al 2019]

In 2017, a new drug, Edaravone, was approved for the treatment of ALS, which appears to play primarily a protective role against oxidizing and nitrosative agents that induce cellular toxicity. To date, however, the mechanisms by which this drug performs its functions are still unclear.

We first conducted cytotoxicity studies in parental SH-SY5Y cells exposed to increasing concentrations, from 1 to 1000 μM of Edaravone, for 24h. In Figure 4 we observe that from the concentration of 100 μM the drug shows the first signs of toxicity that increases in a dose-dependent manner, while concentrations <100 μM do not affect cell viability.

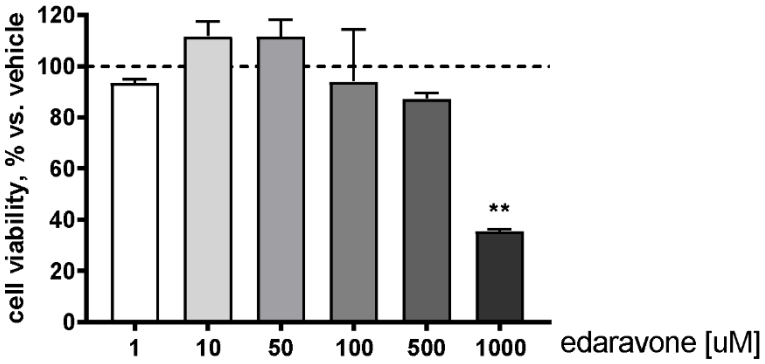


Figure 4. Graphical representation of cell viability in SH-SY5Y cells exposed to increasing concentrations of Edaravone. Values are expressed as mean ± SD vs vehicle (dashed line). N=3; **p<0.001 vs vehicle

In figure 5 we observe a dose-dependent reduction of cell viability induced by H_2O_2 at concentrations of 50 μM (-30% approximately, $p<0.05$) and 100 μM (-70% approximately, $p<0.01$). Both concentrations of Edaravone used (50 and 100 μM) failed to revert the toxicity of H_2O_2.

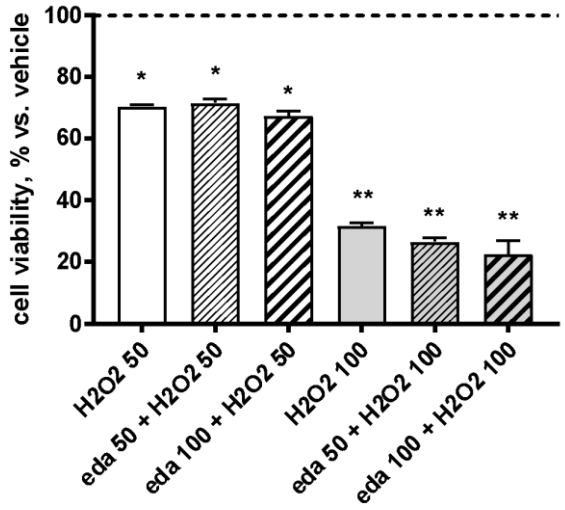


Figure 5. Graphical representation of H_2O_2-induced toxicity (50 and 100 μM) in SH-SY5Y cells. Values are expressed as mean ± SD vs vehicle (dashed line). N=3; *p<0.05; ** p<0.01. vs vehicle.

Expression of DUX4 in ALS cellular models

To understand whether the presence of the G93A SOD1 mutation, typical of familial ALS forms, or the overexpression of wild-type SOD1 could modulate the expression of DUX4, we quantified the gene and protein levels of DUX4 in cells overexpressing the G93A SOD1 mutation (G93A) or overexpressing wild-type SOD1 (WT) as compared to the parental cell line (SH). A significant increase of DUX4 mRNA levels ($p<0.033$) was observed in SOD1 WT cells and a less marked increase in SOD1 G93A cells with respect to parental cells (Figure 6).

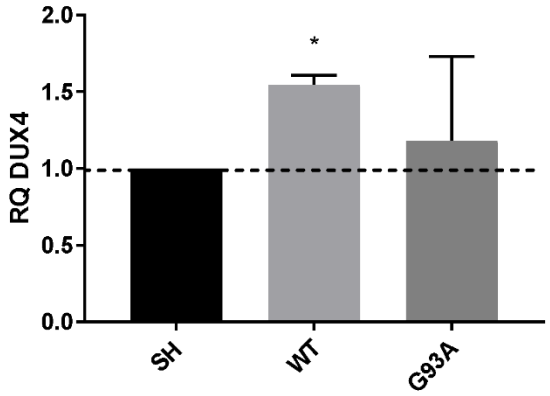


Figure 6. mRNA levels of DUX4 in parental cells (SH), overexpressing WT SOD1 and mutant G93A SOD1. * p<0.033 vs SH.

