NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTAMENT OF LIFE SCIENCES

MARTA ALEXANDRA MARQUES DOS SANTOS

BSc in Biochemistry

THERAPEUTIC POTENTIAL OF INTRATHECAL APPLICATION OF miR-124-BASED SECRETOME IN THE SOD1^{G93A} MOUSE MODEL OF AMYOTROPHIC LATERAL SCLEROSIS

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Adviser: Dora Maria Tuna de Oliveira Brites, PhD
Group Leader – Neuroinflammation, Signaling and Neuroregeneration Lab
Research Institute for Medicines (iMed.ULisboa),
Faculty of Pharmacy, Universidade de Lisboa

Co-advisers: Margarida Casal Ribeiro Castro Caldas Braga, PhD
Assistant Professor, Department of Life Sciences,
NOVA University Lisbon

Examination Committee:

Chair: **Pedro Manuel Br[1] a Costa**,
Assistant Professor, Department of Life Sciences,
NOVA School of Science and Technology, NOVA University Lisbon

Rapporteurs: **Hugo Miguel Vicente Miranda**
Invited Assistant Professor, NOVA Medical School, NOVA University Lisbon

Adviser: **Dora Maria Tuna de Oliveira Brites,**
Group Leader ï Neuroinflammation, Signaling and Neuroregeneration Lab
Research Institute for Medicines (iMed.ULisboa),
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(Dory, from Finding Nemo)

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NEUROSIGNAL

Neuroinflammation Signaling and Neuroregeneration

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease with no effective cure and a short life expectancy, characterized by the degeneration of motor neurons (MNs) and glial cell dysfunction. Inflammatory(inflamma)-associated microRNAs (miRNAs/miRs) were found dysregulated in SOD1^{G93A} (mSOD1) mouse models, as well as in mSOD1 MNs and microglia, and their secretome. Prior studies from our lab detected upregulated levels of miR-124 in mSOD1 NSC-34-MN-like cells and demonstrated its association with their degeneration, including mitochondrial dysfunction and axonal/synaptic dysregulation, together with glial activation. Interestingly, the treatment with anti-miR-124 in mSOD1 MNs prevented their impairment. Furthermore, our group showed that the secretome derived from these modulated mSOD1 MNs (preconditioned secretome) counteracted pathological features and neuro-immune homeostatic imbalance in spinal cord organotypic cultures from early symptomatic mSOD1 mice. This issue was never explored in the *in vivo* model of ALS, though recent evidence sustains that cell secretomes may have therapeutic effects. To decipher the potential therapeutic benefits of such preconditioned secretome, our group performed its intrathecal injection in the mSOD1 mice at the early symptomatic stage of the disease (12-week-old). Notably, this preconditioned secretome prevented the glial reactivity/dysfunction, neurodegeneration, and the altered inflammatory-dynamic balance of the 15-week-old symptomatic mSOD1 mice. At the molecular level, the injection of this engineered secretome enhanced NeuN mRNA/protein expression levels and the *Dlg4*/*Mbp*/*Plp*/*Trem2*/*Arg1*/*Inos*/*Il-10* genes, thus precluding the neuronal/glial cell dysregulation that characterizes the ALS mice. The upregulated GFAP/*Cx43*/S100B/Iba-1 and the inflamma-associated miRNAs (miR-146a/miR-155/miR-21) displayed by the symptomatic mSOD1 mice were also prevented. Overall, this study highlights the intrathecal administration of the anti-miR-124-treated mSOD1 MN preconditioned secretome as a promising cell-free based therapeutic strategy to halt/delay disease progression in the ALS mouse model, supporting its translation potential into the ALS patient as a personalized and autologous treatment.

Keywords: ALS mouse model; Anti-microRNA-124; Secretome-based therapy; Intrathecal delivery route; Human SOD1^{G93A} mutation; Prevention of neurodegeneration and glial dysfunction

RESUMO

A Esclerose Lateral Amiotrófica (ELA) é uma doença neurodegenerativa progressiva sem cura efetiva e com uma expectativa de vida curta, caracterizada pela degeneração dos neurónios motores (NMs) e disfunção glial. A desregulação dos microRNAs (miRNAs/miRs) associados à inflamação foi observada em doentes com ELA e em modelos animais, bem como em NMs mutados (NMs-ELA) e no seu secretoma. Estudos prévios do nosso grupo detetaram níveis elevados do miR-124 em NMs NSC-34 $SOD1^{G93A}$ (SOD1 mutada em G93A, mSOD1) e que os mesmos contribuem para a degeneração e disfunção destes NMs, com comprometimentos a nível mitocondrial, axonal/sináptico, bem como ativação glial. Curiosamente, nós mostrámos que o tratamento com anti-miR-124 nestes NMs-ELA previne a sua desregulação e que o secretoma derivado destes NMs modulados (secretoma precondicionado) impede as características patológicas e o desequilíbrio da homeostasia celular em culturas organotípicas de medula espinhal de ratinhos mSOD1 em estádio sintomático precoce. Esta questão nunca foi explorada no modelo *in vivo* de ELA, embora evidências recentes sustentem que o secretoma derivado de diversos tipos celulares pode ter efeitos terapêuticos. De modo a decifrar o seu potencial terapêutico, o nosso grupo realizou a injeção intratecal deste secretoma precondicionado em ratinhos mSOD1 no estádio sintomático inicial da doença (12 semanas de idade). Este secretoma, com valores de miR-124 regulados para os níveis basais, preveniu a reatividade/disfunção glial, neurodegeneração e o desequilíbrio inflamatório nos ratinhos ELA às 15 semanas de idade (fase sintomática). A nível molecular, a injeção do secretoma elevou os níveis de expressão do NeuN, quer a nível do mRNA, quer da proteína, bem como dos genes *Dlg4*/*Mbp*/*Plp*/*Trem2*/*Arg1*/*Inos*/*Il-10*, impedindo assim a desregulação das células neuronais/gliais que caracteriza os ratinhos ELA. O aumento dos níveis de GFAP/Cx43/S100B/Iba-1 e dos miRNAs associados à inflamação (miR-146a/miR-155/miR-21) exibidos pelos ratinhos ELA sintomáticos, foram também prevenidos. Em resumo, este estudo destaca a administração intratecal do secretoma derivado de NMs-ELA modulados com anti-miR-124 como uma estratégia terapêutica promissora para parar/retardar a progressão da doença no modelo de ratinho ELA, suportando o seu potencial de translação para o doente com ELA, como um tratamento personalizado e autólogo.

