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PATHOGENETIC MECHANISMS IN MOTOR NEURON DISEASES

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ABSTRACT

Amyotrophic Lateral Sclerosis, ALS, and Spinal and Bulbar Muscular Atrophy, SBMA (or Kennedy's disease, KD) are two rare degenerative disorders of nervous system, characterized by early involvement of motor neurons. ALS is characterized by degeneration and death of upper and lower motor neurons, which leads to generalized muscle weakness and atrophy, speech and swallowing disabilities, and progressive paralysis until death is caused by respiratory failure. Its pathogenesis is still unknown. KD is an inherited X-linked motor neuronal disorder caused by an abnormal expansion of a trinucleotide CAG repeat in the first coding exon of the androgen receptor (*AR*) gene and resulting in an extended polyglutamine AR protein. The main clinical features of KD are proximal limb and bulbar muscle weakness and atrophy.

The present study develops two principal topics on these two motor neuron diseases and aims to understand pathogenetic mechanisms for diagnostic, prognostic and treatment purposes.

In our cohort of ALS patients, we evaluate skeletal muscle as possible biomarker site, through the analysis of TDP-43 protein in 30 muscle biopsies; we found no pathological alterations and we concluded that TDP-43 pathology is not to be considered a systemic feature. We also screened 222 ALS patients for *TARDBP* and *FUS/TLS* genes mutations involved in the pathogenesis of ALS; we found two known mutations in *TARDBP* and we could contributed to confirm the frequencies in ALS population. Lastly, we genotyped SNP rs1541160 of *KIFAP3* gene in 228 ALS patients and we could assess a relationship between the minor CC genotype and the upper motor neuron-dominant ALS phenotype.

Concerning KD, we aimed to investigate whether the myopathy could start at muscular level and caused by the mutant polyQ-AR, which is toxic when accumulate into the nucleus. We evaluated differentiation and maturation ability of satellite cells from four KD patients, cultured with or without androgen (Dihydrotestosterone, DHT) treatment; we also assessed polyQ-AR localization in

KD satellite cells and 10-day differentiated myotubes, with/without DHT treatment. The results showed a marked atrophy in KD myotubes that could be assigned to mutant polyQ-AR since it was observed significantly increase in KD myotube nuclei compared to controls. This confirmed the primitive muscular involvement in KD.

ABSTRACT

La Sclerosi Laterale Amiotrofica, SLA, e l'Atrofia Muscolare Spinobulbare (o malattia di Kennedy, KD) sono due rari disturbi degenerativi del sistema nervoso, caratterizzati da un precoce coinvolgimento dei neuroni motori. La SLA è caratterizzata da degenerazione e morte di motoneuroni superiori e inferiori, che comporta debolezza muscolare generalizzata e atrofia, incapacità di parlare e deglutire, e progressiva paralisi fino alla morte, causata da arresto respiratorio. La sua patogenesi è ancora sconosciuta. La KD è un disturbo ereditario legato al cromosoma X dei neuroni motori, causato da un'anormale espansione del trinucleotide CAG nel primo esone codificante del gene del recettore degli androgeni (*AR*), il quale risulta in una proteina con un più esteso tratto di poliglutammine. Le principali caratteristiche cliniche sono debolezza muscolare bulbare e degli arti prossimali.

Il presente studio sviluppa due principali argomenti su queste due malattie dei motoneuroni, allo scopo di comprenderne i meccanismi patogenetici utili per fini diagnostici, prognostici e terapeutici.

Nella nostra coorte di pazienti SLA, abbiamo valutato il muscolo scheletrico come possibile sito per un biomarcatore, attraverso l'analisi della proteina TDP-43 in 30 biopsie muscolari; non sono state trovate alterazioni patologiche e questo ci ha permesso di concludere che le patologie da TDP-43 non si possono considerare sistemiche. Abbiamo anche screenato 222 pazienti SLA per mutazioni nei geni *TARDBP* e *FUS/TLS* che potessero essere coinvolte nella patogenesi della malattia; abbiamo trovato due pazienti con la stessa mutazione in *TARDBP* e abbiamo potuto confermarne le frequenze nella popolazione SLA. Infine, abbiamo genotipizzato lo SNP rs1541160 del gene *KIFAP3* in 228 pazienti SLA e abbiamo potuto stabilire una forte relazione tra il genotipo minore CC e un particolare fenotipo SLA, upper motor neuron-dominant ALS.

Per quanto riguarda la KD, lo scopo dello studio è stato quello di capire se la miopatia poteva scaturire a livello muscolare (anziché essere conseguenza della

denervazione) ed essere causata da AR mutato (polyQ-AR), il quale è noto per essere tossico quando accumula nel nucleo della cellula. Abbiamo valutato le capacità di differenziamento e di maturazione delle cellule satelliti di quattro pazienti KD, tenute in coltura con e senza trattamento androginico con Dihydrotestosterone (DHT); abbiamo anche valutato la localizzazione di polyQ-AR nelle cellule satelliti e nei miotubi dopo 10 giorni di differenziamento, con/senza trattamento con DHT. I risultati hanno mostrato una marcata atrofia nei miotubi KD in confronto con controlli sani, che può essere attribuita alla presenza di AR mutato dato che la proteina polyQ-AR era maggiormente espressa nei nuclei dei miotubi KD rispetto ai controlli. Questo conferma il coinvolgimento primario nel muscolo nella KD.

SUMMARY

MOTOR NEURON DISEASES

Motor Neuron Diseases (MND) is an heterogeneous family of rare degenerative pathologies of nervous system, characterized by early involvement of motor neurons.

Normally, motor neurons control voluntary muscle activity through messages from nervous cells in the brain (*upper motor neurons*, UMN) transmitted to nervous cells in the brain stem and spinal cord (*lower motor neurons*, LMN) and from them to muscles.

MND includes sporadic and familial amyotrophic lateral sclerosis (ALS), spinal muscular atrophy (SMA), hereditary spastic paraplegia (HSP), primary lateral sclerosis (PLS), and spinal and bulbar muscular atrophy (SBMA) or Kennedy disease [1] (Table 1).

	age of onset	prevalence	motor neuron involvement	clinical features
ALS	~45-60 years old	4-6/100.000	UMNs and LMNs	Progressive muscle weakness, atrophy and spasticity
HSP	Early childhood to 70 years old	3-10/100.000	UMNs	Progressive spasticity in the lower limbs
PLS		1/10.000.000	UMNs	Spinal and bulbar spasticity
SMA		1/6.000-10.000	LMNs	Symmetrical muscle weakness and atrophy
SBMA		1-9/100.000	LMNs	Slowly progressive limb and bulbar muscle weakness with fasciculations, muscle atrophy and gynecomastia
LCCS		1/25.000	UMNs and LMNs	Early fetal hydrops and akinesia, degeneration of anterior horns neurons and extreme skeletal muscle atrophy

Table 1. Classification and clinical characteristics of motor neuron diseases. ALS, Amyotrophic Lateral Sclerosis; HSP, Hereditary Spastic Paraplegia; PLS, Primary Lateral Sclerosis; SMA, Spinal Muscular Atrophy; SBMA, Spinal and Bulbar Muscle Atrophy; LCCS, Lethal Congenital Contracture Syndrome.

These syndromes typically manifest clinically by weakness, spastic paralysis, or both, reflecting a functional loss of upper and/or lower motor neurons. ALS includes dysfunction and loss of both upper and lower motor neurons, whereas SMA and SBMA result from the loss of lower motor neurons, [4] and PLS involves loss of upper motor neurons [3]. HSP is a complex group of inherited disorders that includes mutations of at least 20 genes and predominately involves upper motor neurons [2].

These diseases can be sporadic or inherited, with childhood or adult onset. While the genetic alterations that lead to familial forms of these disorders provide a diagnostic measure of the disease, each MND is typically diagnosed by clinical and electrodiagnostic features.

The study develops two principal topics on two MND (Amyotrophic Lateral Sclerosis and Spinal and Bulbar Muscular Atrophy) and aims to understand pathogenetic mechanisms for diagnostic, prognostic and treatment purposes.

Amyotrophic Lateral Sclerosis

INTRODUCTION

ALS is a neurodegenerative disorder characterized by progressive degeneration of motor neurons in the primary motor cortex, brainstem and spinal cord. "Amyotrophy" refers to the atrophy of muscle fibers, which are denervated as their corresponding anterior horn cells degenerate, leading to weakness of affected muscles and visible fasciculations. "Lateral sclerosis" refers to hardening of the anterior and lateral corticospinal tracts as motor neurons in these areas degenerate and are replaced by gliosis [93].

The incidence of sporadic ALS (SALS) is reported to be 1-2 per 100,000 population/year in Europe and North America [128], with a uniform incidence across these countries. A consistent finding in studies is that there is a slight excess of males are affected more than females, with a M:F ratio about 3:2, although more recent data suggests that the gender ratio may be approaching equality [5,70,128,131]. The mean age of onset for SALS varies between 60-70 years. Only 5% of cases have an onset before the age of 30 years [49], although juvenile sporadic onset cases are being increasingly recognised [44]. Bulbar onset is commoner in women and in older age groups. Although most cases of ALS are sporadic, about 10% of cases have a family history of ALS (Familial ALS, FALS) [9]. There is an often Mendelian inheritance and high penetrance, with most cases having autosomal dominant pattern of inheritance (few autosomal recessive pedigrees have also been reported [74,]). The ages of onset of FALS is about a decade earlier than for sporadic cases, affects males and female equally, and have a shorter survival [125].

1.1 Clinical presentation

The features of ALS were first clearly described as a clinicopathological entity by Jean Martin Charcot in 1869 and in subsequent articles in 1874 [125]. However, before that Bell (1824), Aran (1850), Duchenne (1851), and Cruveilher (1853) made important observations that contributed to the understanding of the clinical and pathological syndrome [42,94,103].

1.1.1 Symptoms

Approximately two thirds of patients with typical ALS have a spinal form of the disease. They present with symptoms related to focal muscle weakness where the symptoms may start either distally or proximally in the upper and lower limbs. Rarely, patients may notice focal muscle wasting before onset of weakness, and some patients may present with a spastic paraparesis. Patients may have noticed fasciculations or cramps preceding the onset of weakness or wasting for some months (or years), but rarely these are the presenting symptoms. ALS is usually asymmetrical at onset, the other limbs develop weakness and wasting sooner or later, and most patients go on to develop bulbar symptoms and eventually respiratory symptoms. Gradually, spasticity may develop in the weakened atrophic limbs, affecting manual dexterity and gait. During late stages of the disease patients may develop involuntary spasms due to excess activation of the flexor arc in a spastic limb. Occasionally encountered symptoms include new bladder dysfunction, sensory symptoms, cognitive symptoms and multi-system involvement (*e.g.* dementia, parkinsonism). Patients with bulbar onset ALS usually present with dysarthria of speech, which may initially only be apparent after ingestion of small amount of alcohol. Rarely, patients may present with dysphagia for solid or liquids before noticing speech disturbances. Limbs symptoms can develop almost simultaneously with bulbar symptoms and in the vast majority of cases will occur within 1–2 years. Almost all patients with bulbar symptoms develop sialorrhoea due to difficulty swallowing saliva and mild UMN type bilateral facial weakness which affects the lower part of the face. 'Pseudobulbar' symptoms such as emotional lability and excessive yawning are seen in a significant number of cases.

1.1.2 Variant disorders

Variants of ALS have differing clinical presentations, rate of progression and prognosis. Opinion is divided as to whether these syndromes should be classed as separate entities from ALS, although there is evidence that there may be a common molecular pathology.

- **Progressive Muscular Atrophy, PMA**

The syndrome of PMA accounts for 5–10% of patients with ALS, and indicates a pure lower motor neuron syndrome without accompanying upper motor neurone signs [78]. It is almost always of limb onset, but patients may eventually develop swallowing difficulties. It is reported that up to 50% of patients may develop UMN signs and go on to develop typical ALS picture [114].

- **Flail arm and Flail leg**

The "flail arm" and "flail leg" variants are initially localized forms with a predominantly lower motor neuron presentation. In the flail arm variant, weakness and wasting predominantly affects the proximal upper limb in a symmetrical pattern, leading to severe wasting around the shoulder girdle and the arms hanging flaccidly either side. The lower limbs remain strong for some years but eventually spasticity and wasting develops. Swallowing difficulties and diaphragmatic weakness are usually late features [23,51]. In the flail leg syndrome [125], weakness and wasting begins in the distal lower limbs affecting both lower limbs in a symmetrical manner. The clinical features are of a lower motor neuron syndrome with hypotonia and depressed tendon reflexes. The unusual clinical picture together with lack of neurophysiological evidence of denervation in other regions can lead to considerable diagnostic delays. These two variants characteristically show slower progression compared to more typical forms of ALS [47,51,58,95,111].

- **Primary Lateral Sclerosis, PLS**

Primary Lateral Sclerosis is a clinically progressive pure upper motor syndrome that cannot be attributed to another disease process. There is ongoing debate as to whether this syndrome is in fact an entirely separate disorder to ALS, but there is evidence from pathological studies that hallmarks of ALS such as ubiquitinated inclusions are present in this condition. Patients present with a

pure upper motor neuron syndrome with either absent or minimal lower motor neuron signs. It can be difficult to differentiate PLS from ALS during the early stages as some patients with typical ALS may only manifest UMN signs. For this reason, some authors have suggested that LMN signs must be absent for 3 years from onset to confidently diagnose PLS [84]. However, there may be electrophysiological evidence of LMN involvement in PLS patients despite the absence of clinical LMN signs, and some patients may develop wasting of small muscles of the hands, adding to the diagnostic confusion [67,68], a condition called by some authors as "UMN-dominant ALS" [43,112]. Prognosis for PLS is considerably better than for typical ALS [43].

- **UMN-dominant ALS**

Gordon et al. [43] proposed a new category for diagnostic classification named UMN-dominant ALS. Is defined as predominantly due to UMN signs but with minor electromyogram (EMG) denervation or LMN signs on examination, not sufficient to meet diagnostic criteria for ALS. UMN-dominant patients are reported to have a shorter survival than subjects with clinically pure PLS and a slower disease progression compared to those with typical ALS [18]. As UMN-dominant ALS has only recently been defined, there are no large-scale systematic studies on its course and prognosis. Sorarù et al. (2010) described clinical and laboratory features of 20 patients with UMN-dominant ALS reporting findings that suggest both a different pattern of disability and longer survival in UMN-dominant ALS compared to classic ALS patients [104].

1.1.3 Histopathology

The pathological hallmarks of ALS are the degeneration and loss of motor neurons with astrocytic gliosis and the presence of intraneuronal inclusions in degenerating neurons and glia. Upper motor neuron pathology in ALS is indicated by depopulation of the Betz cells in the motor cortex, variable astrocytic gliosis affecting both the grey matter and underlying subcortical white matter of the motor cortex, and axonal loss within the descending pyramidal motor pathway associated with myelin pallor and gliosis of the corticospinal tracts [54,55]. Lower motor neuron pathology primarily affects the ventral horn motor neurons of the spinal cord and brainstem. The number of lower motor

neurons can be reduced by up to 50% at autopsy but there is considerable variation both between cases and between different spinal levels within cases [55]. The remaining neurons are atrophic and contain intraneuronal inclusions such as Bunina bodies, ubiquitinated or ubiquitin-immunoreactive inclusions and Hyaline conglomerate (Neurofilament) inclusions. Contrary to early belief that ALS was a disease exclusive to the motor system, there is now significant evidence to suggest that ALS is in fact a multisystem disorder. Extra motor pathology is found in regions such as the frontotemporal cortex, hippocampus, thalamus, substantia nigra, spinocerebellar pathways, dorsal columns and peripheral sensory nerves[125]. ALS variant syndromes seem to share a common molecular pathology as suggested by the findings of ubiquitinated inclusions in PLS, PMA, Flail arm syndrome, Flail leg, ALS-dementia and Guam ALS-PDC [125]. A recent finding is that TDP-43 has been shown to be a major protein constituent in the ubiquitin positive (tau and a-synuclein negative) inclusions found in upper and lower motor neurons in ALS (see Par. 1.5.1), frontotemporal lobar degeneration with MND (FTLD-MND) and frontotemporal lobar degeneration with ubiquitin inclusions (FTLD-U). TDP-43 positive inclusions were also detected in one of two cases of PLS but appear to be negative in the inclusions seen in SOD1 positive familial ALS [30,71,90].

1.2 Diagnosis

ALS is diagnosed based on the presence of very characteristic clinical findings in conjunction with investigations to exclude "ALS-mimic" syndromes. The clinical finding of signs suggestive of combined upper motor neuron (UMN) and lower motor neuron (LMN) that cannot be explained by any other disease process (evident on electrophysiological, imaging, cerebrospinal fluid (CSF) or serological studies), together with progression compatible with a neurodegenerative disorder, is suggestive of ALS. Thus, investigation results alone (*e.g.*, evidence of chronic denervation on electromyography are not adequate for achieving a diagnosis, and must be interpreted in light of the patient's history and clinical findings. The World Federation of Neurology (WFN) Research Group on Motor Neuron Diseases have developed the 1994 'El Escorial' diagnostic criteria to aid

diagnosing and classifying patients for research studies and drug trials. Based on these criteria patients can be classified into 'Clinically definite', 'Clinically probable', 'Clinically probable-Laboratory supported' and 'Clinically possible' categories.

1.3 Etiology

The cause of ALS is unknown although some genetic risk factors have been identified. Recent reviews on the role of environmental risk factors in the causation of ALS have concluded that there is no consistent association between a single environmental factor and risk of developing ALS. Most authors favor a hypothesis of complex genetic-environmental interaction as the causal factor for motor neuron degeneration [24,102]. Putative exogenous risk factors associated with development of ALS investigated in case-control studies have been reviewed [10]. By applying an evidenced based approach, it was found that only smoking is likely to be associated with developing ALS, while other risk factors were weakly related. More recent case-control studies have estimated the relative risk (RR) of ALS of 0.8–1.67 in smokers compared to nonsmokers [33,123], and an odds ratio (OR) of 1.6 independent of age, level of education and occupation [109].