Subsequently, we evaluated the DUX4 protein levels in the same cell lines. The analysis in Western blot showed no statistically significant change in the WT and G93A cell lines compared to parental cells, although in both lines we found a slight increase in DUX4 protein expression (Figure 7).

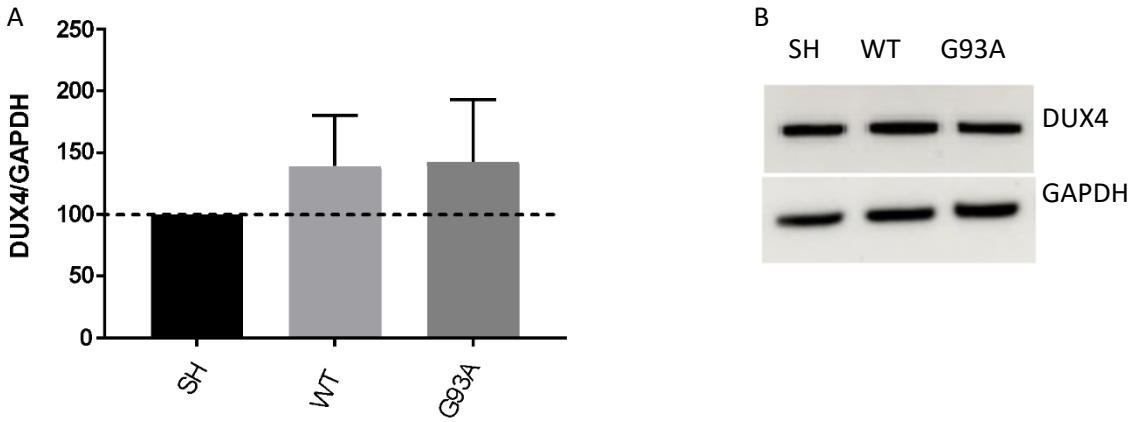


Figure 7 A-B: Protein levels of DUX4 in parental cells (SH), overexpressing WT SOD1 and mutant G93A SOD1

Effect of Edaravone and Riluzole on DUX4 expression

From the results of the cytotoxicity studies, we chose the concentration of 50 μM for Edaravone and 10uM for Riluzole to continue our analysis. We have extended the analysis of the effects of Edaravone and Riluzole on DUX4 gene and protein levels to cell lines overexpressing SOD1 wild-type (WT) and SOD1 with mutation G93A (G93A), both in basal conditions and after an acute oxidative insult represented by exposure to H_2O_2 (50 μM, 24 h).

Analysis of mRNA levels of DUX4 shows that exposure to H_2O_2 causes an increase in DUX4 levels in all three cell lines and that co-treatment with Edaravore or Riluzole significantly reduces the gene transcription of DUX4 in all lines. To further understand the possible effect of the two drugs on DUX4 expression, we also evaluated gene expression levels after exposure to Edaravone or Riluzole only. From our analysis it appears that in the parental line and SOD1 WT there is a significant reduction in the expression of DUX4 in response to treatment with Edaravone, this occurs partially but not at significant levels even in G93A cell line. Regarding treatments with Riluzole only, in all cell lines, we found a not significant decrease in DUX4 levels (Figure 8 A-B-C).