Palavras-chave: Modelo de ratinho ELA; Anti-microRNA-124; terapia baseada no secretoma; via de administração intratecal; mutação $SOD1^{G93A}$ humana; prevenção da neurodegeneração e disfunção glial

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III. MATERIALS AND METHODS

ABBREVIATIONS

AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
ARG1	Arginase 1
ASO	Antisense oligonucleotide
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BSA	Bovine serum albumin
C9ORF72	Chromosome 9 open reading frame 72
cDNA	Complementary DNA
CNS	Central nervous system
CSF	Cerebrospinal fluid
CX3CL1	C-X3-C chemokine ligand 1
CX3CR1	C-X3-C chemokine receptor 1
Cx43	Connexin 43
DAM	Disease-associated microglia
DGCR8	DiGeorge syndrome critical region 8
DIV	Days *in vitro*
Dlg4	Discs large MAGUK scaffold protein 4
DNA	Deoxyribonucleic acid
Dyn	Dynein
EAAT2	Excitatory amino acid transporter 2
ER	Endoplasmic reticulum
EVs	Extracellular vesicles
fALS	Familial amyotrophic lateral sclerosis
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FTD	Frontotemporal dementia
FUS	Fused in sarcoma

G93A	Substitution of a glycine to an alanine at position 93
GFAP	Glial fibrillary acidic protein
GLT-1	Glutamate transporter 1
GM	Gray matter
Gpr17	G protein-coupled receptor 17
hSOD1^{G93A}	Human SOD1^{G93A}
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
HSA	*Homo sapiens*
iAstrocytes	Induced astrocytes
Iba-1	Ionized calcium-binding adaptor molecule 1
ICV	Intracerebroventricular
IL	Interleukin
IL-1β	Interleukin 1 beta
IN	Intranasal
Inflamma-miRNAs	Inflammatory-associated miRNAs
iNOS	Inducible nitric oxide synthase
iNPCs	Induced neuronal progenitor cells
iPSC	Induced pluripotent stem cell
IT	Intrathecal
IV	Intravenous
Kif5b	Kinesin family member 5B
MBP	Myelin basic protein
MFG-E8	Milk fat globule-epidermal growth factor 8
miRNA/miR	MicroRNA
MMU	*Mus musculus*
MN	Motor neuron
mRNA	Messenger RNA
mSOD1	Mutant superoxide dismutase 1
NeuN	Hexaribonucleotide binding protein 3
NMDA	N-methyl-D-aspartate
NMJ	Neuromuscular junction
NO	Nitric oxide
NSC-34	Neuroblastoma/spinal cord-34
OCs	Organotypic cultures
Ol	Oligodendrocyte
OPCs	Oligodendrocyte precursor cells
P2RY12	Purinergic receptor P2Y12
PBS	Phosphate-buffered saline
PLP	Proteolipid protein

Pol II	Polymerase II
Pre-miRNA	Precursor miRNA
Pri-miRNA	Primary miRNA
PSD-95	Postsynaptic density protein 95
rAAV	Recombinant adeno-associated virus
RNA	Ribonucleic acid
RNAseq	RNA sequencing
RISC	RNA-induced silencing complex
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
Rpl19	60S ribosomal L19
RS	Reactive species
RT	Room temperature
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
S100B	S100 calcium-binding protein B
sALS	Sporadic amyotrophic lateral sclerosis
SC	Spinal cord
SCOCs	Spinal cord organotypic cultures
SDS	Sodium dodecyl sulfate
sEV	Small extracellular vesicle
SOD1	Superoxide dismutase 1
TAR	Trans active response
TARDBP/TDP-43	TAR DNA-binding protein 43
TBS	Tris-Buffered Saline
TBS-T	TBS-0.1% Tween 20
TG	Transgenic
Timp2	Tissue inhibitor of metalloproteinases 2
TNF-α	Tumor necrosis factor-alpha
TMEM119	Transmembrane protein 119
TRBP	TAR RNA-binding protein 2
TREM2	Triggering receptor expressed on myeloid cells 2
UPS	Ubiquitin-proteasome system
WB	Western blot
WT	Wild type

I
INTRODUCTION

1. Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic lateral sclerosis (ALS) is a multisystem fatal neurodegenerative disease characterized by the rapid dysfunction and progressive loss of upper and lower motor neurons (MNs) in the motor cortex and the brainstem, and in the anterior horn of the spinal cord (SC), respectively **(Figure I.1)** (Batra et al., 2019; Masrori et al., 2020). The term "amyotrophic" means "no muscle nourishment"; "lateral" refers to the lateral corticospinal tracts (nerves in the SC that signal muscles); and "sclerosis" indicates the hardening of this lateral region (Hulisz, 2018).

It is the most frequent and aggressive form of MN disease (Brenner et al., 2019) and the third most common neurodegenerative disorder worldwide (Bennett et al., 2019), with an estimated global mortality of 30,000 patients per year (Petrov et al., 2017). Once degeneration and death of MNs occur, neuromuscular communication becomes compromised, leading to muscle weakness and atrophy throughout the body due to lack of nourishment. The loss of MNs is accompanied by a significant reaction of neighboring glia, namely microglia and astrocytes. Indeed, the neurodegenerative process in ALS is accompanied by a sustained inflammation in the brain and SC (Brites et al., 2014).

Although ALS often has a focal onset, the symptoms generally spread to distinct body regions. Most patients die within 3 to 5 years after diagnosis due to progressive limb paralysis, eventually leading to respiratory insufficiency (Bennett et al., 2019; Yun et al., 2020).

Despite being studied for several years and the significant research efforts made in the last decades, all this effort has not yet yielded effective therapeutic strategies or cure for the disease, mainly due to the difficulties brought by disease heterogeneity at the neuropathological, genetic, and clinical levels.

