Loss of C9orf72 Function Impairs the Peripheral Neuromuscular System and Anticipates Symptoms in ALS Mice.

Thesis

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Loss of C9orf72 function impairs the peripheral neuromuscular system and anticipates symptoms in ALS mice.

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For the degree of Doctor of Philosophy
Life and Biomolecular Science
The Open University

October 2021
ABSTRACT

Hexanucleotide repeat expansions in the C9orf72 gene is the prevalent genetic cause of ALS, causing neurodegeneration through two gain-of-function mechanisms due to the accumulation of RNA foci and dipeptide-repeat proteins, and a loss-of-function mechanism due to C9orf72 protein reduction.

The loss-of-function has never been examined in the peripheral compartment of neuromuscular system, precociously affected by ALS. In this work we observed that C9orf72 is expressed by Schwann cells and skeletal muscle in mice and the constitutive ablation of C9orf72 leads to mild muscle denervation, alterations of autophagy, increased immune cell infiltration and inflammation in muscle and peripheral nerve, typical features of ALS. Moreover, demyelination, activation of Schwann cells in a repair mode and autoimmunity were found in peripheral nerves of C9orf72−/− mice.

Evidence demonstrates that the loss-of-function synergizes with other toxic stimuli to induce neurodegeneration. Moreover, C9orf72 mutation often occurs in combination with other ALS or FTD-linked genes, suggesting oligogenicity of ALS. Therefore, to verify whether the C9orf72 loss-of-function could act as ALS modulator, we investigated the effect of C9orf72 constitutive ablation in SOD1G93A mice, the best characterized model of ALS. We found that the C9orf72 loss-of-function anticipated the onset of symptoms exacerbating denervation, autophagy impairment and inflammation in muscle, and enhancing axonal transport disruption in SOD1G93A mice. Conversely, increased anti-inflammatory gliosis in the spinal cord and extended survival were detected in SOD1G93A C9orf72−/− mice.

For the first time, in this work we demonstrated that C9orf72 is expressed in the peripheral compartment of neuromuscular system where it is pivotal in maintaining the homeostasis.
Despite the C9orf72 ablation had detrimental effects on this district and anticipated the symptom onset in ALS mice, unexpectedly it prolonged their lifespan. This suggests that reducing the C9orf72 expression in the later phase of the disease, for example through antisense oligonucleotides, might have a therapeutic relevance.
PREFACE

The work described herein was performed at the IRCCS - Istituto di Ricerche Farmacologiche “Mario Negri” – Milan, Italy from October 2017 to October 2021.

The PhD research project was conducted under the supervision and direction of Dr. Caterina Bendotti (Director of Studies) and Dr. Laura Ferraiuolo (External Supervisor).
CANDIDATE PUBLICATIONS EMANATING FROM WORK NOT PERTAINING WITH THIS THESIS

Multiple intracerebroventricular injections of human umbilical cord mesenchymal stem cells delay motor neurons loss but not disease progression of SOD1G93A mice.


RNS60 exerts therapeutic effects in the SOD1 ALS mouse model through protective glia and peripheral nerve rescue.


CXCL13/CXCR5 signalling is pivotal to preserve motor neurons in amyotrophic lateral sclerosis


Novel P2X7 antagonist ameliorates the early phase of ALS disease and decreases inflammation and autophagy in SOD1G93A mouse model


Boosting the peripheral immune response in the skeletal muscles improved motor function in ALS transgenic mice


Molecular Therapy (accepted).
LIST OF ABBREVIATIONS

4-HNE 4-Hydroxynonenal
7,8-DHF 7,8-Dihydroxyflavone
8OH2’dG 8-Hydroxy-2'-deoxyguanosine
AALSRS Appel Amyotrophic Lateral Sclerosis Rating Scale
AChR Acetylcholine Receptor gamma subunit
AD Autosomal Dominant
ADARB2 Adenosine Deaminase RNA Specific B2
AIFM1 Apoptosis Inducing Factor Mitochondria Associated 1
ALS Amyotrophic Lateral Sclerosis
ALSFRS Amyotrophic Lateral Sclerosis Functional Rating Scale
ALSFRS-R Amyotrophic Lateral Sclerosis Functional Rating Scale-Revised
ALYREF Aly/REF Export Factor
AMPA Alpha-Amino-3-Hydroxy-5-Methyl-4-Isxazole Propionic Acid
AR Autosomal Recessive
Arp Actin-Related Protein
ASO Antisense Oligonucleotide
ATG Autophagy-related Genes
Bcl2 B-cell lymphoma 2
bvFTD behavioural variant of Frontotemporal Dementia
C9orf72 Chromosome 9 open reading frame 72
CCL2 C-C motif chemokine Ligand 2
CD4 Cluster of Differentiation 4
CD8 Cluster of Differentiation 8
CD11b Cluster of Differentiation 11b
Cas9 CRISPR associated protein 9
ChAT Choline Acetyltransferase
CNPase 2',3'-Cyclic-nucleotide 3'-phosphodiesterase
CNS Central Nervous System
CRE Causes Recombination
<table>
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<tr>
<th>Acronym</th>
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<tbody>
<tr>
<td>CRISPR</td>
<td>Clustered Regularly Interspaced Short Palindromic Repeats</td>
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<tr>
<td>DENN</td>
<td>Differentially Expressed in Normal and Neoplasia</td>
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<tr>
<td>dNTP</td>
<td>Deoxynucleotide Triphosphate</td>
</tr>
<tr>
<td>DPR</td>
<td>Dipeptide Repeat Protein</td>
</tr>
<tr>
<td>DTI</td>
<td>Diffusion Tensor Imaging</td>
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<tr>
<td>EAAT</td>
<td>Excitatory Amino Acid Transporter</td>
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<td>EEC</td>
<td>El Escorial Criteria</td>
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<td>Electromyography</td>
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<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>ERAD</td>
<td>Endoplasmic Reticulum-Associated protein Degradation</td>
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<tr>
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<tr>
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<td>Familial Amyotrophic Lateral Sclerosis</td>
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<td>FUSGCM</td>
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<td>GDNF</td>
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<td>Glutamate Transporter 1</td>
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<td>GFAP</td>
<td>Glial fibrillary acid protein</td>
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<tr>
<td>GOF</td>
<td>Gain Of Function</td>
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<td>GPNMB</td>
<td>Glycoprotein transmembrane Neuromedin B</td>
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<td>hnRNP</td>
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<td>hnRNPA1</td>
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<td>hnRNPH</td>
<td>heterogeneous nuclear Ribonucleoprotein H</td>
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<td>HRE</td>
<td>Hexanucleotide Repeat Expansion</td>
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<td>human Superoxide Dismutase 1</td>
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<td>HuR</td>
<td>Human antigen R</td>
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<tr>
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<td>iMN</td>
<td>induced Motor Neuron</td>
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<td>LMN</td>
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<td>MN</td>
<td>Motor Neuron</td>
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<td>NDS</td>
<td>Normal Donkey Serum</td>
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<tr>
<td>NES</td>
<td>Nuclear Export Signal</td>
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<tr>
<td>NF</td>
<td>Neurofilament</td>
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<tr>
<td>NF-H</td>
<td>Neurofilament Heavy polypeptide</td>
</tr>
<tr>
<td>NF-L</td>
<td>Neurofilament Light polypeptide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Term</td>
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<tr>
<td>NF-M</td>
<td>Neurofilament Medium polypeptide</td>
</tr>
<tr>
<td>NF200</td>
<td>Neurofilament Heavy polypeptide 200 kilodalton</td>
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<tr>
<td>NGS</td>
<td>Normal Goat Serum</td>
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<tr>
<td>NLS</td>
<td>Nuclear Localization Signal</td>
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<td>NMDA</td>
<td>N-Methyl-D-Aspartate</td>
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<tr>
<td>NMJ</td>
<td>Neuromuscular Junction</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
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<td>NogoA</td>
<td>Neurite Outgrowth Inhibitor A</td>
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<td>NPC</td>
<td>Nuclear Pore Complex</td>
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<td>Non-Transgenic mice</td>
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<tr>
<td>O/N</td>
<td>Over Night</td>
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<td>Optineurin</td>
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<td>OXPHOS</td>
<td>Oxidative Phosphorylation</td>
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<td>PBP</td>
<td>Progressive Bulbar Palsy</td>
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<td>PBS</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>PEG</td>
<td>Percutaneous Endoscopic Gastrotomy</td>
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<td>PHB</td>
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<tr>
<td>PLS</td>
<td>Primary Lateral Sclerosis</td>
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<td>PMA</td>
<td>Progressive Muscle Atrophy</td>
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<tr>
<td>PNS</td>
<td>Peripheral Nervous System</td>
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<td>RAGE</td>
<td>Receptor for Advanced Glycation End</td>
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<td>RBP</td>
<td>RNA Binding Protein</td>
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<tr>
<td>RCF</td>
<td>Relative Centrifugal Force</td>
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<td>RNS</td>
<td>Reactive Nitrogen Species</td>
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<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
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<tr>
<td>sALS</td>
<td>sporadic Amyotrophic Lateral Sclerosis</td>
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</table>
SC  Schwann Cell
SEM  Standard Error of Mean
SN  Sciatic Nerve
SMCR8  Smith–Magenis chromosome region 8
SOD1  Cu/Zn Superoxide Dismutase 1
SPECT  Single Photon Emission Computed Tomography
SRSF1  Serine and arginine Rich Splicing Factor 1
TA  Tibialis Anterior
TARDBP  Transactive Response DNA-binding Protein
TDP43  Transactive Response DNA-binding Protein 43 kDa
TkrB  Tropomyosin receptor kinase B
TLI  T-Lymphocytes Irradiation
TLS  Translocated in Liposarcoma
TNFα  Tumor Necrosis Factor ALPHA
Treg  T regulatory
UBQLN2  Ubiquilin 2
ULK1  Unc-51 Like Autophagy Activating Kinase 1
UMN  Upper Motor Neuron
UPS  Ubiquitin-Proteasome System
VAPB  Vesicle-associated membrane protein (VAMP)-Associated Protein B
VCP  Valosin-Containing Protein
VDAC1  Voltage-Dependent Anion-selective Channel 1
VEGF  Vascular Endothelial Growing Factor
WDR41  WD40-Repeat containing protein 41
WT  Wild Type
XD  chromosome X Dominant
ΔFS  deltaFS (ALS progression rate)
DECLARATION

This PhD project has not been submitted in whole or in part for a degree or diploma or other qualification to any other University. The experimental work described here was performed by me.
ACKNOWLEDGMENTS

I want to express my sincere gratitude to my Director of Studies, Dr. Caterina Bendotti, for providing me the opportunity to attend the Open University PhD program as well as for her guidance given me in all these years spent in her laboratory.

I want to thank my External Supervisor, Dr. Laura Ferraiuolo, for her encouraging words.

Special thanks go to all the former and current member of the Molecular Neurobiology Lab., especially Chiara, Paola, Massimo, and Giovanni, who supported me and contributed to my personal and professional growth during all these years.

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INTRODUCTION

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Amyotrophic Lateral Sclerosis (ALS)
1.1 A BRIEF INTRODUCTION TO THE DISEASE

Amyotrophic lateral sclerosis (ALS) is the most common type of motor neuron disease (MND), a group of rare neurodegenerative disorders that selectively affect motor neurons (MNs) that undergo progressive degeneration and death. MNs are a subclass of neurons whose cell body is located in the motor cortex, (upper motor neurons, UMNs), brainstem and spinal cord (lower motor neurons, LMNs), and whose axon projects to the spinal cord or muscles, controlling the voluntary motor functions. In ALS, the degeneration of the UMNs and LMNs causes the loss of skeletal muscle innervation, leading progressively to muscle weakness, atrophy, complete paralysis and eventually death due to the denervation and dysfunction of respiratory muscles within 5 years after the clinical onset (Cleveland and Rothstein, 2001; Shneider and Rowland, 2001). MND comprises also progressive muscle atrophy (PMA), primary lateral sclerosis (PLS) and progressive bulbar palsy (PBP).

The first research on ALS dates back to 1850, when the English scientist Augustus Waller described the appearance of withered nerve fibres in cadavers. In 1869 the French neurobiologist and clinicians Jean-Martin Charcot described for the first time the principal clinical signs and the anatomopathological features in a single clinical picture, when the clinical diagnosis was still rudimentary and the distinction between UMNs and LMNs neurons was still unknown (Charcot et al., 1885; Charcot and Joffroy, 1869). Initially, the disease was called “Charcot’s sclerosis”, then Charcot coined the term ALS, suggesting both the typical symptomatology and the anatomopathological features of the disease. "Amyotrophic" refers to the muscle atrophy that characterizes the disease, due to the progressive MN degeneration and "Lateral Sclerosis" to the hardening of the lateral horns of the spinal cord observed on palpation during the autopsy, due to the proliferation and hypertrophy of different types of glial cells (gliosis) (Rowland and Shneider 2001). In the USA and Canada, ALS is familiarly known as “Lou Gehrig's disease”, in honour of the great baseball player, first certified victim, who developed the disease in the 1930s.
1.2 EPIDEMIOLOGY

Epidemiologic studies provide not only the knowledge of disease burden but also how it manifests itself within populations with different characteristics. ALS is considered a rare disease with a reported incidence between 0.6 and 3.8 per 100,000 person-years (Longinetti and Fang, 2019). Geographical differences are noted: a higher incidence of ALS was found in Europe, ranging from 2.1 to 3.8 per 100,000 person-years, while the few study studies published from outside Europe reported a lower incidence of ALS in Asia (1.2 per 100,000 person-year in South Korea, 0.8 per 100,000 person-year in China and 1.9 per 100,000 person-year in Turkey) (Zhou et al., 2018; Jun et al., 2019; Turgut et al., 2019) and in Canada (0.6 per 100,000 person-year) (Rose et al., 2019). This could be attributed to the lower prevalence of known ALS genes in ALS population (Kim et al., 2016), but also the absence of population-based studies (Logroscino et al., 2018). The origin of the geographic difference of ALS is a matter of debate since it is partly due to the prevalence of ALS-associated genes and partly to the environmental risk factors. Several environmental factors have been associated with ALS, such as viral and bacterial infections, chemicals containing organophosphates, β-N-methylamino-L-alanine neurotoxin, heavy metals, electromagnetic fields, smoke, and excessive physical activity (Bozzoni et al., 2016; Yu and Pamphlett, 2017). In particular, soccer has been proposed as a risk factor for ALS due to the intense physical activity, repeated head trauma, exposure to pesticides employed on soccer fields and dietary supplement/illegal substances (Chiò et al., 2005). However, none of these environmental risk factors have been confirmed (Yu and Pamphlett, 2017). The delay in the diagnosis hinders the establishment of ALS incidence. Indeed, the diagnosis of ALS is made clinically and requires evidence of a progressive spread of symptoms, not only to avoid misdiagnosis, but also due to the lack of valid diagnostic biomarkers of ALS and the complexity of the clinical feature (Brooks et al., 2000; Agrawal and Biswas, 2015). Moreover, the type of onset and the heterogeneous clinical manifestation can postpone the diagnosis. Patients
with bulbar onset are diagnosed earlier compared to those with spinal onset (Zhou et al., 2018), and male patients are diagnosed sooner than females (Longinetti and Fang, 2019).

Recent population-based studies reported a prevalence between 4.1 and 8.4 per 100,000 person (Longinetti and Fang, 2019). A difference in ALS prevalence due to ethnicity has also been recently reported. Using the National ALS Registry the prevalence of European-American ALS patients was found to be more than double the prevalence of African-American ALS patients (5.4 versus 2.3 per 100,000) (Mehta et al., 2018). Furthermore, a male to female ratio between 1 and 2 was reported, except for Africa (Longinetti and Fang, 2019).

Despite the predominance of spinal onset (58-82%) in all countries, bulbar onset seems to be prevalent in subjects characterised by different traits (females, cognitive impairment, elderly, etc.). In addition to the spinal or bulbar onset, recently have been reported other types of onset that might alter the incidence of the disease in ALS registers: mixed (spinal and bulbar), thoracic, cognitive and respiratory (Longinetti and Fang, 2019).

European patients usually have a later age onset compared to Asia and Latin America (Longinetti and Fang, 2019).

1.3 SYMPTOMATOLOGY

Clinical manifestations of the disease are indicative of the degeneration and subsequent death of UMNs and LMNs. The loss of these MNs leads to innervation failure of skeletal muscles, which undergo severe atrophy.

The main clinical signs that occur in ALS are:

- fasciculations: spontaneous and uncontrolled contraction of single contiguous muscle fibres
- hyperreflexia: increase of osteotendon reflexes
• muscle wasting: reduction of muscle mass which causes a partial or complete loss of muscle functions, resulting in an asymmetrical and progressive weakness.

The involvement of UMs and/or LMNs determines the initial symptoms (Kiernan et al., 2011). The degeneration of UMs causes weakness, coordination difficulties, stiffness, and slowness of movement, associated with spasticity, increased tendon reflex and clones (alternating contraction and relaxation). The Babinski sign, which identifies an anomaly of the cutaneous plantar reflex following a lesion of the corticospinal tract, is present in a variable manner in the early stage of the disease. The degeneration of LMNs causes exhaustion and fatigue, associated with progressive muscular atrophy, fasciculations and fibrillations (spontaneous contractions of isolated muscle fibres, not visible through the intact skin). There is also a reduction of muscle tone and the absence of tendon reflex.

The symptomatology depends on the body region initially affected by the degeneration that determines (Table 1.1):

• Spinal or limb onset: it occurs in 75% of ALS cases, in which the disease first affects the upper and lower limbs with distal asymmetric weakness, muscle atrophy, difficulties in execution and coordination of fine movements, and spasticity, as determined by UMs alterations. (Kiernan et al., 2011). In the same anatomical regions, the loss of LMNs induces fasciculation and muscle wasting. It is possible to note a consistent and appreciable decrease in strength, with the loss of about 50% of the MNs; before this threshold, the compensatory innervation of neighbouring MNs allows good maintenance of muscle function, through an increase in the size of the motor units. For this reason, in the early stages of the disease, MN loss is detectable only through electromyography (EMG). With the progression of the disease, the degeneration spreads to contiguous segments of the body and the patient loses the ability to swallow (dysphagia) and slurred speech (dysarthria), until the complete loss of verbal communication (anarthria).
Bulbar onset: it occurs in 25% of cases in which the bulbar impairment leads first to a difficulty in swallowing and slurring speech, until the total loss of verbal communication; the spastic dysarthria is ascribed to UMN degeneration, while tongue wasting weakness and fasciculation result from the loss of LMNs in the cranial nuclei of the brainstem. There is also an early respiratory failure due to the involvement of the diaphragm and respiratory muscles.

<table>
<thead>
<tr>
<th>UMN</th>
<th>LMN</th>
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<tr>
<td><strong>SPINAL OR LIMB ONSET</strong></td>
<td><strong>BULBAR ONSET</strong></td>
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<tr>
<td>• Weakness</td>
<td>• Spastic dysarthria</td>
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<td>• Lack of coordination</td>
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<td>• Rigidity</td>
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<td>• Spasticity</td>
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<td>• Increased tendon reflex</td>
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<td>• Extensor plantar responses</td>
<td>• Fasciculation</td>
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<tr>
<td></td>
<td>• Upper and lower limb wasting</td>
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<td></td>
<td>• Weakness</td>
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Table 1.1: ALS related symptoms depending on the type of onset and MN subtype involvement.

The onset of the symptoms is highly variable among patients and, as demonstrated in animal models of ALS, the loss of MNs probably starts before the clinical manifestation of the disease (Kennel et al., 1996). With the progression of the disease, the two phenotypes merge and in advanced stages of the disease the patient presents a marked broad disability, which often culminates in the need for ventilatory support (tracheostomy) and forced nutrition interventions (percutaneous endoscopic gastrostomy, PEG). In most cases, death occurs for respiratory failure, due to the progressive paralysis of the diaphragm and muscles that control breathing. Respiratory failure is the principal cause of death in ALS patients without tracheostomy (Salameh, Brown and Berry, 2015).

In the second half of the XX century, the commonly used instrument for the assessment of the disease status and progression in ALS included i) the Norris scale, ii) the Baylor (Appel) scale and iii) the Tufts Quantitative Neuromuscular Examination (Cedarbaum and Stambler, 1997). However, these instruments were not very operational.
There are two scales used in clinical practice for the evaluation of the progression of ALS:

- **ALS Functional Rating Scale (ALSFRS),** developed in 1996 by the World Federation of Neurology, is used almost exclusively. It is an ordinary rating scale consisting of 10 sets of 5 questions. These domains comprise 3 bulbar sets (speech, salivation, and swallowing), 6 motor sets (3 upper and 3 lower limbs) and 1 breathing set used to evaluate the status of patients. For each task, a score from 0 to 4 is assigned. The total score can range from 40 (normal function) to 0 (unable to perform the tasks). A few years later, the ALSFRS was revised (ALSFRS-R) adding 3 additional respiratory sets, addressing dyspnoea, orthopnoea and use of mechanical respiratory aids (Cedarbaum et al., 1999).

- **Appel ALS Rating Scale (AALSRS),** based on objective testing in five categories (bulbar, respiratory function, arm and leg function, and muscle strength) and ranges from 30 (normal) to 164 (maximally impaired) (Appel et al., 1987).

Up to 50% of ALS patients develop cognitive impairment, often leading to Frontotemporal dementia (FTD), characterized by the degeneration of frontal and temporal lobes in the brain, with consequent impairment of cognitive function and personality changes (Abramzon et al., 2020). For years, the idea that the ALS was a selective disease of the motor system dominated, with no involvement of the cognitive system. The evidence of a cognitive-behavioural impairment in ALS patients led to the hypothesis that there is a link between the two diseases, representing a *continuum* of the same neurodegenerative process (Christidi et al., 2018). Even the diagnostic criteria of the disease must take inevitably the cognitive impairment into account: in 1994 it was declared as an exclusion criterion for the diagnosis but since 1998 it is recognized as a possible accompanying framework of the motor deficit (Phukan, Pender and Hardiman, 2007).

As well as the site and the age of onset of the pathology can be highly variable, even the progression of the disease can be a strong element of heterogeneity. The duration of the illness
is usually between 2 and 5 years since diagnosis, even if 20% of the patients live longer and 10% of patients also live more than 10 years (Chiò et al., 2009). A late onset, especially when bulbar with an early respiratory involvement, may be associated with a very aggressive disease progression. On the contrary, a spinal onset at a younger age with a slower onset of specific symptoms, which delays the diagnosis, is often indicative of a longer survival (Kiernan et al., 2011). Moreover, a simple prognostic algorithm based on a multivariate model indicated an association between upper limb or bulbar weakness, executive dysfunction and ALSFRS-R slope before first evaluation as negative prognostic indicators (Elamin et al., 2011). The biological basis for these differences has not yet been fully understood. Even in families with specific genetic mutations, affected members may follow a significant clinical heterogeneity, thus reinforcing the hypothesis that there may be genetic polymorphisms and specific pathways that influence the disease progression (Camu et al., 1999).

1.4 DIAGNOSIS

The diagnosis of ALS is often difficult due to the heterogeneity in clinical manifestations; for this reason, numerous pathologies that mimic the ALS characteristics exist, such as Kennedy disease or multifocal motor neuropathy. To date, there is neither a diagnostic test nor biomarkers that can identify ALS with absolute certainty; the diagnosis relies mainly on the clinical evaluation of patients, which is based on a history of progressive, painless weakness and examination findings of both LMN and UMN dysfunction. However, the symptom manifestation varies among patients depending on the subtype of neurons primarily affected (LMN or UMN) and the body regions involved. Furthermore, the lack of sensitive biometric parameters and specific radiological and neurophysiological tests prevents an early diagnosis.

In the last 25 years numerous functional systems have been developed to standardize observational findings, including the AALSRS (Appel et al., 1987), the Norris ALS scale (Hillel et
and the ALSFRS (The ALS functional rating scale, 1996). The criteria for the clinical diagnosis and pathology of ALS have been defined by the World Federation of Neurology during the FTA meeting at El Escorial (Spain) in 1994 (Brooks, 1994) and then revised by the World Federation of Neurology Research Group on Motor Neuron Disease in 1998, to make them more sensitive and effective. According to El Escorial criteria (EEC), a diagnosis of ALS is based on:

- evidence of LMN degeneration found through clinical, electrophysiological, and neuropathological evaluation
- evidence of UMN degeneration, as detected by clinical examination
- progressive extension of the symptoms or signs within the same region or to other regions, as determined by a survey on patient history or through clinical examinations
- absence of pathological and electrophysiological findings regarding the development of related diseases (multiple sclerosis, Guillain-Barre Syndrome, dystonia, Lambert-Eaton myasthenic Syndrome) that could explain the alterations of the upper MNs and/or lower MNs.

According to EEC, the diagnosis of ALS may be divided into different levels of certainty: definite, probable, possible, and suspected (Silani et al., 2011). In 1998, in Airlie House (Warrenton, VA, US) an experienced group of clinicians revised the EEC adding a level of certainty “probable ALS-laboratory supported”, defined after the proper application of clinical laboratory protocols and neuroimaging. Besides, the “suspected ALS” level was removed (Brooks et al., 2000; Oliveira and Pereira, 2009) (Table 1.2).
LEVEL OF CERTAINTY | CLINICAL MANIFESTATION
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**DEFINITE ALS** | • UMN and LMN signs in the bulbar region and at least two spinal regions, or  
• UMN signs in two spinal regions and LMN signs in three spinal regions
**PROBABLE ALS** | • UMN and LMN signs in at least two regions, with some UMN signs rostral to LMN signs
**PROBABLE LABORATORY-SUPPORTED ALS** | • Clinical evidence for UMN or LMN signs in only one region, or  
• UMN signs alone in one region and LMN signs defined by EMG criteria in at least two muscles of different root and nerve origin in two limbs
**POSSIBLE ALS** | • UMN and LMN signs in only one region, or  
• UMN signs in two or more regions, or  
• LMN signs rostral to UMN signs

*Table 1.2: Revised El Escorial criteria for the ALS level classification*

However, despite efforts to define a rigorous method of diagnosis, 10% of patients may suffer a wrong diagnosis and the period from symptom onset to confirmation of the diagnosis is still more than 12 months in most cases (Oliveira and Pereira, 2009). The EEC has been criticised for being overly restrictive in the usage of electrophysiology data and for being insensitive to ALS diagnosis based on convention clinical evaluation. Indeed, in ~10% of cases, even at the death, the EEC-based diagnosis is categorised as “possible”, and only ~31% of patients meet the criteria of “definite ALS” at the time of diagnosis (Traynor et al., 2000).

A rationalization of revised EEC was made in 2006 at the Awaji-Shima Japan conference (de Carvalho et al., 2008). In contrast to the previous criteria, useful primarily for research purposes and in clinical trials, the new ones fit the application in clinical practice, aligning the electrophysiological parameters to the clinical evidence (de Carvalho, Costa and Swash, 2005).

After more than 100 years from the description of the disease, the diagnosis is still essentially clinical, and the final confirmation takes place only at the autopsy table. The current lines of research, as well as the study of the pathogenesis and possible therapies, are also directed to the discovery of diagnostic and disease progression markers. The proposed methodologies are listed in Table 1.3:
Table 1.3: Proposed clinical and molecular biomarkers for ALS diagnosis.

Although the clinical examination supports ALS diagnosis, several challenges remain: i) the considerable time elapses between the appearance of the first symptoms and the reaching of the definite diagnosis (Palese et al., 2019); ii) the high number of false-negative (26-42%)/positive (8-10%) (Chio, 2000); iii) the misdiagnosis due to the “ALS-mimic syndromes” (Quarracino, Segamarchi and Rodríguez, 2019) and iv) the heterogeneity in symptoms manifestation and speed of disease progression (Bendotti et al., 2020). For these reasons, it is necessary to look for new biomarkers that allow for an early and reliable diagnosis.
1.5 NEUROPATHOLOGY

To date, the neuropathological process of ALS is not fully clarified. The studies in tissues obtained post mortem provided information on neuropathological and histopathological characteristics of the disease; in particular, the principal findings relate to alterations in UMN and LMN and demyelination of the corticospinal tract.

No macroscopical alteration has been found in the brain of most ALS patients, albeit some analyses reported atrophy of the precentral gyrus (Qin et al., 2018). Most significant frontal or temporal cortex atrophy was found in the brain of patients with overlap ALS/FTD (Mioshi et al., 2013). The spinal cord shows atrophy of anterior nerve roots (Chang et al., 2005; Murphy et al., 2007). A reduction of the white matter was observed, particularly in the corticospinal tract (Roccatagliata et al., 2009).

The microscopic hallmark of ALS is represented by the loss of MNs, accompanied by astrocytosis and the presence of intracellular inclusions both in neurons and in the glial cells. The involvement of UMN was observed in almost all cases. In more severe cases there was a marked degeneration of neurons in layer V of the cerebral cortex in the primary motor area. In particular, the Betz cells, the largest of this layer, are more involved than the other pyramidal cells present (Hammer, Tomiyasu and Scheibel, 1979; Kiernan and Hudson, 1991; Nihei, McKee and Kowall, 1993). This is associated with reactive astrogliosis, axonal loss and myelin changes in the descending motor pathway, which leads to myelin pallor of the corticospinal tract, together with the typical sclerosis of the anterior and lateral columns. The presence of astrocytosis and the loss of motor axons in the descendent motor tract, associated with demyelination of the corticospinal tract and the sclerosis of the anterior and lateral columns of the spinal cord, are further evidence demonstrating the involvement of UMN. In the spinal cord and brainstem, shrinkage and atrophy of the LMN cell bodies precede pronounced neuronal loss (Kiernan and Hudson, 1991), in association with a reduction in the calibre of axons and dendritic structures (Nakano and Hirano, 1987). Other pathological features of ALS include vacuolisation, vast empty
spaces near neurons, spongiosis and the presence of Bunina bodies mostly within MN and occasionally in dendrites (Tomonaga et al., 1978; Kuroda et al., 1990; Piao et al., 2003; Won et al., 2016).

Not all MNs are affected by the disease, for example, nuclei of Onufrowicz in the segment S2 of the spinal cord, involved in the control of the pelvic floor muscle (faecal and urinary continence), and cranial MNs that innervate the extrinsic ocular muscles (Iwata and Hirano, 1978) are excluded. However, these neurons are characterized by different intracytoplasmic alterations.