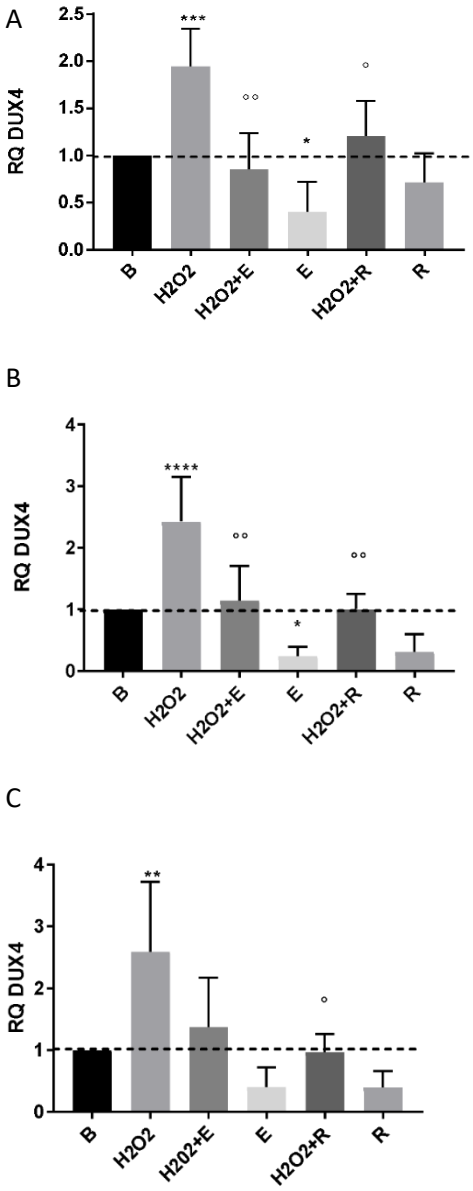
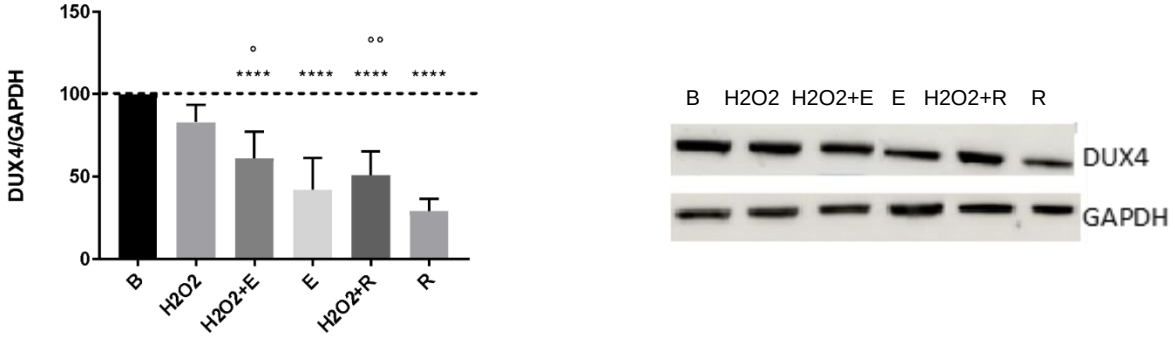


Figure 8. Effect of Edaravone (50 uM) and Riluzole (10 uM) on DUX4 mRNA levels in parental cells, cells overexpressing wild-type SOD1 (WT) and G93A SOD1 mutation under basal conditions and after exposure to H2O2 (50 μM). The values are expressed as the mean ± DS of the fold change vs. untreated cells (RQ=1). N=5. A) Parental line: ***p<0.0006 H_2O_2 vs basal; * p<0.03 Edaravone vs basal; °°p<0,002 H_2O_2+E vs H_2O_2; °p<0,02 H_2O_2+R vs H_2O_2. B) SOD1 WT line: ****p<0.0001 H_2O_2 vs basal; * p<0.03 Edaravone vs basal, °°p<0,008 H_2O_2+E vs H_2O_2. °°p<0,003 H_2O_2+R vs H_2O_2. C) SOD1 G93A line: **p<0,001 H_2O_2 vs basal; °p<0,02 H_2O_2+R vs H_2O_2

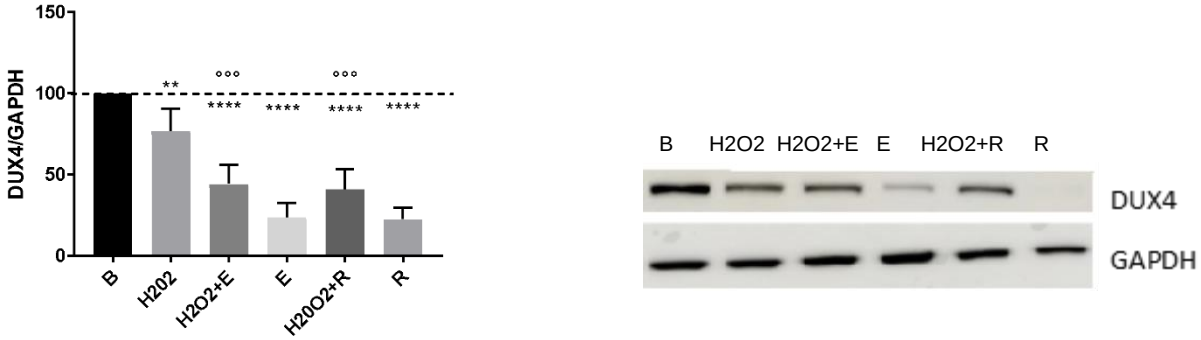
Contrary to what observed in qPCR, a slight decrease (not significant in parental cell line) in DUX4 protein expression was found in cells exposed to H_2O_2. Co-treatment with Edaravone or Riluzole induced a further reduction of DUX4 protein expression in all cell lines with