1.4 Pathogenesis of motor neuron degeneration in ALS

The exact molecular pathway causing motor neuron degeneration in ALS is unknown, but as with other neurodegenerative diseases, is likely to be a complex interplay between multiple pathogenic cellular mechanisms which may not be mutually exclusive [24,102]. These include:

1.4.1 Genetic factors

20% of cases with autosomal dominant FALS and 2% of patients with SALS show mutations in the Copper-Zinc superoxide dismutase (*SOD1*) gene [91]. Mutations in this gene are thought to cause disease through a toxic gain of function rather than causing impairment of the antioxidant function of the SOD1 enzyme [102]. Other genes causing familial MND include *alsin* (ALS2), *senataxin* (ALS4), Vesicle associated membrane protein (*VAPB*, ALS8), *Angiogenin* and a mutation in the p150 subunit of dynactin (*DCTN1*) [125]. Recently, mutations in *TARDBP* and *FUS/TLS* genes (encoding TDP-43 and FUS respectively, see Par. 1.5).

FALS type	locus	gene	inheritance	clinical pattern	mutations	cause SALS
ALS1	21q	SOD1	AD	Classical	> 120	yes
ALS2	2q33	ALSIN	AR	young onset, UMN	10	no
ALS3	18q21		AD	classical		
ALS4	9q34	SETX	AD	young onset, slow	3	probably not
ALS5	15q15		AR	young onset		probably not
ALS6	16q21		AD	classical		
ALS7	20ptel-13		AD	classical		
ALS8	20q13.3	VAPB	AD	varied	1	
ALS-FTD	9q21-22		AD	with FTD		
ALS-FTD	9q21.3		AD	with FTD		
ALS	14q11.2	AGN	AD	classical	6	yes
FTD (FTD3)	3	CHMP2B	AD	FTD	2	
ALS	1	TDP43	AD	ALS	14	yes
LMND	2p13	DCTN1	AD	LMND	1	yes

Table 1.1. FALS gene mutations and clinical features of ALS disease. (AD) autosomal dominant, (AR) autosomal recessive, (SETX) senataxin, (VAPB) vesicle membrane protein, (CHMP2B) chromatin modifying protein 2B, (DCTN1) dynactin-1, (UMN) upper motor neuron disease, (FTD) fronto temporal dementia, (LMND) lower motor neuron disease

Several other gene mutations have been identified in sporadic cases which may increase susceptibility to ALS, such as mutations in the KSP repeat region in the *NEFH* gene (encoding neurofilament heavy subunit) [36,113], apolipoprotein E Σ4 genotype (*APOE*) [7], decreased expression of EAAT2 protein [73] and alterations in the Vascular endothelial growth factor (*VEGF*) gene [64] (Table 1.1).

1.4.2 Excitotoxicity

This is the term for neuronal injury induced by excessive glutamate induced stimulation of the postsynaptic glutamate receptors such as cell surface NMDA receptors and AMPA receptors [82,102]. This over stimulation of glutamate receptors is thought to result in massive calcium influx into the neurons, leading to increased nitric oxide formation and thereby neuronal death. Glutamate levels in CSF are elevated in some patients with ALS [101]. This elevation has been attributed to the loss of the glial cell excitatory amino acid transporter EAAT2 [92].

1.4.3 Oxidative stress

Oxidative stress has longed been linked to neurodegeneration and it is known that accumulation of reactive oxygen species (ROS) cause cell death. As mutations in the anti-oxidant enzyme superoxide dismutase 1 (*SOD1*) gene can cause familial ALS, there is significant interest in this mechanism underlying neurodegenerative process in ALS. This hypothesis is supported by the finding of biochemical changes reflecting free radical damage and abnormal free radical metabolism in CSF and *post mortem* tissue samples of ALS patients [35]. In addition, fibroblasts cultured from ALS patients shows increased sensitivity to oxidative damage controls [6].

1.4.4 Mitochondrial dysfunction

Abnormalities in mitochondrial morphology and biochemistry have been reported in sporadic ALS patients, SOD1 transgenic mice and cellular models [59,60]. Mitochondria from ALS patients show elevated calcium levels and decreased activity of respiratory chain complexes I and IV, implicating defective energy metabolism [124]. Mitochondrial DNA mutations have been described in ALS patients [29].

1.4.5 Impaired axonal transport

Motor neuron axons may reach up to one meter in length in humans, and rely on efficient intracellular transport systems. These systems consist of anterograde (slow and fast) and retrograde transport systems, and rely on molecular 'motors', the kinesin complex of proteins (for anterograde) and the dynein-dynactin complex (for retrograde). SOD1 transgenic mouse models of ALS show evidence of slowed anterograde transport and retrograde transport [14]. Although no such findings have been observed in humans with ALS, mutations in the kinesin genes are known to cause neurodegenerative motor nerve diseases in humans such as hereditary spastic paraplegia and Type 2A Charcot-Marie-Tooth disease [87,130]. Mutations in the dynactin complex cause a lower motor neuron disorder with vocal cord paralysis in humans [85].

1.4.6 Neurofilament aggregation

Abnormal assembly with accumulation of neurofilaments are commonly seen in several neurodegenerative conditions including ALS [50]. In addition, mutations in KSP repeat region of the neurofilament heavy (*NFH*) gene are found in about 1% of SALS cases [36,113]. Neurofilament proteins together with Peripherin (an intermediate filament protein) are found in the majority of axonal inclusions motor neurons of ALS patients [21]. A toxic isoform of peripherin (peripherin 61), has been found to be toxic to motor neurons even when expressed at modest levels and is detectable in spinal cords of ALS patients but not controls [89].

1.4.7 Protein aggregation

Intra-cytoplasmic inclusions are a hallmark of both SALS and FALS (see Par. 1.1.3). However, it is still unclear as to whether aggregate formation directly causes cellular toxicity and have a key role in pathogenesis, if aggregates may be innocent products of the neurodegeneration process, or if formation of the aggregates may be a defense mechanism to reduce intracellular concentrations of toxic proteins [24,102].

1.4.8 Inflammatory dysfunction and contribution of non neuronal cells

Although ALS is not primarily a disorder of autoimmunity or immune dysregulation, there is considerable evidence that inflammatory processes and non-neuronal cells may play a part in pathogenesis of ALS. Microglial and dendritic cell activation is a prominent pathology in human ALS and transgenic SOD1 mice [48]. These activated non-neuronal cells produce inflammatory cytokines such as interleukins, COX-2, TNFα and MCP-1, and evidence of upregulation is found in CSF or spinal cord specimens of ALS patients or *in vitro* models [88,98]. Despite this evidence, immunomodulatory therapies are not good neuroprotective agents in clinical trials of ALS.

1.4.9 Deficits in neurotrophic factors and dysfunction of signalling pathways

Decreased levels of neurotrophic factors (*e.g.* CTNF, BDNF, GDNF and IGF-1) have been observed in ALS patients post-mortem and in *in vitro* models [8,32]. In addition, deletion of the hypoxia-response element in the vascular endothelial growth factor (*VEGF*) gene was found to cause a motor neuron disease in mice [79]. In humans, three mutations in the *VEGF* gene were found to be associated with increased risk of developing SALS [64], although a recent meta-analysis by the same authors failed to show an association between *VEGF* haplotypes and increase risk of ALS in humans [65]. The final process of cell death in ALS motor

neurons is thought to closely resemble a programmed cell death pathway (apoptosis). Biochemical markers of apoptosis are detected in the terminal stages of human and models of ALS [46,69]. Key elements of the normal apoptotic pathway are found to be involved in cell death in ALS, including the caspase family of proteolytic enzymes, the Bcl2 family of oncoproteins (anti-apoptotic and proapoptotic oncogenes) and the apoptosis inhibitor family of proteins (IAPs) [82,96,102].

1.5 RNA processing and neurodegeneration

1.5.1 *TARDBP* and TDP-43

A major shift into the understanding of ALS pathogenesis started in 2006 [75] with the identification of the 43 kDa transactive response (TAR) DNA-binding protein (TDP-43) as the main component of ubiquitinated protein aggregates found in SALS patients and in patients with frontotemporal lobar degeneration (FTLD), a neurodegenerative disease characterized by behavioral and language disorders.

TDP-43 is a 414 amino acid protein encoded by six exons, located in Chromosome 1 (1p36.2), and containing two RNA recognition motifs (RRM1 and 2) and a C-terminal glycine-rich region, often found to mediate protein-protein interactions [80].

TDP-43 was initially described as a regulator of HIV-1 gene expression and it was reported in the past to affect both normal and pathological RNA splicing events. Now it is described with roles in transcription and splicing regulation, microRNA biogenesis, apoptosis, and cell division (major roles are summarized in Figure 1.1). In particular, it plays a fundamental role in the occurrence of several monosymptomatic/full forms of Cystic Fibrosis caused by pathological skipping of CFTR exon 9 from the mature mRNA [17].

TDP-43 is mainly localized within the nucleus, but abnormal TDP-43 distribution such as neuronal cytoplasmic or intranuclear inclusions and dystrophic neurites [75], as well as glial cytoplasmic inclusions [30,76] have been reported. TDP-43 inclusions are not restricted to motor neurons but can be widespread in brain in

ALS patients with or without dementia [120,41]. A significant TDP-43 nuclear clearance in a proportion of neurons containing cytoplasmic aggregates, suggesting that pathogenesis may be driven, at least in part, by loss of one or more nuclear TDP-43 functions [75,120,30,52]. Immunoblotting of detergent-insoluble protein extracts from affected brain and spinal cord has defined a biochemical signature of disease that includes hyperphosphorylation and ubiquitination of TDP-43, and the production of several C-terminal fragments (CTFs) around 25 kDa (Figure 1.2) [75].

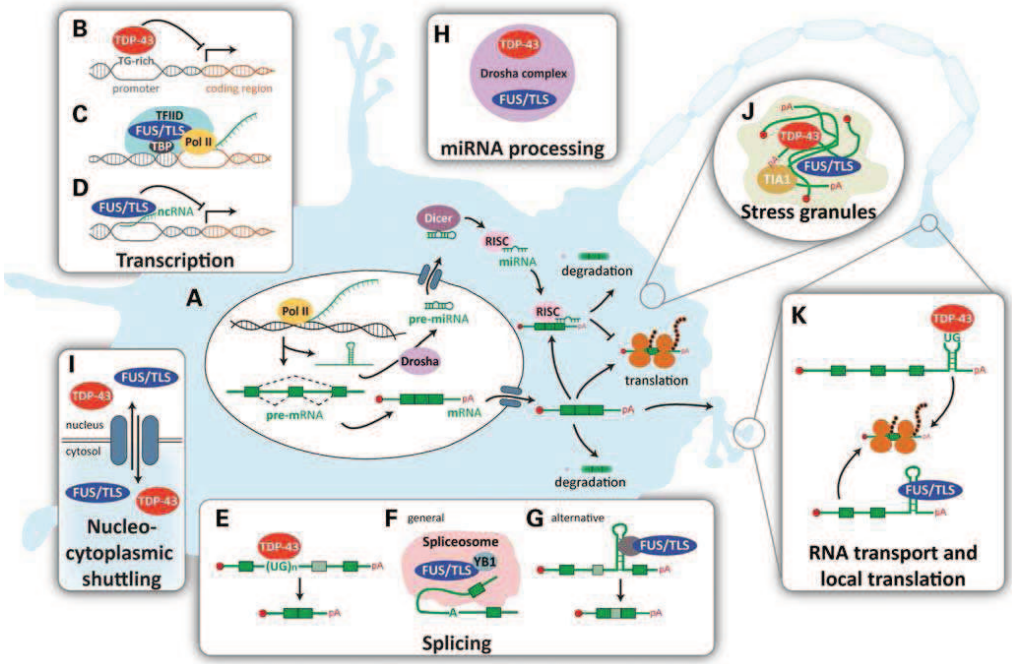

Figure 1.1. Proposed physiological roles of TDP-43 and FUS/TLS. (A) Summary of major steps in RNA processing from transcription to translation or degradation. (B) TDP-43 binds single-stranded TG-rich elements in promoter regions thereby blocking transcription of the downstream gene (shown for TAR DNA of HIV and mouse SP-10 gene). (C) FUS/TLS associates with TBP within the TFIID complex suggesting that it participates in the general transcriptional machinery. (D) In response to DNA damage, FUS/TLS is recruited in the promoter region of cyclin D1 (CCND1) by sense and antisense non coding RNAs (ncRNAs) and represses CCND1 transcription. (E) TDP-43 binds a UG track in intronic regions preceding alternatively spliced exons and enhances their exclusion shown for CFTR and apolipoprotein A-II. (F) FUS/TLS was identified as a part of the spliceosome and (G) was shown to promote exon inclusion in H-ras mRNA, through indirect binding to structural regulatory elements located on the downstream intron. (H) Both proteins were found in a complex with Drosha, suggesting that they may be involved in miRNA processing. (I) Both TDP-43 and FUS/TLS shuttle between the nucleus and the cytosol and (J) are incorporated in SGs where they form complexes with mRNAs and other RNA binding proteins. (K) TDP-43 and FUS/TLS are both involved in the transport of mRNAs to dendritic spines and/or the axonal terminal where they may facilitate local translation. Examples of such cargo transcripts are the low molecular weight NFL for TDP-43 and the actin-stabilizing protein Nd1-L for FUS/TLS.

Interestingly, the composition of TDP-43 inclusions seems to differ between cortical brain and spinal cord in ALS patients, as inclusions from cortical regions are preferentially labeled by C-terminal antibodies, whereas spinal cord inclusions display equivalent immunoreactivity between N- and C-terminal-specific antibodies [52].

Mislocalization of TDP-43 observed by pathology motivated direct sequencing of the gene encoding it (*TARDBP*) in cohorts of ALS patients. In early 2008, the successful identification of dominant mutations as a primary cause of ALS provided evidence that aberrant TDP-43 can directly trigger neurodegeneration [56,106,120].

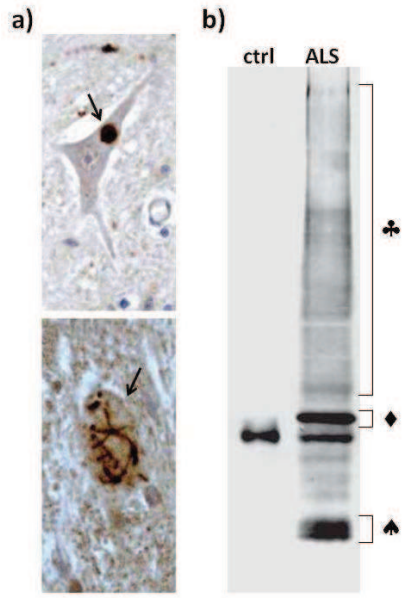

Figure 1.2. TDP-43 pathological alterations in ALS brains. a) Characteristic TDP-43 immunoreactive neuropathological features of ALS (arrows); b) WB probed with TDP-43 antibody demonstrating the disease-specific biochemical profile of TDP-43 in urea extracts from ALS brains, with the presence of pathologic TDP-43 species detected as high-molecular-weight smear (♣), approximately 45-kDa band (♦) and additional approximately 25-kDa bands (♠), which are not detectable in control (ctrl) brains.

A total of 38 mutations (Figure 1.3) have been described in ALS patients with or without apparent family history, corresponding to ~4% of familial ALS (and ~1% of sporadic ALS) [27,56,106,120].

Most patients with TDP-43 mutation develop a classical ALS phenotype without cognitive deficit, with some variability within families in the site and age of onset. Most of the mutations identified are localized in the glycine-rich region encoded by exon 6. All the mutations are dominant missense changes with the exception of a truncating mutation at the extreme C-terminal of the protein (Y374X) [27].

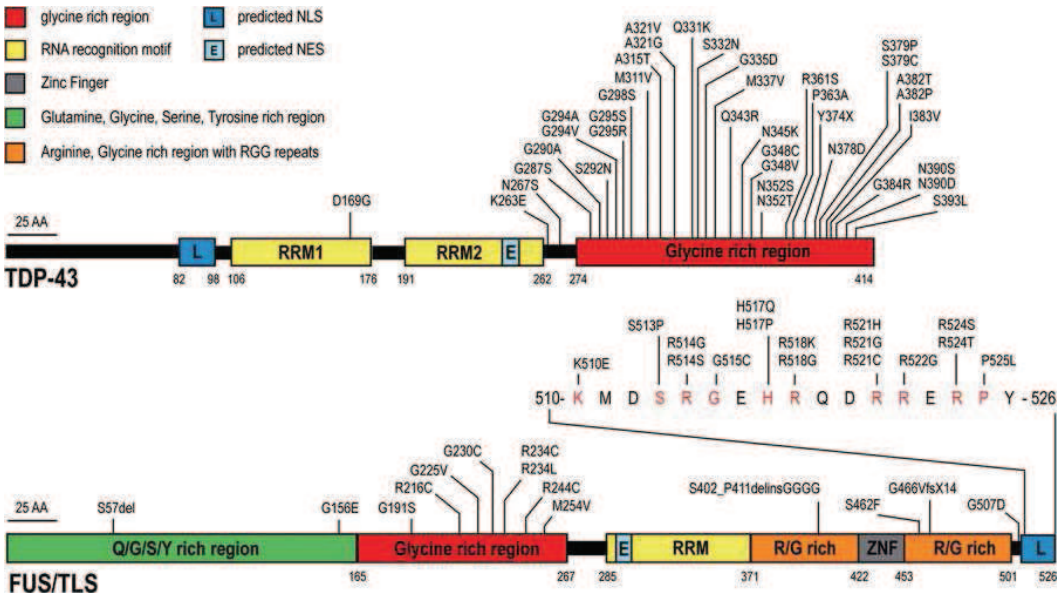

Figure 1.3. TDP-43 and FUS/TLS mutations in ALS patients.