1.5.1 Cytoplasmic inclusions

ALS is often considered a proteinopathy since a common feature of both the sporadic (sALS) and familial ALS (fALS) forms is the presence of protein aggregates in the damaged MNs due to the accumulation of misfolded or abnormal proteins (Strong, Kesavapany and Pant, 2005; Kato, 2008). Ubiquitinated inclusions are found very frequently in spinal cord and brainstem MNs of most fALS and sALS cases and are classified as:

- skein-like inclusions: form intracellular filamentous aggregates composed of microtubules, phosphorylated NF, ubiquitin and p38-MAPK (Bendotti and Carri, 2004) specific for ALS
- agglomerates Lewy body-like: exhibit a compact spherical body and they are similar to the Lewy bodies described in Parkinson's disease (Jellinger, 2008). They consist mainly of alpha-synuclein associated with other proteins such as ubiquitin, NF and alpha β crystalline
- hyaline conglomerates: formed by phosphorylated or non-phosphorylated NF, associated with other proteins and cytoplasmic organelles (Schochet, 1969; Sasaki and Maruyama, 1991). They have been described also in other neurodegenerative disorders (Sobue et al., 1990).
The main element of the ubiquitin-positive inclusions is represented by the nuclear factor TDP43 (Neumann et al., 2006), also present in the skein-like aggregates and hyaline conglomerates (Tan et al., 2007).

The only cytoplasmic inclusions ubiquitin-negative are the Bunina bodies: eosinophils, consist of microtubules and vesicular structures, located in the cytoplasm and dendrites (Kato, 2008); they are most frequently found in the neurons of the spinal cord and brainstem rather than in the Betz cells.

TDP43-positive neuronal inclusions are evident in the brain and spinal cord of sALS patients (up to 97%) and fALS cases linked to multiple gene mutation, including transactive response DNA binding protein (TARDBP), Progranulin (GRN), Optineurin (OPTN), Valosin-containing protein (VCP) and Ubiquilin 2 (UBQLN2) (Tan et al., 2017). Patients with mutations in superoxide dismutase 1 (SOD1) and fused in sarcoma (FUS) genes exhibit respectively SOD1 and FUS protein aggregates, but not TDP43-positive inclusions (De Boer et al., 2021).

1.5.2 Axonal cytoskeleton and transport dysregulation

A typical feature found in tissue derived from ALS patients is the axonal cytoskeleton dysregulation that causes impairment in axonal transport. A phosphorylated NF accumulation can be observed in the perikaryon (Hirano et al., 1984) and axonal swellings of MNs (Corbo and Hays, 1992). NF is implicated in axonal transport, and they can determine the shape of the cells and the axonal calibre. Axonal transport allows the flow of organelles and proteins to (anterograde) and from (retrograde) the cell body. Motor axons with a large calibre rich in NF are preferentially affected by ALS; thus, the level of NF can be an important parameter to understand neuronal vulnerability. This cytoskeletal dysregulation leads to a deterioration of the axonal transport, histologically represented by the formation of spheroids, large bulging structures, mainly located in the proximal dendrites and axons, and globules, smaller and located in a peripheral position in the ventral horn. The mechanism through which the aberrant
expression of NF can cause MN degeneration is however not fully understood. The most supported hypothesis regards the obstacle produced by NF to the transport of molecules needed for axon integrity. It was observed that the accumulation of NF can lead to a slowing of anterograde transport, which in turn causes an early event of denervation (Zhang et al., 1997; Williamson and Cleveland, 1999). Electron microscopy studies conducted in ALS patients (Sasaki and Iwata, 1996) and murine models (Sasaki et al., 2007) showed granules of lipofuscin, Lewy body-like inclusions and accumulations of mitochondria and lysosomes in axons, able to cause abnormalities in the anterograde transport. In mice with mutant SOD1 (SOD1\(^{G93A}\), SOD1\(^{G37R}\) and SOD1\(^{G85R}\) the lag of the anterograde transport already appears at the pre-symptomatic stage, and it gets worse with the progression of the disease (Zhang et al., 1997; Williamson and Cleveland, 1999). Since alterations in anterograde transport appear early, it must be assumed that they are involved in the pathogenesis of ALS. In a more recent study, the alterations of retrograde transport (Ligon et al., 2005) were also highlighted, as it was assumed an interference of the mutant SOD1 in the formation of the dynein-dynactin complex (Kawaguchi et al., 2003).

### 1.5.3 Mitochondrial dysfunction

Many studies showed alterations in the mitochondria of ALS patients (Dupuis et al., 2004; Kawamata and Manfredi, 2010). The first evidence arose from ultrastructural studies in which the presence of aggregates was reported in the sub-sarcolemma of patients (Afifi et al., 1966), while subsequent studies showed the presence of dense conglomerates in the mitochondria of neurons located in the anterior horn of the lumbar spinal cord of ALS patients. These changes were observed in the neurons that are not degenerated yet (Sasaki and Iwata, 1996). More recently, several studies supported the idea that the axonal transport and the movement of mitochondria along nerve endings are altered in ALS; these alterations are accompanied by decreased number of mitochondria and their accumulation in clusters along the neurites (Bendotti, Calvaresi, et al., 2001; De Vos et al., 2008; Magrané et al., 2009; Carri and Cozzolino,
Indeed, mitochondria are altered in MNs of ALS patients and appear swollen, vacuolated and containing aggregates (Hirano et al., 1984; Beal, 2000). Furthermore, studies in cell cultures and transgenic animals have shown alterations in the oxidative metabolism, modifications due to changes in the activity of the electron transport chain, compromised ATP production and programmed cell death (Menzies et al., 2002; Knott et al., 2008; Martin, 2011; Ruffoli et al., 2015). These studies show that the function abnormalities and mitochondrial dynamics are a common and central feature in the pathogenesis of ALS. However, it is not yet clear whether the dysfunction of mitochondria is a primary event or secondary effect due to neurodegeneration.

1.5.4 Golgi apparatus fragmentation

Morphologically the organelles appear smaller, disconnected and more numerous (fragmented) (Gonatas et al., 1992; Mourelatos et al., 1994). These alterations are accompanied by loss/gain of function (LOF and GOF, respectively) in protein sorting, processing, and transport along the axons. Moreover, the organelles fragmentation allows the activation of pro-apoptotic pathways that contributes to MN loss (Haase and Rabouille, 2015).

1.5.5 Other cell involvement

Several other cells of the nervous system, in addition to MNs, appear altered in subjects with ALS. It is now widely demonstrated that ALS is a "non-cell autonomous" disease in which non-neuronal cells, such as glial cells and cells of the immune system, can play an important role in the neurodegenerative process (Nagano et al., 2005; Henkel et al., 2009; Ilieva, Polymenidou and Cleveland, 2009; Barbeito, Mesci and Boillée, 2010; Philips and Robberecht, 2011). For example, astrocyte and microglia activation was reported in the brain, brainstem and spinal cord (Kawamata et al., 1992), surrounding degenerating MNs (Boillée, Vande Velde and Cleveland, 2006; Lasiene and Yamanaka, 2011). Astrocytic activation is notable in the grey matter of the
ventral horn of the spinal cord, which is accompanied by hyaline inclusions and oxidative and nitrative stress markers (Philips and Rothstein, 2014). Microglia activation represents a critical aspect of ALS neuropathology. Indeed, once activated, microglia respond to the neuronal distress releasing a plethora of pro-inflammatory factors increasing the phlogosis (Philips and Rothstein, 2014). Moreover, the degree of microglial activation is correlated to the severity of UMN degeneration (Lasiene and Yamanaka, 2011). In addition, a substantial loss of interneurons in the spinal cord has been demonstrated (Stephens et al., 2006). Infiltrating dendritic cells and T lymphocytes have been widely documented in post mortem tissues of many patients (Henkel et al., 2004) and deficiencies in hematopoietic cells have been identified, regarding the levels of circulating monocytes and T lymphocytes (Mantovani et al., 2009) and modifications of specific proteins of peripheral blood mononuclear cells, which have also been proposed as specific markers of the pathology (Nardo et al., 2011).

Several studies showed that ALS muscles suffer from oxidative stress, mitochondrial dysfunction and bioenergetic disturbance. However, the contribution of muscles to the degenerative process is still debated (Loeffler et al., 2016). Furthermore, the knowledge of the processes underlying the degeneration/regeneration mechanisms and the myogenic potential of ALS muscles is still limited (Jensen et al., 2016). Indeed, studies are still ongoing to clarify the different susceptibility of the muscular compartment to the disease (Jensen et al., 2016; Nijssen, Comley and Hedlund, 2017; Di Pietro, Lattanzi and Bernardini, 2018).

One of the peculiar characteristics of ALS patients is that, even if forced to a state of immobility, rarely present bedsores. This is probably due to an alteration in the dermis collagen network (Kolde, Bachus and Ludolph, 1996).
1.6 PATHOGENIC MECHANISMS

To date, there are different cellular mechanisms that appear to be involved in MN death in ALS (Figure 1.1), but it is still not clear how we can formulate a unifying hypothesis that considers the specific vulnerabilities of each. Most of the acquired knowledge about the aetiology of ALS derived from the study of family forms, in which mutations in single genes are identified. Autoptic studies on ALS patients or animal models suggest that the disease can be considered multifactorial, with the involvement of several mechanisms that work together causing progressive degeneration of MNs. A key step in understanding the molecular mechanisms involved in ALS was the discovery of the SOD1 gene mutation in 1993 (Rosen et al., 1993a) and the subsequent production of the mouse model SOD1$^{G93A}$ in 1994 (Gurney et al., 1994). In fact, given the uniformity of the pathological and clinical profiles of familial and sporadic ALS, it can be stated that the evidence that has emerged from the study of genetic mutations linked to fALS may also be predictive for sALS.

Figure 1.1: Proposed pathogenic mechanisms and pathology in ALS (modified from Mezini et al., 2019).
1.6.1 Protein aggregation

Among the most significant pathological features of several neurodegenerative diseases, including ALS, there is the accumulation of aberrant proteins (Figure 1.2) within MNs and, sometimes, surrounding astrocytes (Migheli et al., 1990; Kato, 1999; Soto, 2003). In physiological conditions, the protein quality control machinery of neurons can efficiently activate adaptative mechanisms to maintain the protein homeostasis (proteostasis) (Balch et al., 2008). However, they are prone to defects in proteostasis since they are long-lived post-mitotic cells that are not able to dilute out protein aggregates during cell divisions (Yue et al., 2009). Moreover, it has been demonstrated that the neuron ability to maintain the proteostasis declines during ageing, that might explain why most of the neurodegenerative diseases occurs in the adulthood (Hipp, Kasturi and Hartl, 2019).

The quality control machinery includes:

- **Chaperones**: proteins that assist others to fold properly during or after the synthesis and help misfolded proteins to correctly fold or target them to degradation
- **Ubiquitin-proteasome system (UPS)**: it involves the covalent conjugation of ubiquitin (ubiquitination) to the substrate protein and its recognition and degradation by the 26S proteasome, the proteolytic core of the UPS
- **Endoplasmic reticulum (ER)-associated protein degradation (ERAD)**: a cellular pathway that leads misfolded proteins from the ER to ubiquitination and degradation by the proteasome
- **Autophagy-lysosomal pathway (ALP)**: damaged proteins are targeted and isolated within a double-membrane vesicle called autophagosome which fuses with a lysosome for the degradation
- **Stress granules**: cytoplasmic membrane-less aggregates composed of untranslating messenger ribonucleoproteins that form from mRNAs stalled in translation
Most of the gene mutations associated with fALS are translated into altered proteins characterized by aggregation which are widely present within the MNs of ALS patients. Moreover, it has been recently reported that the product of mutant genes can change their native conformation and acquire prion-like features (McAlary et al., 2019).

Protein aggregates are also observed in sALS patient in which aberrant post-translational modifications may induce an abnormal conformation and a consequent protein aggregation (Migheli et al., 1990; Neumann et al., 2006). Pieces of evidence suggest that the impairment of the proteostasis quality control can be a common feature of both familial and sporadic ALS. Protein aggregates have also been found in disease models, particularly within dendrites, periaxonal processes of oligodendrocytes, neurons and astrocytes perikarya (Stieber, Gonatas and Gonatas, 2000; Watanabe et al., 2001). Studies performed showed that these inclusions are composed of several proteins, such as SOD1 (Bosco et al., 2010), ubiquitin (Basso et al., 2009), chaperones (Marino et al., 2015), TDP43 (Sanelli et al., 2007), Optineurin (Korac et al., 2013), NF (Beaulieu, Jacomy and Julien, 2000), and many more.

Interestingly, glial cells and muscles seem mostly spare from the misfolded protein accumulation. Recently it has been suggested that these cell types are better equipped in activating chaperones and protein degradation system than neurons, resulting in a more efficient response to counteract the altered proteostasis (Galbiati et al., 2014). It is not yet known if the altered proteostasis in ALS is caused by an excessive protein accumulation or by an overwhelmed protein clearance.
The normal cellular metabolism produces reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as hydroxyl radical (-OH), superoxide anion (-O₂⁻), hydrogen peroxide (H₂O₂), nitric oxide (NO) and peroxynitrite (ONOO⁻). These are mainly the waste products of aerobic metabolism of the cell due to the loss of electrons in the mitochondrial respiratory chain, but they can also be produced by cytochrome P450 in the endoplasmic reticulum, or by immune cells as a second messenger (Knight 2000; Barber and Shaw, 2010). Therefore, at moderate levels, ROS have beneficial effects and are involved in various physiological functions, such as immune functions (i.e. defense against pathogens), cellular signaling pathways, mitogenic response and redox regulation (Valko et al., 2007). The cells have specific antioxidant mechanisms, including the SOD1 enzyme. Oxidative stress derives from the imbalance between the production of oxidative insults and the ability to remove them or repair the damage they
cause in the cell. An increase in oxidative damage markers, such as 3-nitrotyrosine, was observed in patients with both sALS and fALS (Beal et al., 1997; Ferrante et al., 1997; Ihara et al., 2005; Nardo et al., 2009a; Barber and Shaw, 2010; Wang et al., 2019) and in disease models (Beal et al., 1997; Andrus et al., 1998; Nardo et al., 2009b; Cacabelos, Prakash Reddy and Aliev, 2016).

It is not yet known whether oxidative stress is a primary cause of the degeneration or a consequence of other toxic insults. Several pieces of evidence reported that aging increases the production of ROS (Beal, 2002; Liguori et al., 2018), and for this reason it is considered a risk factor for ALS (Barber and Shaw, 2010; Wang et al., 2019). The increased production of ROS is able to cause the degeneration of MNs by different mechanisms which include:

- damage to the mitochondria, highly susceptible to oxidative damage (Barber and Shaw, 2010)
- reduced glutamate uptake through the astroglial glutamate transporter (Rao, Yin and Weiss, 2003)
- nitration of proteins that determines aggregation and aberrant folding (Squier, 2001)
- nitration of specific subunits of the cytoskeleton, which inhibits their normal assembling (Crow et al., 1997)
- activation of glial cells and consequent release of pro-inflammatory cytokines (Banati et al., 1993)
- oxidation of DNA and RNA which leads to a reduction of protein expression (Chang et al., 2008)

1.6.3 Mitochondrial dysfunction

Mitochondrial dysfunction is common to many neurodegenerative diseases. Several studies have reported alterations of morphology (Sasaki and Iwata, 2007), Ca$^{2+}$ homeostasis (Siklós et al., 1996), the respiratory chain (Vielhaber et al., 2000) and antioxidant response (Wood-Allum
et al., 2006) in mitochondria of MNs, muscles and nerves of ALS patients, most representing a pre-clinical sign of disease (Kong and Xu, 1998; Bendotti, Calvaresi, et al., 2001; Wood-Allum et al., 2006). The mitochondrial functions can be dysregulated by the aggregating products of ALS-related genes and/or by the aberrant RNA processing and that products cannot be eliminated by cell due to the impaired autophagic mechanism (Carri, D’Ambrosi and Cozzolino, 2017; Smith, Shaw and De Vos, 2019). A possible correlation between mitochondrial dysfunction and mutant SOD1 has been reported, especially in early stages of the disease (Higgins, Jung and Xu, 2003). Not only the decrease/loss of antioxidant activity of mutant SOD1 is involved in ALS pathogenesis, but also the acquirement of a toxic GOF. The toxicity mediated by SOD1 can act through different routes:

- the accumulation of SOD1 in the intermembrane space leads to the formation of conglomerates (Sturtz et al., 2001) that compromise the electron transport chain (Ferri et al., 2006) and alter the Ca²⁺ homeostasis (Damiano et al., 2006)
- misfolded SOD1 binds voltage-dependent channel (VDAC1) reducing its conductance and leading to a reduced production of ATP. The reduced activity of VDAC1 is associated with reduced survival in SOD1G93A mice, also accelerating the onset of paralysis (Israelson et al., 2010)
- SOD1 interacting with anti-apoptotic protein Bcl2 determines the acquisition of a toxic function (Pedrini et al., 2010) and promotes the release of cytochrome c and apoptosis
- SOD1 alters mitochondrial transport along axons by changing the levels of cytosolic Ca²⁺, favoring the hyperphosphorylation of kinesin and interacting with the cytoplasmic dynein. Such alterations cause energy deprivation in a region with high demand as the synapses, leading to cellular degeneration (Magrané et al., 2012)
1.6.4 Excitotoxicity

The excitatory amino acids, in particular glutamate, can have a significant role in the progressive degeneration of the MNs in ALS. Glutamate is the most abundant excitatory neurotransmitter in the CNS. Glutamate is synthetized in the presynaptic terminal, stored in vesicles, and released in response to depolarization into the synaptic cleft through a calcium-dependent mechanism. Once released, glutamate binds receptors localized on the dendrites of post-synaptic terminal, astrocytes, and oligodendrocytes (Steinhäuser and Gallo, 1996). In mammals, four groups of glutamate receptors have been discovered, three ionotropic and one metabotropic, classified based on their pharmacology. Ionotropic glutamate receptors (AMPA, Kainate and NMDA) form a transmembrane ion channel pore that allow the flow of K⁺, Na⁺ and Ca²⁺ in response to glutamate binding, leading to the depolarization of the neuron. Metabotropic glutamate receptors are G protein-coupled receptors that induce a signaling cascade following glutamate activation. The concentration of glutamate within the synaptic cleft is finely regulated through efficient glutamate transporters (EAATs) on glia and neuron membranes. They re-uptake the excess of the neurotransmitter, to avoid its prolonged persistence in the synaptic space that causes an irreversible neurotoxicity process called excitotoxicity. This phenomenon occurs through a mechanism that involves the increase of the intracellular free Ca²⁺ and the resulting broad-spectrum enzyme activation, inducing degeneration and cell death.

The first evidence of a possible role of glutamate in the pathogenesis of ALS derives from the fact that the levels of this amino acid in the plasma and CSF are about four times higher in ALS patients compared to controls (Rothstein et al., 1995). It was observed that approximately 60-70% of sALS patients shows a reduced expression of the EAAT2 protein which is responsible for astroglial recovery of glutamate from the synaptic cleft in the motor cortex and spinal cord (Rothstein et al., 1995; Fray et al., 1998). Studies in SOD1G93A mice overexpressing EAAT2 showed a delay in the onset of the disease, a prolongation of survival and an improvement in symptoms (Bendotti et al., 2001). Alterations in the level of AMPA receptors were also reported.
The AMPA receptor includes four subunit calls GluR1, 2, 3 and 4; in MNs isolated from the spinal cord of ALS patients there was a defective mRNA editing of GluR2, responsible of the calcium impermeability of the channel (Takuma et al., 1999; Kwak and Kawahara, 2005). The editing defect, which consist in a substitution of an arginine residue with a glutamine, leads to increased permeability to calcium, resulting in the accumulation of the cation in the cytoplasm and activation of toxic processes for the cell. Increased expression of Ca\(^{2+}\)-permeable AMPA receptors could determine the selective activation of degenerative processes in MNs. In addition, the proteins capable to buffer an excessive increase in intracellular Ca\(^{2+}\) are less expressed in MNs, which are then particularly susceptible to the toxicity mediated by activation of these AMPA receptors (Carriedo, Yin and Weiss, 1996). The use of selective AMPA inhibitors can attenuate the progression of the disease and to prolong survival of SOD\(_1^{G93A}\) mice (Tortarolo et al., 2006).

1.6.5 Axonal transport alterations

The axonal transport is a process mediated by motor proteins that allows the intracellular displacement of proteins, lipids, mRNA, vesicles, and organelles along the axon. It is essential for nerve development, function, and survival. Kinesin is responsible for the anterograde transport toward the synapse, while dynein and dynactin allow the retrograde transport toward the cell body. Polarized microtubules act as tracks on which motor proteins deliver cargoes and together with microfilament and intermediate filaments (IFs) compose the eukaryotic cell cytoskeleton. There are three types of IFs in adult MNs: NF, internexin \(\alpha\) and peripherin. NFs are the major IFs within neurons, maintaining the cell morphology, and represents the most abundant cytoplasmic structure in the myelinated axons, leading to the expansion of their calibre. The NFs derive from the co-polymerization of a heavy (NF-H), a medium (NF-M) and a light (NF-L) chain. The three subunits are assembled in the cell body of MN and then transported anterogradely.
along the axon through a slow process during which a progressive phosphorylation of NF-H occurs.

The presence of hyaline conglomerate inclusions formed of an abnormal accumulation of NFs, mitochondria and lysosome within MN soma of patients suggests the involvement of axonal transport in the development of ALS (Hirano et al., 1984). However, the role of these alterations in the disease is still debated: it is unclear whether the accumulation is a consequence of neuronal dysfunction or may instead have pathogenetic relevance. It is well known that neurons are dependent on mitochondria to maintain their function (Schwarz, 2013). However, lack of mitochondrial adaptors or regulators (e.g. Miro, Milton, Kif5C, etc.) has been likewise connected to ALS mutation (Chen, Sakowski and Feldman, 2016) suggesting that mitochondrial deficit could be the causative reason of axonal transport deficit. Similarly, other pathogenic mechanisms such as ER stress (Woehlbier et al., 2016), protein aggregation (Oberstadt et al., 2018; Huai and Zhang, 2019), autophagy dysregulation (Lie and Nixon, 2019) and DNA damage (Naumann et al., 2018) are strictly associated to axonal transport deficit. Nevertheless, further studies are ongoing to clarify whether these pathways are localised upstream or downstream to axonal transport defects in ALS.

The mechanism driving the formation of NF aggregates in ALS is still unclear. Mutation in NF genes have been found in fALS and sALS patients (Figlewicz et al., 1994) and seems to be correlated to post-translational protein modification, particularly hyperphosphorylation (Dale and Garcia, 2012). However, NF gene mutations are not a common cause of ALS but could represent a risk factor for MNs vulnerability (Bonafede and Mariotti, 2017).

Furthermore, it was not established whether the consequences of this accumulation are negative for the possible alteration of the axonal transport or may have a protective function. An excess of NF in the cell body could have a buffering action on other deleterious processes, for example by offering phosphorylation sites for intracellular deregulated kinases such as p38MAPK or reducing axonal transportation loading (Tortarolo et al., 2003; Ackerley et al.,
2004). Surprisingly, the overexpression of the NF-L and NF-H was able to slow down the disease in ALS mice, suggesting a protective effect of NFs accumulation when occurring within neuron cell body (Kong and Xu, 2000).

Besides the accumulation, NFs can also release after neuroaxonal damage and thus can be titrated in biofluids (CSF and serum). NF-L dosage is currently used in clinical practice as a diagnostic and informative biomarker since it has been demonstrated its increased level ~12 months preceding the manifestation of the first signs of the disease (Benatar et al., 2018, 2019). Further studies also showed its predictive value in ALS prognosis (Poesen and Van Damme, 2019).

In addition to NF, peripherin has also been observed in most of patient axonal sferoids, particularly within the large calibre axon of αMNs, the most susceptible to ALS (Sobue et al., 1981; Corbo and Hays, 1992). Peripherin levels are increased in response to cellular damage or to inflammatory cytokines; thus, formation of the inclusions could be a generic response by MNs to stress insults and chronic inflammation (Strong, Kesavapany and Pant, 2005). Mutations in the gene coding for peripherin have been proposed as a possible cause of a small percentage of ALS cases (Gros-Louis et al., 2004; Leung et al., 2004).

1.6.6 Nucleocyttoplasmic transport defects

The nucleocyttoplasmic transport refers to the movement of molecules across the nuclear membrane of the cell, regulated by the nuclear pore complexes (NPC) and nuclear transport receptors like importins and exportins, respectively for the entry and the exit of RNA, proteins and assembled ribosomal subunits. The protein Ran, a small Ras related GTPase, allows importins and exportins to transport their cargo. Importins and exportins recognize and bind respectively nuclear localization signals (NLS) and nuclear export signals (NES) carried by the proteins that must be transported through the nuclear membrane. The mislocalisation of nuclear proteins like TDP43 (Winton et al., 2008), FUS (Ling et al., 2019) and heterogeneous
nuclear ribonucleoprotein A1 (hnRNPA1) (Q. Liu et al., 2016) into cytoplasmic inclusions and the ALS-linked mutations in components of the NPC and NLS prove the contribution of nucleocytoplasmic transport defects to the disease initiation or progress, through both the loss of nuclear functions and the gain of toxicity in cytoplasm (Kim and Taylor, 2017).

1.6.7 RNA dysregulation

The involvement of the main ALS-related genes TDP43, FUS, SOD1 and C9orf72 in RNA metabolism (mRNA transcription, alternative splicing, RNA transport, mRNA stabilization, and miRNA biogenesis) points out the participation of RNA dysregulation to ALS pathogenesis. Particularly, TDP43 plays a role in regulating the expression of genes encoding for proteins that are involved in formation and function of synapses, neurotransmitter processes (Godena et al., 2011; Sephton et al., 2011; Colombrita et al., 2012; Narayanan et al., 2013), and in ALS, such as FUS, ATXN2 and progranulin (Polymenidou et al., 2011; Sephton et al., 2011; Tollervey et al., 2011). Moreover, as splicing regulator, TDP43 is able to reduce its own expression by interacting with the 3' UTR of its own pre-mRNA (Ayala et al., 2011). Interestingly, it was demonstrated to bind the 3' UTR of NF-L mRNA to stabilize it (Strong et al., 2007).

Similarly to TDP43, FUS plays an essential role in regulating splicing events for neuronal maintenance and survival (Lagier-Tourenne et al., 2012), cytoskeletal organization, axonal growth and guidance (Ishigaki et al., 2012; Orozco et al., 2012; Rogelj et al., 2012). FUS is also found within stress granules under cellular stress conditions (Sama et al., 2013). Although SOD1 is not an RNA-binding protein (RBP), pieces of evidence demonstrate its involvement in the regulation of the metabolism of VEGF and NF-L subunit transcripts (Chen et al., 2014).
1.6.8 Non-cell autonomous mechanisms and neuroinflammation

The pathogenic mechanisms so far described are cell-autonomous, occurring within the MNs. Although the selective degeneration of MNs is the distinctive hallmark of ALS, it is widely demonstrated that non-cell autonomous processes participate to the development of the disease and various non-neuronal cell types, such as glial and immune cells, actively contributes to MN death (Thonhoff, Simpson and Appel, 2018; Chiot, Lobsiger and Boillée, 2019). Indeed, the activation of astrocytes and microglia in CNS, Schwann cells in PNS and the infiltration of peripheral immune cells (T lymphocytes, mast cells, dendritic cells, macrophages) in CNS and PNS of both ALS patients and models, with consequent release of inflammatory mediators (cytokines and chemokines), dysregulate the environment surrounding MNs by driving a cascade of events collectively known as “neuroinflammation” (Glass et al., 2010; A. McCombe and D. Henderson, 2011; Philips and Robberecht, 2011).

To further understand the contribution of each cell subtype in the disease pathogenesis, mutant SOD1 (mSOD1) has been expressed or silenced in neurons, glial and skeletal muscle cells of rodents. The exclusive expression of mutant SOD1 in MNs cannot trigger the disease in murine models (Pramatarova et al., 2001; Lino, Schneider and Caroni, 2002), and even when mutant SOD1 is expressed at high levels within neurons, it causes minor injury to MNs and a late-onset disease (Jaarsma et al., 2008). Moreover, the silencing of SOD1 in SOD1G93A mutant mice only in MNs produce a moderate effect on onset without interfering with the disease progression (Boillée et al., 2006). These findings indicate that the degeneration and MN cell death require the participation of non-neuronal cells.

The selective depletion of mutant SOD1 in astrocytes does not affect disease onset nor the early phase in mice but leads to a delayed late disease progression and a significant extension of survival. This effect was accompanied by a reduced microglial activation leading to the conclusion that damage in astrocytes determines the timing of microglial activation amplifying the inflammatory events (Yamanaka et al., 2008).
The selective depletion of mutant SOD1 also from microglia of SOD1\textsuperscript{G93A} mice is protective, slowing the disease progression and decreasing MN degeneration (Beers \textit{et al.}, 2006; Boillée \textit{et al.}, 2006). Interestingly microglia lacking mSOD1 is still activated in these mice, supporting the hypothesis of an active neuroprotective role for this cell population. In contrast, depleting proliferating microglia from SOD1\textsuperscript{G93A} mice, has no effect on disease phenotype (Gowing \textit{et al.}, 2008). The divergent results are explained by studies demonstrating that microglia may adopt a protective anti-inflammatory or a neurotoxic pro-inflammatory phenotype. Indeed, at early stages of the disease, it has been hypothesized that M2 polarized microglia, releasing anti-inflammatory cytokines, are predominant, inducing a neurons protection. Whereas, at late stages, microglia is transformed, by different toxic insults, into the pro-inflammatory and neurotoxic M1 phenotype (Henkel \textit{et al.}, 2009; Appel, Beers and Henkel, 2010; Appel \textit{et al.}, 2011; Liao \textit{et al.}, 2012). Thus, a positive effect on disease progression could be obtained by the selective depletion of neurotoxic microglia.

Recently also a key role for oligodendrocytes has been hypothesized. Although the simultaneous expression of mutant SOD1 in oligodendrocytes and motoneurons does not determine an ALS-like phenotype (Clement \textit{et al.}, 2003; Yamanaka \textit{et al.}, 2008), the selective removal of mutant protein from oligodendrocyte progenitors slows down the disease progression (Kang \textit{et al.}, 2013). The authors proposed that genetic mutations make oligodendrocytes more vulnerable to stress stimuli produced by other cells. Indeed, they demonstrated an early degeneration of oligodendrocytes in fALS mice. The attempt to replace these cells from NG2+ progenitors produces oligodendrocytes functionally impaired both in terms of myelination and metabolic support which might contribute in a non-cell autonomous manner to death of MNs (Kang \textit{et al.}, 2013; Philips \textit{et al.}, 2013).

While B lymphocytes do not seem to have an important role in the disease, a correlation between T cells and ALS phenotype has been demonstrated. Indeed, independently from SOD1 expression, SOD1\textsuperscript{G93A} mice lacking T lymphocytes, and in particular CD4+ cells, have been
reported to have a worse phenotype, suggesting a protective role of this immune cell population (Beers et al., 2008; Chiu et al., 2008).