respect to cells treated with H_2O_2 only. Either Edaravone or Riluzole alone was able to markedly reduce DUX4 protein levels in all cell lines, showing a greater effect in WT and G93A lines. (Figure 9 A-B-C)

A



B



C

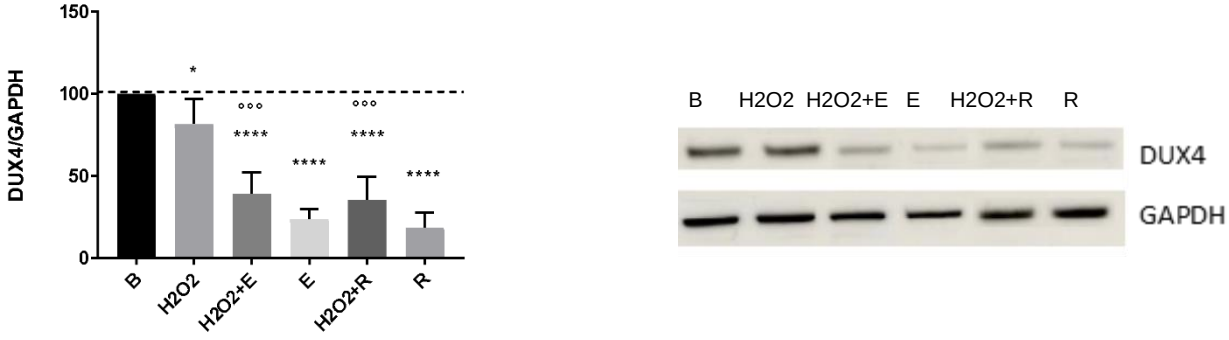


Figure 9. Effect of Edaravone (50 uM) and Riluzole (10 uM) on DUX4 protein levels in parental cells, cells overexpressing wild-type SOD1 (WT) and G93A SOD1 mutation under basal conditions and after exposure to H2O2 (50 μM). The values are expressed as the mean ± DS of the relative expression vs. untreated cells (relative expression=100). N=7. A) Parental line: ****p<0.0001 H_2O_2+E/ only E/ H_2O_2+R/ only R vs basal; °p<0,02 H_2O_2+E vs H_2O_2, °°p<0,001 H_2O_2+R vs H_2O_2. B) SOD1 WT line: ***p<0.0006 H_2O_2 vs basal; ****p<0.0001 H_2O_2+E/ only E/ H_2O_2+R/ only R vs basal, °°°p<0,0004 H2O2+E vs H_2O_2, °°°p<0.0001 H_2O_2+R vs H2O2. C) SOD1 G93A line: *p<0,01 H_2O_2 vs basal. ****p<0.0001 H_2O_2+E/ only E/ H_2O_2+R/ only R vs basal, °°°°p<0.0001 H_2O_2+E/H_2O_2+R vs H_2O_2

Validation experiments in differentiated SH-SY5Y cells

Considering recent literature evidence of the involvement of DUX4 in oncology (Chew et al 2019, Mocciaro et al 2021), and considering the tumor phenotype of the human neuroblastoma SH-SY5Y cell line, we added a set of experiments to verify the possible effect of the tumorigenic nature of used cells on the obtained results.

We differentiated parental SH-SY5Y cells for 7 days with 10 uM retinoic acid, in presence of culture medium with 3% serum and then we exposed to 50 uM H_2O_2 for 24 hours in order to evaluate the effect on DUX4 protein expression. After H_2O_2 exposure, we found a DUX4 protein reduction like what found in non- differentiated cells and reported in Figure 10, thus excluding a possible role of the tumorigenic nature of cells on the observed modulation of DUX4.