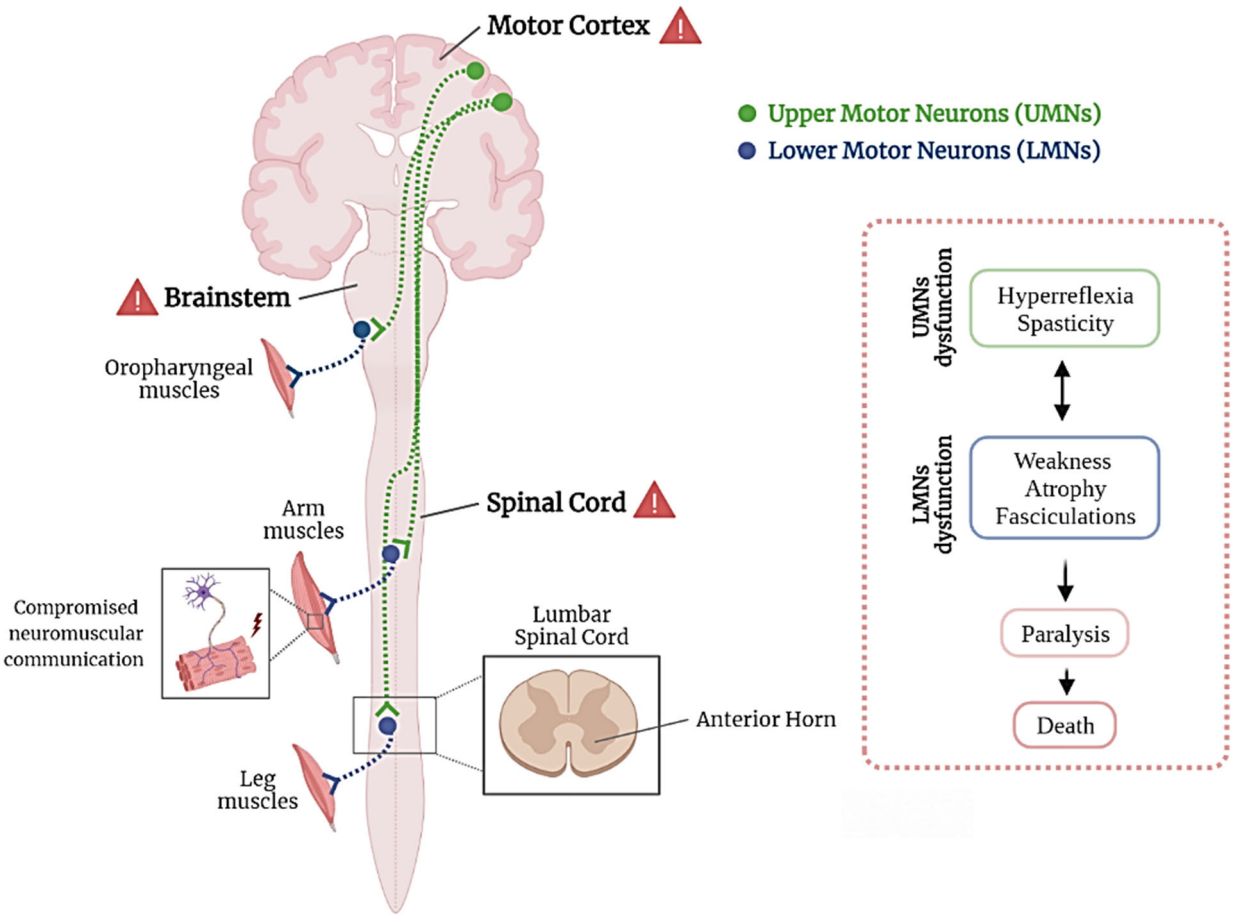

Figure I.1 — Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disorder characterized by progressive degeneration of lower and upper motor neurons (MNs). This disease affects both MNs across the corticospinal tract (upper MNs), which originate in the motor cortex and project to the brainstem and the anterior horn of the spinal cord (SC), while the lower MNs project from the brainstem or SC to innervate effector muscles and glands all over the body. Dashed lines represent impaired innervation in ALS (Created with BioRender.com).

1.1 Epidemiology and Clinical Presentations

ALS is an adult-onset disease with a median age of onset between 51 and 66 years, even though younger individuals may be affected. ALS has a prevalence of 4.1-8.4 cases per 100,000 individuals, with an estimated incidence of 0.6-3.8 per 100,000 per year, despite its non-uniform distribution across the globe. For instance, in Europe, the incidence is higher, with 2.1-3.8 cases per 100,000 people annually (Longinetti et al., 2019). ALS is more common in men than women, affecting 1.2-1.5 men for every woman, with an overall lifetime risk of about 1 in 350 for men and 1 in 400 for women (van Es et al., 2017).

Regarding the clinical onset of the disease, ALS has two main initial presentations that can vary between patients. Spinal-onset ALS (or limb-onset ALS), representing approximately two-thirds of all cases (Longinetti et al., 2019), is characterized by symptoms such as muscle weakness, cramps, spasticity, fasciculations, and atrophy in the limbs and trunk. On the other hand, one-third of patients develop the bulbar-onset disease with speech (dysarthria), breathing, and swallowing (dysphagia) difficulties (Gomes et al., 2020). Clinically, bulbar-onset is the most severe form of ALS, presenting a worse prognosis and a shorter survival, with an average of 2 years (Amin et al., 2020; Qin et al., 2022).

Although the main clinical manifestations of ALS are associated with motor dysfunction, up to 50% of patients develop extra-motor symptoms such as cognitive and behavioral impairment during the

course of the disease, and 13% of patients present with concomitant behavioral-variant frontotemporal dementia (FTD) (van Es et al., 2017). Regarding the behavioral symptoms that affect 10% of all patients with ALS, apathy, and loss of sympathy are the most prevalent, whereas, in the cognitive part, fluency, language, social cognition, and executive functions are the most affected (Abrahams et al., 2014).

Usually, a delay of 9-24 months from the first symptoms to diagnosis is observed (Longinetti et al., 2019). Some factors predict a fast progression and a worse prognosis, including an older age of onset, a short period between first symptoms and diagnosis, bulbar site of onset, presence of cognitive impairment, and genotypic features. Collectively, these parameters lead to reduced patient survival (Al-Chalabi et al., 2013).

1.2 ALS Genetic Basis and Risk Factors

ALS is a complex, heterogeneous, and multifactorial disorder caused by genetic susceptibility factors, and environmental and lifestyle risk factors (Masrori et al., 2020).

There are two types of ALS. Around 10% of patients are diagnosed with familial ALS (fALS) that are due to inherited mutations with more than 30 different causative genes already identified being superoxide dismutase 1 (*SOD1*), chromosome 9 open reading frame 72 (*C9ORF72*), fused in sarcoma (*FUS*), and trans active response (TAR) deoxyribonucleic acid (DNA)-binding protein 43 (*TARDBP*) the most commonly mutated genes in ALS, accounting for the majority of all cases (Amin et al., 2020). Although these genes confer a significant risk of developing ALS, there is evidence of oligogenic inheritance, in which a phenotypic manifestation is determined by several distinct genes, and of genetic pleiotropy, in which a single gene affects multiple phenotypic traits. For instance, *C9ORF72*, the most established example of pleiotropy, is clearly linked to ALS and FTD but is also associated with Parkinsonism, Alzheimer's disease, schizophrenia, and bipolar disorder (Hardiman et al., 2017). The remaining 90% are diagnosed with sporadic ALS (sALS) without any known cause **(Figure I.1)** (Layalle et al., 2021; Yun et al., 2020). Nevertheless, in a substantial proportion of sALS cases, the influence of genetic factors must also be considered (Brenner et al., 2019).