Nevertheless, another group obtained opposite results and demonstrated that the removal of T lymphocytes delayed the onset in SOD1<sup>G93A</sup> mice (Tada et al., 2011). The reason for such different results is not yet known, but a possible explanation is related to the role of T lymphocytes in activating microglia (Appel, Beers and Henkel, 2010). Thus, the presence of T lymphocytes could have a different effect, depending on which subset of microglial cells they activate. In particular, regulatory T lymphocytes (Treg) has been shown to be protective for SOD1<sup>G93A</sup> mice, stimulating the microglia protective phenotype (M2) through the release of IL4 (Beers et al., 2011; Zhao et al., 2012).

An accelerated disease progression was observed by reducing the mutant protein in Schwann cells, but the outcome can be attributed to the loss of the anti-oxidant activity of the SOD1<sup>G37R</sup> mutant, whose expression is elevated in these particular cells type (Lobsiger et al., 2009). Although the selective removal of mutant SOD1 from skeletal muscle had no important effects on ALS phenotype (Miller et al., 2006), the exclusive muscle expression of mutant SOD1 results in muscle atrophy and a late neuropathological phenotype accompanied by MN loss (Dobrowolny et al., 2008; Wong and Martin, 2010).

1.7 THERAPY

To date, ALS has a poor prognosis and no effective therapy, given its heterogeneity and multi-pathogenic nature (Bendotti et al., 2020). Plenty of compounds targeting different mechanisms of the disease have been studied, among which only two have been recognized as “disease-modifying and approved by FDA: Riluzole and Edaravone.
1.7.1 Anti-excitotoxicity

Riluzole, approved in 1995, is an anti-glutamatergic agent which produces only a modest extension of survival of 3-6 months in patients (Bensimon, Lacomblez and Meiningher, 1994; Lacomblez et al., 1996; Miller et al., 1996) and about 2 weeks in animal models (Gurney et al., 1998; Kennel et al., 2000). Riluzole is able to limit the release of glutamate by inactivation of voltage-gated Na+ channel localized in nerve endings, thus delaying the MN death, without being able to stop the progression of the disease. However, the real effectiveness of the molecule has been shown to be so small that its use is not uniformly accepted. Moreover, other glutamate antagonists showed no effect (Tandan et al., 1996; Gredal et al., 1997; Miller et al., 2001; Ryberg, Askmark and Persson, 2003).

Another drug of this type is the Cobalamin (vitamin B-12) which has multiple protective effects, potentially relevant in ALS. It inhibits NMDA-induced cytotoxicity and protects cultured neurons from glutamate cytotoxicity (Akaike et al., 1993); it also shows antioxidant and anti-apoptotic properties (Zhang et al., 2008). In a clinical trial conducted on 24 Japanese ALS patients, the short-term treatment with high-dose methyl Cobalamin was effective in slowing the progression of disease in patients with a good response to the treatment. However, the clinical benefits were transient and followed by a decline in the following months (Izumi and Kaji, 2007). Currently, in Japan there is a large-scale study to evaluate the safety and efficacy of long-term treatment with high dose of methyl Cobalamin (Kaji et al., 2019).

In addition, Ceftriaxone, an antibiotic used in the treatment of bacterial pneumonia and meningitis, interferes with the glutamate-mediated excitotoxicity, upregulating the glutamate transporter 1 (GLT1)-mediated uptake of glutamate in ALS subjects (Rothstein et al., 2005). Other β-lactam antibiotics were also demonstrated to stimulate the expression of glutamate transporters in astrocytes, thereby increasing the absorption and reducing the action of glutamate in animal models of ALS and ischemia (Rothstein, Martin and Kuncl, 1992).
1.7.2 Anti-oxidant

Unlike Riluzole, Edaravone has been approved by FDA but not EMA (European medicines agency). It is an anti-oxidant agent able to eliminate lipid peroxides and hydroxyl radicals (Bhandari, Kuhad and Kuhad, 2018).

Several preclinical and clinical studies have focused on Dexpramipexole (RPPX), an antioxidant R enantiomer (+) of Pramipexole, already approved for the symptomatic treatment of Parkinson's disease and Restless Legs Syndrome. The Dexpramipexole reduces the production of free radicals and neuronal death in models of oxidative stress (Gu et al., 2004) and also reduces apoptosis (Abramova et al., 2002) and prolongs survival in SOD1 mice (Bordet et al., 2007). Its ability to stabilize the ion conductance and maintain the gradient needed for ATP production leads to its neuroprotective effect (Alavian et al., 2012). The results of phase I and phase II clinical trials were promising, but the Phase III study, conducted on 943 ALS patients showed no efficacy of the treatment in the survival and improvement of neurological functions.

1.7.3 Anti-aggregation

Another key mechanism in the ALS pathogenesis is the misfolding and the accumulation of some proteins in MNs. Arimoclomol is able to increase the production of heat shock proteins to protect cells from cellular stress, interfering with protein aggregation and apoptosis. Treatment with Arimoclomol has shown to prolong survival and improve muscle function in a mouse model of fALS when administered at pre-symptomatic stage (Kieran et al., 2004), but a fading effect has been seen if the drug is administered at a symptomatic stage (Kalmar et al., 2008). Arimoclomol has shown a good safety and tolerability profile in a clinical study of ALS patients (Cudkowicz et al., 2008). Another therapeutic strategy considers the use of vaccines or the infusion of immunoglobulins to remove misfolded proteins: vaccination of mice with mutant
SOD1 postpones the onset of the disease and increases survival (Urushitani, Ezzi and Julien, 2007; Takeuchi et al., 2010).

1.7.4 Anti-apoptotic

Apoptosis is largely influenced by mitochondria: if mitochondria are distressed, they release cytochrome C initiating caspase to propagate a cascade of cellular mechanisms to cause apoptosis. Creatine and Minocycline were tested at different concentrations showing beneficial effects in animal models (Klivenyi et al., 1999; Zhu et al., 2002; Shefner et al., 2004) but in humans they have not produced acceptable results (Groeneveld et al., 2003; Gordon et al., 2007; Atassi et al., 2010). Ursodeoxycholic and Tauroursodeoxycholic acids showed moderate positive results (Min et al., 2012; Elia et al., 2016).

1.7.5 Anti-inflammatory

Several immunomodulatory therapies have been hitherto tested in ALS patients (Wosiski-Kuhn et al., 2019). Mentioned among treatments targeting the innate immune system: Celecoxib, cyclooxygenase 2 (COX2) inhibitor that protects from excitotoxicity blocking the prostaglandins synthesis (Cudkowicz et al., 2006); Minocycline, antibiotic that reduces microglial activation and polarises the macrophages toward an anti-inflammatory phenotype (Gordon et al., 2007); Glatiramer Acetate and RN560, compounds able to tip the balance toward the alternative (Th2, M2) inflammatory response (Paganoni et al., 2020).

Conversely, some treatments have been tested to attempt in modulating the adaptive immune response, chiefly T lymphocytes. An example is the total lymphoid irradiation (TLI), which, through the selective target of lymphoid organs, abolish the circulating lymphocytes. However, this treatment did not obtain the expected outcome in ameliorating the disease course of patients but rather increased the number of circulating CD8+ cytotoxic T cells (Drachman et al.,
Another drug able to inhibit the egress of lymphocytes from lymph nodes and recently tested in ALS is Fingolimod, which, differently from TLI, does not affect the level of circulating T regulatory cells (Berry et al., 2017).

Efforts were also made to target the link between the innate and adaptive immune system: the cytokines. Examples of this class of drugs are Anakinra, a selective interleukin 1 receptor (IL1R) antagonist (Maier et al., 2015), and Tocilizumab, a monoclonal antibody against IL6 (Mizwicki et al., 2012). The Pioglitazone, an oral antidiabetic agent with anti-inflammatory effects that stimulates the transcription factor PPAR-γ (Peroxisome Proliferation Activated Receptors γ), resulted effective, increasing survival in ALS mouse models (Kiaei et al., 2005; Schütz et al., 2005). However, the clinical phase II study did not confirm the effectiveness of the drug, which has no beneficial effect on survival of ALS patients (Dupuis et al., 2012).

1.7.6 Neuroprotection

Besides inhibiting the numerous pathological mechanisms underlying neurodegeneration in ALS, efforts have been made to identify therapies that could stimulate the repair of damage MN or promote the growth of new ones. Recently have been proposed as promising compounds: 7,8-dihydroxyflavone (7,8-DHF), an agonist of tyrosine kinase receptor B (TkrB) that mimic the effects of BDNF (brain-derived neurotrophic factor) (Korkmaz et al., 2014) and GPNMB (glycoprotein non-metastatic protein B), which seems to reduce the TDP43-mediated stress (Tanaka et al., 2012).

Another drug proposed was lithium, a mood stabilizer with neuroprotective action in several diseases (Cappuccio et al., 2005). Although a first preclinical study in mouse model SOD1<sup>G93A</sup> has shown a survival increase of 300% associated with a significant delay in the onset of paralysis (Fornai et al., 2008), the results were not confirmed when lithium was tested in two different strains of SOD1<sup>G93A</sup> mice, in which was observed a further deterioration of motor performance (Pizzasegola et al., 2009).
1.7.7 Muscle strength

Many of the therapies in recent years are focusing on skeletal muscle whose involvement in the development and pathogenesis of ALS is becoming increasingly recognized. Among these are: activators of muscle troponin (CK-2017357), myostatin inhibitors (ACE-031) or factors that promote muscle development as GDF8 and Nogo-A (Zinman and Cudkowicz, 2011).

1.7.8 Cell-based therapy

Stem cell therapy is a promising potential treatment option for ALS, given stem cell remarkable plasticity and ability to differentiate into multiple neuronal lineages. Currently available cell therapies may take advantage of a variety of stem cells to modify disease pathophysiology, slow down or even halt the progression of the disease, possibly by providing protective factors to surrounding cells, modulating the host immune environment, inhibiting inflammation or even replacing injured cells (Mao et al., 2015). As replacing MNs in ALS is not currently practical, the focus instead is on the ability to improve functional outcomes through bystander mechanisms, such as neurotrophism (Barnabé-Heider and Miller, 2003; Lu et al., 2003), immunosuppressive effect (Aharonowiz et al., 2008; Ben-Hur, 2008; Einstein and Ben-Hur, 2008) and reducing astrogliosis and inflammation (Teng et al., 2012). There is also some evidence that providing new interneurons that connect with degenerating MNs may have some beneficial effects (Xu et al., 2006; Yan et al., 2006). However, the demonstration that only a small amount of the injected cells can engraft and differentiate within the injury site suggested that their beneficial effect was indirect and thus mediated by the several factors released. Subsequent studies demonstrated that stem cells produce a broad spectrum of extracellular vesicles (EVs) containing an enormous number of factors (cytokines, growth factors, nucleic acids, etc.). This evidence indicated that stem cells could exert their beneficial effect through the EVs secretions, which promote the wound healing releasing their content within the damaged area (Baglio, Pegtel and Baldini,
Thus, EVs (i.e. microvesicles and exosomes) could be used as a novel therapeutic tool, avoiding the ethical and immunogenic risks of stem cells (Bonafede and Mariotti, 2017).

1.7.9 Gene therapy

The progress of medicine brings alternative and innovative approaches for the treatment of so far incurable neurodegenerative diseases, including ALS.

Targeting ALS-associated genes, genetic modifiers or related disease molecules have shown promising results (Cappella et al., 2019). Indeed, it has been shown that antisense oligonucleotides (ASOs) against SOD1 were able to eliminate the mutant protein without adverse effects (Miller et al., 2013). One disadvantage of ASOs is the need for repetitive infusions or the identification of the correct dose. The delivery of ASO or short hairpin RNA to knockdown mutant SOD1 through a viral vector (e.g. AAV9, adeno-associated virus serotype 9) circumvents this issue (Foust et al., 2013; Iannitti et al., 2018). Another approach currently under consideration is the delivery of an AAV9 expressing a single-chain antibody against misfolded SOD1, which demonstrated its efficacy postponing the disease onset and extending survival in ALS mice (Maier et al., 2018).

1.8 GENETICS

ALS is considered a multifactorial disease caused by the interaction of environmental and genetic factors. In most cases (90-95%) it has a sporadic origin (sALS), while in 5-10% of patients the disease is caused by the inheritance of specific mutation (fALS) (Kiernan et al., 2011; Ajroud-Driss and Siddique, 2015). The clinical phenotypes of the two forms are indistinguishable (Pratt, Getzoff and J. J. P. Perry, 2012), but fALS shows an earlier average age of onset compared to the sALS (Camu et al., 1999), and no sex influence on disease susceptibility (Ticozzi et al., 2011), while in sALS a male preponderance was reported (1.5:1) respect to fALS (1:1), although this
ratio tends to decrease after age 70 (Haverkamp, Appel and Appel, 1995; Gros-Louis et al., 2004; Naruse et al., 2019).

### 1.8.1 Sporadic ALS

The etiology of sALS is largely unknown; however, there are many indications from epidemiological and familial data showing that genetic factors contribute to the pathogenesis. Indeed, mutations found in patients with fALS were also found in patients with the sporadic form. There are also several genes, referred to as "susceptibility genes", which could increase the risk of ALS interacting with other risk factors, genetic or environmental (Table 1.4). Genetic variants known and related to sporadic ALS explain only a part of the cases, approximately 10%.

The causes of the difficulty in finding new genetic determinants may be associated to a complex pattern of inheritance with a very low penetrance, a high degree of variability and the existence of environmental factors that would be decisive as regards the predisposition to ALS.

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<th>Gene</th>
<th>Protein</th>
<th>Function</th>
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<tr>
<td>PRPH</td>
<td>Peripherin</td>
<td>Neuronal intermediate filament involved in axonal growth</td>
<td>(Gros-Louis et al., 2004; Leung et al., 2004)</td>
</tr>
<tr>
<td>DCTN1</td>
<td>Dynactin</td>
<td>ER-Golgi transport, lysosome and endosome trafficking, chromosome movement and axonogenesis</td>
<td>(Münch et al., 2004)</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
<td>Vascular permeability, neuronal growth, and repair</td>
<td>(Lambrechts et al., 2003)</td>
</tr>
<tr>
<td>NFH</td>
<td>Neurofilament heavy subunit</td>
<td>Provides a supporting network for neuronal axons</td>
<td>(Figlewicz et al., 1994)</td>
</tr>
<tr>
<td>SMN1</td>
<td>Survival motoneuron</td>
<td>Neuronal protein involved in RNA processing</td>
<td>(Corcia et al., 2006)</td>
</tr>
<tr>
<td>CTNF</td>
<td>Ciliary neurotrophic factor</td>
<td>Neurotransmitter synthesis, neurite outgrowth and neuronal trophic factor</td>
<td>(Giess et al., 2002)</td>
</tr>
<tr>
<td>PON</td>
<td>Paraoxonase</td>
<td>Serum enzyme involved in detoxification of organophosphate and neurotoxins</td>
<td>(Ticozzi et al., 2010)</td>
</tr>
</tbody>
</table>
Apolipoprotein E (APOE) is involved in lipoprotein metabolism (Praline et al., 2011).

APEX is a DNA repair enzyme that is apurinic/apyrimidinic endonuclease. It regulates gene expression and cellular response to stress (Kisby, Milne and Sweatt, 1997).

HFE is a homeostatic iron regulator that is involved in cellular response to oxidative stress (Wang et al., 2004).

Table 1.4: Most common sALS susceptibility genes.

<table>
<thead>
<tr>
<th>ALS type</th>
<th>Onset</th>
<th>Inheritance</th>
<th>Gene</th>
<th>Protein</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALS 1</td>
<td>Adult</td>
<td>AD</td>
<td>SOD1</td>
<td>Cu/Zn superoxide dismutase</td>
<td>(Rosen et al., 1993a)</td>
</tr>
<tr>
<td>ALS 2</td>
<td>Juvenile</td>
<td>AR</td>
<td>KIAA1563</td>
<td>Alsin</td>
<td>(Hadano et al., 2010)</td>
</tr>
<tr>
<td>ALS 3</td>
<td>Adult</td>
<td>AD</td>
<td>-</td>
<td>-</td>
<td>(Hand et al., 2002)</td>
</tr>
<tr>
<td>ALS 4</td>
<td>Juvenile</td>
<td>AD</td>
<td>SETX</td>
<td>Senataxin</td>
<td>(Suraweera et al., 2007)</td>
</tr>
<tr>
<td>ALS 5</td>
<td>Juvenile</td>
<td>AR</td>
<td>KIAA1840/SPG11</td>
<td>Spatacsin</td>
<td>(Ticozzi et al., 2011)</td>
</tr>
<tr>
<td>ALS 6</td>
<td>Adult</td>
<td>AD</td>
<td>FUS/TLS</td>
<td>Fused in sarcoma</td>
<td>(Kwiatkowski et al., 2009)</td>
</tr>
<tr>
<td>ALS 7</td>
<td>Adult</td>
<td>AD</td>
<td>-</td>
<td>-</td>
<td>(Sapp et al., 2003)</td>
</tr>
<tr>
<td>ALS 8</td>
<td>Adult</td>
<td>AD</td>
<td>VAPB</td>
<td>Vescicle-associated membrane protein B &amp; C</td>
<td>(Nishimura et al., 2004)</td>
</tr>
<tr>
<td>ALS 9</td>
<td>Adult</td>
<td>AD</td>
<td>ANG</td>
<td>Angiogenin</td>
<td>(Greenway et al., 2006)</td>
</tr>
<tr>
<td>ALS 10</td>
<td>Adult</td>
<td>AD</td>
<td>TARDBP</td>
<td>TAR DNA-binding protein (TDP-43)</td>
<td>(Kabashi et al., 2008)</td>
</tr>
</tbody>
</table>

1.8.2 Familial ALS (fALS)

Table 1.4 summarizes all the subtypes of ALS with the respective identified mutations. The aberrant functions of mutated proteins associated with various forms of fALS can be due to both increased functionality of the protein that becomes neurotoxic GOF and/or loss of the normal physiological function LOF (Sau et al., 2007). Most cases of fALS are characterized by an autosomal dominant transmission with age-dependent penetrance (Kurland and Mulder, 1955), but also recessive forms and X-linked mutations have been described (Ben Hamida et al., 1990; Ince et al., 2011). In any case the peculiar characteristic of ALS, and thus the primary mechanism that leads to the progressive neuronal death, is the abnormal accumulation of insoluble proteins that form intracellular aggregates with different morphology (Watanabe et al., 2001).
| ALS 11 | Adult | AD | FIG4 | PI(3,5)P(2)5 phosphatase | (Chow et al., 2009) |
| ALS 12 | Adult | AD-AR | OPTN | Optineurin | (Maruyama et al., 2010) |
| ALS 13 | Adult | AD | ATXN2 | Ataxin2 | (Elden et al., 2010) |
| ALS 14 | Adult | AD | VCP | Valosin-containing protein | (Johnson et al., 2010) |
| ALS 15 | Adult-Juvenile | XD | UBQLN2 | Ubiquilin 2 | (Deng et al., 2011) |
| ALS 16 | Juvenile | AR | SIGMA R1 | 1 non-opioid intracellular receptor | (Al-Saif, Al-Mohanna and Bohleps, 2011) |
| ALS 17 | Adult | - | CHMP2B | Charged multivesicular body protein 2B | (Cox et al., 2010) |
| ALS 18 | Adult | AD | PFN1 | Profilin1 | (Wu et al., 2012) |
| ALS 19 | Adult | AD | ERBB4 | Erb-b2 receptor tyrosine kinase 4 | (Takahashi et al., 2013) |
| ALS 20 | Adult | AD | HNRNPA1 | ROA1/hnRNPA1 (Heterogeneous nuclear riboprotein A1) | (Kim et al., 2013) |
| ALS 21 | Adult | AD | MATR3 | Matrin 3 | (Johnson et al., 2014) |
| ALS 22 | Adult | AD | TUBA4A | Tubulin α-4a | (Topp et al., 2017) |
| ALS 23 | Adult | AD | ANXA11 | Annexin A11 | (Topp et al., 2017) |
| ALS (Other) | Adult | AD | DAO | D-amino acid oxidase | (Mitchell et al., 2010) |
| ALS (Other) | Adult | AD | DCTN1 | Dynactin | (Puls et al., 2003) |
| ALS/FTD 1 | Adult | AD | C9ORF72 | - | (DeJesus-Hernandez et al., 2011) |
| ALS/FTD 2 | Adult | AD | CHCH10 | Coiled-coil-helix-coiled-coil-helix domain containing protein 10 | (Bannwarth et al., 2014) |
| ALS/FTD 3 | Adult | AD | SQSTM1/p62 | Sequestosome 1 | (Fecto et al., 2011) |
| ALS/FTD 4 | Adult | AD | TBK1 | TANK-binding kinase 1 | (Cirulli et al., 2015) |
| LAHCD (lateral anterior horn cell disease with artrogryphosis) | - | AR | GLE1 | GLE1, RNA export mediator | (Kaneb et al., 2015) |

Table 1.5: Classification of inherited forms of ALS (modified from Mathis et al., 2019). AD=Autosomal Dominant; AR=Autosomal Recessive; XD=X-linked Dominant.

1.8.2.1 ALS1 – SOD1 (superoxide dismutase 1)

ALS 1 is the best characterized and the first form identified in the 90s (Rosen et al., 1993b) it represents 20% of fALS cases and 1-2% of the total patients. It is characterized by mutations of the gene encoding SOD1, mapping on the queue arm of chromosome 21 (Siddique et al., 1991).
This gene, with different mutations, is widely used to produce transgenic animal models to study the disease.

The gene is 9.3 kb long and consists of 5 exons. The product is the copper-zinc superoxide dismutase 1 (Cu/Zn SOD1), an ubiquitary homodimeric enzyme of 32 kDa, which consists of 153 aminoacids and containing a binding site for metals. Localized predominantly into the cytosol, it is present also in the mitochondrial intermembrane space (Okado-Matsumoto and Fridovich, 2001), nucleus, lysosomes, peroxisomes (Chang et al., 1988) and it is very abundant in cells of the CNS, in particular in MNs (Pardo et al., 1995).

Each monomer presents a β-sheet conformation and has two important functional loops, called "electrostatic loop" and "metal-binding loop" (residues 49-84) surrounding the region designed to the bond with metals (Tainer et al., 1982). The metal-binding loop with catalytic function contains residues binding Cu²⁺ and Zn²⁺, while the electrostatic loop confers stability to the protein. Copper is required for the enzymatic activity of SOD1, while the zinc is thought to have a role in maintaining the stability of the protein structure (Antonyuk et al., 2005). The human SOD1 contains four cysteine residues, in particular Cys57 and Cys146 forming a disulfide bridge that is abnormal in a reducing environment such as the cytosol where the enzyme is localized. The maturation of the protein is very complex and requires several steps: the insertion of copper and zinc, the formation of disulfide bridges and dimerization.

The structural characteristics of the SOD1 confer stability even in denaturant conditions, while the reduction of the disulfide bridge weakens the bond between the subunits and promotes aggregation (Furukawa and O’Halloran, 2005). The protein belongs to the family of dismutase and carries out a red-ox activity that allows to catalyze the transfer of electrons from one molecule reductant (donor) to a molecule oxidant (acceptor). Thus, it performs an important antioxidant action: it is able to transform oxygen free radicals, highly toxic for the cell, in less reactive intermediates. The SOD1 protein converts the superoxide anion (·O₂⁻) into oxygen and hydrogen peroxide (H₂O₂), which is subsequently transformed into water by glutathione
peroxidase and catalase. Catalysis is mediated by copper in two asymmetrical steps in which it
is alternately reduced and oxidized by superoxide. The zinc instead does not participate in this
reaction, but it is essential for the structure of the active site (Strange et al., 2003). The
detoxification processes are vital for the cell: the superoxide is a potent ROS produced in all
aerobic cells and in high concentrations it is capable to damage DNA, membrane lipids and
proteins.

To date, more than 177 different mutations have been identified in exons 5 of SOD1 that may
give rise to the disease. Most mutations are missense or substitutions of a single amino acid,
while deletions and non-sense mutations are rare. Moreover, there were highlighted 8 silent
and 9 intronic mutations which are presumably pathogenic (Al-Chalabi et al., 2012). In general,
mutations are transmitted in an autosomal dominant manner and individuals with two copies
of the mutated gene also show an anticipation of the symptoms (Hayward et al., 1999). An
exception is represented by sporadic mutations D90A and D96N, responsible for both dominant
and recessive forms (Hand et al., 2002; Siddique and Ajroud-Driss, 2011). The mutations are
located in all regions of the primary structure and in all the functional domains of the protein.
The mutated forms are different for catalytic activity, charge, or affinity with metals.
Researchers categorized two groups of SOD1 mutants: the ones which present structural
alterations, but the affinity with metals does not change (SOD1$^{G93A}$), and the ones which present
mutations in the binding site with metals, so they have a lower affinity for copper and zinc ions.
Generally, the mutation may cause changes in the various steps of protein maturation and also
in the interaction with metals, which leads to an unstable enzyme (Lindberg, Tibell and
Oliveberg, 2002), characterized by an abnormal folding due to the formation of interactions
which are not present in the native structure. Furthermore, changes in the interaction with other
proteins can occur (heat shock proteins, proteins of the cascade of electrons, etc...), making
them incapable of performing their functions and inducing the formation of aggregates (Wang
et al., 2011).
The mutant SOD1 results to be more sensitive to thermal variations (Rodriguez et al., 2002) and reduction of the disulfide bridge compared to the native form (Lindberg, Tibell and Oliveberg, 2002). The reduction of the disulfide bridge and the loss of metal from the active site induces the dissociation of the two subunits and the balance shifting from the dimeric to the monomeric form (Lindberg et al., 2005). The monomer without Cu/Zn is considered as the precursor of the toxic form.

One of the hypotheses initially formulated to explain the mechanism by which the SOD1 mutated causes ALS supposed the loss of normal catalytic activity of the enzyme (hypothesis loss-of-function) (Deng et al., 1993). This hypothesis was supported mainly by the accumulation of radicals in the cerebrospinal fluid of ALS patients. Then, it has been disproved by a series of evidence, which showed that the onset of the disease was due to the acquisition of new toxic functions of the mutated enzyme (hypothesis GOF) (Watanabe et al., 2001; Wang et al., 2008). Transgenic mice over-expressing the human SOD1 mutated, for example, develop a MN disease ALS-like, despite the presence of the corresponding wild-type (WT) murine enzyme (Gurney et al., 1994). In these models the dismutase activity levels are comparable, and even superior to those of the non-transgenic mice, supporting the hypothesis that there would be a GOF at the basis of the pathology. Moreover, mice deficient in the SOD1 murine gene do not show MN degeneration (Reaume et al., 1996; Shefner et al., 1999). Even mice expressing the WT human SOD1 do not develop ALS phenotype, highlighting that the mutation determines the toxicity of this protein. On the contrary, the WT hSOD1 showed neuroprotective effects in various assays of oxidative stress (Bruijn et al., 1998). Recently, researchers demonstrated the link between the cytotoxicity associated with SOD1 aggregation in ALS to a non-native trimeric SOD1 species. They showed that SOD1 mutants designed to promote trimerization increase cell death and the cytotoxicity of this mutants correlates with trimer stability, providing a direct link between the presence of misfolded oligomers and neuron death. Thus, the SOD1 trimer causes cell death in
MN s and those non-native trimeric interfaces appear to be shared by multiple cytotoxic SOD1 species (Proctor et al., 2016).

1.8.2.2 ALS6 – FUS/TLS

ALS 6 is an autosomal dominant form of ALS, characterized by onset in the upper limbs, which expands to the lower limbs, but never involves bulbar regions (Kwiatkowski et al., 2009). Recent studies individuated the FUS/TLS gene, likely responsible for the development of ALS 6 (Kwiatkowski et al., 2009; Vance et al., 2009). This gene was originally identified as a product of the translocation t(12;16) that causes the round-cell liposarcoma (Valdmanis et al., 2009). The gene FUS/TLS, consisting of 15 exons, produces a ubiquitous protein of 526 amino acids normally localized in the nucleus. Although the precise role of FUS/TLS has not yet been fully elucidated, structural studies led to the evidence of a RRM domain (RNA Recognition Motif) and other specific motifs which describe it as a multifunctional protein able to bind RNA and implicated in several phases in the regulation of gene expression, including transcription, splicing, RNA transport and translation (Ito and Suzuki, 2011; Al-Chalabi et al., 2012; Ishigaki et al., 2012). Indeed, FUS/− mice present deficits in DNA repair mechanism, resulting in chromosomal instability. Moreover, given the regulatory action on the transport of mRNA Nd1-L (stabilizing-actin protein), it is also able to adjust the neuronal arborization (Kwiatkowski et al., 2009; Valdmanis et al., 2009). To date, 15 different mutations of FUS/TLS have been described, mostly missense, that produce an ALS phenotype (Kwiatkowski et al., 2009; Valdmanis et al., 2009). Analysis conducted in the CNS of patients revealed the presence of abnormal aggregates of FUS/TLS in the cytoplasm of neurons, which are absent both in people with sporadic ALS and in subjects presenting mutant SOD1 (Deng et al., 2010).
1.8.2.3 ALS10 – TDP43

TDP43 is a 43 kDa protein of 414 amino acids encoded by TARDBP gene on 1p36.22 chromosome. It is a multifunctional protein that binds DNA and RNA which belongs to the ribonucleo-family proteins. It shows RRM1 and RRM2 and a C-terminal region rich in glycine able to mediate interactions with other proteins (Lagier-Tourenne and Cleveland, 2009; Da Cruz and Cleveland, 2011). It has a role in the regulation of splicing and transcription, but also in the biogenesis of microRNAs, apoptosis, and cell division (Buratti and Baralle, 2010)(Buratti and Baralle, 2008). In vivo, the depletion of TPD43 causes the reduction of transcripts, splicing errors and the production of non-coding mRNA (Polymenidou et al., 2011). It has been shown that TDP43 is the main component of the cytoplasmic ubiquitinated inclusions present in neurons and glia, in sALS and fALS cases, except ALS 1 (Mackenzie et al., 2007) and ALS 6 (Vance et al., 2009). To date 44 mutations have been found in TARDBP (Da Cruz and Cleveland, 2011), as a primary cause of sALS and fALS (Sreedharan et al., 2008). Under physiological conditions, TDP43 is mainly localized in the nucleus of neurons, but it is also present in the cytoplasm. In stress conditions or in case of mutations it is hyper-phosphorylated, fragmented, ubiquitinated and sequestered in cytoplasmic aggregates (Neumann et al., 2006). Inclusions of TDP43, colocalized with ubiquitin and p62 (Maekawa et al., 2009), are considered neuropathologic characteristic of ALS, however it is unclear whether TDP-43 mutations result in the loss of MNs via the acquisition of a toxic GOF or through the loss of the physiological role of the protein (LOF) (Lagier-Tourenne and Cleveland, 2009).