1.5.2 *FUS/TLS* and FUS

The identification of TDP-43 mutations in ALS was rapidly followed by the discovery of mutations in another RNA/DNA binding protein, FUS/TLS. Thirty mutations (Figure 1.2) have now been reported in ~4% of FALS and in rare SALS patients [12,13,45,61,110,121,122]. The inheritance pattern is dominant except for one recessive mutation (H517Q) [61]. It is noteworthy that in-frame deletions and insertions in polyglycine tracts initially identified in ALS patients [61] were subsequently found in several control individuals, challenging their pathogenic effect [12,121]. The site and age of disease onset are variable within families, and incomplete penetrance has been documented for several *FUS/TLS* mutations [61,13,45]. Most patients carrying these mutations develop a classical ALS phenotype without cognitive defect; however, there are few exceptions.

FUS/TLS is a 526 amino acid protein encoded by 15 exons, located in Chromosome 16 (16p11.2), and characterized by an N-terminal domain enriched in glutamine, glycine, serine and tyrosine residues (QGSY region), a glycine-rich region, an RRM, multiple arginine/glycine/glycine (RGG) repeats in an arginine- and glycine-rich region and a C-terminal zinc finger motif [53]. Most of the mutations are clustered in the glycine-rich region and in the extreme C-terminal

part of the protein. The FUS/TLS nuclear localization signal (NLS) is likely to reside in this conserved C-terminal region.

Like TDP-43, FUS protein is a transcriptional factor ubiquitously expressed, predominantly nuclear in most cell types [9], involved in regulation of transcription, RNA splicing and export to the cytoplasm (Figure 1.1).

Postmortem analysis of brain and spinal cord from patients with *FUS/TLS* mutations found abnormal FUS/TLS cytoplasmic inclusions in neurons and glial cells [61,122]. These inclusions were reported to be immunoreactive for FUS/TLS, GRP78/BiP, p62 and ubiquitin, but strikingly not for TDP-43, implying that neurodegenerative processes driven by *FUS/TLS* mutations are independent of TDP-43 mislocalization [122,110]. Moreover, ubiquitination of FUS/TLS inclusions has not always been detected, but these inclusions do label strongly with antibodies recognizing the Nterminus, mid-region or C-terminus of the FUS/TLS protein [77]. Immunoblots confirmed the increased levels of full-length FUS/TLS protein in cytoplasmic and insoluble fractions, but no other evidence of biochemical abnormality such as hyperphosphorylation or ubiquitination was found [61].

1.6 Genetic variant associated with ALS phenotype

The hypothesis that genetic variants could be associated with SALS susceptibility has been recently tested in several genome-wide association (GWA) studies [20,25,31,38,97,99,100,118,119]. In their GWA, Landers and coll. [66] reported a 14 months survival advantage conferred by the CC genotype of single nucleotide polymorphism (SNP) rs1541160 mapping within the intron 8 of kinesin-associated protein 3 (*KIFAP3*) gene on Chromosome 1.

KIFAP3 works in a complex which is a fast anterograde translocator of membranous organelles.

Further analysis revealed linkage disequilibrium between rs1541160 and rs522444 within the *KIFAP3* promoter and that the favorable alleles of rs1541160 and rs522444 correlate with reduced *KIFAP3* expression in both lympoblasts and brain tissue of ALS patients. This suggested an explanation for the influence of rs1541160 on KIFAP3 regulation.

AIMS

Considering ALS, there is no biomarker that univocally suggests ALS disease and only few point mutations in few genes are surely involved in ALS pathogenesis.
To contribute to shed light on some aspects of ALS diagnosis and its subtype classification we aimed to:

1. Evaluate skeletal muscle of ALS patients as biomarker site, through immunohistochemical and biochemical analysis of TDP -43

2. To identify new mutations or genetic variants in ALS patients, by a screening of *TARDBP* and *FUS/TLS* genes, known to be involved in ALS pathogenesis

3. Assess the relationship between the *KIFAP3* rs1541160 genotype and ALS patients' survival.

MATERIALS AND METHODS

3.1 Patients

We considered ALS patients diagnosed in accordance with the El Escorial criteria [16] attending the Motor Neuron Disease Clinic of the University of Padova Medical Center.

3.1.1 Patients for skeletal muscle study

In order to look for a disease biomarker, we considered skeletal muscle biopsy of 30 patients (18 males and 12 females). As controls, muscle specimen of 15 patients affected with a denervative disease (4 cases with spinal and bulbar muscular atrophy, 2 cases with spinal muscular atrophy, 3 cases with hereditary motor neuropathy and 6 cases with hereditary sensory-motor neuropathy), 2 patients with primitive myopathy (one case with Becker's muscular dystrophy and one with polymyositis) and 13 subjects free from neuromuscular diseases were used. For ALS patients, the mean age at onset was 61,9 years (range 34-81 years) and the mean delay from first symptoms to time of biopsy was 12,3 months (range 3-28 months). The mean age at biopsy was 48,2 years for neurogenic controls (range 5-84 years), 31 years for the two patients with myopathy (16 and 43 years, respectively), and 51 years for normal controls (range 34-66 years). The routine histochemical analysis of all biopsies showed chronic neurogenic changes ranging from mild to severe.
Post mortem brain samples obtained from one ALS patient and one subject free from neurological diseases were also considered.

3.1.2 Patients for genomic DNA screenings

The presence of mutations in *TARDBP* and *FUS/TLS* were analyzed in DNA from 222 ALS patients, consecutively recruited and classified principally in sporadic ALS (SALS, 208 patients) and familial ALS (FALS, 14 patients). All FALS cases were also screened for mutations in *SOD1* before their inclusion in the present study and only one carried a mutation.

Conversely, the correlation study between genetic variants and ALS phenotype was performed in 228 subjects, classified as follow:

- typical ALS;
- progressive muscular atrophy, PMA;
- upper motor neuron-predominant ALS (UMN-ALS), including both cases of primary lateral sclerosis, PLS, and upper motor neuron-dominant ALS, UMNd-ALS;
- flail arm syndrome, FA;
- and flail leg syndrome, FL.

Of each patient we considered the following clinical data: sex, age at onset, the site of onset (bulbar or spinal, the latter was further classified as upper limb, lower limb or trunk/respiratory), the disease duration at the diagnosis of motor neuron disease (MND) and at the first assessment at our clinic (presentation), forced vital capacity (FVC, expressed as percentage of the expected value) at presentation, and survival defined as the time from onset to death or tracheostomy (as death would have occurred without such intervention). A concomitant diagnosis of frontotemporal lobar dementia defined according to Neary's criteria was also recorded.

3.2 TDP-43 characterization in skeletal muscle

3.2.1 Immunohistochemistry analysis, IHC

Serial 10μm cryostat sections of fresh frozen tissue were fixed in acetone at -20°C for 20min, blocked for endogenous peroxidase activity with 0,1% hydrogen peroxide at RT for 8min, incubated overnight at 4°C with primary monoclonal TDP-43 antibody (2E2-D3, Abnova) diluted 1:100 in PBS. Secondary HRP-conjugated-antibody (DAKO) was incubated at RT for 45min. Peroxidase labelling was detected by 3.3-diaminobenzidine. To detect nuclei, slides was stained with 4-6-diaminine-2-phenylindole (DAPI).

3.2.2 Western Blot analysis, WB

10μm cryostat sections of muscle biopsies and brain samples were dissolved in loading buffer (0,05M DTT, 0,1M EDTA, 0,125M Tris, 10% SDS, 0,001% bromophenol blue, pH 8), boiled for 3 min, centrifuged, and the supernatants were resolved in 1,5mm-thick 4-12% polyacrylamide gel. Brain samples were also digested in a loading buffer added of λ-phosphatase (35 μl/ml of loading buffer) (gift of Dr. Mario Pagano, University of Padua). As positive control a commercial nuclear extract from HeLa cells (Cilbiotec) was used. Proteins were then blotted into nitrocellulose membrane (Whatman) for 3 hours with cooling. Post-transfer gels were stained with Coomassie blue, whereas the membranes were air dried until blocked for 1 hour at RT in 5%millk/T-TBS solution. The membranes were incubated overnight at 4°C with primary monoclonal TDP-43 antibody (2E2-D3, Abnova) diluted 1:5000 in 5%milk/T-TBS and then for 1 hour at RT with secondary HRP-conjugated-antibody (DAKO), diluted 1:2000 in T-TBS. The recognized bands were visualized by chemiluminescence (GE Healthcare, Freiburg, Germany) and exposition to films (Kodak).

3.3 Genetic analysis of *TARDBP* and *FUS/TLS*

Genomic DNA was isolated from peripheral blood according to standard protocols. The coding region of *TARDBP*, exons 2–6, was amplified by PCR (see primers in Table 3.1) and directly sequenced (BMR-genomics sequencing facility). Moreover, 72 SALS and 14FALS patients were specifically screened only for the four FUS mutated exons (5, 6, 14, 15).

	exon	forward primer	reverse primer
TARDBP	2	TCAGAACTCTGACATGGTTTGG	CCATGGATGAGACACACACC
	3	TTCTAAAAGGTTTCTGCTCGTTTT	GAAAAACTGTTTGATGCAATCG
	4	TTGGGAATGGAGTGTGTGAG	AAATTCCCAAAGCACAGACG
	5	CCAAGGCGAATGATTTTGTT	CTCCAAAGTGCTGGGATTGT
	6	TGAATCAGTGGTTTAATCTTCTTTG	AACCAACCACAACCCCACG
FUS/TLS	5	GCTATGCTGGGATTGTGATTGTG	CTGCAGACAAAGCTGAAGACATC
	6	GTCCTTCATTGCCTGGCACTTG	CCACCAAAGATACTCCCTGGCTC
	14	GGGAATGGGAATATGATAGATCTTG	CTATGGCCTCTGTTCAACTG
	15	TATCTAGGCTTGGAGAGGCTG	GGTGATCAGGAATTGGAAGGTTAC

Table 3.1. Primers used for sequence analysis.

3.4 Genetic variants analysis

3.4.1 Genotyping

Genomic DNA was isolated from peripheral blood according to standard procedures. Genotypes of SNP rs1541160 were obtained by enzymatic restriction (DdeI, Invitrogen) of the region containing the SNP, previously amplified by PCR (table 3.2). The genotypes of SNP rs522444 within the KIFAP3 promoter was determined by ARMS-PCR using the following primers: rs522444 outer primer for, rs522444 inner primer rev, rs522444 inner primer for, rs522444 outer primer rev.

		forward primer	reverse primer
rs1541160		TGCATGAATTATGGGATTGC	AAGCCAAGAATCCAGAGACG
rs522444	inner	CCCGGTGCGTCAAACTCTGAGGCGAC	TTGTATGCGCAGGCTCAGTCTGAGGGGC
	outer	CATCCTCAAGGGGGCGGTGACTACGGAG	GGCGCTGTGGTTACCACGGTGAAGCCTC

Table 3.2. Primers used for polymorphism analysis.

3.4.2 Gene expression analysis

Total RNA obtained from patients' lymphocytes was analyzed for *KIFAP3* expression. 1 μg of total RNA was reverse-transcribed to cDNA (SuperScript III First-Strand Synthesis System, Invitrogen) and the expression levels, compared to the expression of two housekeeping genes (TBP and GADPH), were measured by Sybrgreen Real Time-PCR (DyNAmo HS SYBR GREEN, Finzyme) with ABI PRISM7000 sequence detection system. Three replicates per sample were amplified (Table 3.3).

	forward primer	**reverse primer**
KIFAP3	ACAGGCTCCAGCATATCTCATAG	CATCTGACGACTCTCTACCATCTC
TBP	GAACCACGGCACTGATTTTC	CCCCACCATATTCTGAATCT
GADPH	ACATCATCCCTGCCTCTACTG	ACCACCTGGTGCTCAGTGTA

Table 3.3. Primers used for gene expression analysis.

3.4.3 Statistical analysis

Normally distributed variables were analyzed using T test for independent groups. Ordinal variables were analyzed using the Mann Whitney U test, while for categorical variables the $\chi 2$ test was performed. Overall survival was calculated using the Kaplan-Meier method, and differences in survival in relation to genotype was evaluated with the log-rank test. Significance level was set at $p<0.05$.

RESULTS

The study takes advantage of biochemical and molecular methodologies to find a biomarker that univocally identify ALS disease or its subtypes, in order to have early diagnosis and discrimination of clinical variants. Moreover, it is to understand how disease expression in ALS is so variable once the motor neuron degeneration develops. In addition, we set genetic analysis to define new mutations and speculate on their involvement in ALS pathogenesis.
Nervous system is clearly not an approachable tissue for this aim and other tissues must be considered. So far, skeletal muscle, whole blood and lymphocytes have been evaluated to such purpose.

4.1 TDP-43 profile in skeletal muscle

We tested the hypothesis that TDP-43 could be a biomarker whether found in ALS skeletal muscle with the same pathological alterations as in ALS brain [75].
Skeletal muscle biopsies from *Vastus Lateralis* of 30 patients affected with ALS (18 males and 12 females) and 30 controls were used to establish biochemical and morphological features of TDP-43. We first performed immunohistochemistry (IHC) analysis on 10µm fresh frozen skeletal muscle slides and TDP-43 immunoreactivity was detected only in muscle fiber nuclei as confirmed by the double-labeling with DAPI (Figure 4.1). TDP-43-positive nuclei were found in all muscle fibers, both ALS patients and controls.
We did not observe any sarcoplasmic TDP-43 accumulation.

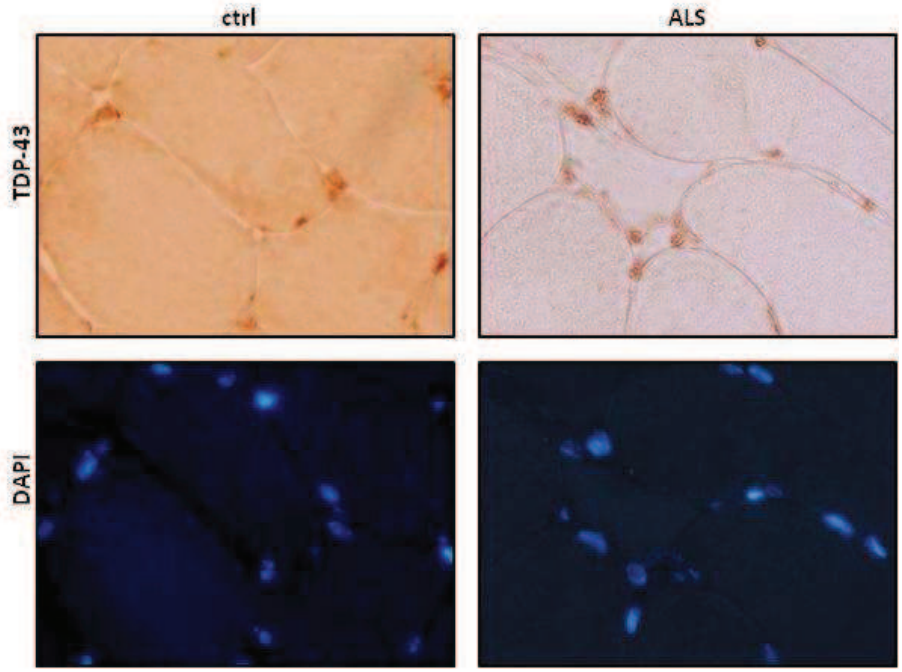

Figure 4.1. Immunohistochemistry, IHC, of 10μm cryostatate skeletal muscle slices of ALS patients and controls. TDP-43 is peroxidase labeled (brown) while nuclei are detected with DAPI.

Accordingly, biochemical analysis of TDP-43 by Western Blot showed the presence of a unique band corresponding to a molecular weight of 43 kDa both in ALS patients and controls (Figure 4.2a).

To disprove that this result is not a matter of the technique, we repeated WB analysis on *post mortem* ALS brain and control. The same TDP-43 monoclonal antibody was used and showed the hyperphosphorylated TDP-43 specie as an additional ~45-kDa band in ALS brain, not detected in the brain sample from the subject with no neurological disease (Figure 4.2b). Dephosphorylation of the ALS brain sample was performed to confirm the character of the additional band: we added λ-phosphatase to the ALS brain sample and the 45 kDa supplementary TDP-43 band became fainter thus confirming the hyperphosphorylated nature of the band.

Neither in muscle nor in brain from ALS patients we could detect the specific ~25 kDa band suggestive of the TDP-43 cleavage products.

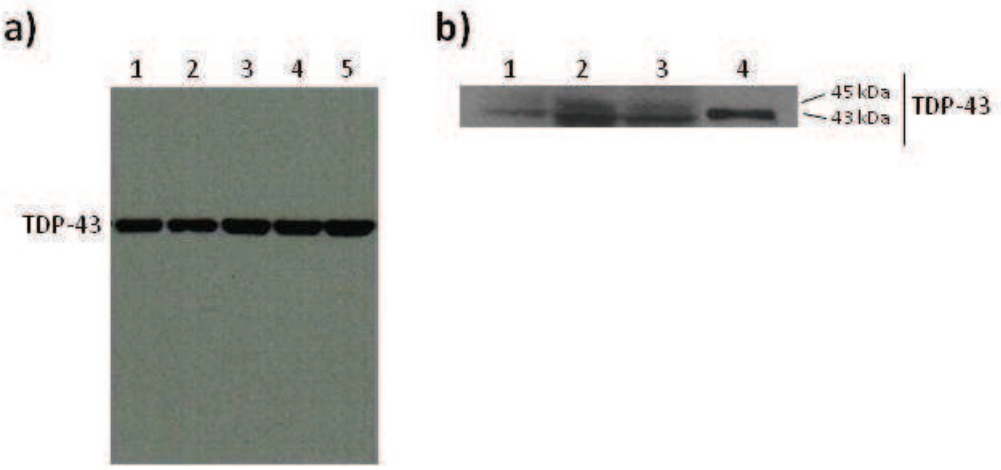

Figure 4.2. TDP-43 WB analysis a) from skeletal muscle of an ALS patient (lane 1), a neurogenic control (2), a myopathic control (3), a normal control (4) and the TDP-43 positive control, HeLa cells, (5); and b) from *post mortem* brain tissue of a negative control (lane 1), an ALS patient (2) showing the additional ~45-kDa band, the λ-phosphatase-treated ALS brain (3) and the TDP-43 HeLa cells (4).