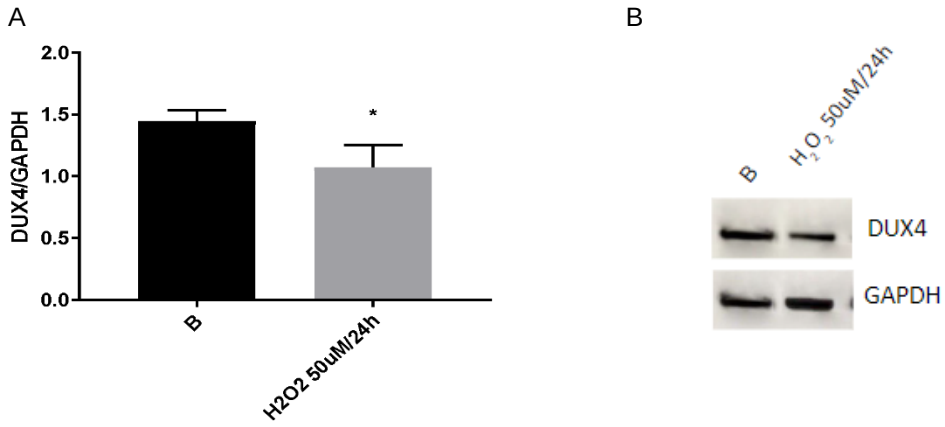


Figure 10. Analysis of protein levels of DUX4 in differentiated SH-SY5Y cells exposed to H_2O_2 50uM/24h *p<0,05, vs. Basal.

3. Discussion

In this study we wanted to verify the findings obtained in a previous study (Homma et al 2015), which demonstrated in FSHD myoblasts how the expression of DUX4 leads to an accumulation of nuclear TDP-43 with a tendency to aggregation, hypothetically via a mechanism of alteration of protein homeostasis via inhibition of the ubiquitin-proteasome system. To directly verify the effect of DUX4 overexpression on TDP-43, we transfected DUX4 in a human neuroblastoma SH-SY5Y cell line and we showed that DUX4 overexpression was able to induce a significant increase of TDP-43 protein insoluble fraction. Our results confirm previous data of Homma et al and suggest a possible role for DUX4 in TDP-43 accumulation and aggregation in ALS pathology, although further studies are needed to clarify the underlying mechanisms of this possible interaction between these two proteins.

To date, it is known that ALS is a multifactorial neurodegenerative pathology, that is, various causative pathogenetic mechanisms of the disease that aren't yet fully known are involved. Alongside the sporadic forms of disease that are the most frequent, there are the genetic forms that represent a small percentage of patients. The first mutated gene to be identified is the one coding for superoxide dismutase type 1 (SOD1), which makes up 10-20% of cases of autosomal dominant familial ALS (Rosen, D. R. et al. 1993). The most studied mutation is G93A (Prudencio, M. et al 2009) which causes the antioxidant enzyme SOD1 to acquire a toxic gain-of-function that leads to increased oxidative stress. Therefore, in our study was used human neuroblastoma SH-SY5Y cellular line transfected with the mutated SOD1 protein (G93A) or with SOD1 wild-type (WT).

However, it is known that one of the most important objectives of scientific research is the identification of effective therapy to treat ALS. The two drugs so far approved by the Food

and Drug Administration are Riluzole and since 2017 Edaravone, which are only able to slow the progression of the disease. In the literature, it is reported that Riluzole has an anti-glutamatergic action and is probably also an antioxidant (Reynolds, D. J. M. 1996, Storch, A., et al 2000). Edaravone, one of the latest investments in pharmaceutical and scientific research in ALS, is able to reduce lipid peroxides but also hydroxylic and peroxynitrite, acting as an antioxidant (Yamamoto, Y et al 1996). In this study we assessed the effect of Riluzole and Edaravone on DUX4 expression in ALS cellular models.

We started by evaluating the cytotoxicity of the drugs in order to identify the best concentrations for the planned experiments. We observed that concentrations >100 μM of Edaravone show the first signs of cytotoxicity, so we choose the 50 μM concentration, which does not affect cell viability. This data are consistent with that reported in a study conducted in PC12 cells (rat pheochromocytoma cells) in which Edaravone was used with similar concentrations to verify the protective effect against different oxidizing agents (Zhang, G. et al. 2013). Previous data from our group demonstrated the insensitivity of SH-SY5Y cells to glutamate and exploited this peculiar characteristic of the model to confirm the antioxidant properties of Riluzole which has been shown to counteract an acute oxidative insult represented by exposure of cells to H_2O_2 (Sala et al 2019). Riluzole co-incubation was able to prevent the H_2O_2-induced (200 μM/24 hr) cell death, with maximal efficacy in the concentration range of 1-10 μM.