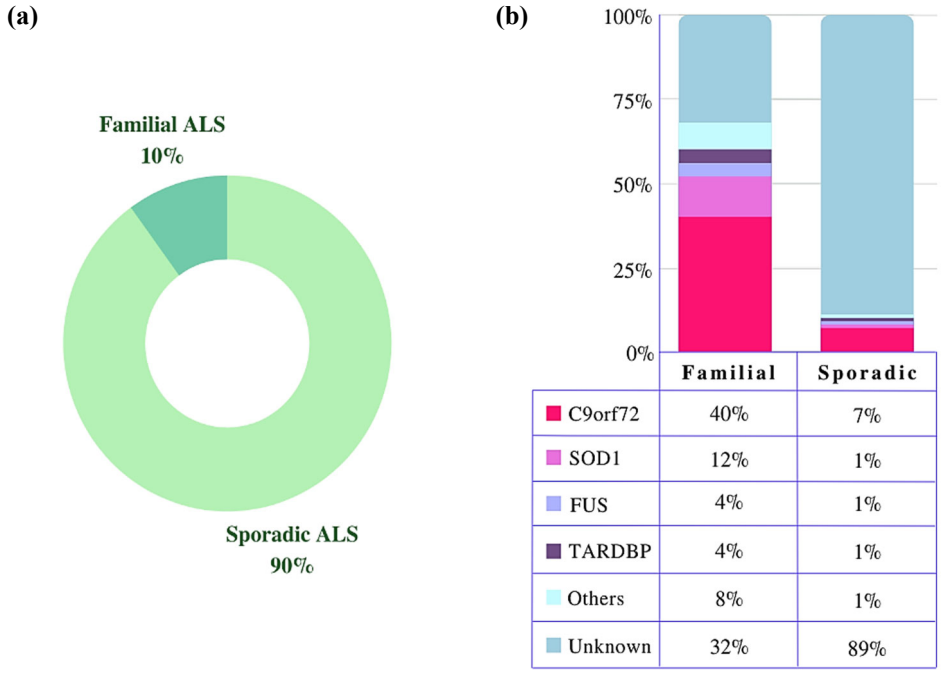

Figure I.2 — Genetic alterations in *SOD1*, *C9ORF72*, *FUS*, and *TARDBP* account for 60% of familial amyotrophic lateral sclerosis (fALS) and 10% of sporadic ALS (sALS). (a) Prevalence of fALS and sALS cases. **(b)** Most common mutated genes in ALS disease and proportion of fALS and sALS cases for each causative gene. *Adapted from* (Yun et al., 2020) (Created with Canva.com).

Clinically and pathologically, fALS is frequently indistinguishable from sALS, both showing the same neuropathological pattern and presenting overlapping pathogenic downstream pathways, including axonal transport, selective autophagy, ribonucleic acid (RNA) metabolism, and DNA repair (Brenner et al., 2019).

Although there are no known environmental risk factors irrefutably linked to ALS, suspected risk factors include smoking and alcohol consumption, the neurotoxic amino acid β-N-methylamino-L-alanine, traumatic brain injury, electromagnetic fields, pesticides and insecticides, and exposure to heavy metals, such as lead and mercury (Talbott et al., 2016).

FUS encodes a multifunctional RNA/DNA-binding protein that undergoes nucleocytoplasmic shuttling to perform various cellular functions, including transcription, protein translation, RNA splicing, and transport. However, in ALS, mutant FUS accumulates in the cytoplasm, exerting a toxic gain of function (Lin et al., 2021; Sama et al., 2014). Mutations in this gene are associated with the young-onset disease with prominent bulbar involvement and fast progression (Al-Chalabi et al., 2013).

The most common genetic cause underlying ALS is the GGGGCC (G_4C_2) hexanucleotide repeat expansion located in the first intron of the *C9ORF72* gene, which is frequently associated with cognitive and behavioral deficits and shorter survival of ALS patients (Balendra et al., 2018; Irwin et al., 2013). *C9ORF72* is known to have several biological functions, such as regulation of lysosome biogenesis and maturation, autophagy, RNA and lipid metabolism, innate immune inflammatory pathways, axon growth, and trafficking, and it seems to have an involvement in synaptic activity (Hardiman et al., 2017; Pang et al., 2021). Healthy people usually have between 2 and 23 repeats, whilst affected individuals may have over hundreds. Although the pathogenesis of *C9ORF72*-linked ALS is currently unknown, both loss- and gain-of-function of *C9ORF72* seem to have a pathogenic role in neuroinflammation and neurodegeneration (Lall et al., 2017; Scarrott et al., 2015). Interestingly, repeat expansions in the *C9ORF72* gene are a frequent cause of both ALS and FTD. The discovery of C9FTD/ALS – the collective term for *C9ORF72*-associated diseases with clinical features of FTD, ALS, or both – has heightened the realization that ALS and FTD are intimately linked on a clinical, genetic, pathological, and mechanistic spectrum (Balendra et al., 2018).

TAR DNA-binding protein 43 (TDP-43) is a nuclear DNA/RNA-binding protein of the family of heterogeneous nuclear ribonucleoproteins encoded by the *TARDBP* gene (Buratti, 2015). It is implicated in the regulation of messenger RNA (mRNA) splicing, transport, stability, translation, and microRNA (miRNA/miR) processing (Buratti et al., 2012; Ratti et al., 2016). Some studies have shown that TDP-43 also plays an essential role in regulating mRNAs involved in neuronal development (Prasad et al., 2019). As in other neurodegenerative diseases, the accumulation and aggregation of intracellular protein inclusions are one of the major hallmarks of ALS, including TDP-43 cytoplasmic aggregates previously found in *post-mortem* tissues of ALS patients (Suk et al., 2020). Furthermore, over 50 mutations in the *TARDBP* gene have already been linked to ALS (Buratti, 2015). Although mutations in *TARDBP* are a rare cause of fALS, TDP-43 is the principal protein constituent of these inclusions in most ALS subtypes. Indeed, about 97% of ALS patients have features of a TDP-43 proteinopathy with depletion of TDP-43 in the nucleus but the formation of cytoplasmic aggregates in MNs (Hardiman et al., 2017). Under physiological conditions, TDP-43 is predominantly found in the nucleus, performing different functions related to RNA processing, such as transcription regulation and alternative splicing. However, under pathological conditions, namely as in ALS, aberrant post-translational modifications can occur, leading to mislocalization of these misfolded TDP-43 aggregates from the nucleus to the cytoplasm, causing deleterious effects to the cell. Once in the cytoplasm, it will accumulate, forming pathological aggregates that disrupt physiological functioning, thereby exacerbating pathology and promoting neuronal degeneration. The deposition of these hyperphosphorylated and ubiquitinated TDP-43 proteins into inclusion bodies can even propagate to neighboring cells (Jo et al., 2020; Prasad et al., 2019).