4.2 Gene sequence analysis

4.2.1 Screening of *TARDBP* gene

Total DNA from 222 ALS patients (208 SALS and 14 FALS) was used for sequence analysis of the five coding exons (2-6) of *TARDBP*, revealing the same heterozygous missense mutation in two unrelated ALS patients (1%), c.1278G>A (p.A382T) (Figure 4.3a). This mutation in located within exon 6 in a highly conserved region of TDP-43.

One of these patients was a carrier coming from a family with a clear autosomal dominant transmission (Figure 4.3b). The proband was a 53-year-old Northern Italian man, who developed a typical ALS phenotype with disease onset in the spinal region at the age of 50 years. His father was diagnosed as affected with ALS at 50 years. The mutation was identified also in his son and his daughter (respectively 22 and 27-year-old) and his 45-year-old sister, all healthy at the time of our study.

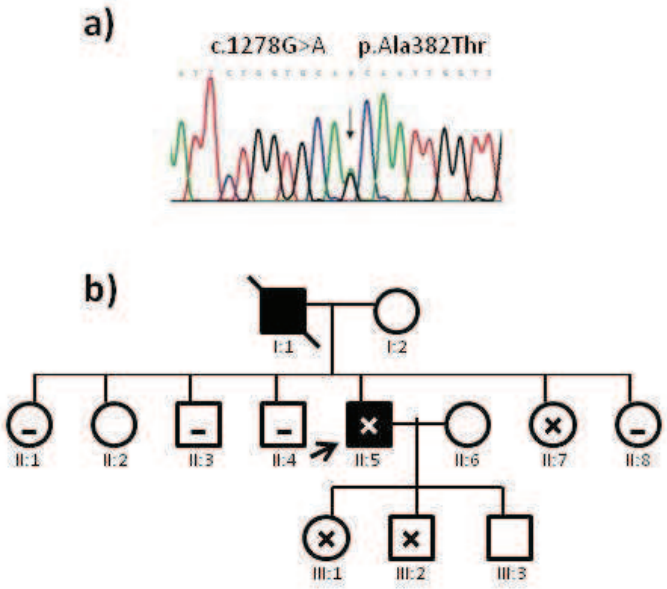

Figure 4.3. a) p.A382T heterozygous missense mutation chromatogram from sequenced exon 6 of *TARDBP*. b) mutated FALS patient's family: II:5 is the proband, II:5, II:7, III:1 and III:2 are positive to p.A382T mutation (**X**), while II:1, II:3 and II:8 are negative; the other members did not agree to be tested.

The same p.A382T mutation has been observed in a 38-year-old Southern Italian male presenting a left spastic hemiparesis, widespread fasciculations, Hoffman sign and plantar extensor response present bilaterally, and difficulties of speech and swallowing. Diffuse bradykinesia, dystonic contraction of the platysma and the lower facial musculature on the left, a resting tremor of the head, facial hypomimia and infrequent blinking were also present. Furthermore, the patient showed Parkinson-like microphagia, anosodiaphoria and bradyphrenia. Hisfamily history was negative for neuromuscular diseases. Patient's parents at age 68 and 65, and the 36-year-old brother were healthy. DNAs from these family members were unavailable.

4.2.2 Screening for *FUS/TLS*

Previous reports described exons 5, 6, 14, 15 of FUS/TLS gene as the only regions affected by mutations in FALS patients [61,122]. Therefore, we specifically screened these four exons in 72 SALS and 14 FALS patients. We detected one deletion in 1 FALS patient involving 3bp in a $(GGN)_{10}$ motif in exon 6, encoding for a Gly_{10} tract. This variant is predicted to result in an in-frame deletion of 1 Gly residue.

4.3 Polymorphic variants analysis

Two-hundred and twenty-eight ALS patients were eligible for this protocol (135 males and 93 females, with a mean age at onset of 57,7 years (range 23-85). One-hundred and seventy-one (75%) had the spinal onset form and 57 (25%) the bulbar form of the disease. According to SNP rs1541160 genotyping, 118 (51%) cases had the TT genotype, 95 (42%) the TC genotype, and 15 (7%) the CC genotype. All the CC but none of the patients carrying the TT or the TC genotypes were also homozygous for the CC SNP rs522444 genotype. There were no significant differences in the mean age at onset, the median diagnostic delay, the median disease duration at presentation and the bulbar/spinal onset ratio in the three groups (Table 4.1).

	TT	**TC**	**CC**
n	118	95	**15**
Bulbar onset, n	29	25	**3**
Spinal onset, n	89	70	**12**
lower limbs, n	*65*	*42*	***10***
upper limbs, n	*22*	*24*	***2***
trunk/respiratory, n	*2*	*4*	***0***
Mean age at onset (± SD), ys	58.14 (±11.9)	57.69 (±11.77)	**56.86 (±11.46)**
Median diagnostic delay, mo	10	13	**12**
Median time from onset to presentation, mo	15	18.5	**18**
Mean fVC at presentation(± SD), %	80.95 (±19.56) (n = 86)	83.1 (±22.35) (n = 60)	**94.18 (±11.91) * (n = 15)**
Dementia, n	6	10	**2**
Clinical variant			
typical ALS, n	*91*	*73*	***9***
UMN-ALS, n	*13*	*10*	***6 *****
PMA, n	*9*	*9*	***0***
FA, n	*1*	*2*	***0***
FL, n	***4***	***1***	***0***

Table 4.1. Clinical features of the ALS cohort according to SNP rs1541160 genotype. (n) number of patients; (SD) standard deviation; (ys) years; (mo) months; (fVC) forced vital capacity expressed as percentage of the expected value; (UMN-ALS) upper motor neuron-predominant ALS; (PMA) progressive muscular atrophy; (FA) flail arm syndrome; (FL) flail leg syndrome; (*) and (**) data analysis yielded a significant difference ($p < 0.05$) comparing CC with TT, and with both TT and TC patients, respectively.

The presence of dementia was equally distributed in the three patient groups. A higher mean FVC value at presentation was noted in the patients with the CC genotype compared to those with the TT one (94.1 vs 80.95%, respectively; $p < 0.05$). The difference was found to be independent of the disease duration at presentation. Analysis of ALS clinical variants in the total cohort of patients showed a frequency of 75% (173/228) for typical ALS, 6.4% (18/228) for PMA, 10.5% (29/228) for UMN-ALS, 0.01% (3/228) for FA and 0.02% (5/228) for FL. A higher incidence of the UMN-ALS phenotype was found in the patients with the CC genotype (6/15, 40%) and the difference was significant in the TT (13/118, 11%, $p < 0.005$) and TC (10/95, 10%, $p < 0.005$) patients. The median survival of the entire cohort (n, 211) was 33 months. At the time of data entering, 123 patients were died or had undergone tracheotomy whereas 105 were still alive.
We found no effect of SNP rs1541160 genotype on survival: median survival of TT patients (n, 111) = 48 months (95% CI 29.4-66,5); median survival of TC patients (n, 83) = 59 months (95% CI 42,5-75,4); median survival of TT patients (n, 15) = 48 months (95% CI 21.4-74,5) (Figure 4.4). The ratio between the dead (or with tracheostomy) and the living patients was not significant in the three groups.

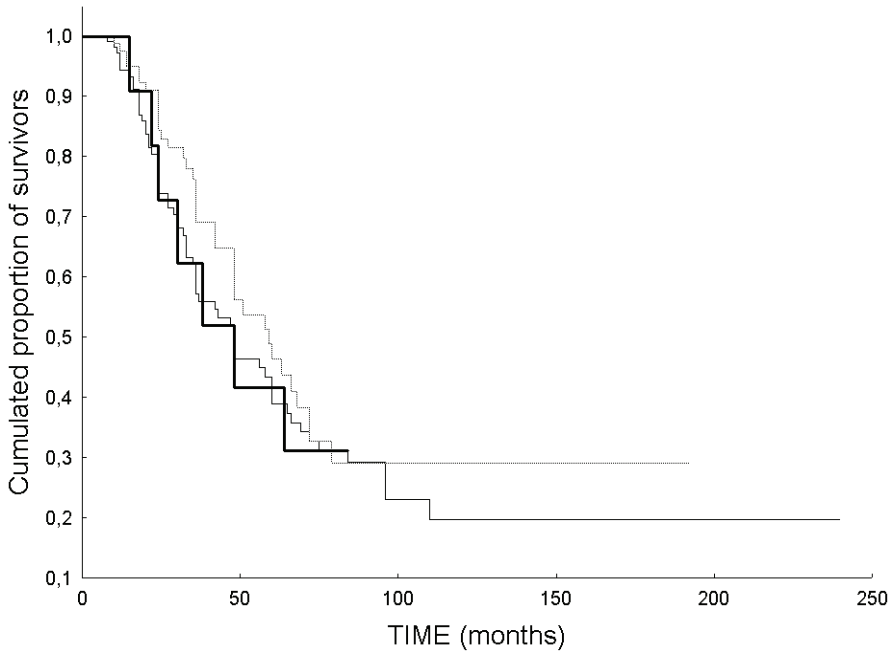

Figure 4.4. Kaplan-Meier plot of survival from disease onset in ALS patients with TT (n=111; thin line), TC (n=83; dotted line), and CC genotype (n=15; thick line) for SNP rs1541160.

To understand whether SNP rs1541160 could be implicated in an altered presence of KIFAP3 within the cell, gene expression analysis was performed on lymphocytes of 18 ALS patients (6 harboring CC and 12 harboring TT genotype) (Figure 4.5). The comparison of KIFAP3 expression in the CC and TT ALS patients revealed no significant differences and high variability.

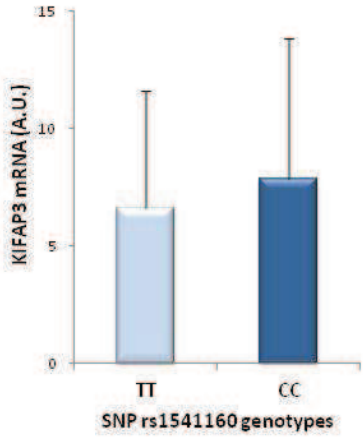

Figure 4.5. Real-Time PCR of *KIFAP3* in lymphoblasts of 18 ALS patients stratified by genotype at SNP rs1541160. No significant difference of *KIFAP3* expression between patients with TT (n=12) and CC (n=6) genotype was observed. The values of mRNA amounts are given as arbitrary units (A.U.) of ratio with *GADPH* and *TBP* housekeeping genes.

DISCUSSION

5.1 TDP-43 profile in skeletal muscle

The discovery of TDP-43 as the major disease protein in ALS and FTLD-U (2) has prompted research in the field of ALS pathogenesis. The identification of a specific TDP-43 expression pattern (ubiquitination, iperphosphorilation and cleavage [75]) gave great significance to TDP-43 analysis for improving ALS diagnosis. In skeletal muscle biopsies from ALS patients we observed that TDP-43 is constantly present in an intranuclear localization, and TDP-43 WB showed the expected 43 kDa band as well as controls [105]. According to these results we can speculate that TDP-43 pathology is probably confined to central nervous system and despite its presence is not only confined on motor neurons but spreads out the CNS, it is not to be considered a systemic feature in ALS.

Skeletal muscle has had a limited role in biomarker discovery but it is conceivable that protein or genetic alterations that occur in ALS may be reflected in this tissue due to its direct connection with lower motor neurons via neuromuscular junction. Few studies on the usefulness of muscle in ALS diagnosis and pathogenesis have been reported. Using the ALS animal model SOD1 mutant mouse, it has been suggested that skeletal muscle tissue is a potential player in the motor neuron degeneration. Indeed, Fischer and coll. demonstrated that motor neuron pathology begins at the distal axon and proceeds in a “dying back” pattern [37] raising a pathogenetic role for muscle. Pradat and coll. assessed muscle biopsies for Nogo-A expression, a protein involved in preventig outgrowth of neurites and regeneration in the central nervous system, and he related lower expression to the progression of ALS disease [83]. However, this phenomenon turned to be unspecific for ALS, since it appears in any denervated muscle [127].

Lymphoblastoid cell lines from ALS individuals have been demonstrated to show a detergent-insoluble TDP-43 product of ~28 kDa, but only after cells were treatment with a proteasome inhibitor [56].

It is therefore conceivable that some abnormal process occurring exclusively in nervous tissue would lead to TDP-43 modifications. Recently, Zhang et al.

showed in cell culture models that a reduction of progranulin, a secreted growth factor, promotes the proteolytic caspase 3 and 7 cleavage of TDP-43, thus generating low-molecular-weight TDP-43 species which accumulate in the cytoplasm [129]. As even mutations in the progranulin gene (PRGN) are responsible for some cases of familial FTLD-U [39], the progranulin-caspases pathway and its link with TDP-43 neuropathology is worthy of further studies.

The hypothesis that TDP-43 abnormalities are restricted to central nervous system limits the possibilities of using TDP-43 study to derive ALS diagnostic tests. Nervous tissue is not accessible for diagnostic sampling in ALS patients. Nonetheless, we still can speculate on the eventual presence of TDP-43 neuronal pattern in cerebrospinal fluid of ALS patients [108]. The discovery of the TDP-43 pathology in neurodegenerative disorders other than ALS or FTLD-U [117] further weakens the value of TDP-43 as an ALS early biomarker.

In conclusion, we found a normal TDP-43 expression in skeletal muscle of ALS patients and suggest TDP-43 proteinopathy to be tissue-specific. To our knowledge, this is the first report of TDP-43 analysis in a tissue of ALS patients different from central nervous system or lymphoblastoid cell lines derived from patients.

5.2 Gene sequence analysis

5.2.1 Screening for *TARDBP*

In this study, We report on a mutation screening of *TARDBP* gene in a cohort of 222 ALS patients (208 SALS and 14 FALS), consecutively recruited. We have detected the previously reported p.A382T mutation in a single SALS case, as well as in one patient with positive family history of ALS. FALS patients carrying *TARDBP* mutations are 7% (1/14) and SALS are 0,5% (1/208). The percentage of incidence appear higher in FALS and lower in SALS when compared to other studies but it may be influenced by the small sample size.

The p.A382T mutation has been observed in a family (Figure 4.3b); the pedigree showed several affected members within three generations and with variable clinical features: the proband showed typical ALS and no clinical relevant bulbar symptoms or signs of cognitive impairment, two children were asymptomatic

probably because of their young age; the sister also was without clinical symptoms, suggesting that there could be *TARDBP* mutants who do not develop a motor neuron disorder. This strengthens the hypothesis of ALS as multifactorial disease.

The same mutation has also been reported in a single SALS case with an atypical phenotype due to a prominent upper motor neuron (UMN) involvement and extrapyramidal features. This suggests that mutated TDP-43 may exert a pathological effect also in brain regions associated with extrapyramidal functions of ALS patients. Since previously reported ALS patients with p.A382T mutation had a typical ALS phenotype, it is conceivable that other pathological factors occurred together with mutated TDP-43.

Our study contribute to estimate frequencies in a larger mutational screening within SLAGEN consortium [28], identifying two novel (p.G294V, p.G295S) and two known mutations (p.G294C, p.A382T), and confirming 1% mutations for SALS and 9% for FALS.

p.A382T has been previously reported in two French FALS patients with similar age of onset (55 and 57 years) but different duration (28 and 73 months) and site of onset (bulbar and spinal, respectively) [56]. The A382T variant might be associated with increased phosphorylation of the TDP-43 protein. Furthermore, the p.A382P mutation, affecting the same amino acid, has been reported in a 68-year-old French patient with spinal site of onset [27].

So far, the comprehension of the pathophysiological mechanism underlying central nervous system neurodegeneration associated with *TARDBP* mutations is limited and needs further studies. As with other neurodegenerative disorders characterized by intracellular protein accumulation, the cause and the effect relationship between TDP-43 inclusions and disease is unknown. The substitutions might cause a toxic gain of function through novel protein interactions or intracellular accumulation. Recently, Kabashi et al. [57] investigated the role of three mutations (A315T, G348C and A382T) in primary motor neurons cells and zebrafish and proved that they confer toxic properties to TDP-43, in which motor neurons are particularly vulnerable, and that their expression causes a motor neuron phenotype in zebrafish embrios. In particular, he observed that mutant A382T-TDP-43 diffusely localizes in the nucleus, cytosol and neuronal processes, while mutant A315T and G348C-TDP-43 have perinuclear localization and aggregates.

Furthermore, the A90V variant, previously reported in a FTLD/ALS patient with a family history of dementia, has been considered as a genetic risk factor; the authors suggested that it might predispose nuclear TDP-43 to redistribute to the cytoplasm and to form pathological aggregates [126]. A predisposing role of this variant to SALS is not supported by our data [28].
It should therefore be concluded that different mechanisms lead to a very similar motor neuron degeneration in humans. The identification of two ALS probands carrying *TARDBP* alterations extends the spectrum of *TARDBP* mutations and supports the pathological role of TDP-43 in both sporadic and familial ALS. The accumulating data on clinical and molecular epidemiology associated with *TARDBP* mutations may serve to define genotype-phenotype correlations, and help genetic counseling in affected families.