From the FSHD literature, it emerged that DUX4 would appear sensitive to oxidative stress and could subsequently be involved in pro-inflammatory pathways following this kind of muscle cell damage (Bosnakovski et al 2014, Dmtriev et al 2016). For this reason, after confirming that DUX4 is overexpressed in ALS patients (Duranti et al submitted paper), we wanted to assess whether these two drugs with antioxidant properties approved for ALS can affect the expression of DUX4.

Our results obtained in human neuroblastoma cell line overexpressing wild-type (WT) or the fALS-associated G93A mutant (G93A) SOD1 showed an increase of DUX4 mRNA and protein levels in these two cell lines compared to the parental line in basal condition. Furthermore, both Edaravone and Rliuzole reduce mRNA and protein levels of DUX4 in all investigated cell lines.

DUX4 mRNA levels in cells exposed to the oxidant agent H_2O_2 (50uM for 24h) is significantly increased, while DUX4 protein levels are reduced. We found a marked effect of both Edaravone and Rliuzole in counteracting DUX4 gene increase induced by H2O2 in all cell lines, particularly in WT cells. In presence of H2O2, both drugs further reduced DUX4 protein levels in all cell lines.

Collectively, the obtained results demonstrate the validity of the antioxidant properties of these two drugs, with a slightly higher effect of Edaravone with respect to Riluzole, and confirm the hypothesis that DUX4 is sensitive to oxidative stress and involved in oxidative stress mechanisms, as reported in the literature (Bosnakovski et al 2014). Futhermore, the tested drugs have shown a higher effect in mutant G93A SOD1 cells, accordingly to the knowledge that this line possesses a high oxidative stress condition due to the presence of SOD1 mutation. Our data also suggest that Riluzole and Edaravone can regulate DUX4 expression in cellular models. However, further studies in patient-derived cell lines (skin fibroblasts, myoblasts) could be helpful to better clarify the antioxidant potential of these two drugs in ALS patients.

It would also be useful to understand if these two drugs can prevent the mislocalization of the DUX4 protein in the cytoplasm, as shown in ALS PBMCs (see Chapter 3).

4. Additional preliminary results

Effect of Edaravone and Riluzole on TDP-43 expression

To test whether, as observed after other treatments, the exposure to Edaravone and Riluzole elicits similar effects on DUX4 and TDP-43, further reinforcing our hypothesis of a possible interaction between these two proteins, in a subgroup of samples we also analyzed the effect of Edaravone and Riluzole on the expression of TDP-43.

Our preliminary results indicate that both mRNA (Figure 11 A-B-C) and protein levels (Figure 12 A-B-C) of TDP-43 show in all cell lines a modulation pattern similar to that observed for DUX4, both following Edaravone or Riluzole exposure and after co-treatment with the pro-oxidant H_2O_2.