To date, 40 different mutations have been identified that determine the 6.5% of the cases of fALS and 5% of sALS, most of which were in the C-terminal region (Sreedharan et al., 2008; Pratt, Getzoff and J. P. Perry, 2012). They are all missense mutations with dominant inheritance, with the sole exception of the replacement Y374X, which gives rise to a truncated protein (Daoud et al., 2009).
1.8.2.4 ALS/FTD 1 - C9orf72

The hexanucleotide repeat expansion (HRE) of 4 guanines and 2 cytosines in the intron located between the non-coding exons 1a and 1b of the C9orf72 gene is the most prevalent cause of both sporadic and familial ALS (DeJesus-Hernandez et al., 2011; Renton et al., 2011; Majounie et al., 2012). C9orf72 is the topic of this work, therefore it will be discussed more in detail in the next chapter.
INTRODUCTION

Chapter 2

Chromosome 9 open reading frame 72 (C9orf72)
2.1 A BRIEF INTRODUCTION

In 2011, separate studies of next-generation sequencing on two families showed that the massive HRE of 4 guanines and 2 cytosines (GGGGCC) within the non-coding region (i.e. intron) between exons 1a and 1b of the C9orf72 gene is the cause of 9p21-linked ALS/FTD (DeJesus-Hernandez et al., 2011; Renton et al., 2011; Ash et al., 2013), leading to the hypothesis that ALS and FTD are parts of a single clinical spectrum (DeJesus-Hernandez et al., 2011).

The concomitance of ALS with FTD has been extensively described for decades arising on both sporadic and familial bases (Mitsuyama and Takamiya, 1979; Hudson, 1981; Mitsuyama and Tobo, 1981; Mitsuyama, 1984, 2000; Morita et al., 1987; Neary et al., 1990). The comorbidity of ALS and FTD syndromes in patients is estimated to occur in 50% of the patients (Murphy et al., 2000; Lomen-Hoerth, Anderson and Miller, 2002; Ringholz et al., 2005; Phukan, Pender and Hardiman, 2007).

From the 2000, linkage analyses in several autosomal dominant families in which individuals develop either ALS or FTD or both, identified a genetic locus on chromosome 9p21 that confers susceptibility to ALS/FTD (Hosler et al., 2000; Morita et al., 2006; Vance et al., 2006; Valdmanis et al., 2007; Luty et al., 2008; Ber et al., 2009; Gijselinck et al., 2010; Laaksovirta et al., 2010; Boxer et al., 2011; Pearson et al., 2011). Moreover, genome-wide association studies linked the same chromosomal region also to sporadic ALS (van Es et al., 2009; Shatunov et al., 2010), and the same risk haplotype, covering MOBKL2B, C9orf72, and IFNK genes, was found in all the affected members of families of 9p21-linked ALS/FTD (Mok et al., 2012).
2.2 C9orf72 GENE

The human C9orf72 gene is located on the reverse strand of chromosome 9 and consists of two non-coding exons (1a and 1b) and 10 coding exons (from 2 to 11). It gives rise to three transcript variants:

- variant 1 (V1) includes exon 1a and exons 2-5
- variant 2 (V2) includes exon 1b and exons 2-11
- variant 3 (V3) includes exon 1a and exons 2-11

V2 is the most abundant transcript in tissue from non-mutation carriers (van Blitterswijk et al., 2015; Rizzu et al., 2016). Alternative splicing of these three RNA variants results in the production of two different isoforms (DeJesus-Hernandez et al., 2011; Renton et al., 2011) (Figure 2.1):

- isoform a: the short (C9orf72-S) isoform, a 222-amino acids protein of 24 kDa, encoded by V1
- isoform b: the long (C9orf72-L) isoform, a 481-amino acids protein of 54 kDa, encoded by V2 and V3.

The long isoform is the most abundant (Frick et al., 2018). The C9orf72 gene is highly conserved in primates and across different species commonly used as model systems, suggesting that the protein(s) encoded by C9orf72 have fundamental biological functions. The similarity between the sequences of the orthologous genes and the human gene, expressed as a percentage of identity, is very high in chimpanzee (99.58%), rhesus macaque (99.58%), mouse (98.13%), rat (97.71%), rabbit (98.54%), Xenopus (83.96%), and zebrafish (75.97%), while for the nematode Caenorhabditis elegans, the similarity is very poor (14.71%), and there is no ortholog of C9orf72 in Drosophila. However, while C9orf72-L appears well conserved in evolution, the short isoform is reported only in humans and is not known in other non-human primates or other species. In humans and mice, both the transcript and protein are expressed mainly in the brain, spinal cord,
and immune system, and at lower levels in other organs such as lung, heart, liver, kidney, and skeletal muscle (Frick et al., 2018). Regarding cell type distribution, C9orf72 expression is higher in myeloid cells (monocytes/macrophages, microglia, and dendritic cells) compared to lymphoid cells, and neurons. In human brain tissues, C9orf72-L is found in cytoplasm, while C9orf72-S appeared localized at the nuclear membrane. Generally, C9orf72 appears to be diffuse in the cytoplasm with speckle-like structures in neurites and synapses of neurons. Moreover, it is found in several organelles like the Golgi apparatus (Aoki et al., 2017), stress granules (Maharjan et al., 2017; Chitiprolu et al., 2018), mitochondria (Wang et al., 2021), and mostly in the compartments of the endolysosomal pathway (Farg et al., 2014; Sellier et al., 2016; Amick and Ferguson, 2017; Frick et al., 2018; Shi et al., 2018). In myeloid cells, C9orf72 is strongly expressed in phagosomes and phagolysosomes (Smeyers, Banchi and Latouche, 2021).

Figure 2.1: C9orf72 structure, transcript variants and protein isoforms (modified from Balendra and Isaacs, 2018)
2.3 C9orf72 FUNCTION

C9orf72 shows homology to the DENN (differentially expressed in normal and neoplasia) family proteins (Yoshimura et al., 2010; Marat, Dokainish and McPherson, 2011; Levine et al., 2013) consisting of an N-terminal longin domain, a DENN domain and C-terminal alpha domains (Zhang et al., 2012). The C9orf72-S isoform contains only the longin domain, while the C9orf72-L isoform includes all three domains (De Franceschi et al., 2014; Xiao et al., 2016). DENN domain proteins act as guanosine diphosphate (GDP)-guanosine-triphosphate (GTP) exchange factors (GEFs) which activate small Rab GTPases, the main regulator of membrane trafficking, and allow them to be recruited to membranes (Yoshimura et al., 2010; Guadagno and Progida, 2019). C9orf72 has been reported to interact with Rab1, Rab5, Rab 7 and Rab 11 to regulate endosomal trafficking, lysosomal biogenesis, and autophagy in cell culture models (Farg et al., 2014; Sellier et al., 2016; Tang, 2016; Webster et al., 2016; Shi et al., 2018). All these Rabs are at the intersection of endocytosis and macroautophagy (Nassif, Woehlbier and Manque, 2017; Shi et al., 2018; Guadagno and Progida, 2019). Rab1 is involved in endoplasmic reticulum–Golgi transport, and Rab5 is necessary for endocytosis and is present at early endosomes, but these two Rabs also regulate the initiation of autophagy. Rab11 associates with recycling endosomes and is involved in autophagosome elongation, while Rab7 associates with late endosomes, is involved in autophagosome maturation and closure, and drives the fusion of late endosomes and autophagosomes to lysosomes. The interaction of C9orf72 and Rab7L1 was also shown to regulate extracellular vesicle secretion (Aoki et al., 2017). It was further shown that C9orf72 interacts with the Unc-51-like kinase 1 (ULK1) autophagy initiation complex and Rab1a and controls the initiation of autophagy by acting as a Rab1a effector (Webster et al., 2016). C9orf72 binds to the activated ULK1 and recruits active Rab1a to the ULK1 complex to promote translocation of the ULK1 complex to the phagophore during autophagy initiation.
C9orf72 interacts with other DENN domain-containing proteins, SMCR8 (Smith–Magenis chromosome region 8) and WDR41 (WD40-repeat containing protein 41) (Blokhuis et al., 2016; Sellier et al., 2016; Sullivan et al., 2016; Ugolino et al., 2016; Yang et al., 2016; Amick and Ferguson, 2017). This complex was reported to act as GEF for Rab8a and Rab39b (Sellier et al., 2016) and to interact with the proteins of the autophagy initiation complex FIP200/Ulk1/ATG13/ATG101 to control autophagic flux (Sellier et al., 2016; Sullivan et al., 2016; Yang et al., 2016). The interaction of the complex with Rab8a and Rab39b also mediates the recruitment of p62 (Sellier et al., 2016).

Mass spectrometry-based studies revealed that C9orf72 interacts with several actin binding proteins such as cofilin, Arp2 and 3, coronin and ARF6 that regulates actin dynamics of filament assembly and disassembly, essential for axonal maintenance and synaptic strength (Flynn et al., 2012; Huang et al., 2013), hypothesizing a role in axon growth.

C9orf72 was very recently shown to regulate the activity of mitochondrial oxidative phosphorylation (OXPHOS) complexes. C9orf72 is imported to the mitochondrial intermembrane space through the interaction with AIFM1. Then C9orf72 interacts with TIMMDC1 and the prohibitin (PHB) complex to stabilize OXPHOS complex I (CI) subunits inducing their assembly (Wang et al., 2021).

It has been also demonstrated that C9orf72 regulates stress granule formation and degradation (Maharjan et al., 2017; Chitiprolu et al., 2018). C9orf72-L is recruited to stress granules upon stress-related stimuli. The lack of C9orf72 impedes the formation of these granules and downregulates other stress granules-associated proteins like TIA-1, G3BP1, and HuR (Maharjan et al., 2017). C9orf72 associates with p62 to target stress granules for the degradation by autophagy (Chitiprolu et al., 2018) after the stress is removed to recover the cell. Through interaction with the cytosolic chaperone Hsc70 (heat shock cognate protein 70), C9orf72 may also be involved in chaperone-mediated autophagy, or aggrephagy, a mechanism that permits
the clearance of aggregating proteins without requiring the presence of autophagic vesicles (Sellier et al., 2016).

C9orf72 is a strong genetic interactor of FIS1, a mitochondrial membrane protein involved in mitochondrial fission and mitophagy. Both C9orf72 and FIS1 interact with ligands of the receptor for advanced glycation end (RAGE), involved in modulating inflammation and chemotaxis. C9orf72 and FIS1 repress the secretion of inflammatory cytokines by preventing the binding of these ligands to RAGE (Chai et al., 2020).

It has been demonstrated that C9orf72 is necessary to maintain immune cell homeostasis in response to immune-stimulating gut microbiota (Burberry et al., 2020). Moreover, in myeloid cells, C9orf72 suppresses the expression of type I interferons, which is activates through the stimulator of interferon gene (STING) pathway, in response to cytosolic DNA (McCauley et al., 2020). STING signalling is modulated by the autophagolysosomal pathway. Thus, blocking its degradation leads to sustained type I interferon production. Given the role of C9orf72 in autophagy, it is possible that C9orf72 regulates STING-dependent inflammation by controlling its degradation (McCauley et al., 2020).

C9orf72-S is localized to at the nuclear membrane where interacts with Importin β1 and Ran-GTPase, major proteins involved in nucleocytoplasmic import (Xiao et al., 2015).

### 2.4 EPIDEMIOLOGY

Approximately 5%-10% of all patients with FTD or ALS, and up to 30% of the patients with both diseases carry the C9HRE (Majounie et al., 2012; Marogianni et al., 2019). A strong heterogeneity in the frequency of C9orf72 mutations is reported between ethnic groups. The highest rates are found in Caucasians and the lowest in Asians (Marogianni et al., 2019): the C9HRE represents the most frequent cause of ALS and FTD in white Europeans, Americans and Australians,
accounting for 6.3% of sALS and 37.6% of fALS, and 5.8% of fFTD and 25.1% of sFTD (DeJesus-Hernandez et al., 2011; Majounie et al., 2012). A significant heterogeneity also in reported incidence and prevalence was observed within and between countries and geographic regions, with generally higher prevalence and incidence rates in Europe and North America than in Asia and Latin America (Brown et al., 2021). The total estimated number of prevalent C9ALS cases is 4,545 (1,198 [26%] fALS and 3,347 [74%] sALS), and the number of incident C9orf72 cases is 1,706 (450 [26%] fALS and 1,256 [74%] sALS) (Brown et al., 2021). Although the proportions of C9orf72 mutations are higher among fALS patients, the higher prevalence results in sALS cases, indicating that the classification of fALS based on reported family history does not capture the full picture of ALS genetic origin (Brown et al., 2021).

An age-dependent penetrance was observed: the pathogenic expansion was non-penetrant in individuals younger than 35 years, 50% penetrant by 58 years, and 90.9%-99.5% penetrant by 83 years (Majounie et al., 2012). The first manifestation and the progression also vary largely (Glasmacher et al., 2020).

### 2.5 CLINICAL MANIFESTATION

The onset of C9ALS usually manifests in the fifth decade (the median age at the onset is 57 years), similarly to non-C9ALS disease. The clinical phenotype covers the entire ALS spectrum, with a higher frequency (30%-40%) of bulbar onset compared to non-C9ALS that explains the shorter overall survival reported in C9ALS (Cammack et al., 2019; Glasmacher et al., 2020). C9ALS patients show signs of cognitive impairment and dementia, mostly of the behavioural variant FTD (bvFTD), more frequently than non-C9ALS patients (Snowden et al., 2012). Accordingly, C9ALS patients show more extensive atrophy of non-motor areas of the frontal cortex than those with non-C9ALS. Although only 20% of ALS patients fulfil FTD diagnostic criteria, an additional 20% has cognitive impairment, usually in executive functioning, social cognition, or
language, and 10% shows behavioural changes. Concomitant parkinsonism and psychiatric symptoms occur more frequently in C9ALS/FTD than in non-C9ALS/FTD (Snowden et al., 2012; Devenney et al., 2014; Wilke et al., 2016; Carneiro et al., 2020). Patients with ALS and the pathogenic repeat expansion were more likely to be female (Majounie et al., 2012).

The G4C2 length in healthy individuals ranged from 2-23 hexanucleotide units, whereas in ALS patients the repeat length is estimated to be 700-1600 units (DeJesus-Hernandez et al., 2011), although the precise threshold of repeats required for disease penetrance is unclear (DeJesus-Hernandez et al., 2011; Renton et al., 2011). A small percentage of patients have shorter expansions (30-80) (Gijselinck et al., 2016). Conflicting results have been reported concerning the association between repeat size and penetrance of ALS phenotypes, due to the great instability of the expansion that causes difficulties in determining the correct repeat size and variability across and within tissues of the same individual (Van Blitterswijk, Dejesus-Hernandez and Rademakers, 2012; Beck et al., 2013; Dols-Icardo et al., 2014; Nordin et al., 2014; Waite et al., 2014; Fratta et al., 2015; Gijselinck et al., 2016; Jackson et al., 2020). Due to somatic mosaicism, HRE size in blood, used for the detection and sizing of HRE, might not reflect size in CNS (Van Blitterswijk, Dejesus-Hernandez and Rademakers, 2012; Jackson et al., 2020) and direct comparisons of blood and brain tissue from the same individual demonstrate similar sizes in some subjects, but completely different sizes in others (Van Blitterswijk, Dejesus-Hernandez and Rademakers, 2012; Nordin et al., 2014; Jackson et al., 2020). Somatic mosaicism opposes to the previous hypothesis that all the patients with the sporadic and familial forms carrying the HRE share the same founder risk haplotype (Majounie et al., 2012), and suggests multiple origins of the C9 expansion (Fratta et al., 2015). Moreover, in addition to mosaicism, the repeat number varies in blood with age at collection and over time in successive blood collections from C9orf72 mutation carriers (Suh et al., 2015; Fournier et al., 2019).
2.6 NEUROPATHOLOGICAL FEATURES

C9ALS/FTD patients show neuronal cytoplasmatic and intranuclear ubiquitin- and p62-positive and, rarely, phosphorylated TDP43 (p-TDP43)-containing inclusions in the granular cell layer and Purkinje cells of cerebellum and pyramidal cell layer of hippocampus. Typical skein-like and globular inclusions positive for both p62 and phosphorylated TDP43 were frequently found in MNs of spinal cord (Al-Sarraj et al., 2011; Murray et al., 2011; Troakes et al., 2012).

In C9ALS/FTD patients, sense and antisense RNA foci containing C9-repeat RNA are found in the frontal and motor cortices, hippocampus, cerebellum and in spinal cord, predominantly in MNs and less frequently in interneurons, astrocytes, microglia and oligodendrocytes (Mizielinska et al., 2013).

Another peculiar feature is the presence of aggregating dipeptide-repeat proteins (DPRs) originating from repeat-associated non-ATG (RAN) translation of the repeats within a proportion of inclusion bodies which are p62-positive and TDP43-negative. DPRs are found predominantly in the cerebellum, hippocampus and neocortex, less frequently in subcortical regions, and rarely in the brainstem and spinal cord (Mori et al., 2013).

2.7 PATHOLOGICAL MECHANISMS

It is well demonstrated that C9HRE causes neurodegeneration through cooperativity between a LOF and two GOF mechanism. Three non-exclusive pathogenic mechanisms have been proposed (Figure 2.2):

- haploinsufficiency (LOF)
- formation of RNA foci (GOF)
- accumulation of DPRs (GOF)
Figure 2.2: Proposed pathological mechanisms of C9orf72 mutation. (A) The massive GGGGCC HRE in the intron between the non-coding exons 1a and 1b of the C9orf72 gene is the most common genetic cause of ALS/FTD. Three mechanisms have been hypothesized through which the HRE could be pathogenic. (B) The expanded repeats could interfere with transcription, leading to a downregulation in the C9orf72 gene and loss of C9orf72 protein function. (C) Sense and antisense transcription of repeats forms RNA foci that accumulate in the nucleus or cytoplasm of cells and sequester RNA binding proteins (RBP), subtracting them to their physiological function. (D) RNA repeats undergo an unconventional form of translation that generates DPRs which accumulate in the brain and spinal cord of C9HRE carriers (modified from Gitler et al., 2016).

2.7.1 Haploinsufficiency

C9ALS patients show decreased levels of C9orf72 mRNA in frontal cortex, cerebellum, lymphoblast and fibroblast (DeJesus-Hernandez et al., 2011; Renton et al., 2011; Belzil et al., 2013; Ciura et al., 2013), and decreased protein expression in frontal cortex, as well as a trend to decrease of the protein expression in hippocampus and spinal cord (Waite et al., 2014). Moreover, a reduction in C9orf72 transcript in brain tissue and lymphoblast of patients that carried or not the C9HRE was found (Ciura et al., 2013). The uniformity of the G4C2 sequence and the abundance of guanine nucleotides in HRE promotes the formation of higher-order DNA structures called G-quadruplexes (Haeusler et al., 2014; Šket et al., 2015). These structures generate truncated RNA transcripts that are aborted in the hexanucleotide repeat region that
can also form G-quadruplexes and RNA hairpin structures (Fratta et al., 2012; Reddy et al., 2013). Moreover, C-rich sense and antisense strands can assemble as i-motifs and hairpin structures (Kovanda et al., 2015). HRE-containing RNA can form hybrids with HRE-containing DNA called R-loops (Reddy et al., 2014). Together, these higher-order structures of DNA and RNA are thought to act as promoters and regulatory elements affecting replication, transcription, and translation of the surrounding region (Brooks, Kendrick and Hurley, 2010; Kendrick and Hurley, 2010), and to exert deleterious effect on cells by causing nucleolar stress and impeding RNA processing (Haeusler et al., 2014). Moreover, in 20–40% of cases, the CpG island in the C9orf72 promoter region upstream of the pathogenic repeats is hypermethylated and in 97% of cases the expanded hexanucleotide repeat itself is methylated. This (hyper)methylation is associated with increased repeat length and reduced transcription of C9orf72 (Belzil et al., 2013; Xi et al., 2013, 2015; Gijselinck et al., 2016). Moreover, hypermethylation correlates with reduced disease duration in patients with C9ALS (Xi et al., 2013). On the other hand, several bodies of evidence show amelioration of disease phenotypes by C9orf72 hypermethylation, associated with reductions in RNA foci and DPRs in patients with C9ALS/FTD (Liu et al., 2014) and C9orf72 cell models (Bauer, 2016), as well as reduced neuronal and grey matter loss (McMillan et al., 2015). Furthermore, hypermethylation has been linked to a prolonged survival in C9ALS/FTD patients (Russ et al., 2015) and a later age of onset in ALS and FTD (Gijselinck et al., 2016). Histone trimethylation is another epigenetic modification that can reduce gene expression (Belzil et al., 2013).

Several independent groups point to a role for C9orf72 in autophagy and endolysosomal trafficking and function. Knockdown of C9orf72 in human cell lines and primary neurons inhibits autophagy, leading to accumulation of p62 (Sellier et al., 2016; Webster et al., 2016) and cytoplasmic aggregation of TDP-43 (Sellier et al., 2016). Accumulation of p62 is observed also in the spleens of C9orf72 knockout (KO) mice (O’Rourke et al., 2016). Accordingly, overexpression of C9orf72 activates autophagy, leading to an increase in autophagosomes in cell lines (Webster
Conversely, other studies demonstrated that the loss of C9orf72 in both cellular models and mice enhances autophagic flux (Ugolino et al., 2016). The relevance of the role of C9orf72 in autophagy for disease pathogenesis is unclear, but neurons from patients with C9ALS/FTD have impaired basal autophagy (Webster et al., 2016; Aoki et al., 2017) and increased sensitivity to autophagy inhibition (Almeida et al., 2013), suggesting that reductions in C9orf72 levels contribute to cellular stress.

In iPSC-derived MNs, C9orf72 primarily localizes to early endosomes, and iPSC-derived MNs from patients with C9ALS/FTD have fewer lysosomes than those from healthy controls (Shi et al., 2018). Both patient neurons and CRISPR–Cas9 C9orf72 KO iPSC-derived neurons have reduced vesicular trafficking, which can be rescued by C9orf72 overexpression (Shi et al., 2018). These cells also have elevated glutamate receptor levels and increased sensitivity to excitotoxicity (Selvaraj et al., 2018; Shi et al., 2018). Consistent with this observation, increased glutamate receptor levels were found in spinal cord tissue of C9orf72 KO mice and in spinal cord (Selvaraj et al., 2018; Shi et al., 2018) and cortical tissue (Shi et al., 2018) from patients with C9ALS/FTD.

Taken together, these data suggest that C9orf72 is involved in multiple cellular trafficking events, and that loss of C9orf72 in both microglia and neurons can sensitize cells to other insults, thereby contributing to neurodegeneration in C9ALS/FTD.

### 2.7.2 Formation of RNA foci

Bidirectional transcription of the mutated C9orf72 gene generates sense and antisense transcripts that form intranuclear RNA foci (DeJesus-Hernandez et al., 2011; Mizielska et al., 2013) that potentially act as new binding sites for specific RNA binding proteins, which may become abnormally sequestered, impairing their function (Gendron et al., 2013; Lee et al., 2013;
Cooper-Knock et al., 2014). A number of RBPs are implicated in the HRE-induced neurodegeneration:

- **Nucleolin**: is one of the main constituents of the nucleolus. When it binds to HRE-containing RNA dislocates from the nucleoli and disperses through the nucleus, resulting in impaired rRNA processing, followed by decreased maturation of ribosomes, and finally, accumulation of untranslated mRNA in the neuronal cytoplasm.

- **Purα**: is a component of the RNA-transport granules—particles that carry mRNAs to the nerve fibres where translation of those mRNAs into proteins occurs—and is involved in the regulation of the cell cycle and in cell differentiation. Sequestration of Purα by the C9orf72 hexanucleotide RNA repeats could impair neuronal mRNA transport thus leading to neurodegeneration.

- **hnRNPs**: HnRNPH is involved in the regulation of RNA processing; thus, hnRNPH sequestration in RNA cause aberrant RNA processing and may enhance neurodegeneration.

- **ADARB2**: is an RNA-editing enzyme. Although the mechanism by which ADARB2 sequestration in RNA foci could contribute to HRE-mediated toxicity is unclear, it was proposed that this protein may be important for RNA foci formation because ADARBP knockdown results in the reduction of neurons that contain RNA foci.

- **SRSF1 and SRSF2**: SRSF1 is a marker of nuclear speckles regions important for storage of splicing factors, its accumulation in RNA foci could disrupt the function of these speckles and lead to aberrant RNA processing. Additionally, binding of the nuclear export adaptor SRSF1 to C9orf72 repeats promotes export of C9orf72 repeats from the nucleus.

- **ALYREF**: is an export factor colocalizing with RNA foci.
2.7.3 Accumulation of DPRs

HRE-containing transcripts can undergo RAN translation that produces DPRs which aggregate and form toxic insoluble cytoplasmatic inclusions (Ash et al., 2013; Gendron et al., 2013; Mori et al., 2013). Poly-Gly-Ala (GA), poly-Gly-Pro (GP), and poly-Gly-Arg (GR) are translated from the sense transcript and poly-Gly-Pro (GP), poly-Pro-Arg (PR), and poly-Pro-Ala (PA) from the antisense transcript (Figure 2.3).

**Figure 2.3:** Sense- and anti-sense DPRs (modified from Balendra and Isaacs, 2018).

Toxicity of DPR proteins seems to be mainly dependent on arginine-containing DPR proteins, particularly poly-PR (Kwon et al., 2014; Wen et al., 2014; Jovičič et al., 2015). Arginine-rich DPRs disrupt nucleocytoplasmic transport and RNA processing (Jovičič et al., 2015), which can cause dysregulation of translation (Kanekura et al., 2016), nucleolar stress (Tao et al., 2015), disturb ubiquitin proteasome system (Yamakawa et al., 2015), affect the formation of stress granules (Boeynaems et al., 2017), and influence the Notch signalling pathway (Yang et al., 2015). Moreover, poly-GA DPR proteins are shown to disturb the ubiquitin proteasome system and cause endoplasmic reticulum stress (Zhang et al., 2014) and enhance the formation of toxic amyloid fibrils (Chang et al., 2016) (Figure 2.4). Although there is molecular evidence of DPRs toxicity in *in vitro* and *in vivo* C9ALS/FTD models (Kramer et al., 2018; Swaminathan et al., 2018), *post mortem* analyses of human brain revealed inconsistent results.
Figure 2.4: Physico-chemical properties of amino acids comprising the DPR species in C9orf72 mediated ALS/FTD (top). The structure and charge of individual DPR species and their influence on their interactions and toxicity (bottom left). Cellular functions known to be impaired by DPR toxicity (bottom right) (modified from Freibaum and Taylor, 2017).

No correlation of DPR proteins with clinical phenotype, severity of disease, and neurodegeneration was observed, and the abundance of DPR proteins was low in the brain regions most affected in ALS and FTD (MacKenzie et al., 2013; Davidson et al., 2014; Gomez-Deza et al., 2015; Mackenzie et al., 2015). Furthermore, there were discrepancies in the outcome of the studies comparing the distribution of DPR proteins between ALS and FTD cases (Davidson et al., 2014; Gendron et al., 2015).

2.8 C9orf72 ANIMAL MODELS

Several animal models have been generated to understand the contribution of LOF and GOF to ALS pathogenesis and progression.

2.8.1 LOF models

The partial (knockdown-KD) or total (KO) depletion of C9orf72 was studied in different in vitro and in vivo model to study both the function of C9orf72 protein and the role of its reduction in ALS.
KD of the zebrafish C9orf72 ortholog causes axonal degeneration of MNs, that can be rescued by expressing human C9orf72 mRNA (Ciura et al., 2013). Deletion of C. elegans ortholog results in MN degeneration and stress sensitivity (Therrien et al., 2013). However, given the low rate of homology (zebrafish 75.97% and C. elegans 14.71%) the use of murine models has been preferred. The mouse C9orf72 orthologue is located on the reverse strand of chromosome 4 and consists of 13 exons with a similar intron-exon structure to the human gene with which it shares the 98% homology (only nine amino acids differ between the predicted protein sequences encoded by the mouse and the human genes). Like human, C9orf72 murine protein is expressed within brain and spinal cord neurons and glia, immune cells (monocytes/macrophages, dendritic cells, and T-cells) and other non-neuronal cells (muscle, spleen, kidney, liver, and testes) (Suzuki et al., 2013; Koppers et al., 2015; Atanasio et al., 2016; Ferguson, Serafeimidou-Pouliou and Subramanian, 2016; O’Rourke et al., 2016). Constitutive heterozygous KO did not show any kind of phenotype (Atanasio et al., 2016; Burberry et al., 2016). Transient reduction of C9orf72 expression in the CNS by ASOs (Lagier-Tourenne et al., 2013) and conditional homozygous KO of C9orf72 in neurons and glia (Koppers et al., 2015) resulted in reduced body weight, but did not induce MN degeneration, denervation, defects in motor function or altered survival. Moreover, no gliosis or TDP43 mislocalisation were found. Constitutive homozygous KO or CRISPR-Cas9-mediated KO of C9orf72 developed a robust immune-phenotype characterized by lymphadenopathy and splenomegaly, increased percentages of myeloid lineage cells in lymphoid organs, leukocyte expansion and fatal autoimmune disease (Atanasio et al., 2016; Burberry et al., 2016; Jiang et al., 2016; O’Rourke et al., 2016; Sudria-Lopez et al., 2016; Sullivan et al., 2016). Some of these studies reported mild motor or cognitive impairment (Atanasio et al., 2016; Jiang et al., 2016), decreased body weight (Atanasio et al., 2016; Burberry et al., 2016; Jiang et al., 2016; Sudria-Lopez et al., 2016) or reduced lifespan (Atanasio et al., 2016; Burberry et al., 2016; Jiang et al., 2016; Sudria-Lopez et al., 2016; Ugolino et al., 2016). However, none
exhibit a neurodegenerative phenotype, neuronal loss, or denervation, suggesting that C9orf72 LOF is insufficient to trigger the disease.

2.8.2 GOF models

Bacterial artificial chromosome (BAC) transgenic mice express the mutated human C9orf72 gene with surrounding regulatory regions.