5.2.2 Screening for *FUS/TLS*

Here I report a mutational screening of *FUS* gene conducted in a cohort of 86 ALS patients (72 SALS and 14 FALS). We identified one deletion in 1 FALS patient involving 3bp in-frame in a $(GGN)_{10}$ motif in exon 6, encoding for a Gly_{10} tract.
Our study contribute to estimate frequencies in a larger mutational screening within SLAGEN consortium [22], identifying six novel (p.R216C, p.G191S, p.G225V, p.G230C, p.R234C, p.G507D) and one known mutations (p.R521C), and confirming 0,7% mutations for SALS and 4,4% for FALS. This frequencies are similar to those reported in SALS patients for the other genes mutated in an appreciable number of patients, such as SOD1, ANG [19,34,40] and the more recently identified *TARDBP* [62]. With the exception of p.G507D and p.R521C, the mutations identified in sporadic cases are all localized in the Gly-rich region encoded by exon 5 and 6, differently from the mutations reported in FALS cases that clustered in the C-terminal of the protein, encoded by exon 15.
In this study the presence of different deletions was observed in the two distinct $(GGN)_n$ stretches localized in exons 5 and exon 6 encoding polyglycine residues, normally containing Gly_9 and Gly_{10} residues, respectively. Triplet repeat length variations in these motifs in ALS was previously described by Kwiatkowski and co-authors [61], who reported two FALS patients carrying an in-frame deletion and insertion of 6bp in exon 5, resulting in a change of glycine number. The genetic

analysis of a large number of individuals allowed them to further characterize these two stretches of glycines and to detect their deletions also in healthy controls. These findings strongly suggest that these are not causal mutations for ALS, but are rare polymorphic tandem repeats. Nevertheless, we cannot exclude the involvement of these variations as a susceptibility factor to ALS, since the deletions in exon 6 were more frequently observed in patients than in controls. Evidence that poly-glycine tract length variants could be associated to disease susceptibility was reported for Androgen Receptor (*AR*) gene for which a correlation with protein activity was also described [15].
Further studies are needed to verify the real involvement of the glycine stretch length on FUS protein function.

5.3 Polymorphic variants analysis

Landers findings sustained the hypothesis that the SNP rs1541160 within the *KIFAP3* gene is a genetic modifier of ALS survival [66]. On the basis of the SNP genotype analysis we carried out, no significant difference in survival was found in the ALS patients. While the number of patients studied here is smaller, there were nevertheless several similarities in the ALS populations studied by our group and that by Landers [66]:

1. an almost identical frequency of each SNP rs1541160 genotype (TT: 51% vs. 50%, TC: 42% vs. 40%; CC: 6% vs. 9%)
2. no association between the SNP rs1541160 genotype and some clinical parameters such as the site and age at onset
3. the type of patients being studied, almost all attending ALS Clinics. It can be argued then that the patient samples are comparable despite the difference in size.

No relationship was, however, found in our patients between *KIFAP3* mRNA expression and SNP genotype; the evaluation was performed using RNA extracted from patient lymphoblasts, in which a significant reduction in *KIFAP3* expression was reported by Landers in their CC genotyped ALS patients. Our present findings are in agreement with those of a recent work by Traynor et al. [115] demonstrating that the 140 SNPs genotyped within the *KIFAP3* locus

(including rs1541160) had no effect on survival or on gene expression in a population-based cohort of ALS patients.

Analysis of our patients' clinical features revealed that almost half of the subjects with the CC genotype fell into the UMN-ALS classification. Conversely, there was approximately a 10% frequency of UMN-ALS in both the TT and the TC patient groups reflecting the frequency of the entire cohort considered. Clinical features with a predominance of UMN involvement predict a more benign disease course of ALS [115,116]. We have recently confirmed that there is a longer survival in patients with the UMN-dominant ALS compared to the typical ALS form and have hypothesized that the better prognosis is associated to the persistence of normal respiratory function [104]. Patients genotyped as CC in the present study had in fact a higher mean FVC value at presentation, a parameter that many consider a clinically meaningful predictor of survival and of disease progression in ALS [11,26,107]. On the basis of these clinical data, a longer survival should be logical in the CC patients compared to that in the other rs1541160 variants. The discrepancy of these results with survival figures raises the question if other factors may be involved. Among these, the time the patient cohort was ascertained may have played a relevant role. Indeed, since all UMN-ALS patients analysed in this study are still alive and reasonably supposed to have a prolonged disease duration, assessing the survival of this cohort in a few years time might produce very different results. It is conceivable that the higher prevalence of longer living UMN-ALS in the CC genotype group could be the variable underlying the controversial results on survival inside of this work and with respect to the original paper [66]. The demonstration of UMN-ALS phenotype recurrence in the CC patients of our cohort provides some evidence for considering the SNP rs1541160 within the gene *KIFAP3* a potential modifier of ALS phenotype.

KIFAP3 is part of the trimeric motor kinesin-II complex or KIF3 that is involved in multiple functions including the intracellular transport machinery [72]. Motor proteins have been implicated in the pathogenesis of motor neuron disorders by a number of studies. First, a specific down-regulation of two kinesin-related protein, KIF1bβ and KIF3Aβ by quantitative mRNA analysis was detected in motor cortex specimen of sALS patients [81]. Second, a loss-of-function mutation in the motor domain of the KIF1B gene is the cause of the autosomal dominant form of Charcot-Marie-Tooth disease (CMT) type 2A [130]. Third, point mutations of the gene encoding the p150 subunit of dynactin (DCT1), which is the major subunit of the dynactin motor protein complex, was observed to

segregate with disease in a family with a slowly progressive lower motor neuron disease [86]. Finally, an UMN disorder such as hereditary spastic paraplegia (SPG10) was found to be associated with a missense mutation in the motor domain of the neuronal kinesin heavy chain KIF5A gene [87]. These studies as well as our and Landers' findings seem to suggest that KIFAP3 is potentially involved in both the function and the pathology of motor neurons. Although we have confirmed the association of the C alleles at the SNPs rs522444 and rs1541160, we did not observe any *KIFAP3* expression abnormalities in our patients.

In conclusion, while an association between the SNP rs1541160 within the *KIFAP3* gene and survival in ALS has not been confirmed here, a potential relationship between the CC rs1541160 genotype and the UMN phenotype could indicate that *KIFAP3* is involved in ALS. These promising findings may open the path to new insights concerning the pathogenesis and perhaps therapy for this devastating disease.

Since the mechanism by which SNP rs1541160 controls gene function has not been confirmed, this should be the objective of future studies concerning the role of KIFAP3 in ALS.

Spinal and Bulbar Muscular Atrophy

INTRODUCTION

Spinal and Bulbar Muscular Atrophy, SBMA (also called Kennedy's Disease, KD, from who first described it in 1968 [155]) is an inherited X-linked motor neuronal disorder caused by an abnormal expansion of a trinucleotide CAG tandem-repeat in exon 1 of the Androgen Receptor (*AR*) gene (chromosome Xq11-12) [174]. The genetic defect was discovered by La Spada et al. in 1991 [156].
KD is a rare disease that usually occurs at adult age and it is reported to be 1,6/100.00 in whole population (2,8/100.000 in male population). The main clinical features of KD are proximal limb and bulbar muscle weakness and atrophy; patients occasionally demonstrate signs of androgen insensitivity.

1.1 Clinical presentation

Because of the X-linked transmission, KD almost exclusively affects males but is transmitted by female carriers.

1.1.1 Affected males

The main neurological manifestations of BSMA are weakness and wasting of bulbar, facial, and limb muscles [180]. Weakness and wasting usually follow an asymmetric distribution. Some patients also develop sensory disturbances [180]. Endocrinologic disturbances include androgen resistance with gynecomastia, elevated testosterone and progesterone, or reduced fertility [144,182]. Age of onset is between 20 and 40 years. Presenting symptoms are postural tremor, proximal flaccid weakness predominantly of the lower limbs, dysarthria, dysphagia, hanging jaw, fasciculations, or flexor muscle cramps or tremor, which will run a slow progressive course [144]. Some patients may also present with perioral fasciculations with variable bulbar paresis [157,183]. One third of the patients develops easy fatigability [157]. The phenotypic features vary between

asymptomatic hyper-CK-emia to severe muscle disease with bulbar involvement, requiring artificial ventilation [142]. There may be marked phenotypic heterogeneity within an affected family, concerning age at onset, phenotype expression, and severity of disease [151].

1.1.2 Female carriers

Female carriers of the mutation are usually clinically unaffected. Rarely, they are slightly affected [133,151]. Clinical affection is attributed to skewed inactivation of the X-chromosome according to the Lyon hypothesis [167]. Though skewed inactivation occurs in the majority of the female carriers only few female carriers are clinically affected. Female carriers show no abnormalities on electromyography in most studies [167] but exhibit skewed methylation of the wild-type allele [167]. Clinical manifestations in female carriers include hyper-CK-emia, fasciculations, minimal distal weakness, or muscle cramps [183]. Muscle biopsy in these female carriers may show mild myopathic or neurogenic alterations [178].

1.1.3 Histopathology

The cardinal histopathologic findings in BSMA is loss of anterior horn cells in the brain stem and spinal cord [153]. A further histopathologic hallmark of BSMA is diffuse nuclear accumulation and nuclear and cytoplasmic inclusions of the mutant poly-Q AR in residual motor neurons of the brainstem or spinal cord and other visceral organs [133]. In BSMA mice additionally accumulation of neurofilaments and synaptophysin can be found in distal motor axons and skeletal muscle [153]. The retrograde axonal transport is impaired, as expressed by reduced levels of mRNA levels of Dynactin-1, an axonal motor for the retrograde transport [153].

1.2 Diagnosis

The golden standard of diagnosing the disease is the molecular demonstration of a CAG-repeat expansion in the AR gene [184]. Genetic analysis allows a precise diagnosis on an individual basis and reliable genetic counseling. Beside, KD is diagnosed upon the history, clinical neurologic examination, blood chemical investigations, nerve conduction studies, electromyography, evoked potentials and transcranial magnetic stimulation. Creatine-kinase (CK) is usually elevated only in clinically manifesting male mutation carriers [Sorenson AJ, Klein CJ 2007]. However, recently non-manifesting male mutation carriers have been reported, who presented with idiopathic hyper-CK-emia 10 years before onset of the clinical manifestations [179]. Nerve conduction velocities of motor and sensory nerves, compound muscle action potentials and sensory nerve action potentials may be reduced in KD, indicating that axonal degeneration in motor and sensory nerves is the primary pathogenetic process [180]. These abnormalities are usually more pronounced in the upper limbs as compared with the lowers [180]. Muscle biopsy predominantly shows neuropathic alterations [178], but myopathic features are occasionally seen particularly prominent in muscles with high disability, either attributed to severe denervation or to primary myopathic changes, [178]. MRI of skeletal muscle may show a hyperintense signal on T1-weighted images [146]. Scrotal skin biopsy may show the degree of nuclear accumulation of mutant AR, and may be used to assess the efficacy of therapeutic trials [133,136].

1.3 Etiology

1.3.1 Androgen Receptor, AR

AR is a 110 kDa receptor responsible for the biological actions of androgenic steroids. AR is a ligand-activated transcription factor, which is responsible for the androgen responsiveness in target cells [164] and belongs to the Heat Shock Protein (HSP) 90 client protein family [133].

AR is encoded by *AR* gene (8 exons), located on the long arm of Chromosome X, Xq11-12.

The AR protein , like other members of the steroid receptor superfamily, is structurally composed of three main domains (Figure 1.1):

- Ligand binding domain, is located in the C-terminal region of the protein. This domain folds to give rise to an hydrophobic pocket in which the lingads are incorportaed;
- DNA binding domain, consisting of two Zn fingers;
- N-terminal domain, where a highly polymorphic polyGln tract (usually consists of a poly-Q repeat of 9–36 residues [140]) may act as a protein-protein interaction (169).

The AR undergoes to post−translational modifications: phosphorylation (at least seven phosphorylation sites are present in the AR, six out of seven in the N−terminal domain), sumoylation and acetylation. In absence of ligand, the AR is confined in the cytoplasm of androgen−responsive tissues.

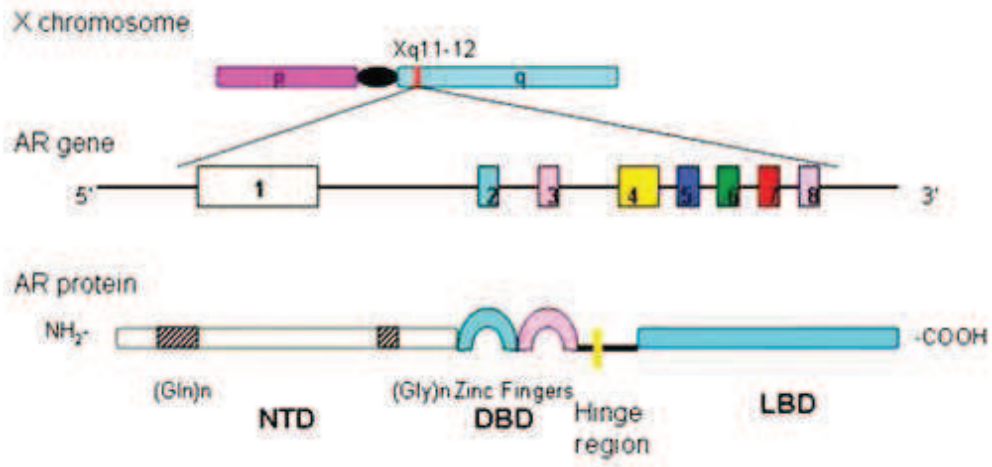

Figure 1.1. Human Androgen Receptor gene and protein.

In basal conditions, AR is confined in the cytoplasm associated with Heat Shock Proteins (HSPs). The binding with testosterone or dihydrotestosterone (DHT, active form of testosterone) induces AR conformational changes and its dissociation from HSPs; this allows AR dimerization and translocation of the receptor to the cell nucleus, where AR binds to androgen-responsive elements in the promoter region of AR target genes activating their transcription (Figure 1.2).

The CAG repeat encodes the polyglutamine tract (polyQ) located in the N-terminal transactivation domain of the AR protein, the mediator of testosterone action in the cells. In brain, AR-mediated androgenic effects are involved in the developmental fate of motor neurons, in the formation of neuromuscular synapses, and in the growth of adult dendrites and axons.

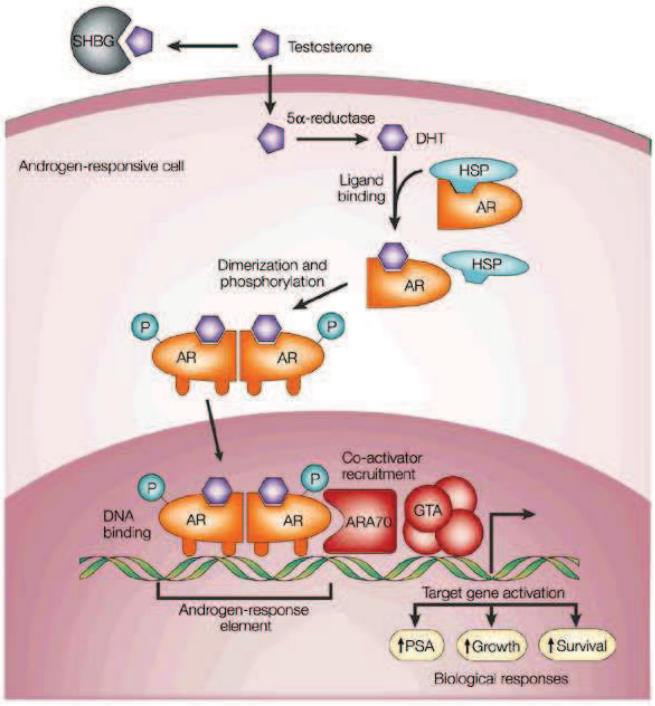

Figure 1.2. Ligand-dependent activation of the androgen receptor. AR (androgen receptor); DHT (dihydrotestosterone); HSP (heat shock protein) [143].

1.3.2 Polyglutammine tract, polyQ

Neurodegeneration in KD appears to be mainly caused by a toxic gain of property conferred by the expanded polyQ tract to the AR protein. In the normal population, the polymorphic glutamine region ranges between 9 and 36 residues, with an average size of about 22 glutamines. In KD patients, more than 38 contiguous glutamines causes neurotoxicity, through a gain-of-function misfolded protein toxicity or loss of normal protein function [140,164,182].

Expanded CAG-repeat size is negatively correlated with age at onset, shows somatic and germline instability, and has a slight tendency to further expand in successive generations (anticipation) [154].

Structurally, the elongated polyQ confers an abnormal protein conformation, with β-sheets structures, facilitating protein aggregation.
Affection of motor nerves seems to be associated with longer CAG-repeat size, whereas affection of sensory nerves is associated with shorter CAG-repeat expansions [180]. Also the physiologic polymorphism of the CAG-triplet repeat expansion of the AR gene is involved in endocrine disease, such as prostate cancer susceptibility, hirsutism, male infertility, cryptorchism, or depression [164]. Repeat sizes between 11 and 30 repeats are associated with high androgen sensitivity or high sperm counts [159]. Very low repeat numbers are associated with mental retardation and repeat numbers between 30 and 40 with reduced cognition [159]. Neuronal speed may reduce with repeat numbers below 11 or above 30 [159]. Whether individuals carrying 35-39 repeats are ever affected is unclear, but there are reports about patients with 37 repeats, which were asymptomatic.
KD is a ligand-dependent disorder, so the ligand-activated polyQ-AR must exert its effect by a toxic gain of function only after testosterone-induced conformational changes, which occur during the activation process of the receptor.