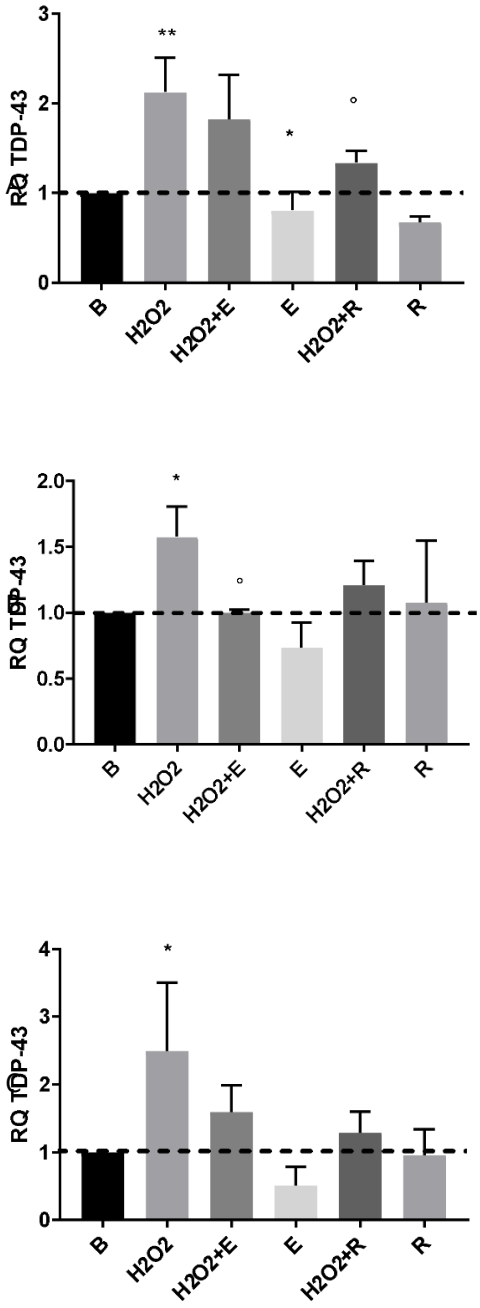


Figure 11. Effect of Edaravone (50 uM) and Riluzole (10 uM) on TDP-43 mRNA levels in parental cells, cells overexpressing wild-type SOD1 (WT) and G93A SOD1 mutation under basal conditions and after exposure to H_2O_2 (50 μM). The values are expressed as the mean ± SD of the fold change vs. untreated cells (RQ=1). N=3. A) Parental line ***p<0.001 H_2O_2 vs basal; * p<0.01 H_2O_2+E vs basal; °p<0,01 H_2O_2+R vs H_2O_2. B) SOD1 WT line: *p<0.04 H_2O_2 vs basal; °p<0.01 H2O2+E vs basal. C) G93A line: *p<0,01 H_2O_2 vs basal.

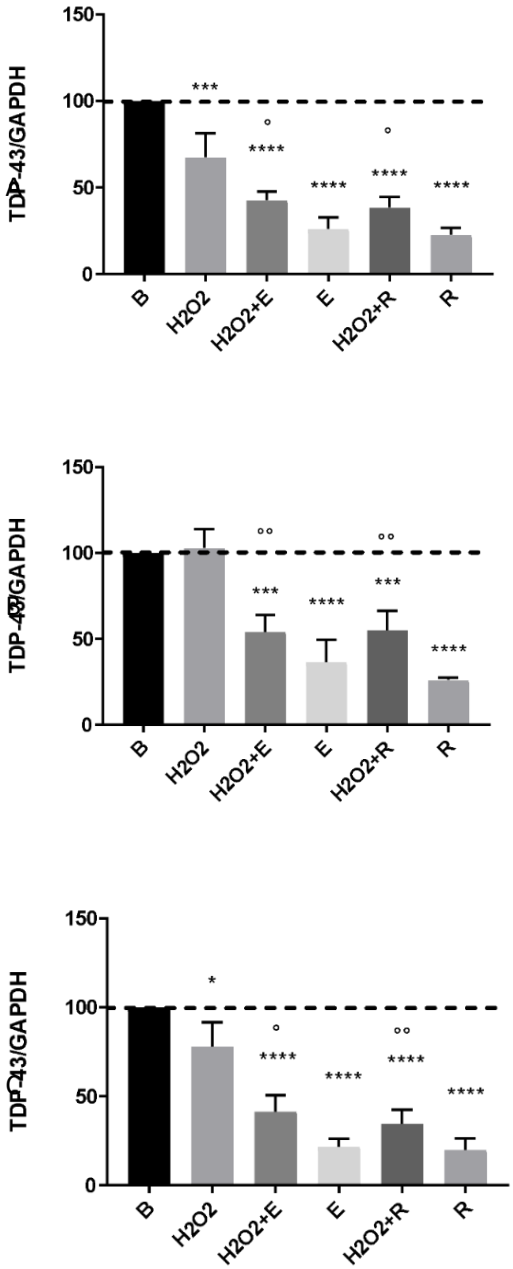


Figure 12. Effect of Edaravone (50 uM) and Riluzole (10 uM) on TDP-43 protein levels in parental cells, cells overexpressing wild-type SOD1 (WT) and G93A SOD1 mutation under basal conditions and after exposure to H_2O_2 (50 µM). The values are expressed as the mean ± SD of the relative expression vs. untreated cells (relative expression=100). N=3. A) Parental line: ***p<0.0007 H_2O_2 vs. basal, ****p<0.0001 H_2O_2+E/ only E/ H_2O_2+R/ only R vs basal; °p<0,04 H_2O_2+E vs H_2O_2, °p<0,02 H_2O_2+R vs H_2O_2. B) SOD1 WT line: ***p<0.0006 H_2O_2 vs basal; ***p<0.0003 H_2O_2+E/ H2O2+R vs basal, ****p<0,0001 only E/only R vs basal, °°p<0,003 H_2O_2+E/H_2O_2+R vs H_2O_2. C) SOD1 G93A line: *p<0,02 H_2O_2 vs basal. ****p<0.0001 H_2O_2+E/ only E/ H_2O_2+R/ only R vs basal, °p<0.01 H_2O_2+E vs H_2O_2, °°p<0.005 H_2O_2+R vs H_2O_2.