O’Rourke and colleagues developed a C9BAC mouse model on a C57Bl/6 background by using the full C9orf72 gene with repeats from 100 to 1000 (O’Rourke et al., 2015), while Peters et al., used only exons 1-6 with 300/500 repeats on an SJL/B6 background (Peters et al., 2015). Both the C9BAC mouse models expressed RNA foci and DPRs, without signs of TDP43 pathology. Moreover, no motor or cognitive deficits were observed, and survival of the mice was not reduced. Jiang and colleagues developed multiple BAC transgenic lines using a construct containing exons 1-5 of the C9orf72 gene (with the 5 ’10-kb flanking sequence) (Jiang et al., 2016). Four lines were characterized, one of which expresses 110 repetitions while the other three 450 repetitions (which show an increase in expression levels). These mice showed no evidence of a neuromotor pathology. No deficits in weight and motor performances were observed beyond 18 months of age, and no deficits were found in EMG at 12 months. There was no loss of MNs or gliosis in the spinal cord or motor cortex. However, some of these animal models developed memory impairment and loss of hippocampal neurons, with an increase in levels of p-TDP43. In this model, a single administration of ASO targeting C9orf72 RNA led to an improvement in cognitive deficits, and a reduction of RNA foci and DPRs. Only the BAC model reported by Liu and colleagues (Liu et al., 2016) developed an overt neurodegenerative phenotype and expressed RNA foci, DPR, TDP43 pathology and neurodegeneration. However, only a subset of female mice showed an acute motor phenotype, paralysis, weight loss and decreased survival, while the other mice developed a slower progressive neurodegeneration.
No MN loss nor glial activation was observed in the lumbar spinal cord of these mice (Liu et al., 2016). Recently, the phenotype of these C9BAC mouse model has been debated, since two different research group failed to reproduce the same evidence (Mordes et al., 2020), while two others did (Nguyen et al., 2020). One of the reasons of this discrepancy was attributed to an environmental factor since the experiment were carried out in different laboratories. Moreover, these mice had an FVB/N background, known to develop seizure activity, even in absence of genetic manipulation (Nguyen et al., 2020), arguing against the specific toxicity of RNA foci and DPRs. Altogether, these C9BAC mouse models suggest that GOF alone is not sufficient to induce neurodegeneration.

2.8.3 Synergistic models

The studies on human iMNs derived from cells of C9ALS/FTD patients demonstrated that C9orf72 haploinsufficiency hypersensitized these cells to glutamate (Shi et al., 2018). Similarly, C9KO rats treated with a subclinical dose of kainic acid to promote glutamate excitotoxicity developed motor deficits and MN loss (Dong et al., 2021). Abo-Rady and colleagues showed that the combination of HRE and the additional C9KO in iMN from C9ALS/FTD patient cells decreased axonal trafficking of lysosomes and caused significant apoptosis (Abo-Rady et al., 2020). Moreover, the reduction of autophagy due to C9orf72 loss impaired the clearance of neurotoxic DPRs in cellular models (Boivin et al., 2020). In keeping with this, two studies demonstrated that reduction or loss of C9orf72 caused or exacerbated motor deficit in C9BAC mice (Shao et al., 2019; Zhu et al., 2020). Interestingly, Zhu and colleagues reported neurodegeneration and gliosis in hippocampus, but not spinal cord.

All these models strongly sustained the idea that the reduction of C9orf72 levels synergise with C9HRE gain of toxicity.
AIM OF THE THESIS

Chapter 3
The HRE in the intronic region of the C9orf72 gene is the most prevalent genetic cause of both familial and sporadic forms of ALS (DeJesus-Hernandez et al., 2011; Renton et al., 2011; Majounie et al., 2012; Ash et al., 2013). Three non-mutually exclusive mechanisms have been proposed through which the mutation could cause neurodegeneration. First, a GOF might result from the formation of foci from repeat RNA that sequester RNA-binding proteins, subtracting them to their physiological function. A second GOF mechanism was linked to the accumulation of DPRs generated by RAN translation of the HRE. Third, a LOF mechanism might result from the haploinsufficiency of C9orf72 protein, due to reduction of the protein level. Several KD and KO models have been generated to study the function of the C9orf72 protein and the effect of its depletion, in order to understand the contribution of haploinsufficiency to ALS. C. elegans and zebrafish models of C9orf72 LOF showed neurodegeneration and motor deficits (Ciura et al., 2013; Therrien et al., 2013). In contrast, constitutive C9orf72 KO mouse models exhibited a general inflammatory state, immune dysfunction, and altered autophagic-lysosomal pathway, but did not display any overt neurodegenerative phenotype (Atanasio et al., 2016; Burberry et al., 2016; O’Rourke et al., 2016; Sudria-Lopez et al., 2016). However, a deep characterization of the C9orf72 LOF within the neuromuscular system, particularly the peripheral compartment, mainly affected by ALS, is still lacking.

Several pieces of evidence demonstrated that C9orf72 LOF synergizes with other toxic stimuli (Sellier et al., 2016), including the GOF resulting from DPRs (Shi et al., 2018; Abo-Rady et al., 2020; Boivin et al., 2020), to induce neuronal dysfunction and degeneration (Sellier et al., 2016; Lall et al., 2021). Moreover, it has been widely demonstrated that C9orf72 mutation often occurs in combination with other ALS or FTD-linked genes, driving the disease presentation in the direction of one or the other pathology, and influencing the disease progression and severity (Nguyen, Van Broeckhoven and van der Zee, 2018).

For all these reasons, my PhD project aimed to:
1) study the expression and cellular distribution of the C9orf72 protein in the neuromuscular system (spinal cord, sciatic nerve, and skeletal muscle) of mice

2) examine the effect of the C9orf72 ablation within the neuromuscular system, with a major regard to the peripheral compartment, in constitutive C9orf72 KO (C9/−) mice

3) investigate whether, and through which mechanism, the C9orf72 LOF may act as a modulator of ALS by breeding C9/− and SOD1G93A mice.
MATERIAL AND METHODS

Chapter 4
4.1 MURINE MODELS

GenOway company (France) was commissioned to generate a line of heterozygous floxed mice by flanking exons from 3 to 4 of one C9orf72 allele with loxP sites. These exons contained the two ATGs start codons. Floxed mice were bred with C57BL/6J deleter mice that constitutively express Cre-recombinase under the cytomegalovirus (CMV) promoter. The resulting heterozygous mice with a mixed genetic background 129sv and C57BL/6J carrying one KO allele were backcrossed with C57BL/6J female mice for 5 generations (speed congenic) to obtain C57BL/6J congenic heterozygous C9orf72 KO mice, avoiding genetic variability. Heterozygous C9orf72 KO mice were interbred to obtain both the homozygous KO mice (C9−/) that did not express the C9orf72 gene and the relative WT controls with the C57BL/6J genetic background.

Qualitative PCR of the DNA from tail biopsy confirmed the excision of exons 3 and 4.

Figure 4.1: Generation of C57BL/6J C9orf72 KO mouse model. Grey rectangles represent C9orf72 non-coding exon portions, black rectangles indicate coding sequences, solid lines represent chromosome sequences. The neomycin (NEO) positive selection cassette is indicated. loxP sites are represented by green triangles and flippase recognition target (FRT) sites by double red triangles. (A) A targeting vector containing homology regions in 129sv background, two loxP sites flanking C9orf72 exons 3 and 4, and a positive selection neomycin gene flanked by FRT sites was constructed. The targeting vector inserts in the C9orf72 endogenous locus by homologous recombination in 129sv embryonic stem (ES) cells. (B) Recombined ES cells were injected into a C57BL/6J blastocyst, that was then re-implanted into OF1 pseudo-pregnant females. The resulting offspring is a chimera of two different cell types (129sv ES cell-derived cells and C57BL/6J) and the contribution of the recombinant ES cells to each individual was assessed using coat colour markers (129s agouti, C57BL/6J black). (C) Male chimeras with a chimerism rate above 50%
were mated with C57BL/6J flippase (FLP) deleter mice to excise the neomycin selection cassette. (D) Then the resulting progeny was bred with C57BL/6J Cre-deleter mice to excise the loxP flanked sequence and generate heterozygous mice carrying the KO allele. The presence of agouti pups in the F1 generation was the evidence of the germline transmission of the ES cells.

C9orf72 female mice were then crossed with C57BL/6J SOD1\textsuperscript{G93A} (Stock No: 004435; Jackson Laboratories, USA) male mice, expressing approximately 20 copies of human mutant SOD1 with a Gly93Ala substitution (Mark E. Gurney et al., 1994), to obtain transgenic mice null for the C9orf72 gene. Female SOD1\textsuperscript{G93A} mice with or without C9orf72 were used for the analysis.


The animals were housed under Specific Pathogen Free (SPF) conditions at a temperature of 22±1 °C, the relative humidity of 55±10% and 12 hours light/dark schedule, 5 per cage. Food (standard pellets; Altromin, MT, Rieper) and water were supplied \textit{ad libitum}. When the animals showed the first symptoms of the disease (reduced hindlimb adduction after tail suspension), the food was placed at the bottom of the cage and the access to water was facilitated using a bottle with a longer spout. The protocol for the use of laboratory animals was approved by the Italian Ministry of Health and by an internal ethical committee.

4.2 GENOTYPING

Genotyping was performed through Polymerase Chain Reaction (PCR) using genomic DNA extracted from tail biopsies collected from the mice after weaning, within the twenty-first day of age. In the present study the PCR assay was used to genotype mice for the C9orf72 or SOD1\textsuperscript{G93A} gene.
4.2.1 Polymerase chain reaction (PCR) for C9orf72

Caudal biopsies were completely digested using the Maxwell® 16 System, according to the manufacturer’s instruction. The DNA extracted from the tail of each animal was used for qualitative PCR. The amplification solution is composed of Buffer 3 1X (Roche), deoxynucleotide triphosphates (dNTPs) 250 µM (Promega), forward primer (5’- GCCCTCCCCTTCTGTTTTGTCT-3’) 0.5 µM (Invitrogen), reverse primer (5’- AGACGGCAACTCTGTGAGCATAGTTG -3’) 0.5 µM, Expand Long Template Polymerase (Roche) 2.6 U in a final volume of 10 µl. The MJ Research PTC-200 Thermocycler (Bio-Rad Laboratories, Hercules, CA, USA) carried out an amplification program set with the following parameters:

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<th>FUNCTION</th>
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<th>TEMPERATURE</th>
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<tbody>
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<td>2 min</td>
<td>94 °C</td>
</tr>
<tr>
<td>DNA denaturation</td>
<td>30 sec</td>
<td>94 °C</td>
</tr>
<tr>
<td>Primers annealing</td>
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<td>65 °C</td>
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<tr>
<td>Amplification</td>
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<tr>
<td>Final elongation</td>
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<td>Sample maintaining</td>
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(Table 4.1: PCR assay program for C9orf72 gene.)

Each PCR assay requires the presence of a homozygous KO, a heterozygous KO, a WT, and a blank control to verify the efficiency and the cleanliness of the amplification process. The PCR reaction products, added with 2 µl/sample of Dye Blue dye (Biolabs), are resolved in 1% agarose gel (Euroclone), containing 1% GelRed (Biotium) Nucleic Acid Gel Stain in Tris-Acetate-EDTA (40 mM Tris, 0.35% vol/vol acid acetic, 1 mM EDTA). For the evaluation of the molecular weight of the bands, the Ladder 0.1-10 Kb (Biolabs) is loaded. The electrophoretic run is approximately 45 minutes at 140 Volts. The size of the specific bands is revealed by placing the gel on a UV
transilluminator and comparing the bands of the samples with those of the controls. The amplification product results in the presence of one or two bands of the following size (Figure 4.2):

- $C9^{+/+}$ (WT): 3 Kb;
- $C9^{+/−}$ (heterozygous KO): 3 Kb and 1 Kb;
- $C9^{-/-}$ (KO): 1 Kb.

![Figure 4.2: Detection of C9orf72 gene by qualitative PCR](image)

### 4.2.2 Polymerase chain reaction (PCR) for hSOD1

The samples were completely digested by overnight incubation at 55 °C in Direct-PCR Lysis Buffer (Viagen Biotech, Los Angeles, California, USA) containing 0.1 μg/μl of Proteinase K (Promega). The following day, the lysates were incubated at 85 °C for 30-45 min, to inactivate Proteinase K, and then diluted with a proportion of 1:6 in sterile H$_2$O. The DNA of each animal was used as a substrate for qualitative PCR in a mix containing 1X PCR Buffer, Go-Taq DNA Polymerase (0.25U), dNTPs (250μM each), specific forward (5’-3’, CATCAGCCCTAATCCATCTGA) and reverse (5’-3’, CGCGACTAACAATCAAAGTGA) primers (0.5μM each) in a final volume of 10 μl. All reagents were purchased by Promega, except for primers that were synthesised by Invitrogen. PCR is performed using an MJ Research PTC-200 Thermocycler (Bio-Rad Laboratories, Hercules, CA, USA). The conditions for PCR are set as follow:
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<td></td>
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<td>72 °C</td>
</tr>
<tr>
<td>Final elongation</td>
<td>7 min</td>
<td>72 °C</td>
</tr>
<tr>
<td>Sample maintaining</td>
<td>-</td>
<td>4 °C</td>
</tr>
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</table>

*Table 4.2: PCR assay program for the detection of human SOD1 transgene.*

Each PCR assay requires the presence of positive control, negative control, and a blank sample to validate the efficiency and the cleanliness of the amplification process. Amplicons are resolved in 1% agarose gel containing GelRed Nucleic Acid Gel Stain 1:30000 in Tris-Acetate-EDTA (40 mM Tris, 0.35% vol/vol acid acetic, 1 mM EDTA). The presence of the specific band for the transgene is revealed by placing the gel on a UV transilluminator and comparing the bands of the samples with those of the positive control (Figure 4.3).

*Figure 4.3: Detection of human SOD1<sup>G93A</sup> transgene by qualitative PCR.*

### 4.3 EVALUATION OF THE DISEASE PROGRESSION AND END POINT

The onset and disease progression have been evaluated twice a week from 11 to 22 weeks of age, after a training period of 2 weeks (n=18 per experimental group). The parameters measured included bodyweight and latency to fall from a grid (inverted grid test) as previously described...
(Tortarolo et al., 2015). Bodyweight was monitored before behavioural tests. For the inverted grid test to measure the four-limb resistance, mice were placed on a horizontal metallic grid that was slightly shaken to force the mouse grip and then inverted, exploiting their natural propensity to cling. The latency to fall of each mouse was recorded. The test ends after 90 seconds; in case of failure, the measurement was repeated three times. The best performance of the sessions was considered for the statistical analysis.

A cohort of SOD1<sup>G93A</sup> mice (n=12 per experimental group) was euthanized at the end stage of the disease considered when the mice were unable to right themselves within 10 s after being placed on each side.

### 4.4 HISTOLOGICAL ANALYSIS

#### 4.4.1 Tissue collection

For the histological analyses on the lumbar spinal cord, animals (n=4 per experimental group) were deeply anaesthetized with ketamine hydrochloride (150 mg/kg) and medetomidine (2 mg/kg) followed by intracardiac perfusion with phosphate buffer saline (PBS) 0.1 M pH=7.4 and then by 4% paraformaldehyde (PFA, Merck) in PBS. Spinal cords were removed and post-fixed overnight (O/N) in 4% PFA in PBS 0.1 M (Tortarolo et al., 2015). Tissues were dehydrated in 30% sucrose solution in PBS until they sank, embedded in cryostat medium OCT (Sakura, Zoeterwounde, The Netherlands) and then frozen in N-pentane at -45 °C for 3 minutes and conserved at -80 °C. Thirty μm coronal sections were obtained by cutting lumbar spinal cords (LSC) (L2-L5) using a cryostat at -20 °C. The spinal cord sections were collected in PBS 0.01 M and transferred in a cryo-preservation solution (NaH<sub>2</sub>PO<sub>4</sub>, Na<sub>2</sub>HPO<sub>4</sub>, MilliQ, H<sub>2</sub>O, Ethylene Glycol, Glycerol) and stored at -20 °C until use.

For the histological analyses on the sciatic nerve (SN) and tibialis anterior (TA) muscle, animals (n=4 per experimental groups) were perfused with PBS 0.1 M, the tissues were dissected and
respectively frozen on dry-ice or snap-frozen in cooled isopentane. SN (14 μm) and TA muscle (20 μm) sagittal sections were obtained using a cryostat at -20 °C, placed on poly-lysine coated slides and stored at -20 °C until use.

4.4.2 Chat immunofluorescence and MN count in lumbar spinal cord

For MN counting, 20 serial coronal sections (1 every 10) from LSC L2-L5 were analysed. After blocking the non-specific binding sites by incubation with a solution containing 10% normal donkey serum (NDS) and 0.3% Triton X-100 (Sigma) in phosphate-buffered saline (PBS) 0.01 M, the sections were incubated with goat anti-choline acetyltransferase (ChAT) primary antibody (1:200, Merck Millipore AB144P) diluted in a solution containing 5% NDS and 0.1% Triton X-100 O/N at 4 °C. Sections were then washed and incubated with Alexa Fluor 488 donkey anti-goat secondary antibody (1:500, Abcam) diluted in 1% NDS in PBS 0.01 M 1 h at room temperature (RT). After 3 washes in PBS 0.01 M, the sections were mounted on a glass slide and then covered with the coverslip using FluorSaveTM (Calbiochem).

4.4.3 C9orf72 immunofluorescence with tyramide protocol in lumbar spinal cord

For C9orf72 immunofluorescence on LSC, an indirect immunofluorescence protocol with tyramide amplification was applied (Peviani et al., 2007). The sections were pretreated with 1% hydrogen peroxide in TBS (0.1 M Tris–HCl, 0.14 M NaCl, pH 7.4) to inactivate endogenous peroxidases. The blocking of the non-specific binding sites was performed with a solution containing 10% normal goat serum (NGS) and 0.1% Triton X-100 in PBS 0.01 M. Then the sections were incubated with mouse anti-C9orf72 primary antibody (1:200, GeneTex cod. GTX634482) diluted in a solution containing 1% NGS and 0.1% Triton X-100 in PBS 0.01M, O/N at 4 °C. After three washes in PBS 0.01M, the sections were incubated with biotinylated secondary antibody (1:200, Vectastain kit, Vector Laboratories) for 1 hour, washed with TNT (TBS 0.1 M; Triton 0.05%) and incubated for 90 min in TNB (TBS 0.1 M, Blocking reagent 0.5%). Subsequently, the
sections were washed with TNT and treated with streptavidin (1:100, Perkin Elmer, Waltham, MA, USA) in TNB for 30 min, washed with TNT and incubated for 10 min with tyramide conjugated with Cy5 fluorochrome (1:500, Perkin Elmer) in amplification diluent (boric acid 0.1M pH 8.5, hydrogen peroxide 0.003%). Processed sections were mounted on slides and coverslipped with Fluorsave.

4.4.4 Immunofluorescence on lumbar spinal cord

Immunofluorescence was performed on 6 coronal LSC sections (1 every 10) per animal. After blocking the non-specific binding sites by incubation with a solution containing 10% NGS and 0.1% Triton X-100 in PBS 0.01 M, the sections were incubated with the primary antibodies ON at 4 °C, diluted in a solution containing 1% NGS and 0.1% Triton X-100 in PBS 0.01M. The primary antibodies listed in table 4.3 were used. After three washes in PBS 0.01 M, the sections were incubated 1h at RT with appropriate secondary antibodies conjugated to fluorochromes with various wavelengths (Alexa Fluor® 488, 594 or 647, Life Technologies), diluted 1:500 in 1% NGS in PBS 0.01 M. Neurotrace, that recognises the Nissl substance present in the neurons perikaryon, was directly conjugated with Alexa Fluor® 647, diluted 1:500 in PBS 0.01 M at RT and incubated for 30’. After 3 washes in PBS 0.01 M, the sections were mounted on a glass slide and then covered with the coverslip using FluorSaveTM (Calbiochem).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host species</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
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<tr>
<td>PhosphoDetect Anti-Neurofilament H</td>
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<td>1:1000</td>
<td>Sternberger Inc.</td>
</tr>
<tr>
<td>(SMI31)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Ionized calcium-Binding Adapter molecule 1</td>
<td>rabbit</td>
<td>1:500</td>
<td>Wako</td>
</tr>
<tr>
<td>(IBA1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macrosialin (CD68)</td>
<td>rat</td>
<td>1:200</td>
<td>Biorad</td>
</tr>
<tr>
<td>Glial Fibrillary Acid Protein (GFAP)</td>
<td>rabbit</td>
<td>1:2000</td>
<td>Dako</td>
</tr>
</tbody>
</table>
Table 4.3: List of the antibody used for the immunohistochemistry analyses in LSC.

4.4.5 Immunofluorescence on sciatic nerve

Immunofluorescence was performed on 4 sagittal SN sections (1 every 4) per animal. The blocking of the non-specific binding sites was performed with a solution containing 10% NGS and 0.1% Triton X-100 in PBS 0.01 M. Then the sections were treated with unconjugated Fab fragment (1:50, Jackson ImmunoResearch) diluted in PBS 0.01 M for 1h to block endogenous immunoglobulin and suppress the off-target signal. After three washes, the sections were incubated with the primary antibodies ON at 4 °C, diluted in a solution containing 1% NGS and 0.1% Triton X-100 in PBS 0.01M. The primary antibodies listed in Table 4.4 were used. After three washes in PBS 0.01 M, the sections were incubated 1h at RT with appropriate secondary antibodies conjugated to fluorochromes with various wavelengths (Alexa Fluor® 488, 594 or 647, Life Technologies), diluted 1:500 in 1% NGS in PBS 0.01 M.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host species</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosome 9 open reading frame 72 (C9orf72)</td>
<td>mouse</td>
<td>1:100</td>
<td>GeneTex</td>
</tr>
<tr>
<td>Glial Fibrillary Acid Protein (GFAP)</td>
<td>rabbit</td>
<td>1:2000</td>
<td>Dako</td>
</tr>
<tr>
<td>Glial Fibrillary Acid Protein (GFAP)</td>
<td>mouse</td>
<td>1:2500</td>
<td>Merk Millipore</td>
</tr>
<tr>
<td>Growth Associated Protein 43 (GAP43)</td>
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<td>Millipore</td>
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<td>Abcam</td>
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<td>----------------</td>
<td></td>
</tr>
<tr>
<td>Low-molecular Mass Protein 7 (LMP7)</td>
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<td>Abcam</td>
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<td>Abcam</td>
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<tr>
<td>S100β</td>
<td>rabbit</td>
<td>1:200</td>
<td>Swant</td>
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</table>

**Table 4.4:** List of the antibody used for the immunohistochemistry analyses in SN.

### 4.4.6 Neuromuscular junction in tibialis anterior muscle

Detection of the neuromuscular junctions (NMJs) denervation was made in 6 sagittal sections (1 every 8) of TA muscle. Sections were fixed in acetone for 10 min, incubated in a blocking solution (10% NGS, 5% BSA, 1% Triton-X 100 in 0.01 M PBS) for 1 h at 22°C and then left ON at 4°C with anti-synaptic vesicle 2 (SV2) and anti-NF-M (clone 2H3) primary antibodies (mouse, 1:100 and 1:50, respectively, Hybridoma Bank) in 10% NGS, 5% BSA, 1% Triton-X 100 in 0.01 M PBS. The sections were then incubated with goat anti-mouse 647 (1:500, Alexa Fluor® Dyes, Life Technologies) secondary antibody and with bungarotoxin (BTX) (1:500, Invitrogen) conjugated with Alexa Fluor® 488 (Life Technologies).

### 4.4.7 Image acquisition and analysis

The sections were analysed with an IX81 microscope equipped with a confocal scan unit FV500 with three laser lines: Argon-Krypton (488 nm), Helium-Neon red (646 nm), and Helium-Neon green (532 nm) and a UV diode (Olympus, Tokyo, Japan).

For the MN count, only the ChAT-positive neurons with a cell body area ≥ of 400 μm² located in a congruent position (αMNs) were incorporated into the count (Friese et al., 2009). The number of MN was calculated for each hemisection and the means used for statistical analysis. The analysis was performed at 20X magnification using the free software ImageJ (http://imagej.nih.gov/ij/), previously calibrated.
The quantification of GFAP, IBA1 and CD68 intensity was carried out by the same operator, by determining the mean grey value of fluorescent signals in the ventral horns in the relative surface occupied by the staining. The quantification was performed at 20X magnification for GFAP, IBA1 and CD68 immunostaining, using the free software ImageJ (http://imagej.nih.gov/ij/).

Dual-staining co-localization of pre- and post-synaptic markers was analyzed at 20x magnification. Innervated neuromuscular junctions were identified by totally or partially co-localization of SV2/2H3 with BTX. Plaques marked with BTX only were considered denervated and expressed as the percentage of the total plaques.

4.5 WESTERN BLOT

4.5.1 Tissue collection

Mice (n=6 per experimental group) were anaesthetised with a mix of ketamine (1.75 mg/Kg) and medetomidine (1 mg/Kg) and transcardially perfused with 50ml of 0.1M PBS pH7.4. Following the blood removal, the spinal cord was fluxed from the vertebral column employing sterile physiological solution (0.9% NaCl) and dissected in the three main segments (i.e. cervical, thoracic and lumbar). Spinal cord segments and nerve were immediately frozen on dry ice, while the tibialis anterior muscles were dissected out and immediately frozen in cooled isopentane. All tissues collected were stored at -80°C until required.

4.5.2 Protein extraction and quantification

The LSCs were homogenized with a Teflon potter in 10 volumes (w/v) of buffer, 15 mM Tris-HCl, pH 7.6, 1 mM DTT, 0.25 M sucrose, 1 mM MgCl2, 2.5 mM EDTA, 1 mM EGTA, 0.25 M sodium orthovanadate, 2 mM sodium pyrophosphate, 25 mM NaF, 5 µM MG132, and protease inhibitors cocktail (Roche; 1 tablet/10 ml), essentially as described in Basso et al., 2009 (Basso
Samples were centrifuged at 10000 RCF at 4°C for 15 min and supernatant 1 was collected in a new tube. The pellet was suspended in ice-cold homogenization buffer with 2% of Triton™ X-100 and 150 mM KCl, sonicated and shaken for 1 h at 4°C. The samples were then centrifuged twice at 10000 RCF at 4°C for 10 min to obtain the Triton-resistant fraction pellet and supernatant 2. Supernatants 1 and 2 were pooled, as the Triton-soluble fraction, and analysed by Western blot. The fraction insoluble in 2% of Triton X-100 was resuspended in 50 mM Tris-HCl, 1 mM DTT and 2% SDS.

The SNs were homogenized with a Teflon potter in ice-cold homogenization buffer (20 mM Tris-HCl pH 7.4, 2% Triton X-100 1%, 150 mM NaCl, 1 mM EDTA, 5 mM MgCl₂, anhydrous glycerol 10%, protease and phosphates inhibitor cocktail, Roche), then sonicated and centrifuged at 13000 RCF for 30 min at 4 °C. The supernatant was collected and stored at -80 °C until use.

The TA muscles were homogenized with a Teflon potter in ice-cold RIPA buffer (Abcam) with protease and phosphates inhibitor cocktail (Roche), then sonicated, shaken for 30’ at 4 °C and centrifuged at 13000 RCF for 20’ R.T. The supernatant was collected and stored at -80 °C until use.

Protein extracts were quantified using the Pierce™ BCA Protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA) with bovine serum albumin (BSA) as standard. The absorbance of the solution was read at 562nm wavelength using the Infinite®200 multimode reader (Tecan). A simple linear regression analysis of the BSA curve was performed to which the absorbance of samples was interpolated to estimate the protein concentration of samples.

4.5.3 Mono-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Prior to electrophoresis, samples were boiled in SDS sample buffer (6% SDS, 0.1M DTT, 20% glycerol, 0.125M Tris/HCl pH 6.8, 0.025% blue bromophenol) at 95 °C for 5’. Equal amounts of total proteins were separated on Tris-glycine polyacrylamide TGX precast gels and
electroblotted onto a polyvinylidene difluoride (PVDF) membrane using a BioRad midi transfer
system (BioRad Laboratories, Hercules, CA, USA).

For the immunoblotting protocol, membranes were incubated in blocking buffer composed of
5% BSA dissolved in 0.1% Tween20 in Tris-buffered saline pH 7.4 (TBS-T) solution for 1h and then
probed O/N at 4°C with the primary antibody diluted in 3% BSA in TBS-T. The primary antibodies
listed in Table 4.5 were used. After three washes of 5 min in TBS-T, membranes were incubated
with the opportunely diluted HRP-conjugated secondary antibody (Santa Cruz) for 1h at room
temperature (RT) and finally washed three times per 5 min in TBS-T. Immunoreactivity was
visualised with Immobilon Forte Western HRP substrate (Millipore, Billerica, MA, USA) at
ChemiDoc XRS (Biorad).

The optical density of the blots was measured with Image Lab 6.1 software (BioRad). In the first
part of the study, the normalization was made to β-actin, later we acquired the new Biorad Stain-
free technology that allow us to perform accurate total protein normalisation without the need
to perform extra staining steps.

<table>
<thead>
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<th>Antibody</th>
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</tr>
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<td>Chemicon</td>
</tr>
<tr>
<td>Chromosome 9 open reading frame 72 (C9orf72)</td>
<td>mouse</td>
<td>1:100</td>
<td>GeneTex</td>
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<td>Glial Fibrillary Acid Protein (GFAP)</td>
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<td>1:2000</td>
<td>Dako</td>
</tr>
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<td>Glial Fibrillary Acid Protein (GFAP)</td>
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<td>1:2500</td>
<td>Merk Millipore</td>
</tr>
<tr>
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<td>Upstate</td>
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<td>LC3B</td>
<td>rabbit</td>
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<td>Low-molecular Mass Protein 7 (LMP7)</td>
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<td>Abcam</td>
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Table 4.5: List of the antibody used for the Western blot analyses.

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<td>Neurofilament heavy polypeptide (NF200)</td>
<td>rabbit</td>
<td>1:4000</td>
<td>Abcam</td>
</tr>
<tr>
<td>PhosphoDetect Anti-Neurofilament H (SMI31)</td>
<td>rabbit</td>
<td>1:1000</td>
<td>Sternberger Inc.</td>
</tr>
<tr>
<td>P62</td>
<td>mouse</td>
<td>1:500</td>
<td>Abcam</td>
</tr>
<tr>
<td>P75 neurotrophin receptor (p75RT)</td>
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<td>Abcam</td>
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</tbody>
</table>

4.6 REAL-TIME PCR (REAL-TIME PCR)

4.6.1 Tissue collection

Murine tissues were collected as described in paragraph 4.6.1

4.6.2 RNA extraction and cDNA synthesis

The total RNA from spinal cord, nerves and muscles was extracted using the TRIzol™ method (Invitrogen), purified with Ambion PureLink RNA columns (Thermo Fisher Scientific) according to the manufacturer's recommendation, and suspended in RNAse-free water. Extracted RNA was quantified with Nanodrop™ Spectrophotometers (Thermo Fisher Scientific). Prior to the retro-transcription, RNA samples were treated with DNase I (Invitrogen) to avoid genomic DNA contamination, and the reverse transcription was done with the High-Capacity cDNA Reverse Transcription Kit (Invitrogen).