The *SOD1* gene encodes for an antioxidant copper/zinc (Cu/Zn)-binding metalloenzyme of 32kDa that catalyzes the conversion of toxic superoxide radical anions ($O_2^{\bullet-}$), produced during cellular respiration, into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) (Doucette et al., 2004). Therefore, the main functions of SOD1 are to protect cells from reactive oxygen species (ROS) and reduce the concentration of superoxide, avoiding harmful amounts of these toxic molecules (Azadmanesh et al., 2018). In 1993, mutant *SOD1* (mSOD1) was the first gene to be linked to ALS (Kim et al., 2020). Over 170 *SOD1* mutations have been identified as being associated with ALS, most of them found in familial cases of the disease (Yun et al., 2020). The first identified mutations in the *SOD1* gene that affect protein activity were the substitution of alanine to valine at codon 4 (A4V), D90A, where aspartate had been substituted to alanine at codon 90, and the substitution of glycine to alanine at position 93 (G93A). Although the G93A is a relatively rare mutation in humans, it is among the most widely studied, as it was the first mutation to be characterized in the transgenic (TG) ALS mice model. Moreover, *SOD1*-asssociated mutations constitute most of the *in vitro* and *in vivo* studies, since the animal model efficiently reproduces the clinical and pathophysiological symptoms of the disease (Pansarasa et al., 2018). When mutated, the abnormal SOD1 enzyme is thought to gain new deleterious properties. Therefore, oxidative stress due to *SOD1* mutations has been attributed to accumulation and toxic gain of function of the misfolded protein, rather than by the loss of scavenging activity of SOD1. This proposed gain of toxic function mechanism may involve misfolding of mSOD1 to form aberrant intracellular protein aggregates. These SOD1 inclusions are found both in fALS patients with *SOD1* mutations and in mSOD1 TG mice (Hayashi et al., 2016; Mejzini et al., 2019; Rowland et al., 2001). Individuals with *SOD1*-related ALS display some specificities including an earlier age of onset, longer duration of disease, onset of motor symptoms mostly in the lower limbs and rare occurrences of cognitive perturbations (Mathis et al., 2019).

1.3 Molecular and Cellular Mechanisms in MN Degeneration

Although the pathophysiological mechanisms underlying MN degeneration in ALS are still not fully understood, several cellular and molecular processes implicated in ALS pathogenesis have already been described (Filipi et al., 2020). Multiple factors, rather than a single initiating event seem to contribute to the development and progression of the disease (Mejzini et al., 2019). The neuropathological signature of ALS is characterized by the loss of the neuromuscular connection, axonal retraction, and subsequent MN death, surrounded by astrogliosis and microgliosis, with ubiquitin-positive inclusions being observed in surviving neurons (Masrori et al., 2020).

Most of the pathological features observed in sALS cases are similar to those detected in fALS, suggesting common events that lead to MN degeneration (Ferraiuolo et al., 2011). Extensive research on ALS-causing gene products has discovered many pathogenic pathways, proposing multifactorial pathomechanisms in ALS that interact mutually through diverse molecular and genetic pathways (Yamanaka et al., 2018). Effectively, the large number of genes and cellular pathways implicated in ALS has led to the proposal of multiple disease mechanisms associated with loss and degeneration of MNs, including protein misfolding, disturbance in RNA processing and metabolism, inclusion bodies, impaired protein homeostasis, nucleocytoplasmic and vesicular transport deficits, impaired DNA repair, oxidative stress, endoplasmic reticulum (ER) stress, excitotoxicity, axonal transport disruption, mitochondrial dysfunction, prion-like propagation, non-cell autonomous toxicity of neighboring glia, neuroinflammation, and oligodendrocyte (Ol) defects **(Figure I.3)** (Mejzini et al., 2019). Some of these mechanisms are detailed below.