1.4 Pathogenesis

1.4.1 polyQ-AR aggregates

Neurodegeneration in KD is mainly caused by a toxic gain of property of the expanded polyQ tract of AR (153). The expanded polyQ stretch alters the conformation of AR, resulting in nuclear and cytosolic aggregation of the protein, both in neural and non-neural tissues. The polyQ-AR aggregates contain several chaperones, component of the ubiquitin-proteasome system, and chaperone as well as transcription factors, suggesting that they are involved in an attempt to degrade or refold the mutant AR [133]. Several experimental observations indicate that formation of toxic oligomers, or intermediates, of abnormal polyQ-AR results in disruption of cellular functions such as transcription and axonal transport [132,133,154,164,180], which leads to neurodegeneration [181]. Poly-

Q ultimately results in the degeneration of motor neurons and occasionally also of the dorsal root ganglia [164], and clinical onset depends on amount of intra-nuclear accumulation of mutant protein in motor neurons [188]. PolyQ-AR neurotoxicity is highly cell specific because not all the nervous system cell types degenerate in KD. Moreover, there are also findings that challenge the view that poly-Q expanded AR-aggregation is the sole cause of neurotoxicity [166]. Increasing evidences show that testosterone plays a pivotal role in the pathogenesis of neurodegeneration in KD, since that, in endocrine evaluations, an elevated testosterone level occurred in 70% of the studied KD patients [141] and that testosterone reduction in transgenic mice prevents KD phenotypic expression of the disease [152].

1.4.2 Effects on mitochondria

In cell models and animal models mutated AR causes mitochondrial dysfunction through indirect effects on the transcription of nuclearly encoded mitochondrial genes or through direct affection of mitochondria [171]. Expression of the mutant AR in cell cultures results in depolarization of the mitochondrial membrane, generation of reactive oxidative species, or increased levels of key proteins of the apoptosis cascade, such as Bax, caspase 9, or caspase 3 [171]. In cell cultures the mutant AR associates with mitochondria. In BSMA knock-out mice expression of the peroxisome proliferator activated receptor gamma coactivator 1 and the mitochondrion-specific superoxide dismutase 2 is reduced [171]. In leukocytes mtDNA may be depleted and the lower the mtDNA content the more severely the patient is affected and the higher the CAG-repeat number [158].

1.5 Skeletal muscle

1.5.1 Involvement in KD

Histopathological studies of KD patients suggest “neurogenic” responses to denervation, and the etiology of this disease is therefore generally thought to begin with motor neuron pathology [176]. However, skeletal muscle of KD patients are reported [134,147,160] to be characterized by a number of changes suggestive of primary muscle disease together with the expected neurogenic atrophy. The potential for myogenic contributions to KD is also supported by the following observations:

1. mutant AR containing neurofilament inclusions (NIs) are present in skeletal muscle of a transgenic mouse model of KD [152];
2. mutant AR containing NIs formation significantly precedes motoneuron pathology in a polyQ AR knockin mouse model [189];
3. a transgenic mice that overexpress wild type AR exclusively in their skeletal muscle fibers recapitulates KD disease clinical phenotype and pathology [162].

Androgens are known to stimulate myogenesis regulating both satellite cell proliferation and differentiation. In skeletal muscle, satellite cells are the predominant sites of AR expression [175]. Chen et al. (2005) demonstrated AR levels to be up-regulated in satellite cells after androgen administration, in a dose-dependent manner [138]. It is conceivable that the toxic AR protein would impair muscle satellite cells function as well as it does at motoneuronal level leading to a defect of muscle regeneration and, possibly, myogenic neurodegeneration.

Histological and molecular signs of muscle pathology are detectable before the appearance of pathological abnormalities in the spinal cord in a knockin mouse model of KD [189], suggesting that mutant AR may exert a toxic effect directly on skeletal muscle. The observation that overexpression of normal AR in the skeletal muscle induces a phenotype similar to KD [162] supports this hypotesis. Analysis of muscle biopsy samples derived from KD patients suggests a mixed pathology with both myopathic and neurogenic features [178]. Palazzolo et al. (2010) demonstrated the beneficial effects of overexpression of muscle-specific isoform of Insulin-like Growth Factor-1 (mIGF-1) on motor neurons of KD transgenic mice

[165]. The results indicated that AR aggregates appear in the spinal cord of polyQ-AR/mIGF-1 mice at later stages of disease as compared to muscle. Of interest, overexpression of mIGF-1 causes increase Akt activation and AR phosphorylation and decreased AR aggregation. Augumentation of IGF-1/Akt signaling rescues behavioural and histopathological abnormalities, extends the life span, and rescues both muscle and spinal cord pathology of KD mice. Although the extent to which muscle weakness in KD is a consequence of motor neuron degeneration with denervation and secondary muscle atrophy, or primary muscle degeneration with secondary effects on the motor neurons, is unknown, these observations suggest that skeletal muscle may be an important target for disease treatment [149].

1.5.2 Satellite cells and muscle regeneration

Satellite cells are mononucleated cells located between the basal lamina and the plasmalemma of the multinucleated myofiber [137]. They act as myogenic stem cells in vivo, able to supply myonuclei, to grow myofibers and to give rise to many new satellite cells (Figure 1.3) [139]. Because muscle is able to efficiently regenerate after damage, so a viable satellite cell pool must be maintained. Over 30 years ago, self-renewal was proposed as the primary mechanism of regeneration [190]. In normal mature muscle, satellite cells are mitotically quiescent and are activated to muscle regeneration only in response to diverse stimuli, including denervation [173]. The primary myogenic regulator factor (MRF), MyoD, is required for the determination of myoblasts; it continues to be expressed and permits the activation of the secondary MRFs, myogenin and MRF4, that regulate terminal differentiation. Myotubes further fuse to myofiber. The same factors participating in the regulation of myogenic differentiation play, through feedback mechanisms, an important role in the precise control of self-renewal process allowing the maintenance of a quiescent population of satellite cells.

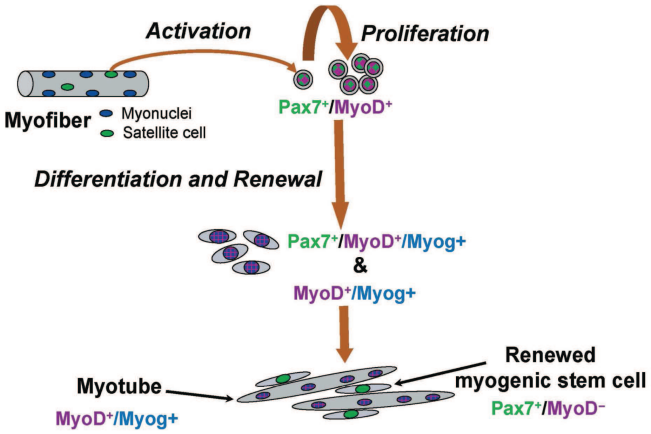

Figure 1.3. Satellite cell activation, proliferation, differentiation, and self-renewal.

When cultured in vitro, satellite cells lose their stem cell potential and behave as myogenic primary cells. They are however able to proliferate for a limited number of passages (up to 20) conserving their myogenic potential and can be induced to differentiate by removing growth factors from the culture medium, so they can fuse together generating myotubes.

AIMS

This study is based on the hypothesis that mutant polyQ-AR could trigger pathologic features of KD directly on skeletal muscle, given that it undergoes to a number of changes suggestive of a primary muscular disease together with the expected neurogenic atrophy.
To verify whether the myopathy could start at muscular level and be due to the elongated polyQ-AR, we aimed to:

1. Evaluate proliferation, differentiation and maturation ability of KD satellite cells, cultured with/without DHT treatment;

2. Assess polyQ-AR localization in KD satellite cells and 10-day differentiated myotubes, with/without DHT treatment.

MATERIALS AND METHODS

3.1 Patients

Biopsies of *vastus lateralis* were obtained from four unrelated KD patients (Table 3.1) and three age-matched healthy controls (one females and two males, age range 30-57 years). KD patients were diagnosed at the Department of Neurology, University of Padua, on the basis of molecular criteria (CAG expansion of *AR* gene over 37 repeats).

	CAG repeats	age of onset	symptoms at onset	myoblast culture	myotubes culture
K1	44	54	cramps	no	yes
K2	46	50	lower limbs weakness, asthenia	no	yes
K3	45	57	limb iposthenia	yes	yes
K4	44	27	upper limbs weakness, lower limbs cramps	yes	yes

Table 3.1. KD patients considered for the study.

3.2 Cell cultures

Satellite cells were obtained from skeletal muscle biopsied from KD patients and controls. As soon as the sample is taken to the lab (no more than one hour after biopsy), it is cultured in Rich medium (Table 3.2) for 1-3 days. The sample is then cut in smaller pieces and cultured in the same medium added with 20% plasma serum to mimic an injure. This step is fundamental to activate satellite cells to get out the muscle fragment and proliferate. The cells are maintained in a mixed culture with fibroblasts (Desmin-positive myoblasts >70%). Cells obtained were cultured in Proliferative medium (Table 3.2) and when 70% confluence was reached, differentiation was triggered by lowering FBS to 2% (Differentiative medium, Table 3.2). All the cell lines were cultured both with and without 10nM Dihydrotestosterone, DHT (Sigma). Samples were collected at 0 and 10 days of differentiation using techniques specific to the various different analyses.

	recipes
Rich medium	70% DMEM medium (Gibco) 30% FBS (Euroclone) added with: 10µg/ml Insulin (Sigma) 100µg/ml Penicillin-Streptomicin (Biochrom) 3,3µg/ml Fungizone (Bristol-Myers-Squibb) growth factors (25ng/ml FGF and 10ng/ml EGF, Pepro Tech EC Ltd)
Proliferative medium	80% Ham's F14 medium (Euroclone) 20% FBS (Euroclone) added with: 10µg/ml Insulin (Sigma) 100µg/ml Penicillin-Streptomicin (Biochrom) 3,3µg/ml Fungizone (Bristol-Myers-Squibb) growth factors (25ng/ml FGF and 10ng/ml EGF, Pepro Tech EC Ltd)
Differentiative medium	98% Ham's F14 medium (Euroclone) 2% FBS (Euroclone) added with: 10µg/ml Insulin (Sigma) 100µg/ml Penicillin-Streptomicin (Biochrom) 3,3µg/ml Fungizone (Bristol-Myers-Squibb)

Table 3.2. Mediums' recipes for muscular cells cultures.

3.3 Morphological analysis

3.3.1 Differentiative ability

Bright-field images of myotubes were collected using a Zeiss IM35 microscope equipped with a standard camera. Differentiation was quantified considering the average number of nuclei per myotube in ~20 images of at least 100 myotubes for each cell line. Myotubes average width at 10 days of differentiation was measured using ImageJ software. The same parameters were measured in all the separate set of experiments with and without DHT treatment.

3.3.2 Immunofluorescence, IF

IF was performed in fixed myotubes (4% PFA, 20min), permeabilized with 0.2% TritonX-100 and incubated for 20 minutes with 4% BSA/PBS. AR primary specific antibody (clone H-280, Santa Cruz) was diluted 1:100 in 2%BSA/PBS and

incubated overnight at 4°C. Secondary Alexa 488 antibody (Invitrogen) was incubated for 1 hour at room temperature. Samples, mounted in Vectashield mounting medium with DAPI (40-6-diamidino-2-phenylindole) (Vector Laboratories), were observed with an Olympus BX60 fluorescence microscope.

3.3.3 Electronic Microscopy, EM

EM was performed in collaboration with Prof. Cenacchi's laboratory, Department of Pathology, University of Bologna. Myoblasts and 10-day differentiated myotubes were first fixed in 2,5% glutaraldehyde in cacodylate buffer for 3 hours and then in 1% osmium tetroxide in cacodylate buffer. All samples were dehydrated and detached from the plastic dish with propylene oxide, centrifuged, and embedded in Araldite. Ultrathin sections were stained with uranyl acetate and lead citrate and observed with a Philips 400T transmission electron microscope (TEM).

3.4 TUNEL assay

Apoptosis detection in 10-day differentiated myotubes was performed using TUNEL assay (DeadEnd Fluorometric TUNEL System, Promega). This method measures nuclear DNA fragmentation of apoptotic cells by catalytically incorporating fluorescein-12-dUTP at 3´-OH DNA ends using the enzyme Terminal Deoxynucleotidyl Transferase (TdT). The fluorescein-12-dUTP-labeled DNA was visualized directly by fluorescence microscopy (Olympus BX60). Cells were also mounted with Vectashield mounting medium with DAPI for the visualization of all nuclei. At least 50 myotubes per cell line were considered.

3.5 mRNA analysis

Total RNA was isolated from myoblasts and 10-day differentiated myotubes using Trizol (Invitrogen) protocol. In all samples, 1 μg of total RNA was reverse-transcribed to cDNA (SuperScript III First-Strand Synthesis System, Invitrogen) and the expression levels of the gene of interest (Table 3.3), compared with the expression of the housekeeping gene (β_2-microglobulin), were measured by SYBRgreen Real Time-PCR (DyNAmo HS SYBR GREEN, Finzyme) with ABI PRISM7000 sequence detection system.

	forward primer	**reverse primer**
AR	TTGTCCACCGTGTGTCTTTCTTCTGC	TGCACTTCCATCCTTGAGCTTGGC
MyoD	GACGGCATGATGGACTACAG	AGGCAGTCTAGGCTCGACAC
myogenin	AAGAGAAGCACCCTGCTCAA	CAGATGATCCCCTGGGTTG
CK-M	CAAGGAACTCTTTGACCCCA	CCACAGAGAGCTTCTCCACC
Atrogin-1	GCTGAACAACATTCAGATCAC	CAGCCTCTGCATGATGTTCAGT
MuRF-1	CCTGAGAGCCATTGACTTTGG	CTTCCCTTCTGTGGACTCTTCCT
β_2-microglobulin (housekeeping)	ATGAGTATGCCTGCCGTGTGA	GGCATCTTCAAACCTCCATG

Table 3.3. Primers used for Real Time-PCR expression analysis.

3.6 Western Blot, WB

Myoblasts and myotubes at 10 days of differentiation, both DHT-treated and untreated, were collected for WB analysis:

- nuclear/cytosolic fractions: an equal amount of cells were scrapered in 500μl PBS and centrifuged 5 minutes at 1000rpm. Cellular pellets were separated into nuclei and citosol fractions by NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Scientific), added with proteases inhibitors cocktail (3,5% PIC, 2% PIC1 and PIC2; Sigma).
- total extracts: cells were harvested in 100μl of lysis buffer (50mM Tris pH 7.5, 150mM NaCl, 10mM $MgCl_2$, 0,5mM DTT, 1mM EDTA, 10% glicerolo, 2% SDS, 1% Triton, proteases inhibitor cocktail (3,5% PIC, 2% PIC1 and PIC2; Sigma)), incubated 10 minutes in ice, heated 10 minutes at 70°C and centrifuged 10 minutes at 13000rpm to collect surnatants.

30μg of proteins were electrophoresed in 7.5% or 10% Criterion precast gels (Bio-Rad Laboratories). Proteins were blotted for 2 hours at constant 400mA into nitrocellulose membrane (Whatman), blocked in 5%milk/T-TBS for 1 hour and probed overnight with specific primary antibodies (Table 3.4). After incubation of 1 hour with secondary HRP-conjugated antibodies, the recognized bands were visualized by chemiluminescence (GE HealthCare). Integrated optical density of each band was calculated with commercial software (Gel Pro Analyzer) and normalized compared with housekeeping protein amount.

		primary antibody	**secondary antibody**
AR	Santa Cruz	1:1000	1:2500
myogenin	Millipore	1:400	1:1000
p-Akt	Cell Signaling	1:1000	1:2500
Akt	Cell Signaling	1:1000	1:2500
lamin (nuclei housekeeping)	Santa Cruz	1:250	1:1000
tubulin (citosol housekeeping)	Santa Cruz	1:1000	1:2500
β-actin (housekeeping)	Chemicon	1:8000	1:2000

Table 3.4. Antibodies brands and dilutions.

3.7 Statistical analysis

Statistical analysis was performed where at least 2 experimental values were available and the comparisons were performed using the *Student's t* test. In every analysis, values of $p<0,05$ were considered significant. Quantitative data are presented as means ± SD.

RESULTS

To test the hypothesis that mutant AR toxic gain of function would impair myogenic capacity of commitment, differentiation and/or muscle maturation of KD satellite cells, we plan to analyze molecular, biochemical and morphological features in 10 days differentiated myotubes derived from muscle cell lines of 4 KD patients and 3 controls (Table 3.1).
In addition we plan to test the effects of testosterone on *in vitro* muscle commitment, differentiation and maturation by culturing all the cell lines with and without 10mM Dihydrotestosterone (DHT, testosterone active form).

4.1 Evaluation of commitment and differentiative ability of KD satellite cells with/without DHT treatment

4.1.1 *In vitro* normal myogenic capacity of KD muscle cells

Muscle KD and control cells grew at the same rate and went through the same number 4-12 of passages. As our culture procedure established a mixture of fibroblasts and myoblasts, in all the cell lines the percentage of Desmin-positive myoblast population was calculated and found to exceed 70% (data not shown).
To compare the myogenic capacity of myoblasts from normal and KD patients and from DHT-treated and untreated cells, we examined morphological aspects together with the expression of several markers of myogenesis at 0 (myoblasts) and 10 days of differentiation (myotubes). In the first 10 days of differentiation normal and KD myoblasts presented a similar myogenic ability in terms of time and number of mytubes. We did not observe delay in differentiation time or reduced number of myotubes in all the DHT-treated and untreated KD muscle cell lines compared to the controls (Figure 4.1). The activation of MyoD followed by the expression of myogenin and CK-M were considered as markers of myogenesis. The levels of myogenin were estimated by a combination of RT-PCR and western

blot assays (Figure 4.2c-d) while the level of early myogenic regulatory factor (MyoD) and muscle-specific creatine kinase (CK-M) was quantified by RT-PCR (Figure 4.2a-b).