The quality of the cDNA obtained was tested with a qualitative PCR using primers for the murine Superoxide Dismutase 2 (SOD2) gene (forward: TGCACAGGAGGTTCAATGGTG; reverse: TAGAGCAGGCAGCAATCTGT).
4.6.3 Gene expression analysis

Real-time PCR was performed using the Taq Man Gene expression assay (Applied Biosystems) following the manufacturer’s instructions, on cDNA specimens in triplicate, using SensiFAST Probe Hi-ROX Kit (Aurogene) and 1x mix containing specific probes (Thermo Fisher Scientific).

The TaqMan™ probes used in this study are listed in table 4.6.

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<tr>
<th>Probe</th>
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</tr>
</thead>
<tbody>
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<tr>
<td>Beta-actin (β actin)</td>
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<tr>
<td>CD4</td>
<td>Mm00442754_m1</td>
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<tr>
<td>CD8a</td>
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<td>Mm01216829_m1</td>
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<tr>
<td>Tumor Necrosis Factor alpha (TNFα)</td>
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Table 4.6: List of the TaqMan™ probes used for Real-time analysis

Relative quantification was calculated from the ratio between the cycle number (Ct) at which the signal crossed a threshold set within the logarithmic phase of the given gene and that of the reference β-actin gene. Mean values of the triplicate results for each animal were used as individual data for 2-ΔΔCt statistical analysis.
4.7 STATISTICAL ANALYSIS

Statistical analyses were performed using the Prism software (Graph Pad Prism 8, Graph Pad Inc), USA. Values are reported as mean ± SEM. Body weight and inverted grid test data were analyzed using ANOVA for repeated measures, followed by Bonferroni Post Hoc test. The onset of symptoms and survival were analysed by Log-rank Mantel-Cox test and Kaplan-Meier plots were generated. Previous Shapiro-Wilk omnibus normality test, the data were statistically analysed using Student’s t test for two groups or One-way ANOVA followed by Fisher’s LSD post Hoc test for three groups. For all analyses, a p-value < 0.05 was considered statistically significant.
RESULTS

Chapter 5

Expression and distribution of C9orf72

in the neuromuscular system of mice
5.1 BACKGROUND AND AIM

The HRE in the C9orf72 gene is the most common cause of both familial and sporadic forms of ALS (DeJesus-Hernandez et al., 2011; Pearson et al., 2011; Renton et al., 2011). Both LOF and GOF mechanisms have been proposed to mediate C9ALS. However, the pathogenic pathways are not fully elucidated, nor the expression, localisation and function of the C9orf72 protein within the neuromuscular system.

Several studies have investigated the expression of C9orf72 in human and mouse tissues, and both the transcript and protein have been found predominantly within CNS (brain and spinal cord) and at lower level in non-nervous tissues (Suzuki et al., 2013; Langseth et al., 2017). However, there has been a lack of consensus about the expression of C9orf72 in different neuronal and non-neuronal cell subtypes, partly due to the limitations of the techniques used and the poor specificity of the available commercial antibodies. Moreover, the distribution of C9orf72 in the PNS and skeletal muscle has never been considered so far, although these compartments are primary targets in the pathogenesis of ALS.

Thus, understanding the expression pattern of C9orf72 in the mouse neuromuscular system may provide important clue for outlining the pathogenesis of C9ALS. Recently, Laflamme and colleagues (Laflamme et al., 2019) identified the most selective C9orf72 antibody for immunoblot and immunohistochemical analyses; thus, we decided to test it in the CNS (LSC), PNS (SN) and skeletal muscle (TA) of WT (C9+/+) mice and verified its selectivity in constitutive C9orf72 KO (C9−/−) mice.
5.2 RESULTS

5.2.1 Expression of C9orf72 transcript and protein and distribution in the mouse neuromuscular system

Real-time and Western blot analyses respectively showed the presence of C9orf72 transcript and protein in LSC, SN and TA muscle of C9^{+/+} mice (Figure 5.1). As expected, the transcript and the protein were absent in all the tissues derived from C9^{-/-} animals.

![Figure 5.1: C9orf72 is expressed in lumbar spinal cord, sciatic nerve, and tibialis anterior muscle of mice. Real-time PCR (A, B, C, D) and Western blot analyses (E, F, G) showed the expression of C9orf72 in LSC (A, D), SN (B, E) and TA muscle of C9^{+/+} but not C9^{-/-} mice. The Ct values (D) indicated that C9orf72 transcript levels are higher in LSC compared to SN and TA muscle. Real-time data are normalized on β-actin and expressed as mean ± SEM (n=6 per experimental group). ****p<0.0001 by Student’s t-test.]

The immunofluorescence on the LSC (Figure 5.2A) and SN (Figure 5.3A) of C9^{+/+} mice showed a specific signal for C9orf72, which was absent in C9^{-/-} tissues. The colocalisation study performed in the LSC showed that C9orf72 was highly expressed in the cytoplasm of MNs, stained with the Choline Acetyltransferase (ChAT) (Figure 5.2A), in IBA1+ microglia in the grey matter (Figure 5.2C) and S100β+ oligodendrocytes in the white matter (Figure 5.2E) of C9^{+/+} LSC. In spinal cord, S100β is also used as a marker of astrocytes; however, it has been recently demonstrated that nearly all the S100β+ cells are myelinating oligodendrocytes in the white matter of the spinal cord (Du et al., 2021). No colocalisation was detected between C9orf72 and the marker of astrocytes glial fibrillary acid protein (GFAP) (Figure 5.2D).
Figure 5.2: C9orf72 is expressed by MNs, microglia and oligodendrocytes but not astrocytes in spinal cord. Immunofluorescence analyses on LSC coronal sections showed the expression of C9orf72 in C9+/+ but not C9−/− mice (A). Specifically, C9orf72 colocalized with the MN marker Choline Acetyltransferase (ChAT) (B), microglial marker Iba1 (C), but not astrocyte marker GFAP (D) in the grey matter, and with the oligodendrocyte marker S100β in the white matter (E). Inset: zoom 3x.
In the SN, C9orf72 was found in colocalization with the calcium binding protein S100β (Figure 5.3B), marker of Schwann cells (SCs), but not with the heavy neurofilament (NF200) (Figure 5.3C), the main component of the axon cytoskeleton.

![Image of Figure 5.3: C9orf72 expression in Schwann cells and axons.](image)

**Figure 5.3: C9orf72 is expressed by Schwann cells but not in axons in sciatic nerve of mice.** Immunofluorescence analyses on SN longitudinal sections showed the expression of C9orf72 in C9+/+ but not C9−/− mice (A). Specifically, C9orf72 colocalized with the Schwann cell marker S100β (B), but not with axonal marker NF200 (C).

Disappointingly, we were unable to determine the distribution of C9orf72 in the TA muscle. Indeed, we obtained an immunofluorescent signal in the TA muscle of both C9+/+ and C9−/− mice (Figure 5.4), probably due to the abundant presence of connective tissue to which antibodies often bind in a non-specific manner.
Figure 5.4: Immunofluorescence analysis on the TA muscle longitudinal sections showed C9orf72 immunofluorescent signal in both C9\textsuperscript{+/+} and C9\textsuperscript{-/-} mice, suggesting its non-specificity.
5.3 DISCUSSION

For the first time, this study showed that both the C9orf72 transcript and protein are expressed not only within the CNS but also in the peripheral tract of the mouse neuromuscular system (SN and TA muscle), which is early affected by ALS (Nardo et al., 2016; Gentile et al., 2019). So far, the expression of C9orf72 in the SN was never investigated. We found that C9orf72 localised in SCs but not in peripheral axons.

We demonstrated that both C9orf72 transcript and protein are expressed in the TA muscle of mice, although we were not able to determine the precise distribution of the protein in this tissue through immunofluorescence. Indeed, despite our efforts, we did not obtain a clear and specific signal for C9orf72. In contrast with our result, a previous work (Suzuki et al., 2013) showed that C9orf72 was not expressed in the TA muscle. The cause of this discrepancy is unknown but could be due to the use of heterozygous LacZ reporter mice, which are unable to detect low levels of gene expression (Sundararajan et al., 2012).

As concerned the expression of C9orf72 in the CNS, we confirmed that both C9orf72 mRNA and protein are abundantly expressed in the mouse lumbar spinal cord, where immunofluorescence showed high protein expression in MN perikarya, modest expression in microglia and the absence of a C9orf72-positive signal in astrocyte. Moreover, we found that C9orf72 is expressed in mature myelinating oligodendrocytes. While there is a strong consensus about the C9orf72 expression in spinal MNs, its production by astrocytes, microglia and oligodendrocytes is debated. Suzuki and colleagues (Suzuki et al., 2013) reported that microglia and astrocytes were respectively mostly and entirely β-gal-negative in the spinal cord of C9orf72-LacZ knock-in mice. By contrast, Langseth et al. (2017) found few β-gal-positive astrocytes and microglial cells using the same reporter mice. Although these studies suggested that microglia express low levels of C9orf72, other studies demonstrated that microglia showed the highest levels of C9orf72 transcript in the brain. These findings highlight the variability of C9orf72 expression across the population of microglial cells in the brain and spinal cord (Langseth et al., 2017).
Moreover, while the study of Suzuki and colleagues did not observe any β-gal-positive signal in the white matter of the spinal cord, other studies reported the C9orf72 expression in the spinal oligodendrocytes (O’Rourke et al., 2015; Jiang et al., 2016; Langseth et al., 2017). This result suggests that C9orf72 may have an essential role in the correct functioning of these cells. SCs are fundamental to maintain the homeostasis of the peripheral nervous system (PNS), providing trophic support and myelination to axons. During ALS, SC function is disrupted since the pre-symptomatic stage, playing an active role in the pathogenesis and progression of the disease (Nardo et al., 2016). Therefore, we cannot exclude that the loss of C9orf72 could alter their physiological function, predisposing to ALS.
RESULTS

Chapter 6

The effect of constitutive C9orf72 ablation

in the neuromuscular system of mice
6.1 BACKGROUND AND AIM

The C9orf72 LOF resulting from haploinsufficiency is one of the three non-exclusively pathogenic mechanisms through which the HRE in the intronic region of the C9orf72 gene may induce neuronal dysfunction and death in ALS, together with the GOF resulting from the formation of both toxic RNA foci and DPRs (Majounie et al., 2012).

The contribution of the GOF mechanisms has been extensively investigated both in vitro and in vivo. However, the emerging evidence is rather inconclusive, especially as concerns the implication for ALS in vivo models. Although several bacterial artificial chromosome transgenic mice expressing the human C9orf72 gene (C9BAC) with various copies of HRE showed RNA foci and DPRs in the neurons (O’Rourke et al., 2015; Peters et al., 2015; Jiang et al., 2016), only one study reported a subset of C9BAC mice exhibiting TDP43 inclusion pathology, neurodegeneration, and premature death (Liu et al., 2016). However, other research groups failed in reproducing this phenotype (Mordes et al., 2020). Moreover, the only neuropathological change with strong clinic-pathological correlations and association with neurodegeneration in C9HRE carriers is TDP43 pathology (MacKenzie et al., 2013; Davidson et al., 2014; Gomez-Deza et al., 2015; Mackenzie et al., 2015), which is rarely associated with DPR pathology and RNA foci formation in the same neuron, suggesting that a direct link between these processes is unlikely (Mackenzie et al, 2014). These results point out that GOF alone is insufficient to induce neurodegeneration.

The evidence regarding the LOF is still inconsistent (Braems, Swinnen and Van Den Bosch, 2020). C. elegans and zebrafish models of C9orf72 LOF showed motor dysfunction, axonopathy and motor neurodegeneration (Ciura et al., 2013; Therrien et al., 2013). Conversely, C9orf72 KO mouse models did not exhibit any neurodegenerative phenotype, probably due to the difference in the homology of the human C9orf72 orthologues. C9orf72 KO mice displayed disruption in the autophagic-lysosomal pathway, altered inflammatory response with aberrant microglia and macrophages and high production of inflammatory cytokines, dysfunction in immune system
with expansion and infiltration of leukocytes, autoimmunity, splenomegaly and lymphadenopathy (Atanasio et al., 2016; Burberry et al., 2016; O’Rourke et al., 2016). Growing evidence suggests that the C9orf72 LOF plays a pivotal role in the mechanisms through which the GOF induces neuronal dysfunction and death in ALS. Boivin and colleagues demonstrated that when the C9HRE are embedded within the natural C9orf72 human sequence instead of the artificial ATG, DPRs are expressed at low levels and consequently display poor toxicity in neuronal cells in vitro, and only when C9orf72 is ablated, DPRs became toxic (Boivin et al., 2020). In line with this, recently Zhu and colleagues (Zhu et al., 2020) showed that the reduction or loss of C9orf72 exacerbates cognitive and motor deficits in C9BAC transgenic mice however only the hippocampal neurons were significantly degenerated by the synergistic effect of the gain of toxicity from repeat containing RNAs and the reduction in C9orf72 function, while lumbar spinal cord MNs were unaffected. Since C9BAC transgenic mice with complete loss of endogenous C9orf72 showed trends of abnormal stride length and reduced general activity in absence of MN degeneration is plausible to hypothesize that other compartment of the neuromuscular system, not investigated in the study, could be affected.

The study of both in vitro and in vivo models of LOF provided knowledge on the physiological function of C9orf72 and the contribution of its loss in ALS. Nevertheless, the effort has been focused mainly on the study of the C9orf72 role and LOF on the immune system and within brain. For these reasons, the aim of this chapter is to examine in depth the effect of the C9orf72 LOF throughout the neuromuscular system, mostly affected by ALS, with particular attention to the peripheral compartment.
6.2 RESULTS

6.2.1 C9orf72 ablation induces splenomegaly and lymphadenopathy but does not impair muscle force in mice

First, we verified whether the ablation of C9orf72 could affect the muscle force or the general health status of mice from 11 to 22 weeks of age and at one year of age (52-54 weeks). Unexpectedly, we detected an increase in body weight in C9⁻/⁻ mice compared to C9⁺/+ controls over time from 11 to 22 weeks of age (p<0.0001) (Figure 6.1A), while we observed decreased body weight in C9⁻/⁻ mice compared to C9⁺/+ controls at 52 weeks of age (p<0.05) (Figure 6.1B). No difference in the inverted grid test performance was found between C9⁻/⁻ and C9⁺/+ mice (Figure 6.1C), as previously reported by other studies (Koppers et al., 2015; Atanasio et al., 2016; O’Rourke et al., 2016). Upon dissection at 22 weeks of age, we observed marked lymphadenopathy and splenomegaly in C9⁻/⁻ mice compared to C9⁺/+ (Figure 6.1C, D), in line with other studies (Atanasio et al., 2016; Burberry et al., 2016; O’Rourke et al., 2016).

Overall, our evidence confirmed that the C9orf72 LOF induces a robust immune dysregulation but does not affect the muscle force in mice.

Figure 6.1: C9orf72 ablation induces increased body weight, splenomegaly and lymphadenopathy, but does not impair muscle force in mice. Evaluation of body weight from 11 to 22 weeks of age (n=18) (A) and at 52 weeks of age (n=5, 9) (B) in C9⁻/⁻ mice compared to C9⁺/+ controls. Evaluation of the muscle force...
by inverted grid test from 11 to 52 weeks of age (B) in C9⁻/⁻ mice compared to C9⁺/- controls (n=18). Body weight from 11 to 22W and inverted grid test data are expressed as mean ± SEM for each time point and analysed by Two-way ANOVA for repeated measure, followed by Bonferroni post-hoc test. Body weight at 52W data are expressed as mean ± SEM and analysed by Student’s t-test. *p<0.05. Visual analysis of lymphnodes and spleen (C) and evaluation of spleen weight (D) in C9⁻/⁻ mice compared to C9⁺/- controls (n=6). Data are expressed as mean ± SEM and analysed by Student’s t-test. ***P<0.001.

6.2.2 C9orf72 ablation does not affect MNs in spinal cord

In line with other groups, in chapter V we observed that C9orf72 is highly expressed in spinal MNs. Therefore, we evaluated whether the ablation of C9orf72 could affect the survival of αMNs, the main target of ALS (Conradi and Ronnevi, 1993; Lalancette-Hebert et al., 2016). For this purpose, we performed immunohistochemistry for Choline Acetyltransferase (ChAT) enzyme, a marker of MNs, and measured the number of the ChAT-positive cells in LSC of C9⁻/⁻ mice and C9⁺/- controls. Only the cells whose area was greater than 400 µm² were identified as αMN and considered for the count (Friese et al., 2009). We observed that the full ablation of C9orf72 did not affect the number of αMN (Figure 6.2A, B). However, we wondered whether the loss of C9orf72 function could induce sub-lethal alterations in MNs. Thus, we examined the distribution of the phosphorylated NF, which normally is localized in dendrites and axons, while in pathological conditions accumulates in altered neuronal structures, such as vacuolized and dystrophic axons and MN perikarya (Tortarolo et al., 2003). Immunohistochemistry for SMI31 showed no difference in phosphorylated NF distribution between C9⁻/⁻ and C9⁺/- mice (Figure 6.2C). Accordingly, Western blot analysis showed that ratio between the portion of phosphorylated neurofilament (SMI31) on the total neurofilament (NF200) was unchanged between C9⁻/⁻ and C9⁺/- mice (Figure 6.2D, E).
6.2.3 C9orf72 ablation does not affect autophagy in lumbar spinal cord of mice

Since it has been widely demonstrated that C9orf72 is involved in autophagy regulation (Farg et al., 2014; Sellier et al., 2016; Ugolino et al., 2016; Webster et al., 2016; Aoki et al., 2017; Boivin et al., 2020), we analysed the autophagy proteins p62 and LC3B. The autophagy substrate p62 shuttles ubiquitinated cargo proteins to the autophagosomes for degradation, being degraded itself (Liu et al., 2016). LC3BI is the cytosolic form of LC3B protein, which is converted in the LC3BII-lipidated form localizing to the autophagosome membrane during autophagy (Mizushima and Yoshimori, 2007; Barth, Glick and Macleod, 2010). While increasing levels of LC3BII prove only an accumulation of autophagosomes without demonstrating the autophagic degradation, decreasing or increasing levels of p62 show a higher degradation or an engulfment of autophagic flux, respectively (Mizushima and Yoshimori, 2007). Immunoblots revealed no change in the levels of p62 (Figure 6.3A, B) and in LC3BII/LC3BI ratio (Figure 6.3A, C) between C9/− mice and C9+/+ controls, confirming that the ablation of C9orf72 does not alter autophagy in spinal cord of mice.
Figure 6.3: C9orf72 ablation does not affect autophagy in LSC of mice. Representative immunoblot of p62 and LC3BII/LC3BI performed on LSC lysates from C9$^{+/+}$ mice and C9$^{-/-}$ controls (D) and relative quantification (E, F). p62 is normalised on total protein. Data are expressed as mean ± SEM (n=6 per experimental group) and analysed by Student’s t-test.

6.2.4 C9orf72 ablation does not affect glia in spinal cord

In our study we confirmed that C9orf72 is expressed by microglia in LSC, where has been demonstrated to play a pivotal role for the proper function (O’Rourke et al., 2016). Moreover, microglial activation, together with astrocytosis, is a hallmark of ALS. The immunohistological and biomolecular analysis did not show any difference in the activation of IBA1+ pan-microglia (Figure 6.4A, B, C) nor CD68+ macrophagic microglia (Figure 6.4D, E, F) between C9$^{-/-}$ and their controls.

Figure 6.4: C9orf72 ablation does not induce microglial activation in LSC of mice. (A) Representative images of LSC coronal hemisections from C9$^{+/+}$ mice and C9$^{-/-}$ controls stained with IBA1, marker of pan-microglia (A), and CD68, marker of macrophagic microglia (D) and relative quantifications (n=4 per experimental groups) (B, E). Real-time PCR analysis on LSC lysates from C9$^{+/+}$ mice and C9$^{-/-}$ controls for IBA1 (C) and CD68 (F) transcripts normalised on β-actin housekeeping (n=6 per experimental groups. Data are expressed as mean ± SEM and analysed by Student’s t-test.
Furthermore, we did not observe any alteration in the levels of GFAP, a marker of astrocytosis, both through immunofluorescence and Western blot analysis (Figure 6.5A-D). Previously, we demonstrated that C9orf72 is localized in oligodendrocytes, which have been demonstrated to contribute to the MN death in ALS (Lowe, 1994; Nonneman, Robberecht and Van Den Bosch, 2014). However, no difference in myelin basic protein (MBP) was found between C9⁻/⁻ and C9⁻/⁻ mice in the LSC (Figure 6.5E, F).

Figure 6.5: C9orf72 ablation does not induce astrocytosis in LSC of mice. Representative images of LSC coronal hemisections from C9⁻/⁻ mice and C9⁻/⁻ controls stained with GFAP, marker of astrocytes (A) and relative quantification (n=4 per experimental groups) (B). Representative immunoblots performed on LSC lysates from C9⁻/⁻ mice and C9⁻/⁻ controls for GFAP (C) and myelin basic protein (MBP) (E) and relative quantifications (n=6 per experimental groups) (D, F). Data are normalised on total protein, expressed as mean ± SEM and analysed by Student’s t-test.

6.2.5 C9orf72 ablation does not induce T-cell infiltration in spinal cord

C9orf72 is involved in the regulation of the immune system response (Atanasio et al., 2016; Burberry et al., 2016; McCauley et al., 2020) and its ablation causes leukocyte expansion and T-cell infiltration in several peripheral organs (Atanasio et al., 2016). Thus, we wanted to verify whether the lack of C9orf72 could induce T-cell infiltration also in spinal cord. We analysed the levels of CD8, CD4 and FOXP3 transcript, markers of cytotoxic, helper and regulatory T-
lymphocytes respectively. However, no difference in the recruitment of T-cells was detected in C9⁻/⁻ mice compared to controls (Figure 6.6A, B, C, respectively). Accordingly, no differences in the transcription levels of several inflammatory cytokines such as interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), interleukin 1 beta (IL1β), chemokine (C-C motif) ligand 2 (CCL2), and interleukin 10 (IL10) were detected between C9⁻/⁻ and C9⁺/+ mice (Figure 6.6 D, E, F, respectively).

Taken together, these results suggest that even if C9orf72 is expressed by different types of cells in the spinal cord, its ablation did not affect the homeostasis of this tissue in mice.

Figure 6.6: C9orf72 ablation does not induce T-cell infiltration nor increased cytokines levels in the LSC of mice. Real-time analysis performed on LSC lysates from C9⁺/+ mice and C9⁻/⁻ controls at 22W for CD8 (A), CD4 (B), FOXP3 (C), IFNγ (D), IL1β (E), TNFα (F), CCL2 (G), and IL10 (H) transcripts (n=6 per experimental groups). Data are normalised on β-actin, expressed as mean ± SEM and analysed by Student’s t-test.
6.2.6 C9orf72 ablation affects Schwann cells but not axon structure in the sciatic nerve

We analysed the effect of C9orf72 ablation on sciatic nerve, where we found that C9orf72 is highly expressed by SCs and rarely in axon. No difference in the axon structure was detected between C9⁻/⁻ and C9⁺/+ mice, as demonstrated by immunohistochemistry for NF200 (Figure 6.7A), the main constituent of the axonal cytoskeleton (Lee and Shea, 2014). Moreover, Western blot analysis revealed that the levels of NF200 and β-importin, a karyopherin involved in transducing damage signals from the axons of injured neurons back to the cell body (Hanz et al., 2003), were unchanged between C9⁻/⁻ and C9⁺/+ mice (Figure 6.7B, C, D), suggesting that the ablation of C9orf72 did not affect the structural integrity nor induced the retrograde injury signalling in motor axons of mice.

![Figure 6.7: C9orf72 ablation does not affect axonal structure nor functionality in SN of mice.](image)

Then, we examined the effect of the constitutive C9orf72 ablation on SCs, which are responsible of the axon myelination, and in case of damage dedifferentiate, proliferate, and assume a progenitor-like state, reducing the expression of myelination-related genes and activating a
repair program (Gaudet, Popovich and Ramer, 2011; Jessen, Mirsky and Lloyd, 2015). Immunoblots showed a significant decrease in myelin basic protein (MBP) levels (Figure 6.8A, B) and 2',3'-Cyclic-nucleotide 3'-phosphodiesterase (CNPase) levels (Figure 6.8C, D), a myelin-associated enzyme expressed in SCs, in C9⁻/⁻ mice compared to controls. Moreover, both immunoblot and immunofluorescence analyses showed an increase in the immunoreactivity for GFAP, a marker of dedifferentiating and proliferating SCs, in C9⁻/⁻ mice compared to controls (Figure 6.8E, F). The levels of p75 neurotrophin receptor (p75NTR), expressed by immature and repair SCs (Jessen, Mirsky and Lloyd, 2015) and implicated in remyelination after injury (Tomita et al., 2007), were unchanged between C9⁻/⁻ and C9⁺/+ mice, as shown by immunoblot (Figure 6.8 G, H). Conversely, immunofluorescence revealed an increased expression of GAP43, a marker for both growing axons and non-myelinating and denervated SCs (Sensenbrenner, Lucas and Deloulme, 1997), by GFAP+ SCs in C9⁻/⁻ mice compared to C9⁺/+ controls (Figure 6.8I).
Figure 6.8: C9orf72 ablation induces SC activation in the SN of mice. Representative immunoblots performed on SN lysates from C9+/+ mice and C9−/− controls for MBP (A), CNPase (C), GFAP (E), and p75NTR (G) and relative quantifications (n=6 per experimental groups) (B, D, F, H). Data are normalised on β-actin (MBP, CNPase and GFAP) or total protein (p75NTR), expressed as mean ± SEM and analysed by Student’s t-test. *p<0.05. Representative images of SN longitudinal sections from C9+/+ mice and C9−/− controls stained for GAP43 (red) and GFAP (green) (I).

These results suggest that the ablation of C9orf72 does not affect the MN axon but induce the SCs in an ineffective repair mode.

6.2.7 C9orf72 ablation affects autophagy in the sciatic nerve of mice

Given the involvement of C9orf72 in autophagy (Sellier et al., 2016; Webster et al., 2016), which is upregulated by repair SCs for myelin clearance (Lutz et al., 2017; Ko et al., 2018; Li et al., 2020),
we decided to analyse autophagy markers in the sciatic nerve. Immunoblots revealed decreased levels of p62 and increased LC3BII/LC3BI ratio, indicating an increase in the autophagic flux in C9\(^{-/-}\) mice compared to controls (Figure 6.9A, B, C).

**Figure 6.9:** C9orf72 ablation affects autophagy in the SN of mice. Representative immunoblots performed on SN lysates from C9\(^{+/+}\) mice and C9\(^{-/-}\) controls for p62 and LC3BII/LC3BI (A), and relative quantifications (n=6 per experimental groups) (B, C). Data are normalised on total protein, expressed as mean ± SEM and analysed by Student’s t-test. *p<0.05; **p<0.01.

**6.2.8 C9orf72 ablation induces immune dysregulation in the sciatic nerve of mice**

Besides alterations in SC functionality, we wanted to verify whether the ablation of C9orf72 could lead to immune cell infiltration in the sciatic nerve of mice. Real-time analysis revealed the recruitment of cytotoxic T lymphocytes in C9\(^{-/-}\) mice compared to C9\(^{+/+}\) controls, as demonstrated by increased levels of CD8 transcript, but not CD4+ helper T lymphocytes and FOXP3+ regulatory T lymphocytes (Figure 6.10A, B, C). Moreover, we observed a decrease in the transcript levels of CD11b, marker of myeloid lineage, in C9\(^{-/-}\) mice compared to controls (Figure 6.10D).
A growing body of evidence suggests that SCs may modulate local immune responses by activating the major histocompatibility complex type I (MHCI) and presenting antigens in pathological conditions (Hörste et al., 2008). Moreover, infiltrating CD8+ T-cells in SN could mediate cell cytotoxicity towards SCs promoting peripheral neuropathy (Tang et al., 2013). For this reason, we analyzed the expression of the major histocompatibility complex class I (MHCI) in the SCs. No expression of MHCI was observed in the SN of C9+/+ mice. Conversely, an immunofluorescent signal of MHCI was detected in the sciatic nerve of C9−/− mice, which colocalized with GFAP and S100β (Figure 6.11).
Figure 6.11: C9orf72 ablation induces the expression of MHCI by SCs in the SN of mice. Representative images of SN longitudinal sections from C9\(^+/+\) mice and C9\(^{-/-}\) controls stained for MHCI, GFAP, and S100β.

We extended the analysis to LMP7, a component of the immunoproteasome that is required for the production of antigenic peptide to be presented by the MHCI. Interestingly, Immunofluorescence revealed an increased signal of LMP7, which partially colocalized with MHCI, in the SN of C9\(^{-/-}\) compared to C9\(^+/+\) mice (Figure 6.12), suggesting the potential production of antigenic peptides by SCs with consequent exposure of a self-peptide on the cell membrane and the possible interaction with CD8+ cytotoxic T-cells.

Figure 6.12: C9orf72 ablation induces the expression of LMP7. Representative images of SN longitudinal sections from C9\(^+/+\) mice and C9\(^{-/-}\) controls stained for LMP7 and MHCI.
Thus, we wanted to evaluate the establishment of an inflammatory environment in the sciatic nerve of C9\(^{-}\) compared to controls. Real-time analysis revealed increased levels of IFN\(\gamma\) and IL1\(\beta\) (Figure 6.13A, B), decreased levels of TNF\(\alpha\) (Figure 6.13C), and unchanged levels of CCL2 transcripts in C9\(^{-}\) mice compared to controls (Figure 6.13 D). Furthermore, we observed increased levels of interferon beta 1 (IFN\(\beta\)1) (Figure 6.13E), a marker of autoimmunity, which expression has been demonstrated to be induced in myeloid cells depleted for C9orf72 (McCauley et al., 2020).