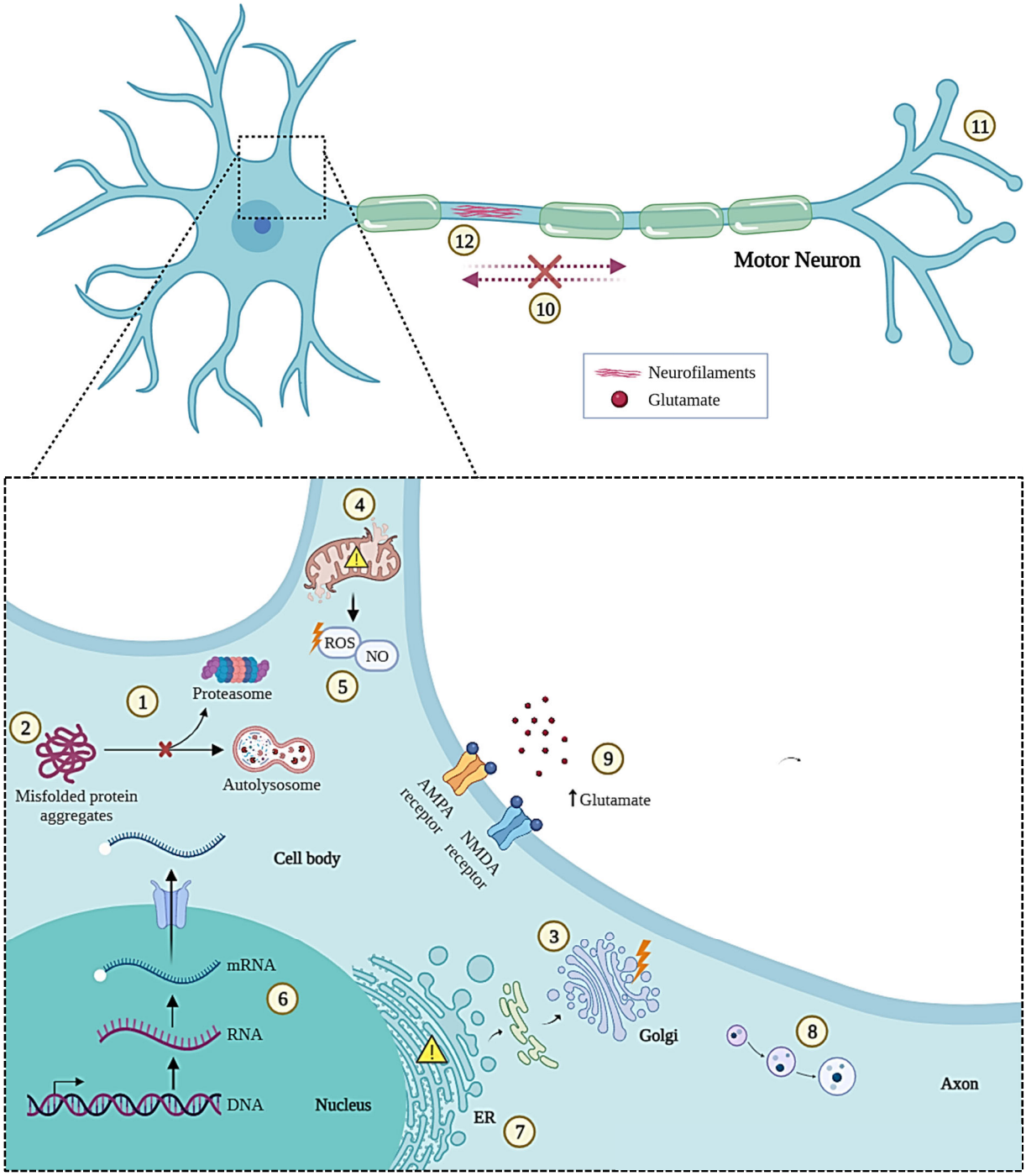

Figure I.3 — Amyotrophic Lateral Sclerosis (ALS) is characterized by a complex interaction between genetic and molecular pathways that are associated with motor neuron (MN) degeneration. The pathophysiological mechanisms underlying neurodegeneration in ALS are linked to an intricate interplay between a combination of several molecular pathways: **(1)** Impaired protein homeostasis resulting from functional defects in autophagy and ubiquitin-proteasome system **(2)** Misfolded protein aggregates **(3)** Golgi fragmentation **(4)** Mitochondrial dysfunction **(5)** Oxidative stress **(6)** Abnormal RNA processing and metabolism **(7)** ER stress **(8)** Vesicular transport defects **(9)** Excitotoxicity **(10)** Impaired axonal transport **(11)** Synaptic failure **(12)** Neurofilament accumulation (Created with BioRender.com). **AMPA**, α-Amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid; **DNA**, deoxyribonucleic acid; **ER**, endoplasmic reticulum; **mRNA**, messenger RNA; **NMDA**, N-methyl-D-aspartate; **RNA**, ribonucleic acid; **ROS**, reactive oxygen species.

The pathological hallmark in ALS is the presence of neurotoxic misfolded protein aggregates within MNs, resulting from impaired protein homeostasis (Amin et al., 2020) **(Figure I.3)**. Some types of pathological aggregates that might be observed are the accumulation of TDP-43 inclusions and misfolded SOD1 in MNs of sALS and fALS cases (Ferraiuolo et al., 2011; Hardiman et al., 2017).

Mutations in some genes lead to the translation of misfolded proteins that have an abnormal cellular localization or are aberrantly formed. Consequently, they can impair the proteasomal or autophagic machinery of the cell, leading to a compromised protein turnover. Indeed, genes associated with fALS encode proteins that can promote dysfunction of the ubiquitin-proteasome system (UPS). For instance, mSOD1 is associated with decreased expression of UPS components. Additionally, SOD1 and TDP-43 are known autophagy substrates, suggesting that defective autophagy contributes to the toxic accumulation of these proteins in ALS (Hardiman et al., 2017).

The cellular protein degradation machinery and autophagy pathways are crucial in clearing misfolded/aggregated proteins and damaged organelles (Prasad et al., 2019). Thus, deficits in these mechanisms lead to the accumulation of neurotoxic misfolded proteins within MNs **(Figure I.3)**. In turn, these protein aggregates are proposed to disrupt cellular processes that ultimately result in neurodegeneration (Amin et al., 2020). Cell-to-cell transmission of misfolded protein aggregates in a prion-like mechanism (a protein conformation capable of replicating without a nucleic acid genome) has been associated with the production of novel aggregates and with the propagation of neurodegeneration to the surrounding cells, suggesting a role in ALS dissemination (Al-Chalabi et al., 2017).

Besides being responsible for the translation, folding, and transport of membrane and secreted proteins, the ER plays a role in pathways associated with unfolded-protein responses by recognizing aberrant proteins and causing their degradation. However, the accumulation of misfolded proteins causes excessive ER stress **(Figure I.3)** and, consequently, dysfunction in protein handling (Ferraiuolo et al., 2011). The involvement of ER stress in the pathogenesis of ALS has been proposed in *SOD1*-related ALS and sALS patients (Hayashi et al., 2016). Since the capacity of the ER to synthesize and process proteins is limited, the accumulation of misfolded proteins may activate different ER stress pathways, exerting a critical role in disease pathogenesis (Berthod et al., 2012).

In a physiological scenario, mitochondria have a crucial role in intracellular adenosine triphosphate (ATP) production, calcium (Ca^{2+}) homeostasis, and control of apoptotic cell death (Ferraiuolo et al., 2011). However, considering the high energetic demand of MNs, the perturbation of mitochondrial function has profound harmful effects on these cells. Dysregulated mitochondrial function can lead to MN death due to inadequate levels of ATP, formation of ROS, and increased Ca^{2+}-mediated excitotoxicity that leads to apoptotic cascades. Mutations in several ALS-associated genes address the involvement of mitochondrial dysfunction and dysregulated energy metabolism as contributory factors to MN injury and disease pathogenesis **(Figure I.3)**. Expression of mSOD1 in the neuroblastoma/spinal cord-34 (NSC-34) MN cell line (more detailed in subsection 3.2.1. of this introduction) results in the development of morphologically swollen mitochondria, compromised activity of complexes II and IV of the mitochondrial respiratory chain and impaired cellular bioenergetic status (Shaw, 2005). Additionally, in mSOD1 mice, mSOD1 aggregates within mitochondria cause mitochondrial vacuolation through expansion of the intermembrane space, which is postulated to induce organelle dysfunction (Chio et al., 2020; Ferraiuolo et al., 2011).

In addition, oxidative damage to mitochondrial proteins and lipids that lead to defects in respiratory chain function has been found in ALS patients and in mSOD1 mice, and several experimental models of ALS present deficiencies in the axonal transport of mitochondria, which could contribute to the axonopathy at the neuromuscular junction (NMJ) (Hardiman et al., 2017). Moreover, other key evidence

for mitochondrial dysfunction in human sALS includes the high frequency of mitochondrial DNA mutations in the motor cortex, muscle, and SC tissues, reduced activity of complex IV in spinal MNs, and increased mitochondrial volume and Ca^{2+} levels within motor axon terminals in muscle biopsies from sALS cases (Shaw, 2005).

Associated with mitochondrial dysfunction is the impairment of mitochondrial dynamics, which includes defects in fusion and fission mechanisms and axonal transport **(Figure I.3)**. MNs are highly dependent on an efficient intracellular transport system with anterograde and retrograde components. Indeed, axonal transport is a crucial mechanism in neuronal function, which is dysregulated in ALS. There are two major microtubule-based motor proteins: kinesin mediates anterograde transport from the cell body to the axon, while dynein drives retrograde transport from the distal axon to the cell body (Hirokawa et al., 2010). These proteins carry several cargos, including lysosomes, mitochondria, endosomes, protein complexes, and mRNAs. The proposed molecular mechanism responsible for the compromised axonal transport in ALS involves mitochondrial damage, alterations in the phosphorylation of kinesin and dynein, and a decline in microtubule stability (De Vos et al., 2017).

Reactive species (RS) are generally categorized as ROS and reactive nitrogen species (RNS). The most relevant RS include $O_2^{\bullet-}$, hydroxyl radical ($^{\bullet}OH$), nitric oxide (NO), H_2O_2, and peroxynitrite. The major sites of RS production involve endogenous enzymatic sources, primarily the mitochondrial electron transport chain, the NADPH oxidase complex, peroxisomes, the cytochrome P450 enzymes or the mitochondrial enzyme monoamine oxidase (Michalska et al., 2020).