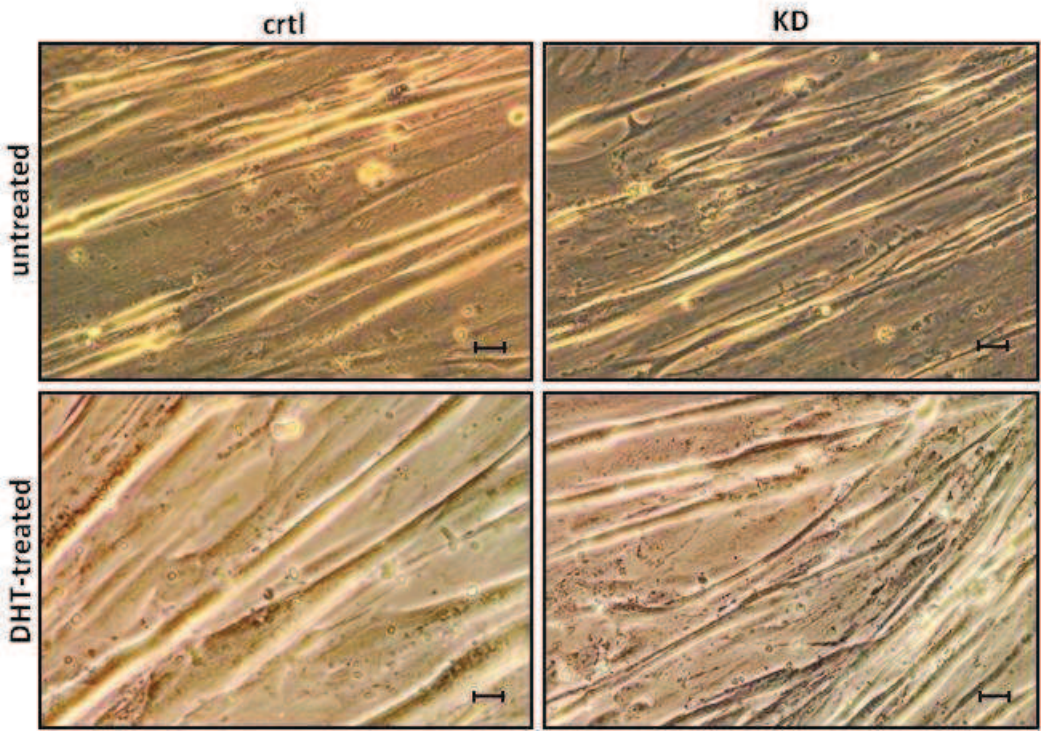

Figure 4.1. Similar differentiative ability of KD myoblasts. Representative bright-field images of 10-day differentiated myotubes from controls and KDs, treated with/without DHT (scale bar 20µm).

In all myoblasts and myotubes from KD patients the expression of MyoD, was similar to controls and unaffected by the treatment with DHT.

Likewise CK-M expression increased significantly as expected in myotubes compared to myoblasts both from KD and controls to a similar extension in presence or not of 10nM DHT. On the contrary DHT treatment provoked a significant ($p<0.03$) increase in myogenin protein in differentiated myotubes where was recorded a similar upward trend both in KD and control myotubes, suggestive of an effect on muscle differentiation-maturation triggered by the androgenic administration (Figure 4.2c).

These data suggest that KD primary muscle cells did not present any impairment in the early steps of myogenesis, behaving as controls until 10 days of differentiation.

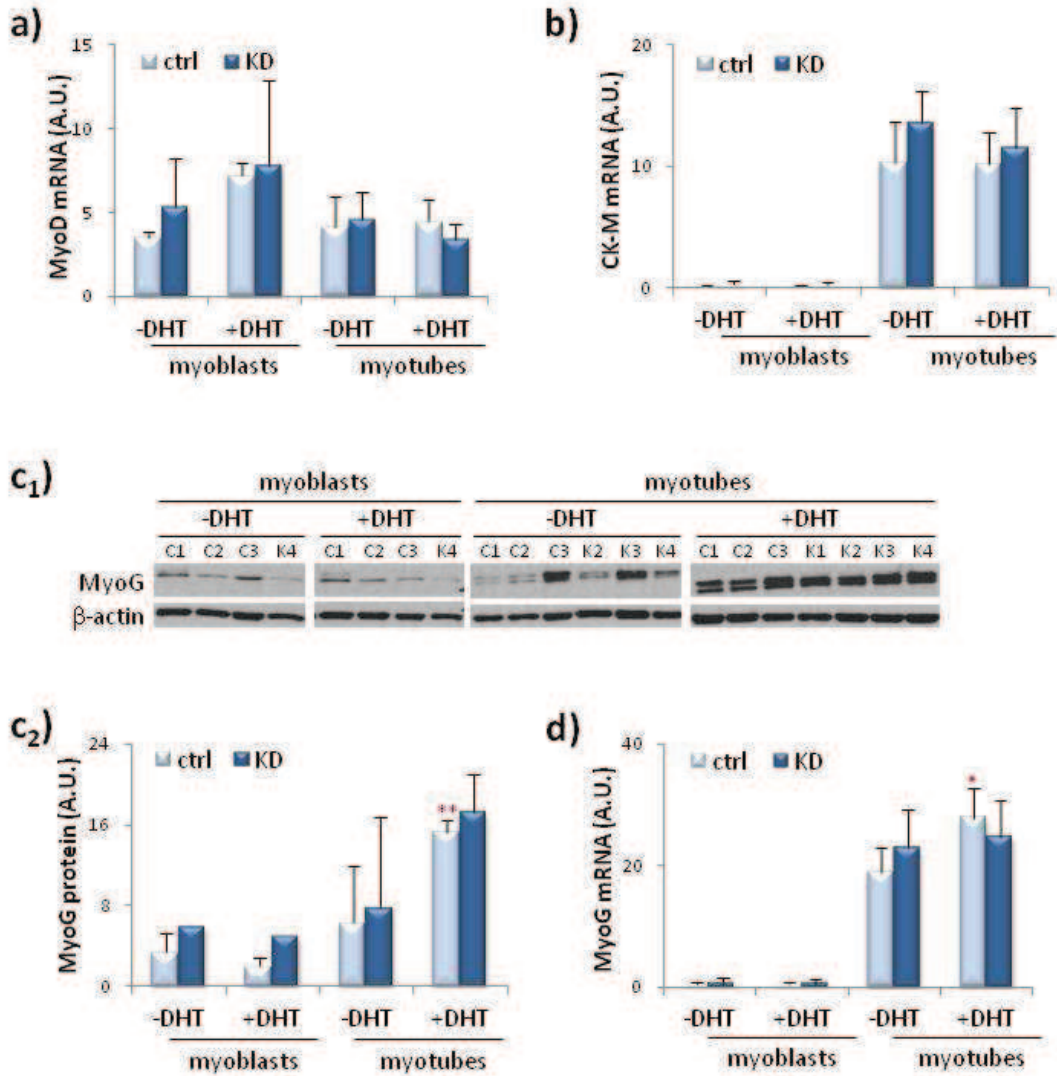

Figure 4.2. Differentiative ability of KD myoblasts. Expression levels of a) MyoD, b) CK-M and d) myogenin in KD and control myoblasts and myotubes; in DHT-treated control myotubes, myogenin mRNA expression was marginally significantly increased compared to untreated control myotubes (* $p<0,08$). $c_{1\text{-}2}$) WB analysis of myogenin in KD and control myoblasts and myotubes (30µg each); in DHT-treated control myotubes, myogenin protein was significantly increased compared to untreated control myotubes (** $p<0,01$). The values of mRNA and protein amounts are given as arbitrary units (A.U.) of ratio with β_2-microglobulin housekeeping gene or with β-actin protein respectively and are expressed as means ±.SD.

4.1.2 Atrophic features in KD myotubes following DHT treatment

The normal myogenic capacity found in KD primary myoblasts were accompanied by the observation that in presence of DHT-treated KD myotube population presented an increased atrophy compared to controls. In fact DHT treatment did not provoke any trophic effect in KD myotubes at difference of control myotubes that appeared larger and bigger than the control myotubes growing in absence of DHT (Figure 4.1). Indeed the DHT treatment did support an increased growth in control myotubes that had a 14% larger width compared to untreated control myotubes. This improvement was not observed in DHT-treated KD myotubes that were as large as the untreated KD myotubes and presented an average width reduced by 9% compared to DHT-treated controls (Figure 4.3a-c). Several studies showed that muscle atrophy is accompanied by a reduction in mean number of myonuclei per fiber.

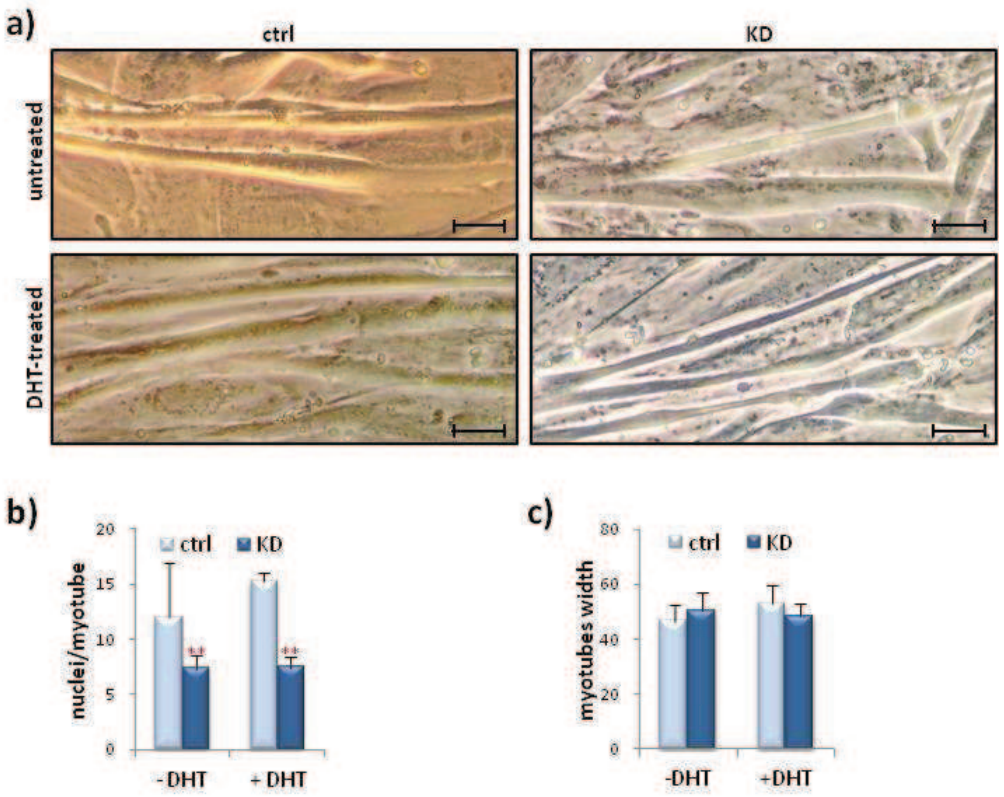

Figure 4.3. Fusion index and average width in 10-day differentiated KD and control myotubes, in untreated and DHT-treated samples. a) Representative bright-field images of 10-day differentiated myotubes from controls and KDs, treated with/without DHT, show different amount of nuclei per myotubes. b) fusion index (FI) given as number of nuclei per myotube; in KD myotubes, both DHT-treated and untreated, the number of nuclei/myotubes is significantly reduced compared to controls (** p<0,01). c) average myotube width. All values were obtained considering at least 100 myotubes/cell line and expressed as means ±.SD.

Consistently, we found a statistically reduced number of nuclei per myotube in KD myotubes of 40% compared to controls, both in DHT-treated and untreated cell cultures (Figure 4.3a-b).

The visual impression of atrophic KD myotubes (Figure 4.3) was confirmed by the electron microscopy (Figure 4.4).

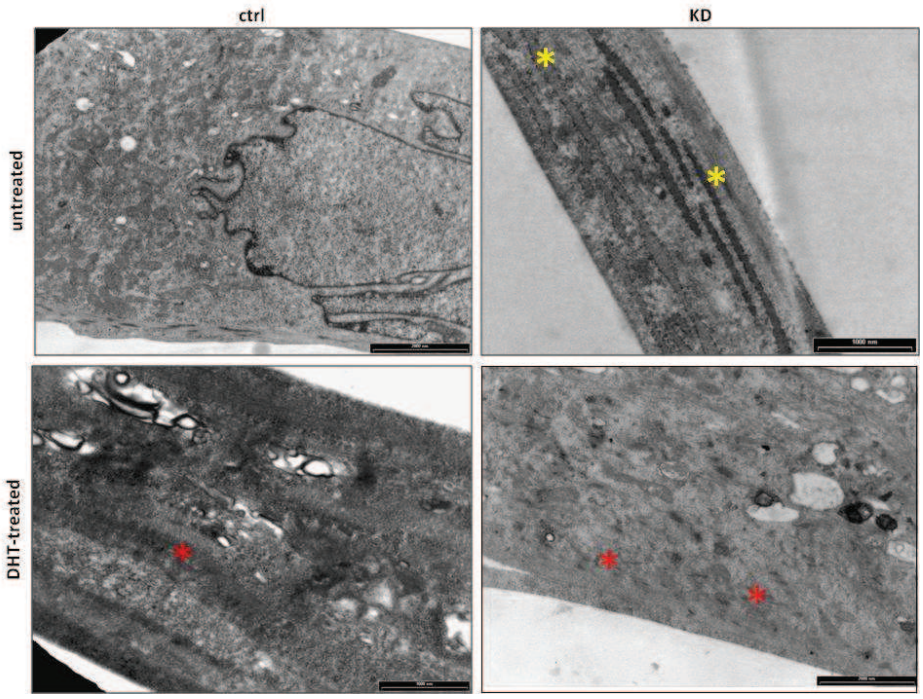

Figure 4.4. Electron microscopy images of KD and control myoblasts and 10-day differentiated myotubes, DHT-treated and untreated (scale bar 2µm).

Ultrastructural analysis of DHT-treated controls clearly showed a well defined myofibrillar structure formation (red asterisk), while in DHT-treated KD myotubes we could notice an increase of the sarcoplasmic volume but only rudimentary miofibrillar structures can be appreciated (red asterisk). The untreated KD myotubes showed an undifferentiated submicroscopical morphology characterized by a fibroblast-like phenotype: elongated cell shape and few rough endoplasmic reticulum, RER, profiles. These features were similar to untreated control myotubes, even if in controls was present a larger sarcoplasm with numerous organelles such as mitochondria, rough endoplasmic reticulum profiles (yellow asterisk) and Golgi apparatus.

Together, these results suggested the activation of catabolic pathways in muscle cells of KD patients after DHT treatment.

To characterize the signaling involved in KD myotube atrophy, we investigated the presence of apoptosis by studying chromatin fragmentation by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL). By this approach any difference in number of apoptotic nuclei between KD myotubes and controls has been found. Indeed, TUNEL assay revealed the same low presence of apoptotic nuclei in all KD myotubes during the differentiation than control samples (Figure 4.5a).

Moreover, in total extract of DHT-treated and untreated myoblasts and myotubes the pro-survival factor p-Akt was measured by WB analysis (Figure 4.5b). Surprising 10nM DHT treatment reduced 10-fold pAKT/Akt ratio in both KD and control myotubes compared to their untreated cells. On the other hand KD differentiated myotubes had an increased pAkt/Akt ratio compare to controls, both in presence or in absence of DHT.

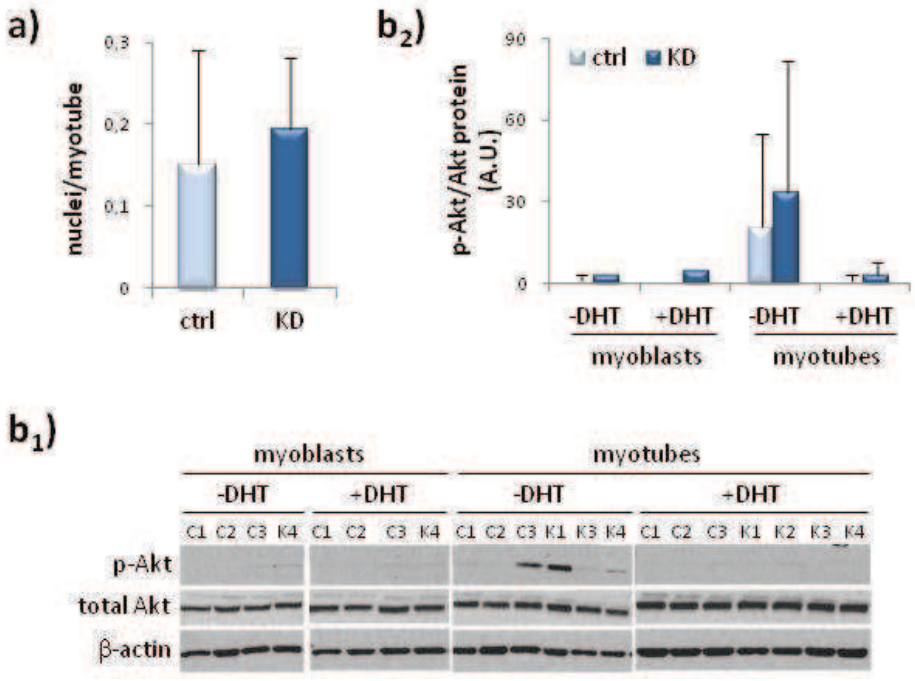

Figure 4.5. a) average number of apoptotic nuclei per DHT-treated myotubes, obtained considering at least 50 myotubes/cell line. b_1) WB analysis of Akt and p-Akt in KD and control myoblasts and myotubes (30µg of total protein), given as arbitrary units (A.U.) of ratio with β-actin protein; b_2) diagram of the ratio p-Akt/total Akt, expressed in A.U. All values are expressed as means ±.SD.

Overall, these data provide evidence that apoptosis do not contribute to the atrophic feature found in DHT-treated KD myotubes, indicating that other mechanisms could be activated.

4.2 Assessment of polyQ-AR localization with/without DHT treatment

The effects of the AR with elongated polyQ tract have never been evaluated in primary human satellite cell cultures from KD patients, so we planed to investigate the *AR* gene expression and the AR protein quantification/localization in proliferating myoblasts and in 10-day differentiated myotubes of KD satellite cells, both in DHT-treated and untreated cultures.