![Figure 6.13: C9orf72 ablation Real-time analysis performed on SN lysates from C9\(^{+/+}\) mice and C9\(^{-}\) controls for IFN\(\gamma\) (A), IL1\(\beta\) (B), TNF\(\alpha\) (C), CCL2 (D), and IFN\(\beta\)1 (E) transcripts (n=6 per experimental groups). Data are normalised on \(\beta\)-actin, expressed as mean ± SEM and analysed by Student’s t-test. *p<0.05; **p<0.01.](image)

6.2.9 C9orf72 ablation provokes a mild denervation without affecting the atrophy in the hind limbs of mice

Recent pieces of evidence show that muscle atrophy and denervation of NMJs are early events in the pathogenic cascade of ALS, anticipating MN degeneration and the onset of symptoms. Since we demonstrated that C9orf72 is expressed in TA muscle, we wanted to investigate the effect of its LOF in this compartment.
We first performed immunoreactivity for synaptic vesicle 2 (SV2) and NF-M (clone 2H3) as pre-synaptic markers and bungarotoxin as a post-synaptic marker on TA muscle. The NMJs were considered innervated when the signal of pre- (red) and post- (green) synaptic terminals co-localised. Conversely, they were considered denervated when there was only bungarotoxin signal. The analysis showed that the percentage of denervated plaques in the TA muscle of C9\textsuperscript{−/−} mice was significantly higher compared to controls (27.5 ± 3.2% vs 11.2 ± 1.4% respectively; p<0.05) (Figure 6.14A, B). Denervation of NMJs is characterized by the acetylcholine receptor epsilon (AChRe)-to-gamma subunit (AChRγ) switch (Dobrowolny, Aucello and Musarò, 2011). Therefore, we examined the transcription levels of AChRγ in TA muscle and also *gastrocnemius caput medialis* (GCM) muscle. We found a slight but significant increase in the AChRγ mRNA levels in both TA and GCM muscles of C9\textsuperscript{−/−} mice compared to C9\textsuperscript{+/+} animals (Figure 6.14 C, D, respectively). However, we did not detect any difference in the muscle weight between C9\textsuperscript{−/−} and C9\textsuperscript{+/+} mice (TA: C9\textsuperscript{+/+} 37.5 ± 2.5 mg vs C9\textsuperscript{−/−} 39.17 ± 1.5 mg; GCM: C9\textsuperscript{+/+} 107.5 ± 5.5 mg vs C9\textsuperscript{−/−} 119.2 ± 5.3 mg; mean ± SEM) (Figure 6.14E, F).
Figure 6.14: Representative images of TA muscle longitudinal sections from C9+/+ mice and C9−/− controls at 22W stained for SV2 and 2H3 as markers of pre-synaptic terminals (red) and BTX as marker of post-synaptic terminals (green) (A), and relative analysis of NMJ denervation (B). Real-time analysis performed on TA muscle (C) and GCM muscle (D) lysates from C9+/+ mice and C9−/− controls for AChRγ transcript normalized on β-actin. Evaluation of TA muscle (E) and GCM (F) weight. Data are expressed as mean ± SEM and analysed by Student’s t-test. *p<0.05; **p<0.01.

6.2.10 C9orf72 ablation induces autophagy alterations in the tibialis anterior muscle of mice

Then, we analysed autophagy in TA muscle, where it regulates synaptic structure and function (Rudnick et al., 2017). Immunoblots revealed unchanged levels of p62, but increased LC3BII/LC3BI ratio, indicating altered autophagy in C9−/− mice compared to controls (Figure 6.15 A, B, C).
Figure 6.15: Representative immunoblots performed on TA muscle lysates from C9\textsuperscript{+/+} mice and C9\textsuperscript{-/-} controls for p62 and LC3BII/LC3BI (A), and relative quantifications (n=6 per experimental groups) (B, C). Data are normalised on total protein, expressed as mean ± SEM and analysed by Student’s t-test. **p<0.01.

6.2.11 C9orf72 ablation caused immune dysregulation in the tibialis anterior muscle of mice

We analysed the immune infiltration and the inflammatory state in the TA muscle of C9\textsuperscript{-/-} and C9\textsuperscript{+/+} mice. Real time analysis showed increased levels of CD8 and CD4, but not FOXP3 transcript, in C9\textsuperscript{-/-} mice compared to controls (Figure 6.16A, B, C, respectively). Also in TA muscle, we observed a decrease in the levels of CD11b transcript (Figure 6.16D).

Figure 6.16: Real-time analysis performed on TA muscle lysates from C9\textsuperscript{+/+} mice and C9\textsuperscript{-/-} controls for CD8 (A), CD4 (B), FOXP3 (C) and CD11b (D) (n=6 per experimental groups). Data are normalised on β-actin, expressed as mean ± SEM and analysed by Student’s t-test. *p<0.05; **p<0.01.
Alongside with CD8+ and CD4+ T-cell infiltration, we found increased levels of pro-inflammatory cytokine transcripts of INFγ, IL1β, TNFα, and CCL2, in TA muscle of C9/− mice compared to controls (Figure 6.17A, B, C, D), while the transcript levels of IL10, anti-inflammatory cytokine, and IFNβ1 transcript, were unchanged between the experimental groups (Figure 6.17E, F).

Figure 6.17: Real-time analysis performed on SN lysates from C9+/+ mice and C9/− controls for IFNγ (A), IL1β (B), TNFα (C), CCL2 (D), IL10 (E) and IFNβ1 (F) transcripts (n=6 per experimental groups). Data are normalised on β-actin, expressed as mean ± SEM and analysed by Student’s t-test. *p<0.05; **p<0.01.

Overall, these results put in evidence the role of C9orf72 in the peripheral nervous system. C9orf72 is expressed in both the CNS and PNS; however, the ablation of the protein produced structural and functional alterations selectively in the peripheral compartment of the neuromuscular system, together with immune infiltrates and an inflammatory state. It remains to be established whether these alterations are the cause or consequence of the immune and inflammatory dysregulation.
6.3 DISCUSSION

This study confirmed previous evidence and provided new insights about the effect of C9orf72 LOF in mice. As demonstrated by other groups, the C9orf72 ablation did not lead to any alterations in the spinal cord of mice (Koppers et al., 2015; O’Rourke et al., 2015). Conversely, for the first time we demonstrated that the ablation of C9orf72 impairs the peripheral compartment of the neuromuscular system.

In the sciatic nerve of C9/− mice, we observed mild demyelination, de-differentiation and proliferation of Schwann cells, as well as increased pro-inflammatory cytokines levels and activation of a low-level repair program, independent of axon alterations or injury. This condition is similar to the “inflammaging” found in uninjured old nerves, characterized by a hyperinflammatory state, reduced myelination and persistence of Schwann cells in an undifferentiated and nonfunctional repair mode, incapable of proper myelination, which contributes to the development of neuropathies (Büttner et al., 2018). Moreover, we found induced the expression of the major histocompatibility complex class I (MHCI) in Schwann cells, that could be mediated by the increase of both immunoproteasome and autophagy (Øynebråten 2020). This evidence suggests that the constitutive ablation of C9orf72 could promote the cross-presentation of auto-antigens to CD8+ T-cells that mediate their cytotoxicity towards SCs via cell-cell signaling and induce inflammation through the production of cytokines such as INFγ and IL1β, similarly to what happens in diabetic peripheral neuropathy (Tang et al., 2013).

McCauley and colleagues reported that myeloid cells from C9orf72 KO mice showed increased levels of stimulator interferon gene (STING) and consequent activation of the type I interferon (comprising IFNα, IFNβ, IFNω, IFNε and IFNκ) response (McCauley et al., 2020), implicated in the pathogenesis of numerous autoimmune diseases. Indeed, type I IFN cytokines can rapidly induce and amplificate both the innate and adaptative immunity, generating a feed-forward loop of IFN
production that can be maladaptive in immune responses directed against host tissues (Hall and Rosen, 2010). Consistent with this, we found that the C9orf72 LOF markedly induced the production of IFNβ1 in the sciatic nerve, that may have driven autoinflammation and autoimmunity against myelin. Moreover, it has been demonstrated that IFNβ1 induces the octamer-binding factor 6 (Oct6) in SCs, exerts activating as well as repressing functions during myelination and correctly timed, transient expression of Oct6 is crucial for the development of myelinating SCs (Jaegle and Meijer, 1998). For this reason, the overproduction of IFNβ1 in the sciatic nerve due to the C9orf72 LOF may be responsible of defective myelination. Nevertheless, whether the inflammation induces SC response and demyelination, or aberrant SCs are critical for inducing the inflammatory state in the sciatic nerve, needs to be elucidated.

In both the TA and GCM muscles we observed a slight but significant NMJ denervation, accompanied by neither atrophy nor muscular impairment, but with alterations in autophagy in TA. Several studies demonstrated that autophagy regulates NMJ structure and function (Rudnick et al., 2017) and autophagy impairment in muscles leads to denervation and early aging (Carnio et al., 2014). Thus, the loss of C9orf72 could affect NMJ innervation through impaired autophagy. The recruitment of T cells, both cytotoxic and helper, is also observed in the TA muscle of C9−/− mice, consistent with the increase of several proinflammatory cytokines such as IL1β, TNFα, IFNγ and CCL2. Interestingly, both in the sciatic nerve and muscle, we observed an inflammatory state without any increase in the infiltration of myeloid cells, which normally migrate to the damage site participating in the inflammatory response (Liu et al., 2019; Walton et al., 2019). Myeloid cells are the cell population that mostly express C9orf72 (O’Rourke et al., 2016). It has been demonstrated that the C9orf72 is required for the proper function of myeloid cells (O’Rourke et al., 2016), and its depletion induce alterations of autophagy in these cells. Autophagy is also essential for the migration of macrophages (Jiang et al., 2019). Thus, the depletion of C9orf72 could affect the recruitment of myeloid cells in SN and muscles through
the alteration of autophagy. Further studies are needed to understand whether the lymphocyte infiltrate is the cause or the consequence of denervation.

From 11 to 22 weeks of age we observed an increase in the body weight of C9\(^{-}\) mice compared to controls. This is consistent with the recent evidence that the loss of C9orf72 dysregulates the lipid metabolism, increasing de novo fatty acid production (Liu et al., 2018). Conversely, at about one year of age, we observed an opposite trend, with a decrease in body weight of C9\(^{-}\) mice compared to controls. This evidence suggests that the constitutive ablation of C9orf72 could lead to progressive worsening of the healthy status of mice, in line with other studies that reported a body weight decrease (Atanasio et al., 2016) in C9KO mice.

Overall, these data provide new insights about the role of C9orf72 in the neuromuscular system. The C9orf72 LOF induced alterations in the SCs and NMJs, accompanied by an inflammatory state, demonstrating that it plays an important role in the maintaining of the peripheral compartment, that is the first to be involved in ALS. Even though these alterations do not produce any muscular impairment, the C9orf72 LOF may synergize with other toxic stimuli associated to ALS, acting as a modifier of the pathology.
RESULTS

Chapter 7

The effect of C9orf72 ablation on the disease progression of SOD1^{G93A} mice
7.1 BACKGROUND AND AIM

The data reported in chapter 6 confirmed the evidence that the C9orf72 LOF is not enough to trigger ALS. However, for the first time we showed that it can impair the peripheral compartment of the neuromuscular system. Several studies demonstrated that the C9orf72 KD or KO on itself is not deleterious, while it could synergize with other toxic stimuli, enlightening a multiple-hit pathological mechanism for ALS. Sellier et al. showed that the knockdown of C9orf72 synergizes with mutation in the ALS-related ATX-2 gene in both cortical neurons and zebrafish (Sellier et al., 2016). Studies on human iMNs derived from cells of C9ALS/FTD patients demonstrated that C9orf72 haploinsufficiency hypersensitized these cells to glutamate (Shi et al., 2018). Similarly, C9KO rats treated with a subclinical dose of kainic acid to promote glutamate excitotoxicity developed motor deficits and MN loss (Dong et al., 2021). Moreover, Abo-Rady and colleagues showed that the combination of HRE and the additional C9KO in iMN from C9ALS/FTD patient cells decreased axonal trafficking of lysosomes and caused significant apoptosis (Abo-Rady et al., 2020). In keeping with this, two studies demonstrated that reduction or loss of C9orf72 caused or exacerbated motor deficit in C9BAC mice (Shao et al., 2019; Zhu et al., 2020). However, Zhu and colleagues reported neurodegeneration and gliosis in hippocampus, but not spinal cord.

In addition to the hypothesis of a synergy between C9orf72 LOF and GOF inducing the disease, increasing shred evidence supports the oligogenicity in ALS, which is characterized by strong heterogeneity in terms of age of onset and clinical manifestation. This heterogeneity is underpinned not only by the presence of different pathogenic mechanisms, but also high overlap with other diseases. The identification of the HRE in the C9orf72 gene as the most prevalent cause of both ALS and FTD, which are considered as part of a disease continuum, pointed out the oligogenic inheritance of ALS (Majounie et al., 2012; Cruts et al., 2013). As a matter of fact, multiple mutations in ALS-FTD-linked genes included C9HRE more frequently
than other mutations (Nguyen, Van Broeckhoven and van der Zee, 2018), and the presence of C9HRE in unaffected individuals (DeJesus-Hernandez et al., 2011; Renton et al., 2011; Gijselinck et al., 2012) suggests the need of other mutation to trigger the pathology (Nguyen, Van Broeckhoven and van der Zee, 2018). Moreover, the mutation in C9orf72 gene can cause ALS or FTD individually, suggesting that additional mutations in other genes may drive disease presentation towards the one or the other symptomatology (Nguyen, Van Broeckhoven and van der Zee, 2018). For instance, the C9-carriers with mutation in GRN gene had only FTD, while co-occurrence of C9HRE and mutation in SOD1 led exclusively to ALS (Nguyen, Van Broeckhoven and van der Zee, 2018).

Taken together, these results suggest that the C9orf72 LOF could act as a modulator of the disease in the presence of other mutations linked to ALS. To verify this hypothesis, we examined the effect of C9orf72 LOF on the pathology of SOD1<sup>G93A</sup> mice, the best characterised ALS model.
7.2 RESULTS

7.2.1 Loss of C9orf72 anticipates the onset of neuromuscular impairment but prolonged survival of SOD1<sup>G93A</sup> mice

We crossbred NTG C9<sup>+/+</sup> mice and SOD1<sup>G93A</sup> mice to generate SOD1<sup>G93A</sup> C9<sup>+/+</sup> mice, in order to examine the involvement of C9orf72 in ALS pathogenesis. We monitored the bodyweight fluctuation and the muscle strength impairment of SOD1<sup>G93A</sup> C9<sup>+/+</sup> mice and SOD1<sup>G93A</sup> C9<sup>−/−</sup> mice through the inverted grid test twice a week from 11 to 22 weeks of age, the symptomatic stage of the disease, when the mice were sacrificed, and tissues were collected for histological and biomolecular analyses (n=18 per experimental group). Another cohort of mice (n=12 per experimental group) was evaluated for the survival. We report only the comparison data between the SOD1<sup>G93A</sup> C9<sup>+/+</sup> and SOD1<sup>G93A</sup> C9<sup>−/−</sup>, as the NTG mice both C9<sup>+/+</sup> and C9<sup>−/−</sup> mice showed no behavioural difference. Henceforth, C9<sup>+/+</sup> mice will be named NTG C9<sup>+/+</sup> to distinguish them from SOD1<sup>G93A</sup> C9<sup>+/+</sup> mice. The assessment of body weight during the disease progression showed no significant difference between SOD1<sup>G93A</sup> C9<sup>+/+</sup> and SOD1<sup>G93A</sup> C9<sup>−/−</sup> mice (Figure 7.1A). However, during the symptomatic stage of the disease the decline in body weight appeared slower in SOD1<sup>G93A</sup> C9<sup>−/−</sup> mice compared to SOD1<sup>G93A</sup> C9<sup>+/+</sup> animals, albeit not significantly different. Conversely, the SOD1<sup>G93A</sup> C9<sup>−/−</sup> mice displayed about two-week anticipation in the onset of the muscle strength impairment (Mean ± SEM: SOD1<sup>G93A</sup> C9<sup>−/−</sup>: 16.69 ± 0.27 vs SOD1G93A C9<sup>+/+</sup> 18.47 ± 0.30 weeks) (Figure 7.1B). This was followed by a worsening of the performance at the inverted grid test compared to SOD1<sup>G93A</sup> C9<sup>+/+</sup> mice (p<0.0001) (Figure 7.1C). Unexpectedly, SOD1<sup>G93A</sup> C9<sup>−/−</sup> mice showed a significant extension of the survival compared to SOD1<sup>G93A</sup> C9<sup>+/+</sup> animals (SOD1<sup>G93A</sup> C9<sup>−/−</sup>: 26.99 ± 0.53 vs SOD1<sup>G93A</sup> C9<sup>+/+</sup> 24.98 ± 0.37 weeks ± SEM) (Figure 7.1D), leading to an overall increase of the disease duration in a paralysis state.
Figure 7.1: Loss of C9orf72 anticipates symptom onset but prolonged survival in SOD1\textsuperscript{G93A} mice. Evaluation of body weight (A) and latency to fall by inverted grid test (B) in SOD1\textsuperscript{G93A} C9\textsuperscript{−/−} mice compared to SOD1\textsuperscript{G93A} C9\textsuperscript{+/+} animal from 11 to 22 weeks of age (n=18 per experimental group). Data are expressed as mean ± SEM for each time point and analysed by Two-way ANOVA for repeated measure, followed by Bonferroni post-hoc test. ***p<0.001. Evaluation of the onset of symptoms (C) and survival (n=12 per experimental group) (D) in SOD1\textsuperscript{G93A} C9\textsuperscript{−/−} mice compared to SOD1\textsuperscript{G93A} C9\textsuperscript{+/+} controls. Data are analysed by Log-Rank test. *p<0.05; ****p<0.0001.

7.2.2 Loss of C9orf72 does not alter the levels of mutant hSOD1 in the lumbar spinal cord of SOD1\textsuperscript{G93A} mice.

First, we examined whether the loss of C9orf72 could alter the levels of mutant human SOD1 (hSOD1) and its aggregation in the spinal cord, a hallmark of SOD1\textsuperscript{G93A} mice due to proteostasis alteration (Bendotti \textit{et al.}, 2012).

Immunoblot analyses showed no difference in the levels of both soluble (SOL) (Figure 7.2A, B) and Triton-insoluble fraction (TIF) (Figure 7.2B, C) of mutant hSOD1 between the two SOD1\textsuperscript{G93A} groups.
Figure 7.2: Loss of C9orf72 does not alter the levels of mutant hSOD1 in the lumbar spinal cord of SOD1<sup>G93A</sup> mice. Representative immunoblot for the soluble (A) and insoluble (C) fraction of hSOD1 performed on LSC lysates from SOD1<sup>G93A</sup> C9<sup>+/+</sup> and SOD1<sup>G93A</sup> C9<sup>-/-</sup> at 22W and relative quantifications (B, D). Data are expressed as mean ± SEM (n=4 per experimental group) and analysed by Student’s t test.

7.2.3 Loss of C9orf72 does not affect MN loss in the lumbar spinal cord of SOD1<sup>G93A</sup> mice.

The degeneration and death of MNs is the main feature of ALS. Thus, we examined whether the more severe pathology in SOD1<sup>G93A</sup> C9<sup>-/-</sup> mice was related to an increased MN loss compared to SOD1<sup>G93A</sup> C9<sup>+/+</sup> symptomatic mice. The immunofluorescence for ChAT and the measurement of MNs revealed a massive loss of αMNs, the most affected by ALS, in LSC of SOD1<sup>G93A</sup> mice compared to NTG C9<sup>+/+</sup> controls. The number of αMNs tended to decrease in LSC of SOD1<sup>G93A</sup> C9<sup>-/-</sup> compared to SOD1<sup>G93A</sup> C9<sup>+/+</sup> mice; however, the effect was not statistically different (Figure 7.3A, B). Interestingly, the immunofluorescence for SMI31 showed a higher accumulation of phosphorylated NF in proximal axons and dendrites which appeared more swollen, indicating a more pronounced MN dysfunction in SOD1<sup>G93A</sup> C9<sup>-/-</sup> mice compared to SOD1<sup>G93A</sup> C9<sup>+/+</sup> animals (Figure 7.3C). This result was confirmed by immunoblot that demonstrated a significant increase in the ratio between the portion of SMI31 on NF200 (Figure 7.3D, E). These results suggest that
the loss of C9orf72 in SOD1\textsuperscript{G93A} mice exacerbates the cytoarchitectural alteration in MNs, leading to higher dysfunction.

![Figure 7.3: Loss of C9orf72 does not affect MN loss in the lumbar spinal cord of SOD1\textsuperscript{G93A} mice.](image)

7.2.4 Loss of C9orf72 does not affect autophagy in lumbar spinal cord of SOD1\textsuperscript{G93A} mice

It is known that both p62 and LC3BII accumulates in the LSC of ALS mice, impairing the autophagic flux (Zhang \textit{et al.}, 2011). Given that C9orf72 is involved in autophagy processes (Sellier \textit{et al.}, 2016), we wanted to verify whether the ablation of C9orf72 could exacerbate the autophagy impairment. Immunoblots revealed a significant increase in p62 levels and LC3BII/LC3B ratio in both SOD1\textsuperscript{G93A} C9\textsuperscript{+/} and SOD1\textsuperscript{G93A} C9\textsuperscript{-/-} mice compared to NTG C9\textsuperscript{+/}
controls. However, no difference was detected between SOD1\textsuperscript{G93A} C9\textsuperscript{+/-} and SOD1\textsuperscript{G93A} C9\textsuperscript{-/-} mice (Figure 7.4A, B, C).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure7_4.png}
\caption{Loss of C9orf72 does not affect autophagy in lumbar spinal cord of SOD1\textsuperscript{G93A} mice. Representative immunoblot of p62 and LC3BII/LC3BI performed on LSC lysates from NTG C9\textsuperscript{+/-}, SOD1\textsuperscript{G93A} C9\textsuperscript{+/-} and SOD1\textsuperscript{G93A} C9\textsuperscript{-/-} at 22W (A) and relative quantifications (B, C). p62 is normalised on total protein. Data are expressed as mean ± SEM (n=6 per experimental group) and analysed by one-way ANOVA followed by Fisher’s LSD post-hoc test. *p<0.05; **p<0.01.}
\end{figure}

7.2.5 Loss of C9orf72 exacerbates gliosis in the lumbar spinal cord of SOD1\textsuperscript{G93A} mice

Neuroinflammation resulting from hyperreactive microglia and astrocytes is a typical feature of ALS, actively contributing to the MN degeneration through a non-cell autonomous mechanism (Zhao, Beers and Appel, 2013). Moreover, microglial hyperactivation was found in C9ALS post-mortem brain regions, as indicated by increased immunoreactivity for IBA1, marker of pan-microglia, and CD68, marker of macrophagic microglia, compared to sporadic ALS cases (Cooper-Knock et al., 2014).

For these reasons, we analysed the effect of C9orf72 ablation on microglia activation in the LSC of SOD1\textsuperscript{G93A} mice at the symptomatic stage of the disease. We observed a high activation of microglia in SOD1\textsuperscript{G93A} mice compared to NTG C9\textsuperscript{+/-} controls, which was significantly exacerbated in SOD1\textsuperscript{G93A} C9\textsuperscript{-/-} mice compared to SOD1\textsuperscript{G93A} C9\textsuperscript{+/-} animals, as demonstrated by the increased levels of IBA1 immunoreactivity (Figure 7.5A, B) and mRNA (Figure 7.5C). Real-time analysis
revealed a significant increase also in the levels of CD68 mRNA (Figure 7.5F); however, immunohistochemistry showed only a trend to increase of CD68 in LSC of SOD1<sup>G93A</sup> C9<sup>−/−</sup> mice compared to SOD1<sup>G93A</sup> C9<sup>+/+</sup> animals (Figure 7.5D, E).

**Figure 7.5: Loss of C9orf72 exacerbates microglial activation in the lumbar spinal cord of SOD1<sup>G93A</sup> mice.** Representative images of LSC coronal hemisections from NTG C9<sup>+/+</sup>, SOD1<sup>G93A</sup> C9<sup>+/+</sup> and SOD1<sup>G93A</sup> C9<sup>−/−</sup> at 22W stained for IBA1 (A) and CD68 (D) and relative quantifications (n=4 per experimental groups) (B, E). Real-time PCR analysis on LSC lysates from NTG C9<sup>+/+</sup>, SOD1<sup>G93A</sup> C9<sup>+/+</sup> and SOD1<sup>G93A</sup> C9<sup>−/−</sup> at 22W for IBA1 (C) and CD68 (F) transcripts normalised on β-actin (n=6 per experimental groups. Data are expressed as mean ± SEM and analysed by one-way ANOVA followed by Fisher’s LSD post-hoc test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.
We also examined the effect of C9orf72 ablation on astrocytosis in the LSC of SOD1<sup>G93A</sup> mice at the symptomatic stage of the disease. Both immunohistochemistry (Figure 7.6A, B) and Western blot showed increased levels of GFAP, marker of astrocyte activation, in SOD1<sup>G93A</sup> mice compared to NTG C9<sup>+/+</sup> controls. However, only immunohistochemistry demonstrated a significant exacerbation of astrocytosis in SOD1<sup>G93A</sup> C9<sup>-/-</sup> mice compared to SOD1<sup>G93A</sup> C9<sup>+/+</sup> animals.

![Figure 7.6: Loss of C9orf72 exacerbates astrocytosis in the lumbar spinal cord of SOD1<sup>G93A</sup> mice.](image)

**Figure 7.6:** Loss of C9orf72 exacerbates astrocytosis in the lumbar spinal cord of SOD1<sup>G93A</sup> mice. Representative images of LSC coronal hemisections from NTG C9<sup>+/+</sup>, SOD1<sup>G93A</sup> C9<sup>+/+</sup> and SOD1<sup>G93A</sup> C9<sup>-/-</sup> at 22W stained with GFAP, marker of astrocytes (A) and relative quantification (n=4 per experimental groups) (B). Representative immunoblots performed on LSC lysates from NTG C9<sup>+/+</sup>, SOD1<sup>G93A</sup> C9<sup>+/+</sup> and SOD1<sup>G93A</sup> C9<sup>-/-</sup> at 22W for GFAP (C) and relative quantifications (n=6 per experimental groups) (D). Data are normalised on total protein, expressed as mean ± SEM and analysed by one-way ANOVA followed by Fisher’s LSD post-hoc test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

7.2.6 Loss of C9orf72 increases immune cell infiltration in the lumbar spinal cord of SOD1<sup>G93A</sup> mice

The recruitment of immune cells in the spinal cord also contributes to the disease progression through a non-cell autonomous mechanism. Thus, we analysed the rate of CD8+ cytotoxic, CD4+ helper and FOXP3+ regulatory T lymphocytes in LSC of SOD1<sup>G93A</sup> mice at the symptomatic stage
of the disease, in absence or presence of C9orf72. We found increased levels of CD8 (Figure 7.7A) and CD4 (Figure 7.7B) transcript in SOD1<sup>GR3A</sup> mice compared to NTG C9<sup>+/+</sup> controls. This phenomenon was significantly enhanced in SOD1<sup>GR3A</sup> C9<sup>-/-</sup> mice compared to SOD1<sup>GR3A</sup> C9<sup>+/+</sup> animals. We also analysed the levels of FOXP3 transcript. However, no difference was detected between the three experimental groups (Figure 7.7C).

**Figure 7.7:** Loss of C9orf72 increases immune cell infiltration in the lumbar spinal cord of SOD1<sup>GR3A</sup> mice. Real-time analysis performed on LSC lysates from NTG C9<sup>+/+</sup>, SOD1<sup>GR3A</sup> C9<sup>+/+</sup> and SOD1<sup>GR3A</sup> C9<sup>−/−</sup> at 22W for CD8 (A), CD4 (B), and FOXP3 (C), IFNγ (D), IL1β (E), TNFα (F), CCL2 (G), and IL10 (H) transcripts (n=6 per experimental groups). Data are normalised on β-actin, expressed as mean ± SEM and analysed by one-way ANOVA followed by Fisher’s LSD post-hoc test. *p<0.05; **p<0.01.

### 7.2.7 Loss of C9orf72 alters the inflammatory milieu in the lumbar spinal cord of SOD1<sup>GR3A</sup> mice

Besides the reactive gliosis and infiltration of T-cells, we characterized the inflammatory milieu in the LSC of SOD1<sup>GR3A</sup> mice. Previous studies demonstrated that isolated microglia from the spinal cord of C9<sup>-/-</sup> mice assume a pro-inflammatory phenotype (O’Rourke et al., 2016). Thus, we wondered whether the increased gliosis and T-cell infiltration in the LSC of SOD1<sup>GR3A</sup> C9<sup>-/-</sup> mice compared to SOD1<sup>GR3A</sup> C9<sup>+/+</sup> animals could enhance the expression of inflammatory factors. Real-time analysis displayed increased levels of pro-inflammatory factors such as IL1β, TNFα and CCL2, produced by M1 polarised microglia in LSC of SOD1<sup>GR3A</sup> mice compared to NTG C9<sup>+/+</sup> controls. However, no difference was observed in SOD1<sup>GR3A</sup> C9<sup>-/-</sup> compared to SOD1<sup>GR3A</sup> C9<sup>+/+</sup> mice (Figure 7.8A, B, C). Conversely, the analysis revealed a significant increase in the transcript...
levels of IL10 and YM1, two anti-inflammatory factors and markers of M2 polarised microglia, in the LSC of SOD1^{G93A} C9^{-/-} mice compared to SOD1^{G93A} C9^{+/+} (Figure 7.8D, E).

**Figure 7.8:** Loss of C9orf72 alters the inflammatory milieu in the lumbar spinal cord of SOD1^{G93A} mice. Real-time analysis performed on LSC lysates from NTG C9^{+/+}, SOD1^{G93A} C9^{+/+} and SOD1^{G93A} C9^{-/-} at 22W for IL1β (A), TNFa (B), CCL2 (C), IL10 (D) and YM1 (E) transcripts (n=6 per experimental groups). Data are normalised on β-actin, expressed as mean ± SEM and analysed by one-way ANOVA followed by Fisher’s LSD post-hoc test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

### 7.2.8 Loss of C9orf72 induces alterations of axon but does not affect Schwann cells in the sciatic nerve of SOD1^{G93A} mice

ALS is recently recognized as a distal axonopathy (Fischer et al., 2004; Moloney, de Winter and Verhaagen, 2014) and axonal degeneration is an early event in the pathology of SOD1^{G93A} mice (Clark et al., 2016) that occurs prior to MN death (Dobrowolny et al., 2008; Dadon-Nachum, Melamed and Offen, 2011). Therefore, we investigated the effect of the C9orf72 ablation on the axon structure and function in the SN of SOD1^{G93A} mice. Immunohistochemistry for NF200 showed an abnormal NF accumulation and swelling of axons in SOD1^{G93A} C9^{-/-} mice compared to
SOD1<sup>G93A</sup> C9<sup>+/+</sup> (Figure 7.9A). Moreover, immunoblot analysis showed a marked increase in the levels of NF200 and importin β in the SN of SOD1<sup>G93A</sup> C9<sup>-/-</sup> mice compared to SOD1<sup>G93A</sup> C9<sup>+/+</sup> animals (Figure 7.9B, C, D).

Figure 7.9: Loss of C9orf72 induces alterations of axons in the sciatic nerve of SOD1<sup>G93A</sup> mice. Representative images of SN longitudinal sections from NTG C9<sup>+/+</sup>, SOD1<sup>G93A</sup> C9<sup>+/+</sup> and SOD1<sup>G93A</sup> C9<sup>-/-</sup> at 22W stained with NF200, marker of neurofilament heavy chain (A). Representative immunoblots performed on SN lysates from NTG C9<sup>+/+</sup>, SOD1<sup>G93A</sup> C9<sup>+/+</sup> and SOD1<sup>G93A</sup> C9<sup>-/-</sup> at 22W for NF200 and Importin β (B) and relative quantifications (n=6 per experimental groups) (C, D). Data are normalised on β-actin, expressed as mean ± SEM and analysed by one-way ANOVA followed by Fisher’s LSD post-hoc test. **p<0.01; ***p<0.001.