Taken together, these first results confirm data on TDP-43 previously seen in our laboratory and not yet published. Above all, they represent further evidence supporting the existence of a possible interaction between DUX4 and TDP-43 proteins, described in facioscapulohumeral dystrophy (Homma et al. 2015) and hypothesized in the first part of this thesis in ALS patients (Duranti et al. submitted paper).

However, the effect of these drugs on TDP-43 remains to be confirmed in a higher sample size, and specific studies are needed to clarify the existence of a specific interaction between TDP-43 and DUX4.

Chapter 6:

Further prospective

In these studies, the existence of an increased expression of DUX4 in ALS patients emerged for the first time, corroborating the hypothesis in the literature according to which DUX4 could have a link with TDP-43, possibly playing a role in the pathogenesis of ALS.

A potential limitation of this study is that we did not include cohorts of patients affected by other pathologies, such as neuromuscular or neurodegenerative diseases, to evaluate the specificity of the increase in DUX4 observed in ALS. Further work is required to investigate the expression of DUX4 in other neuromuscular diseases, to verify its specificity in the differential diagnosis of ALS, and in other neurodegenerative pathologies (Alzheimer's and Parkinson's diseases) characterized by protein accumulation, to understand the exact role of DUX4 in TDP-43 alterations. As hypothesized in a previous study (Homma et al. 2015), DUX4 dysfunction could have a general impact on proteostasis, possibly through the alteration of protein quality control systems. In the future, it may be interesting to deeply study the involvement of DUX4 in autophagy and other protein degradation processes. In this view, we recently started a new set of experiments to assess the effect of autophagy (obtained by 3-MA exposure) or proteasome (obtained by MG132 exposure) inhibition on the expression of DUX4 that need to be completed to reach a conclusion.

Another general limitation of this work is that DUX4 alterations evidenced in peripheral tissues from ALS patients (PBMCs and fibroblasts) do not reflect an already demonstrated alteration of DUX4 in tissues affected by the pathology. To partially overcame this limitation, in this study we analyzed DUX4 expression in central nervous tissues from patients, although more extensive studies in ALS motor neurons are needed.

Anyway, this limitation is at least partially overcome by the clear finding of a molecular endophenotype typical of ALS already demonstrated in PBMCs, which show TDP-43 pathology similar to that observed in motor neurons.

Furthermore, as this can be considered a pilot study, the obtained results in ALS PBMCs need to be confirmed in larger and more homogeneous population of ALS patients. However, even though evidence of an increase in DUX4 levels in patients will be confirmed, the specific significance of this alteration remains to be clarified, especially in terms of the molecular mechanisms involved.

In the future, it will be interesting to use myoblast cell lines obtained from patient biopsies, available in the Telethon Biobank. Muscle tissue is in fact one of the tissues mainly involved in the pathology; therefore, it would be extremely useful to extend our analysis performed in human neuroblastoma cells also in this cell model. This strategy will allow us to have a cell model without the limitations of neuroblastoma cells, which have a tumor phenotype that renders these cells unsuitable for specific studies. In fact, to understand the possible molecular mechanisms underlying DUX4 overexpression, it is essential to identify cellular pathways (including protein kinases pathways) that could be altered but that can not be investigated in tumoral cell lines.

Furthermore, studies on the role of DUX4 in FSHD have shown its possible involvement in inflammatory processes operative in the muscle. Since there is increasing evidence of the great importance of muscle damage in the severity and progression of ALS, it would be interesting to verify in primary cell cultures of myoblasts from ALS patients the possible involvement of DUX4 in pro-inflammatory processes.

However, despite the limitations and the necessary insights, results obtained in this thesis might open the way, in a still incurable disease such as ALS, to new fascinating and effective therapeutic perspectives focused on different strategies, including gene therapy, targeted on DUX4 modulation.

Chapter 7:

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