WB analysis of AR showed a higher band in KD muscle cells protein extracts compared to controls (Figures 4.6 - 4.7 - 4.8), which demonstrated the presence of elongated polyQ-AR in KD patients.

We verified whether the differentiation changes the amount of AR at transcriptional or translational level. Interestingly, the expression level of *AR* in untreated and DHT-treated KD myoblasts did not differ from controls, and was substantially unaffected by DHT treatment. Whereas we could observe that in all the tested samples, differentiation *per se* increased AR expression of 150% compare to myoblasts ($p<0.01$) (Figure 4.6a).

Differently from the expression data, WB analysis revealed that in DHT myotubes the levels of AR protein were statistically increased compared to untreated. Moreover the high AR levels of DHT myotubes were similar to what found in all the myoblasts. (Figure 4.6b). These discrepancies indicated that the levels of AR protein is substantially modified at post translational levels and that DHT treatment influence the stabilisation of AR protein only in differentiated muscle cells.

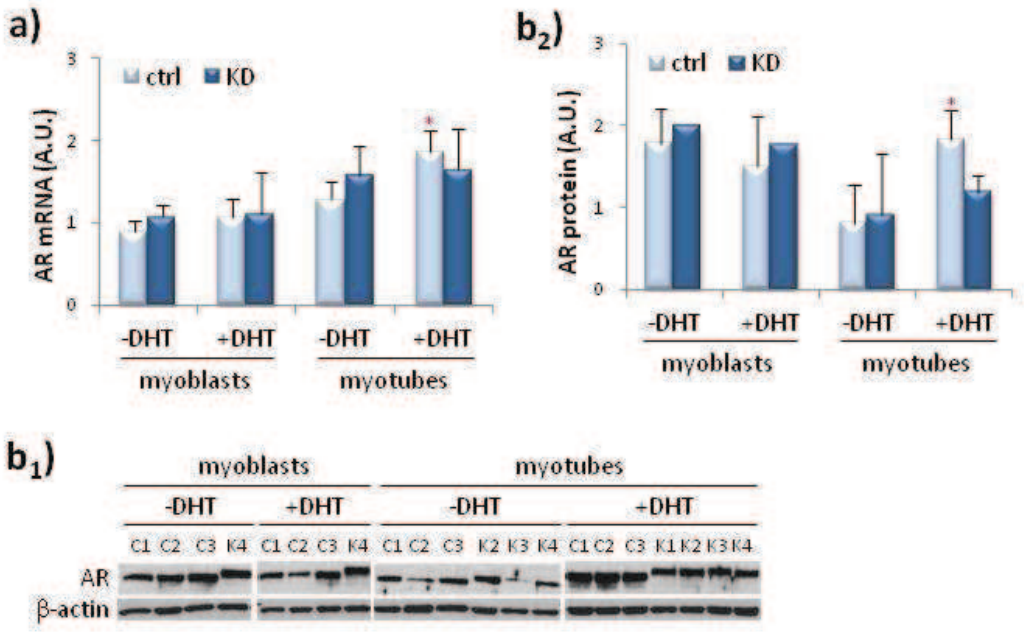

Figure 4.6. AR analysis in total cells of KD and control myoblasts and myotubes. a) expression level of AR given as arbitrary units (A.U.) of ratio with β_2-microglobulin housekeeping gene; in DHT-treated control myotubes, AR mRNA expression was significantly increased compared to untreated controls (* $p<0,05$). $b_{1\text{-}2}$) WB analysis of AR from total extracts, given as arbitrary units (A.U.) of ratio with β-actin protein; in DHT-treated control myotubes, AR protein was significantly increased compared to untreated controls (* $p<0,05$). All values are expressed as means ±.SD.

Since the translocation of AR from cytosol to nucleus occurs following DHT-treatment and since mutated poliQ-AR performs its toxic gain of function when localized into the nucleus, nuclear and cytosolic amount of AR was quantified separately by WB analysis of nuclear and cytosolic fractions of myoblasts and 10-day differentiated myotubes from untreated and DHT-treated KD patients and controls.

We could recorder the physiologic AR translocation after DHT treatment that was demonstrated by the enrichment of AR protein levels in the nuclear fraction (Figures 4.7 - 4.8). The main observation was that DHT treatment led to a substantial greater AR increase in KD myotubes nuclei compared to controls (Figure 4.7a), while in proliferative satellite cells DHT-treated increased to the same extension the nuclear AR protein amount of KD and controls (Figure 4.8a). These data suggest that in presence of DHT, mutated polyQ-AR accumulation occurs after differentiation and not during proliferation of muscle satellite cells.

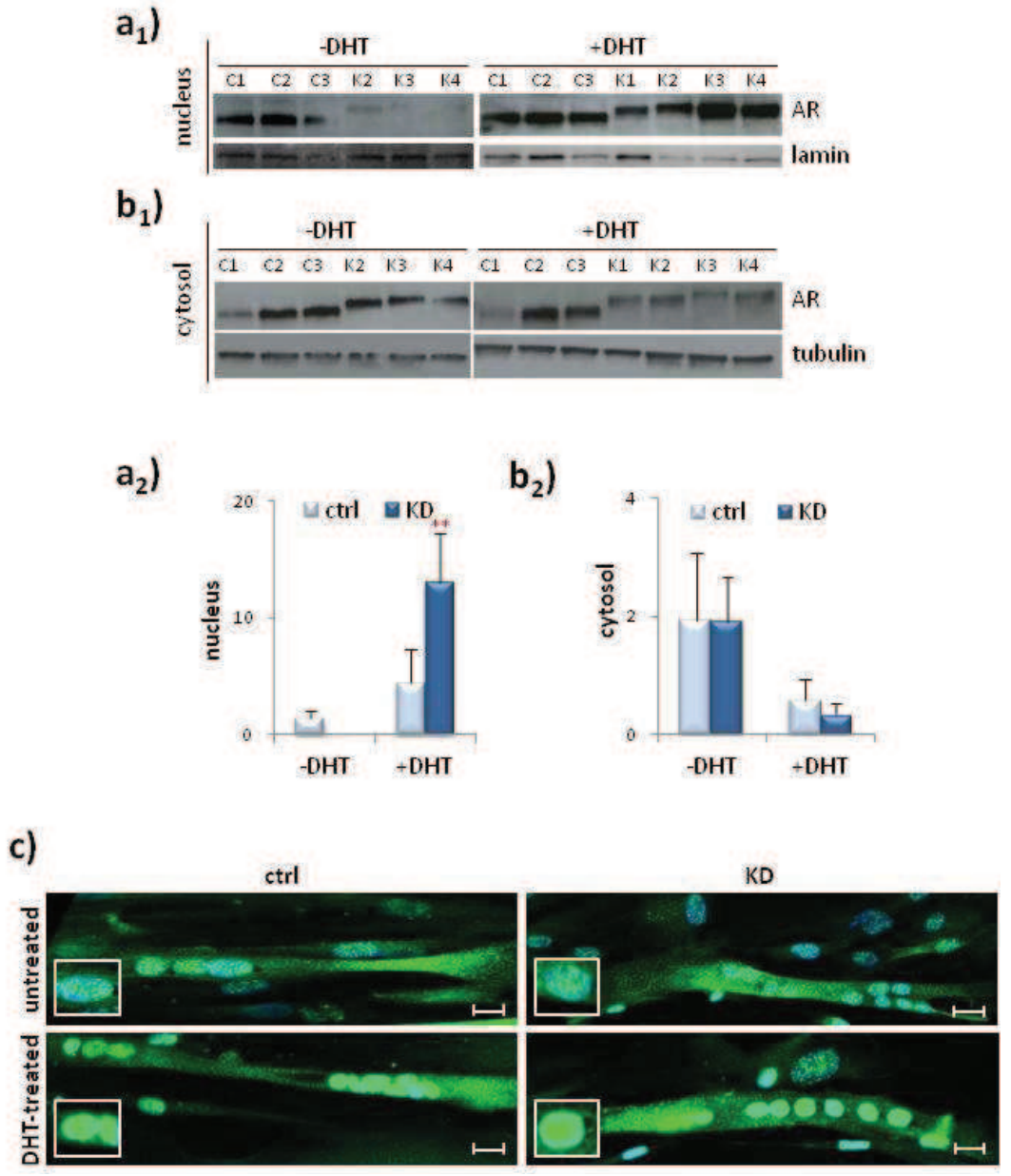

Figure 4.7. Myotubes: AR localization in $a_{1\text{-}2}$) nuclear and $b_{1\text{-}2}$) cytosolic fractions from KD and control, treated with/without DHT; AR protein amount is given as arbitrary units (A.U.) of ratio with lamin protein in the nucleus and tubulin in the cytosol. All values are expressed as means ±.SD and in DHT-treated KD myotubes, AR protein was significantly increased compared to untreated KDs (** $p<0,01$). c) Representative immunofluorescence (IF) images of AR protein (green) and nuclei (DAPI) of KD and control myotubes, treated with/without DHT (scale bar 10µm).

Immunofluorescence morphological data confirmed the different localization of AR, showing that DHT treatment induced AR translocation from cytosol to nucleus. We also detected a greater increase of AR signal in the KD DHT-treated myotubes nuclei compared to controls (Figure 4.7c).

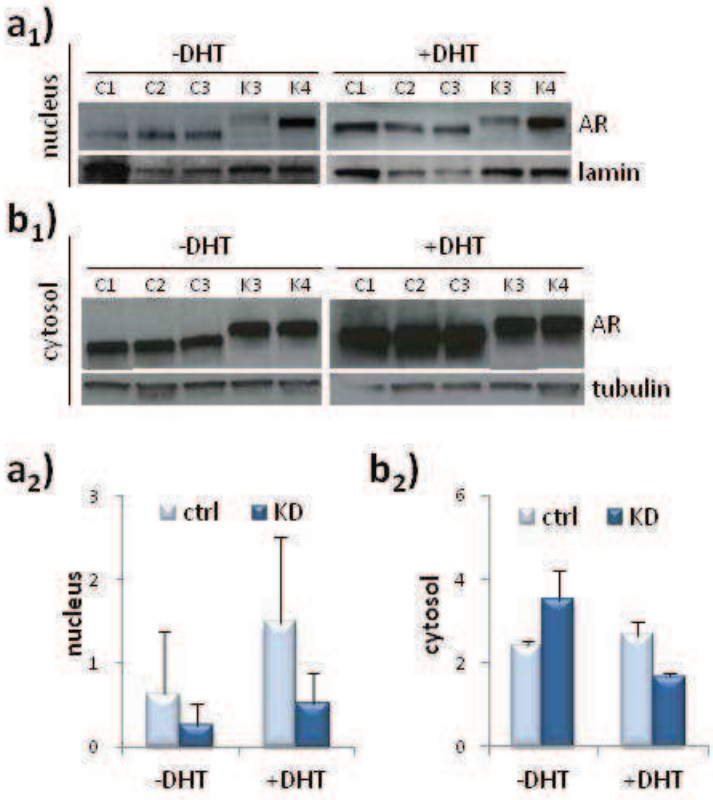

Figure 4.8. Myoblasts: AR localization in $a_{1\text{-}2}$) nuclear and $b_{1.2}$) cytosolic fractions of KD and control myoblasts, treated with/without DHT; AR protein amount is given as arbitrary units (A.U.) of ratio with lamin protein in the nucleus and tubulin in the cytosol. All values are expressed as means ±.SD.

Since DHT treatment in KD myobubes does not affect the transcription of AR (Figure 4.6a), but the nuclear protein level (Figure 4.7a), accumulation in KD nuclei must be due to post-translational events.

DISCUSSION

Neurodegeneration in KD is mainly caused by a toxic gain of property of the expanded polyQ-AR [140,164,182]. The elongated polyQ stretch alters the conformation of AR, resulting in toxic nuclear aggregation of the protein, both in neural and non-neural tissues. The polyQ-AR aggregates contain several chaperones and components of the ubiquitin-proteasome system (UPS), apart from transcription factors, suggesting that they are involved in an attempt to degrade or refold the mutant AR [133]. In particular, polyQ-AR leads to the degeneration of motor neurons. Skeletal muscle of KD patients is reported to be characterized by a number of changes suggestive of primary muscle disease together with the expected neurogenic atrophy [134,147160]. We therefore hypothesized that myopathic changes in KD skeletal muscle may be due to a failure of satellite cells activation or an impairment in differentiation-maturation caused by mutant polyQ-AR, since these cells are the predominant site of AR expression [169] and play a key role in repair processes preventing muscle damage [145].

5.1 Myogenic capacity of KD satellite cells treated with/without DHT

In the present study, we showed that proliferation of KD satellite cells is similar to control and unaffected by DHT treatment. Moreover the expression of myogenic markers such as MyoD, myogenin and CK-M in KD 10-day differentiated myotubes is not significantly different from controls. These data suggest that there is no impairment in differentiative ability of KD muscular cells. In addition we observe that DHT treatment induces myogenin increase both in mRNA and protein levels both in KD and control myotubes in relation to the anabolic effect of testosterone on skeletal muscle mass as reported [187]. Androgens induce increase in muscle mass by enhancing contractile protein synthesis, by promoting increase in the myonuclear number and myogenic

differentiation. The anabolic effects of androgens are mainly mediated by AR even if a non-AR-mediated mechanisms has been observed at supraphysiological doses of testosterone [170,177]. Testosterone is reported to increase number of nuclei per myotube (Joubert et al. reported an 80% increase in number of myonuclei per fiber in the *levator ani* muscle of adult female rats after 30 days of DHT treatment [150]) and fiber diameter [148].Consistently we demonstrated the anabolic effect of DHT in DHT-treated control satellite cells that differentiated in big myotubes, with increased number of nuclei (27%) and of myotube width (14%) compare the untreated myotubes (Figure 4.3), with a well organized myofibrillar structure formation (Figure 4.4). In contrast, we noticed the absence of trophic effect of DHT treatment in KD differentiated myotubes when these were analyzed for morphological features such as fusion index, myotube width, that were 48% and 89,5% respectively compare to controls. Also EM analysis reveal a less-differentiative subcellular organization of KD DHT treated muscle cells (Figure 4-4).

These data are in line with the atrophic features, known to be the principal catabolic pathway in KD [176] and were confirmed by the absence of apoptosis that excluded other causes of muscular distress.

During differentiation we could observed an increase of the pro-survival factor Akt. It was significantly activated in p-Akt in untreated myotubes (more in KD than in controls) compared to myoblasts. Inexplicably, DHT-treated myotubes showed the same p-Akt low levels of myoblasts. It is reported that 0,1 to 1nM DHT administration in prostatic cells induces an increase of p-Akt, while 10 to 100nM DHT administration decreases p-Akt activation [135]. It is difficult to explain the reason of this DHT dose dependent paradoxical effect on p-Akt .

All together these data suggest a toxicity mediated by nuclear mutant polyQ-AR.

5.2 PolyQ-AR localization with/without DHT treatment

To understand the mechanisms of skeletal muscle atrophy in KD, we investigated the involvement of mutant polyQ-AR in muscle KD satellite cells and in 10 days differentiated myotubes compared to controls when treated with/without DHT.
Physiologically, DHT induces AR dissociation from HSPs and allows AR dimerization and translocation to the nucleus, where binds to androgen-responsive elements in the promoter region of AR target genes activating their transcription [163]. In this work we demonstrated the effect of translocation to the nucleus, by an increase in AR nuclear content, in both KD and control myotubes. In addition we observed that KD DHT-treated muscle cells have a greater increase compared to controls. This observation suggests that an AR-mediated pathological effect occurs in the muscle cells, as well as observed in neurons. Consistently we previously found that in KD adult muscle the nuclear AR protein was 5-fold increased ($p<0,05$) compared to controls (unpublished data). Besides, in the nuclei of dividing DHT-treated KD satellite cells (myoblasts), AR protein was at the same level of controls, which suggested that the accumulation of abnormal AR protein starts after differentiation and could explain why proliferation of KD myoblasts is not compromised.
RT-PCR revealed that mRNA amount of AR was unaffected by DHT in KD myotubes. We therefore hypothesized the AR accumulation in KD is due to post-translational events rather than transcription activation. An increase in AR mRNA and protein is seen only in DHT-treated control myotubes. It is now accepted that KD nuclear aggregates are formed by truncated species of polyQ-AR specifically cleaved by caspase-3; this post-transcriptional modification appears to occur only in the expanded polyQ-AR [168]. The nuclear toxicity may be due to different and concomitant events, such as the sequestration of transcriptional factors or transcriptional disregulation [153,169,171] and can start in other tissues rather than CNS. The aggregation process is a consequence of the acquisition of aberrant protein conformation, which occurs when polyQ is pathologically elongated and is capable of starting the oligomerization of the mutant polyQ-AR. Usually, the UPS should prevent the formation of nuclear aggregates, by clearing the cells from misfolded proteins. However, an overload of misfolded substrates may impair UPS activity leading to polyQ-AR accumulation also in the nuclei. This process is accompanied by the

sequestration of important nuclear factors and their removal can have deleterious impact on motor neuronal survival [185]. Rusmini et al (2007) found UPS impairment, resulting in self aggregation of testosterone-activated AR. She postulated that aggregates may represent an initial attempt of the cells to respond to the presence of the neurotoxic species of the mutant polyQ-AR [172], by segregating the mutant protein into physically well-defined subcellular compartment, waiting for their degradation.

As previously, in KD skeletal muscle we were unable to find the presence of the aggregates in satellite cells and in 10-day differentiated myotubes, likely because the detection of AR aggregates needs longer repeats of polyQ expansion. In fact very long polyQ tracts (more than CAG_{100}, which have never been found in KD patients) are required to mimic the formation of nuclear aggregates in cell culture [161,186].

In conclusion we found signs of an impairment in maturation of KD 10-day differentiated myotubes treated with DHT, likely due to the accumulation of mutant polyQ-AR in cell nucleus, confirming the hypothesis of a primitive muscle involvement in KD.

Further analysis are needed to understand this post-translational events of mutant polyQ-AR leading to the KD pathological pathway.

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