Although ROS/RNS play vital physiological roles, being essential for cellular signaling and pro-survival pathways, an imbalance between their production and removal leads to excessive RS generation, called oxidative stress **(Figure I.3)**. In particular, ROS are generated by aerobic metabolism and have the potential to damage cells by oxidizing several biomolecules, including proteins, lipids, and DNA. Indeed, oxidative stress has been suggested as a primary initiating factor in ALS pathogenesis (Mejzini et al., 2019), strongly contributing to MN degeneration. Numerous markers of free radical damage are increased in biofluids from ALS patients, including cerebrospinal fluid (CSF), serum, and urine. Other direct evidence of the involvement of oxidative stress in ALS is the oxidative damage to proteins, lipids, and DNA in *post-mortem* tissue from sALS and *SOD1*-related fALS patients (Ferraiuolo et al., 2011; Hayashi et al., 2016).

A particular interest has been shown in the role of oxidative stress in ALS, given that mutations in *SOD1*, which encodes a key cellular antioxidant protein, account for a considerable percentage of fALS cases (Chio et al., 2020).

RNA metabolism is another cellular process usually altered in ALS pathology **(Figure I.3)**. Indeed, mutations in several RNA-binding proteins are related to familial forms of the disease, namely in those that have mutations for TDP-43 or FUS. These proteins are involved in transcription, splicing, and axonal transport of mRNAs and biogenesis of miRNAs. Thus, such mutations lead to considerable alterations in cell transcriptome, thereafter affecting protein homeostasis and triggering detrimental changes in neuronal function (Brown et al., 2017; Hardiman et al., 2017).

The pathological mechanisms that contribute to MN degeneration also involve the contribution of non-neuronal cells, such as microglia, Ols, and astrocytes. These will be later explored in section 1.5.

1.4 Diagnosis, Screening, and Available Therapies

Unfortunately, no precise and definitive diagnostic test is yet available for ALS, particularly due to the heterogeneous clinical presentations and the varying speed of disease progression. The diagnosis of

ALS includes the presence of upper and lower MN clinical features, disease progression, and the appropriate exclusion of other diseases with overlapping symptoms. The diagnosis relies mainly on the medical history and physical examination, supported by electrophysiologic evidence (needle electromyography and nerve conduction studies) and negative findings in neuroimaging and laboratory studies (blood and CSF tests). For instance, spine and brain magnetic resonance imaging are regularly performed to exclude syndromes that mimic ALS (Hardiman et al., 2011; Masrori et al., 2020). Genetic testing of the most prevalent genes found to be mutated in ALS has progressed in recent years with the use of Sanger sequencing for individual genes, whole-exome sequencing, and multigene next-generation sequencing panels that may be useful for patients with an ALS-positive family history (Roggenbuck et al., 2017).

As there is still no cure nor effective treatment or successful medical intervention which might halt/delay ALS progression, the focus is on symptomatic, rehabilitative, and palliative therapy with an overall goal of improving patients' quality of life (Hogden et al., 2017; Ng et al., 2017). Currently, patient management is largely mediated by symptomatic therapies, including the use of muscle relaxants and physiotherapy for spasticity, speech therapy for dysarthria, pharmacological treatments for muscle cramps and pain, dietary changes, specialized exercises for dysphagia and, in most severe cases, non-invasive mechanical ventilation must be required for respiratory support (Dorst et al., 2018; Hardiman et al., 2017; Masrori et al., 2020).

Only two available therapeutic drugs are already approved by the Food and Drug Administration (FDA). Riluzole (Rilutek), a glutamatergic neurotransmission inhibitor, became the first FDA-approved drug for ALS, in 1995. This glutamate antagonist prevents the excessive glutamate release from presynaptic MNs, stimulates extracellular glutamate uptake, and inhibits postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) and the N-methyl-D-aspartate (NMDA) glutamate receptors, thus preventing glutamate-mediated toxicity (Chio et al., 2020; Dorst et al., 2018; Jaiswal, 2019). Edaravone (Radicava), approved by the FDA in 2017 but not in use in Portugal, is a free-radical scavenger and a potent antioxidant that alleviates oxidative stress by removing oxygen radicals (Oskarsson et al., 2018; Takei et al., 2017). However, neither of them prove to be effective since they do not prevent the rapid progression of the disease and only slightly prolong the lifespan of ALS patients, providing only modest benefits and only in some patients (Filipi et al., 2020; Mejzini et al., 2019).

Thus, the development of new and effective therapies and the identification of potential therapeutic targets for the diagnosis and treatment of the disease are urgently required. For new therapies, the challenge is to define mechanisms of disease that are amenable to drug targeting and to define patients who are likely to respond to these therapeutic agents (Hardiman et al., 2017). Biomarkers can be critical in diagnostic, predictive, or prognostic research studies. They could become crucial for the stratification of patients and monitoring treatment effects in clinical trials (Masrori et al., 2020). Recently, epigenetic targets such as miRNAs have shown to be very promising as potential biomarkers (Bennett et al., 2019).

1.5 The Contribution of Non-Neuronal Cells to ALS Neurodegeneration

Initially, the selective death of MNs expressing the mutant protein was considered the key player in the disease onset. However, aside from all the pathological mechanisms described above that are implicated in the degeneration of MNs, ALS is increasingly being recognized as a non-cell-autonomous disease, meaning that other cell types contribute to disease progression by triggering or aggravating damage to MNs (Raffaele et al., 2021). Nowadays, it is widely accepted that MN degeneration and ALS progression result from a complex interplay between multiple pathogenic mechanisms involving the MNs themselves and their neighboring non-neuronal cells, the glial cells (Parisi et al., 2016). These non-neuronal neighbors are believed to be implicated in the onset and progression of ALS, thus underlying

MN dysfunction and loss (Brites et al., 2014). In fact, it is thought that MN degeneration arises from the toxic effects of the surrounding glial cells, such as microglia, astrocytes, and Ols (Hayashi et al., 2016). Therefore, glial cells and their striking impact on the functioning of MNs during disease progression are currently being studied (Filipi et al., 2020), with accumulating evidence demonstrating that glial cells can modify the course of the disease by affecting neighboring MNs.

Notably, in TG mice expressing different *SOD1* variants, the selective downregulation of m*SOD1* in microglia or astrocytes extended their survival, suggesting that glial cells contribute to ALS pathogenesis (Hayashi et al., 2016). During the course of the disease, the SC of TG mice expressing mSOD1 is the site of a progressive loss of MNs and a dramatic gliosis characterized by reactive astrocytes and activated microglial cells.