Then, we examined the effect of the C9orf72 ablation on the SCs in the SN of SOD1<sup>G93A</sup> mice. Western blot analysis showed a marked decrease in MBP (Figure 7.10A, B) and CNPase (Figure 7.10C, D) levels in the SN of SOD1<sup>G93A</sup> mice compared to NTG C9<sup>+/+</sup> controls, which was not further modified by the C9orf72 depletion. Accordingly, the SC proliferation and dedifferentiation resulted unchanged between the two groups of SOD1<sup>G93A</sup> mice, as demonstrated by the expression levels of GFAP (Figure 7.10E, F) and p75<sup>NTR</sup> (Figure 7.10G, H).
**Figure 7.10: Loss of C9orf72 does not affect Schwann cells in the sciatic nerve of SOD1\(^{G93A}\) mice.**

Representative immunoblots performed on SN lysates from NTG C9\(^{+/+}\), SOD1\(^{G93A}\) C9\(^{+/+}\) and SOD1\(^{G93A}\) C9\(^{-/-}\) at 22W for MBP (A), CNPase (C), GFAP (E), and p75\(^{NTR}\) (G) and relative quantifications (n=6 per experimental groups) (B, D, F, H). Data are normalised on β-actin (MBP, CNPase and GFAP) or total protein (p75\(^{NTR}\)), expressed as mean ± SEM and analysed by one-way ANOVA followed by Fisher’s LSD post-hoc test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001

**7.2.9 Loss of C9orf72 does not affect autophagy in the sciatic nerve of SOD1\(^{G93A}\) mice**

Then, we analysed the effect of the C9orf72 ablation on autophagy also in the SN of SOD1\(^{G93A}\). Western blot analysis revealed increased levels of p62 and LC3BII/LC3BI ratio in the SN of SOD1\(^{G93A}\) C9\(^{+/+}\) mice compared to NTG C9\(^{+/+}\) controls, suggesting an impairment of the autophagic flux. However, no difference in the expression of both autophagic markers was recorded between SOD1\(^{G93A}\) C9\(^{+/+}\) and SOD1\(^{G93A}\) C9\(^{-/-}\) mice (Figure 7.11A, B, C).
7.2.10 Loss of C9orf72 enhances the infiltration of CD8+ T-cells but decreases the levels of myeloid cells in the sciatic nerve of SOD1\textsuperscript{G93A} mice

Massive infiltration of both T cells and macrophages has been reported in the SN of symptomatic SOD1\textsuperscript{G93A} mice in an attempt to promote axonal regeneration (Hu and McLachlan, 2002; Barrette \textit{et al.}, 2008; Liu \textit{et al.}, 2019). Moreover, it has been recently demonstrated that the recruitment of immune cells in the peripheral axons is fundamental to slow down the disease progression (Nardo \textit{et al.}, 2016). Therefore, we examined the effect of C9orf72 ablation on the immune cell infiltration in the SN of SOD1\textsuperscript{G93A} symptomatic mice. Real-time PCR revealed increased levels of CD8 (Figure 7.12A), CD4 (Figure 7.12B), FOXP3 (Figure 7.12C), CD11b (Figure 7.12D) transcripts in SOD1\textsuperscript{G93A} mice compared to NTG C9\textsuperscript{+/+} controls. Moreover, we found an exacerbated infiltration of CD8+ cytotoxic T-cells (Figure 7.12A), but not CD4+ helper T-cells (Figure 7.12B) and FOXP3+ Tregs (Figure 7.12C) in the SN of SOD1\textsuperscript{G93A} C9\textsuperscript{−/−} mice compared to SOD1\textsuperscript{G93A} C9\textsuperscript{+/+} littermates. Intriguingly, the loss of C9orf72 significantly modified the infiltration of CD11b+ myeloid cells (Figure 7.12D), the cell population that mostly express C9orf72 (O’Rourke \textit{et al.}, 2016), in the PNS of SOD1\textsuperscript{G93A} mice.

\textit{Figure 7.11: Loss of C9orf72 does not affect autophagy in the sciatic nerve of SOD1\textsuperscript{G93A} mice.} Representative immunoblot of p62 and LC3BII/LC3BI performed on SN lysates from NTG C9\textsuperscript{+/+}, SOD1\textsuperscript{G93A} C9\textsuperscript{+/+} and SOD1\textsuperscript{G93A} C9\textsuperscript{−/−} at 22W (A) and relative quantifications (B, C). p62 is normalised on total protein. Data are expressed as mean ± SEM (n=6 per experimental group) and analysed by one-way ANOVA followed by Fisher’s LSD post-hoc test. **p<0.01; ***p<0.001.
Figure 7.12: Loss of C9orf72 enhances the infiltration of CD8+ T-cells but decreases the levels of myeloid cells in the sciatic nerve of SOD1<sup>G93A</sup> mice. Real-time analysis performed on SN lysates from NTG C9<sup>+/+</sup>, SOD1<sup>G93A</sup> C9<sup>+/+</sup> and SOD1<sup>G93A</sup> C9<sup>-/-</sup> mice at 22W for CD8 (A), CD4 (B), FOXP3 (C) and CD11b (D) (n=6 per experimental groups). Data are normalised on β-actin, expressed as mean ± SEM (n=6 per experimental group) and analysed by one-way ANOVA followed by Fisher’s LSD post-hoc test. *p<0.05; ***p<0.001; ****p<0.0001

7.2.11 Loss of C9orf72 alters the inflammatory milieu in the sciatic nerve of SOD1<sup>G93A</sup> mice

Since we observed that the loss of C9orf72 altered the immune response in the SN of symptomatic SOD1<sup>G93A</sup> mice, we wondered whether the expression of cytokines which are pivotal for the peripheral nerve regeneration (Dubový, Jančálek and Kubek, 2013; Büttner et al., 2018), could be modified. Real-time PCR showed increased levels of IFNγ (Figure 7.13A), IL1β (Figure 7.13B), TNFα (Figure 7.13C) and CCL2 (Figure 7.14D) transcript, and unchanged levels of IFNβ1 (Figure 7.13E) transcript, marker of autoimmunity, in SOD1<sup>G93A</sup> C9<sup>+/+</sup> mice compared to NTG C9<sup>+/+</sup>. Unexpectedly, we found that the loss of C9orf72 decreased the levels of IL1β (Figure 7.13B) and TNFα (Figure 7.13C) transcript, while did not affect the levels of IFNγ (Figure 7.13A) and CCL2 (Figure 7.13D) transcript, in the SN of SOD1<sup>G93A</sup>. Conversely, the levels of IFNβ1 (Figure
7.13E) transcript, were strikingly increased in the SN of SOD1\textsuperscript{G93A} C9\textsuperscript{−/−} mice compared SOD1\textsuperscript{G93A} C9\textsuperscript{+/+} animals.

**Figure 7.13:** Loss of C9orf72 alters the inflammatory milieu in the sciatic nerve of SOD1\textsuperscript{G93A} mice. Real-time analysis performed on SN lysates from NTG C9\textsuperscript{+/+}, SOD1\textsuperscript{G93A} C9\textsuperscript{−/−} and SOD1\textsuperscript{G93A} C9\textsuperscript{+/−} mice at 22W for IFN\textgamma (A), IL1\textbeta (B), TNF\alpha (C), CCL2 (D), and IFN\beta 1 (E) transcripts (n=6 per experimental groups). Data are normalised on \beta-actin, expressed as mean ± SEM and analysed by one-way ANOVA followed by Fisher’s LSD post-hoc test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

7.2.12 Loss of C9orf72 exacerbates NMJ denervation but not atrophy in the hind limb muscles of SOD1\textsuperscript{G93A} mice

We recently demonstrated that neuromuscular denervation, more than MN loss, contributes to anticipate disease onset in SOD1\textsuperscript{G93A} mice (Nardo et al., 2016). Moreover, in chapter 6 we observed that the ablation of C9orf72 led to a slight but significant NMJ denervation in both TA and GCM muscles. Therefore, we investigated the effect of C9orf72 depletion on denervation and atrophy in TA and GCM muscles of symptomatic SOD1\textsuperscript{G93A} mice. We found that in the TA muscle of SOD1\textsuperscript{G93A} C9\textsuperscript{−/−} mice the percentage of denervated NMJs was significantly higher compared to SOD1\textsuperscript{G93A} C9\textsuperscript{+/+} animals (Figure 7.14A, B). Moreover, we observed an upward trend
in AchRy mRNA levels in TA muscle of SOD1<sup>G93A</sup> C9<sup>/−</sup> mice compared to SOD1<sup>G93A</sup> C9<sup>/+</sup> animals (Figure 7.14C), and significantly increased levels of the same marker were observed in GCM muscle of SOD1<sup>G93A</sup> C9<sup>/−</sup> mice compared to SOD1<sup>G93A</sup> C9<sup>/+</sup> animals (Figure 7.14D). However, the loss of C9orf72 exacerbates neither TA (Figure 7.14E) nor GCM (Figure 7.14F) muscle weight of SOD1<sup>G93A</sup> mice.

Figure 7.14: Loss of C9orf72 exacerbates NMJ denervation but not atrophy in the hind limb muscles of SOD1<sup>G93A</sup> mice. Representative images of TA muscle longitudinal sections from NTG C9<sup>/+</sup>, SOD1<sup>G93A</sup> C9<sup>/+</sup> and SOD1<sup>G93A</sup> C9<sup>/−</sup> mice at 22W stained for SV2 and 2H3 as markers of pre-synaptic terminals (red) and BTX as marker of post-synaptic terminals (green) (A), and relative analysis of NMJ denervation (B). Real-time analysis performed on TA muscle (C) and GCM muscle (D) lysates from NTG C9<sup>/+</sup>, SOD1<sup>G93A</sup> C9<sup>/+</sup> and SOD1<sup>G93A</sup> C9<sup>/−</sup> mice at 22W for AChRy transcript normalized on β-actin. Evaluation of TA muscle (E) and GCM (F) weight. Data are expressed as mean ± SEM and analysed by one-way ANOVA followed by Fisher’s LSD post-hoc test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.
7.2.13 Loss of C9orf72 alters autophagy in the tibialis anterior muscle of SOD1\textsuperscript{G93A} mice

Rudnick and colleagues (Rudnick et al., 2017) demonstrated that autophagy regulates NMJ structure and function. Moreover, it is known that autophagy is impaired in the skeletal muscle of SOD1\textsuperscript{G93A} mice (Crippa et al., 2013). Thus, we verified whether the exacerbated denervation recorded in SOD1\textsuperscript{G93A} C9\textsuperscript{-/-} mice could be attributable to alterations in autophagy. Immunoblots showed a marked increase in the levels of both p62 (Figure 7.15A, B) and LC3B (Figure 7.15A) autophagic protein in the TA muscle of SOD1\textsuperscript{G93A} mice compared to NTG C9\textsuperscript{+/-} mice, even if the LC3BII/LC3BI ratio did not change in SOD1\textsuperscript{G93A} C9\textsuperscript{+/-} mice compared to NTG C9\textsuperscript{+/-} controls (Figure 7.15A, C). We observed that the loss of C9orf72 did not affect the levels of p62 in the TA muscle of SOD1\textsuperscript{G93A} mice (Figure 7.15A, B). Conversely, the LC3BII/LC3BI ratio was strikingly increased in SOD1\textsuperscript{G93A} C9\textsuperscript{-/-} mice compared to SOD1\textsuperscript{G93A} C9\textsuperscript{+/-} animals (Figure 7.15A, C), suggesting an increase or accumulation of autophagosomes.

*Figure 7.15: Loss of C9orf72 alters autophagy in the tibialis anterior muscle of SOD1\textsuperscript{G93A} mice.*

Representative immunoblot of p62 and LC3BII/LC3BI performed on TA muscle lysates from NTG C9\textsuperscript{+/-}, SOD1\textsuperscript{G93A} C9\textsuperscript{+/-} and SOD1\textsuperscript{G93A} C9\textsuperscript{-/-} mice at 22W (A) and relative quantifications (B, C). p62 is normalised on total protein. Data are expressed as mean ± SEM (n=6 per experimental group) and analysed by one-way ANOVA followed by Fisher’s LSD post-hoc test. *p<0.05; **p<0.01.
7.2.14 Loss of C9orf72 enhances T cell but decrease myeloid cell infiltration in the tibialis anterior muscle of SOD1\textsuperscript{G93A} mice.

We recently demonstrated that both innate and adaptive immune responses in the peripheral nervous system may influence NMJ denervation in SOD1\textsuperscript{G93A} mice with a protective role at the very early disease stage followed by a detrimental effect during the disease progression (Nardo \textit{et al.}, 2016, 2018; Vallarola \textit{et al.}, 2020).

An increased expression of immune cell transcripts such as CD8 (Figure 7.16A), CD4 (Figure 7.16B), FOXP3 (Figure 7.16C), and CD11b (Figure 7.16D) was also observed in the TA muscle of SOD1\textsuperscript{G93A} C9\textsuperscript{+/+} mice compared to NTG C9\textsuperscript{+/+} controls. The lack of C9orf72 further increased the levels of CD8, CD4, and FOXP3 (Figure 7.16 A, B, C, respectively) transcripts in the TA muscle of SOD1\textsuperscript{G93A} mice, while decreased the levels of CD11b transcript (Figure 7.16D).

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figure7_16.png}
\caption{Loss of C9orf72 enhances T cell but decrease myeloid cell infiltration in the tibialis anterior muscle of SOD1\textsuperscript{G93A} mice. Real-time analysis performed on TA muscle lysates from NTG C9\textsuperscript{+/+}, SOD1\textsuperscript{G93A} C9\textsuperscript{+/+} and SOD1\textsuperscript{G93A} C9\textsuperscript{−/−} mice at 22W for CD8 (A), CD4 (B), FOXP3 (C), and CD11b (D) (n=6 per experimental groups). Data are normalised on β-actin, expressed as mean ± SEM (n=6 per experimental group) and analysed by one-way ANOVA followed by Fisher's LSD post-hoc test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.}
\end{figure}
7.2.15 Loss of C9orf72 increases inflammation in the tibialis anterior muscle of SOD1<sup>G93A</sup> mice.

Then, we assessed whether the massive infiltration of T-cells in SOD1<sup>G93A</sup> C9<sup>/−</sup> corresponded to increased inflammation factors. Real time analysis showed a significant upregulation of the pro-inflammatory cytokines IFNγ (Figure 7.17A), IL1β (Figure 7.17B), TNFα (Figure 7.17C), and CCL2 (Figure 7.17D) transcript, but not the anti-inflammatory cytokine IL10 transcript (Figure 7.17E), as well as the IFNβ transcript (Figure 7.17F), marker of autoimmunity, in the TA muscle of SOD1<sup>G93A</sup> mice compared to NTG C9<sup>+/+</sup> mice. We found that the loss of C9orf72 enhanced the increase of the IFNγ, IL1β, TNFα, and IFNβ transcripts (Figure 7.17A, B, C, respectively), but not the CCL2 and IL10 transcripts (Figure 7.17D, E), suggesting a more marked toxic fingerprint of inflammation in the TA of SOD1<sup>G93A</sup> C9<sup>/−</sup> mice compared to SOD1<sup>G93A</sup> C9<sup>/+</sup> animals.

Figure 7.17: Loss of C9orf72 increases inflammation in the tibialis anterior muscle of SOD1<sup>G93A</sup> mice. Real-time analysis performed on TA muscle lysates from NTG C9<sup>+/+</sup>, SOD1<sup>G93A</sup> C9<sup>+/+</sup> and SOD1<sup>G93A</sup> C9<sup>/−</sup> mice at 22W for IFNγ (A), IL1β (B), TNFα (C), CCL2 (D), IL10 (E) and IFNβ1 (F) transcripts (n=6 per experimental groups). Data are normalised on β-actin, expressed as mean ± SEM and analysed by one-way ANOVA followed by Fisher’s LSD post-hoc test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.
7.3 DISCUSSION

In this chapter, we demonstrated that the C9orf72 LOF synergizes with the SOD1<sup>G93A</sup> mutation by anticipating the motor onset and worsening the motor performance of mice during the progression of the disease. Unexpectedly, the C9orf72 LOF extended the lifespan of SOD1<sup>G93A</sup> mice, providing evidence that C9orf72 has opposite effects in the early and late phases of disease, and bringing to light a positive therapeutic action of a (further) reduction in the C9orf72 protein levels at a later stage of the disease. This is notable in relation to the ongoing clinical trial (NCT04931862) with ASOs designed to lower the expression of the C9orf72 repeats but that might also decrease the levels of the protein.

We hypothesize that an increase in the accumulation of NFs in both CNS and PNS occurs during the early stage of the disease as a possible consequence of disrupted axonal transport, mechanism in which C9orf72 has been demonstrated to be involved (Abo-Rady <em>et al.</em>, 2020). This phenomenon might have contributed to the exacerbation of the hind limb denervation, leading to the anticipation of muscle strength impairment. Another possible mechanism underlying the increased muscle denervation in SOD1<sup>G93A</sup> C9<sup>-/-</sup> mice is the alteration of autophagy in this compartment. In fact, it has been demonstrated that autophagy regulates the structure and function of NMJ (Rudnick <em>et al.</em>, 2017). Conversely, we found that the loss of C9orf72 did not affect the autophagy in the LSC and SN as detected by immunoblot. However, a deeper analysis of autophagy which includes immunohistochemical staining should be performed to examine possible differences in p62 and/or LC3B accumulation in SOD1<sup>G93A</sup> C9<sup>-/-</sup> mice compared to SOD1<sup>G93A</sup> C9<sup>+/+</sup> controls. In fact, Brenner and colleagues (2019) demonstrated that the levels of the autophagic markers p62 and LC3B evaluated through Western blotting of spinal cord protein lysates did not change between SOD1<sup>G93A</sup> Tbk1<sup>-/-</sup> and SOD1<sup>G93A</sup> Tbk1<sup>+/+</sup> mice, but accumulation of aggregated p62 observed through immunohistochemistry was greater in SOD1<sup>G93A</sup> Tbk1<sup>-/-</sup> compared to SOD1<sup>G93A</sup> Tbk1<sup>+/+</sup> mice (Brenner <em>et al.</em>, 2019). Moreover, the
unchanged levels of the autophagic markers p62 and LC3B in LSC and SN of SOD1
g93A C9−/− mice may indicate that the loss of C9orf72 renders the autophagic pathway unable to compensate for
the accumulation of NF.

What happens during the later phase of the disease progression, that results in increased survival in SOD1
g93A mice depleted for C9orf72, needs to be better investigated. Similarly, an
anticipation of the disease onset and a prolonged survival of the disease has been reported in
SOD1
g93A mice depleted for Atg7 selectively in MNs or constitutively KO for Tbk1 (Rudnick et al.,
2017; Brenner et al., 2019). Like C9orf72, Atg7 and Tbk1 are involved in the regulation of
autophagy.

Besides its role in autophagy and axonal transport, the involvement of C9orf72 in the regulation
of inflammation and immune response has been abundantly demonstrated. Together with an
exacerbated NMJ denervation, we found enhanced inflammation characterized by increased
recruitment of T lymphocytes and a more pronounced toxic milieu in SOD1
g93A C9−/− mice compared to SOD1
g93A C9+/− animals. Further analyses are needed to establish whether the
increased immune cell infiltration is the cause or the consequence of exacerbated NMJ
denervation.

In peripheral axons, the loss of C9orf72 in SOD1
g93A mice induced a massive increase and
accumulation in swollen structures of NF-H, which is a pathological characteristic of several
neurodegenerative diseases, suggesting a disruption of axonal transport (Miller et al., 2002).
This is accompanied by increased levels of importin β, a marker of axonal stress (Perry et al.,
2012). While the loss of C9orf72 in NTG mice affected SCs but not axons, in presence of a toxic
stimuli such as mutant hSOD1 we observed an inverted scenario. Indeed, the depletion of
C9orf72 in SOD1
g93A mice did not seem to affect SC response to injury, as shown by unchanged
levels of MBP, CNPase, GFAP and p75NTR. However, given that the loss of C9orf72 impairs SC
function also in NTG mice, this mechanism could be affected earlier in SOD1<sup>G93A</sup> mice, thus further analyses at an earlier stage of the disease are needed.

As we observed in NTG mice, also in SOD1<sup>G93A</sup> mice the loss of C9orf72 enhanced the recruitment of CD8+ cytotoxic T-cells, while decreased the infiltration of myeloid cells. Myeloid cells support axonal regeneration and functional recovery by creating a growth-permissive milieu for injured axons (Barrette et al., 2008). However, in later phase of ALS disease the excessive activation of an immunity that assume a strong proinflammatory phenotype is detrimental, contributing to axonal degeneration, promoted also by macrophages. In the SN of SOD1<sup>G93A</sup> mice we found that the loss of C9orf72 attenuated the toxic fingerprint of inflammatory milieu, that together with fewer myeloid cells could indicate a switch toward a less cytotoxic phenotype, in accordance with the prolonged survival.

In the LSC, the loss of C9orf72 predisposes to a pro-inflammatory phenotype of microglia (O’Rourke et al., 2016), CNS autoimmunity (EAE) and leukocytes expansion (Atanasio et al., 2016; Burberry et al., 2016; Sudria-Lopez et al., 2016). We found that C9orf72 ablation led to exacerbated microglial activation and astrocytosis, as well as enhanced cytotoxic CD8+ and helper CD4+ T-cell infiltration in LSC which has been demonstrated to contribute to neurodegeneration through the secretion of cytotoxic factors at the symptomatic stage of the SOD1<sup>G93A</sup> pathology, while having a more beneficial role in the earlier phases of the disease (Beers et al., 2011; Liao et al., 2012). Unexpectedly, we found that the loss of C9orf72 prompted neuroinflammation towards an anti-inflammatory phenotype, as demonstrated by increased levels of IL10 and YM1, markers of M2 microglia, and unchanged levels of pro-inflammatory cytokines such as IFNγ, IL1β, TNFα and CCL2. This evidence suggests that the unchanged extent of MN loss in SOD1<sup>G93A</sup> C9<sup>−/−</sup> mice compared to SOD1<sup>G93A</sup> C9<sup>+/+</sup> animal could be the result of a balance between the increased MN dysfunction (accumulation of NF) and the prevailing anti-inflammatory milieu. Also in this case, a switch toward a less cytotoxic phenotype is in accordance with the prolonged survival of SOD1<sup>G93A</sup> C9<sup>−/−</sup> mice.
Another hypothesis for the prolonged survival may relate to the slight attenuation of the body weight loss during the disease progression in SOD1<sup>G93A</sup> mice depleted for C9orf72. Evidence in both ALS patients and animal models demonstrated that the maintenance of the body mass index (BMI) is associated with an improved outcome and increased survival (Ngo, Steyn and McCombe, 2014). Thus, while the increased NMJs denervation induced by C9orf72 LOF causes an exacerbation of neuromuscular deficit in SOD1<sup>G93A</sup> mice, the higher energetic reserves due to the higher body weight might slowing down the rapid deterioration of the general state of the animal, allowing an increase in survival.

Taken together, these results indicate that the loss of C9orf72 exacerbated structural alterations in MNs in both CNS and PNS that lead to functional alterations, acting in a cell autonomous manner in presence of the SOD1<sup>G93A</sup> toxic stimuli. However, only in the PNS, and particularly at the neuromuscular plaque, we observed significant alterations induced by the C9orf72 LOF, that is likely the cause of the onset anticipation. This is accompanied by an exacerbated immune response that led to the establishment of a greater proinflammatory environment, that contributes to the denervation. Conversely, in the SN and clearly in the LSC the loss of C9orf72 mitigates the detrimental action of inflammation at the symptomatic stage of the disease, leading to a prolonged survival. This evidence suggests that reducing the levels of C9orf72 protein might have a therapeutic relevance. We can conclude that the loss of C9orf72 mostly affects the PNS, which is the first compartment of the neuromuscular system to be affect by the disease, enhancing denervation and the disruption of axon cytoarchitecture.
GENERAL DISCUSSION AND CONCLUSIONS

Chapter 8
The mutation in the C9orf72 gene is the most prevalent genetic cause of both ALS and FTD (DeJesus-Hernandez et al., 2011; Renton et al., 2011; Majounie et al., 2012). The reason why the mutation could induce either ALS or FTD, or a combination of the two is unknown. This evidence points out a possible oligogenic nature of C9ALS pathology (Nguyen, Van Broeckhoven and van der Zee, 2018). However, the precise mechanism through which it causes neurodegeneration is still unknown. Both LOF and GOF pathogenic mechanisms have been proposed, that could trigger the disease in cooperation.

Growing bodies of evidence support the “dying back” theory, according to which ALS is a distal axonopathy originating in the peripheral tissues, including skeletal muscle, and spreads to MNs through a retrograde signalling cascade. Furthermore, in previous studies we demonstrated the pivotal role of the immune response in the peripheral nervous system (PNS) in governing the disease progression (Nardo et al., 2016; Gentile et al., 2019).

Given that, it is essential to understand the role of C9orf72 in the neuromuscular system with particular attention to the peripheral compartment, in order to establish the involvement of C9orf72 in ALS.

For the first time, in this study we demonstrate that C9orf72 is expressed not only in the CNS, but also in the peripheral compartment of the neuromuscular system in mice. Our work highlights that besides spinal cord, C9orf72 is expressed also in the sciatic nerve, particularly by Schwann cells and skeletal muscle, suggesting a function in the peripheral compartment. Indeed, we found that the constitutive ablation of C9orf72 in mice induced denervation in the skeletal muscles, accompanied by altered autophagy, infiltration of T-cells and the establishment of a proinflammatory milieu, which are typical features of ALS, even if this is not sufficient to induce MN degeneration. However, when the ablation of C9orf72 occurred in SOD1G93A mice, the best characterized ALS mouse model, we observed an exacerbation of denervation and autophagy alterations, associated to enhanced T-cell infiltration and cytotoxic environment. All these
events led to the anticipation of the symptom onset and worsening of the motor performance, suggesting that C9orf72 has a role in the maintaining of the neuromuscular junction functionality and its loss contributes to ALS through denervation, synergizing with other ALS mutations. On the other hand, the ablation of the C9orf72 gene showed a positive effect on SOD1	extsuperscript{G93A} mice, prolonging their survival. This finding could have an important therapeutic implication, especially in view of the recent clinical trial in which ASOs are used to decrease the expression of C9orf72 repeats but might also lead to a decrease of the C9orf72 “healthy” protein, carrying out an additional positive effect on C9ALS patients.

In the sciatic nerve of mice, C9orf72 is mostly expressed by the SCs and when the protein is depleted alterations in this cell population occur. The ablation of C9orf72 in NTG mice caused demyelination, dedifferentiation, and proliferation of SCs, accompanied by a low-level insufficient attempt of regeneration, even in absence of axon injury, a condition similar to the “inflammaging” in the old nerves, leading to neuropathy (Büttner et al., 2018).

Interestingly, the loss of C9orf72 also induced the expression of MHCI in the SCs and the infiltration of CD8+ T-cells, that are cytotoxic to SCs, contributing to the neuropathy (Tang et al., 2013). Moreover, the loss of C9orf72 favours the establishment of a pro-inflammatory environment in the SN, with signs of autoimmunity as detected by the increase of IFNβ1. Also in the sciatic nerve, we found alterations in autophagy, a pivotal pathway for the myelinating function of SCs. Our results are in line with the evidence that the loss of C9orf72 led to systemic autoimmunity similar to systemic lupus erythematosus (Atanasio et al., 2016) and promotes the development of experimental autoimmune encephalitis through alterations in autophagy (McCauley et al., 2020).

Conversely, the loss of C9orf72 in SOD1	extsuperscript{G93A} mice altered the axonal transport in both central and peripheral axons, another mechanism in which C9orf72 is involved (Abo-Rady et al., 2020), while did not affect SCs. The loss of C9orf72 in SOD1	extsuperscript{G93A} mice caused a lower inflammatory response
but exacerbated the CD8+ T-cells infiltration in sciatic nerve and autoimmunity. Overall, these data showed that C9orf72 is expressed by SCs and has a pivotal role in the proper function of this cell population, and the loss of C9orf72 induced inflammaging with peripheral autoimmune neuropathy, without axon compromission. On the contrary, when in presence of the SOD1G93A toxic stimuli, the loss of C9orf72 acts on the MN functionality and exacerbates the autoimmunity. Although we did not observe any significant alterations in SCs response in SOD1G93A pathology at the symptomatic stage, we cannot exclude that exacerbation of SCs impairment could occur at early stage of the disease, while at the symptomatic stage there is a switch toward a less detrimental environment, in line with the prolonged survival of SOD1G93A mice depleted of C9orf72.

In accordance with other studies, we found that the ablation of C9orf72 in NTG mice did not produce any alterations in MN number, autophagy, or immune response in lumbar spinal cord, suggesting that it is involved in redundant pathway and that it is dispensable for the maintenance of homeostasis in this compartment of the neuromuscular system. On the contrary, in lumbar spinal cord of SOD1G93A mice the loss of C9orf72 induced an enhanced cellular stress in MNs, glial activation and immune response. However, this is accompanied by an increased anti-inflammatory milieu, in line with the prolonged survival, even whether the time of paralysis was not postponed.

In conclusion, C9orf72 is expressed in the peripheral compartment of the neuromuscular system. The loss of C9orf72 induces mild denervation peripheral inflammation and autoimmune peripheral neuropathy, suggesting a pivotal role in the maintaining of the homeostasis of the NMJs and SCs. In SOD1G93A mice, the loss of C9orf72 anticipates the muscle force impairment but prolongs the survival, suggesting a counteracting role of C9orf72 in different stages of the disease. This corresponds to the exacerbation of denervation, axonal alterations, and detrimental inflammation in muscle, and to a switch of the proinflammatory milieu to a more beneficial environment. Overall, these results suggest that the C9orf72 LOF has a pivotal and
dual role in modulating ALS pathogenesis, acting in combination with other ALS-linked mutations, and it might also have a positive therapeutic effect on the extension of lifespan. Moreover, C9orf72 LOF particularly affects the peripheral compartment, which is the earliest affected by the disease.

As future perspectives, we aim to study in depth the role of C9orf72 within SCs. Particularly, we want to produce a conditional C9KO mouse model in which C9orf72 is depleted selectively within SCs to verify whether the C9orf72 LOF directly alters the functionality of SCs, which produce the alterations that we observed in the nerves of C9<sup>+/</sup> mice, or the C9orf72 LOF alterations in immune response are responsible of these alterations.

Moreover, we aim to verify whether the C9orf72 LOF could modulate the disease progression also in other ALS mouse models, by breeding C9orf72 KO mice with mutant TDP43 and FUS mice.
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