1.5.1. The Role of Microglia

Microglia, constituting 5-18% of the total number of glial cells, are the immune-resident cells of the central nervous system (CNS), involved in immune surveillance and maintenance of intercellular homeostasis. They have an essential role in clearing cellular debris through phagocytosis, as well as in controlling immunological homeostasis, while also supporting neurogenesis, neuronal connectivity, synaptic pruning, myelination, vasculogenesis, and blood-brain barrier (BBB) permeability (Brites, 2020). However, when their functions are impaired, or the cells are overactivated, microglia contribute to neurologic pathologies. Some microglia-specific markers include the C-X3-C chemokine receptor 1 (CX3CR1), cluster of differentiation molecule 11B, ionized calcium-binding adaptor molecule 1 (Iba-1), and triggering receptor expressed on myeloid cells 2 (TREM2) (Hickman et al., 2018).

These cells are essential in the inflammatory process of the nervous tissue triggered in response to several harmful stimuli, including infection, neuronal injury, and inflammation. This active response occurs mainly due to the microglial expression of various pattern recognition receptors that respond to danger signals. Thus, they play a neuroprotective role and contribute to tissue maintenance and homeostasis (Korzhevskii et al., 2016).

Despite the heterogeneity of microglial cells and difficulties in establishing phenotype characteristics and respective functions, some concepts have been adopted. In healthy conditions, the denominated steady-state microglia, usually called “surveillant microglia”, is a highly dynamic and motile population with a ramified morphology that constantly surveys their microenvironment, releasing neuroprotective and anti-inflammatory mediators when needed (Geloso et al., 2017). However, in response to injury and neurodegenerative diseases, these cells undergo context-dependent and temporally regulated changes in their morphology and gene expression. Under a CNS insult or homeostatic changes, microglia can become activated and turn into ameboid reactive cells (Clarke et al., 2020), presenting a neurotoxic and pro-inflammatory potential that can cause severe damage to adjacent MNs and astrocytes (Brites, 2020; Filipi et al., 2020). Actually, microglia have a dynamic and complex role in ALS neuroinflammation. After an initial triggering of microglia through factors mainly released by damaged MNs and astrocytes (Parisi et al., 2016), the cell activation process is characterized by rapid proliferation, production, and secretion of high levels of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-1 beta (IL-1β), increased expression of the inducible nitric oxide synthase (iNOS), and cytotoxic factors including NO, ROS, and chemokines. Nonetheless, in a regenerative context, microglia can also induce anti-inflammatory cytokines, including IL-10 and IL-4. In fact, microglia can acquire a pro-regenerative phenotype characterized by the release of some specific neurotrophic factors (Michalska et al., 2020; Prinz et al., 2019). Hence, the cell activation process can next be either counteracted or exacerbated, with an outcome consequently turned from beneficial to toxic (Parisi et al., 2016). Effectively, chronic activation of microglia has detrimental effects on the survival and normal functioning of MNs, astrocytes, and Ols (Subhramanyam et al., 2019).

Many effects of miRNAs have been described in microglia, affecting their homeostasis and pro-inflammatory activity. Microglia can secrete and internalize miRNAs in their free form, bound to proteins or inside small extracellular vesicles (sEVs)/exosomes. Furthermore, miRNAs participate in an intricate microglia-neuronal crosstalk (Brites, 2020). For instance, a crucial role of neuronal miR-124 in regulating microglia activation was described. Indeed, miR-124 upregulation in mSOD1 MNs and in the secretome of these cells was correlated with neurodegeneration and microglia activation (Vaz et al., 2021b).

Recently, a new microglial phenotype termed disease-associated microglia (DAM) has been identified and linked to neurodegenerative conditions, including ALS. In cases of neuronal damage, this DAM subtype is triggered by neurodegeneration-associated molecular patterns, which are thought to activate TREM2 among other immune receptors such as C-X3-C chemokine ligand 1 (CX3CL1, fractalkine). DAM expresses typical microglial markers, including Iba-1, coincident with the downregulation of ''homeostatic'' microglial genes, including the purinergic receptors P2Y12 (*P2ry12*) and *P2ry13*, *Cx3cr1*, *Cd33*, and transmembrane protein 119 (*Tmem119*) (Deczkowska et al., 2018).

In ALS, microglia activation and involvement have been intensively studied and demonstrated over time, both in mSOD1 models and in patients (Zhao et al., 2013). Indeed, mSOD1 microglia accelerates disease onset, and microglial activation exacerbates MN death. Moreover, data suggest that microglia isolated from mSOD1 mice evolve from a neuroprotective phenotype at disease onset to a more neurotoxic and pro-inflammatory phenotype with an altered cytokine release profile at end-stage disease, pointing to changes in microglial phenotypes with disease progression (Hardiman et al., 2017; Hickman et al., 2018). Microglia also exhibit changes in inflammatory-associated miRNAs (inflammamiRNAs), as documented below (see section 2.2).

Pinto and colleagues (2017) demonstrated that miR-124-enriched exosomes derived from mSOD1 NSC-34 MNs and incubated in N9 microglia are responsible for the activation and loss of function of the N9, resulting in reduced phagocytic ability and increased cell senescence. Furthermore, the secretome from mSOD1 NSC-34 MNs determines a shift from steady-state microglia immunophenotype into a pro-inflammatory subtype, overexpressing specific inflammatory-related genes, namely *Il-1β* and *Inos*, while presenting decreased arginase 1 (*Arg1*) and unchanged *Tnf-α* and *Il-10*. These results attest to microglia polarization into a characteristic DAM phenotype due to mSOD1 MN paracrine signaling pathogenicity (Vaz et al., 2021b).

1.5.2. Astrocyte Aberrancies

Astrocytes, the largest glial population in the CNS, constitutes a morphologically and functionally heterogeneous cell group. They are responsible for plentiful processes that are essential to the development, maintenance, and homeostasis in the CNS and present distinct expression of specific markers, including glial fibrillary acidic protein (GFAP) and S100 calcium-binding protein B (S100B), and distinct Ca^{2+} signaling.

In a healthy brain, astrocytes present a neuroprotective phenotype providing trophic support for neurons and performing key roles of neuroprotection, modulation of synaptic activity, uptake and regulation of neurotransmitters, BBB maintenance, and immune functions (Hayashi et al., 2016; Matias et al., 2019). Beyond these, one of the most recognized functions of astrocytes is to control the extracellular concentrations of glutamate at the synapses predominantly through the glutamate excitatory amino acid transporter 2 [EAAT2; Glutamate transporter 1 (GLT-1), the rodent homologous)], once excessive levels of glutamate in synaptic cleft result in toxicity to neurons through overstimulation. Besides this, mitochondrial function and Ca^{2+} homeostasis in astrocytes are also impaired in ALS. Astrogliosis, a pathological condition marked by a proliferation of astrocytes into CNS tissues, also contributes to an

altered immune response and consequent neuroinflammation, which is one of the pathophysiological mechanisms promoting MN degeneration in ALS (Parisi et al., 2016).

In neurodegenerative diseases, astrocytes change their morphology and molecular expression patterns and are referred to as reactive or activated astrocytes. In ALS, these cells lose their beneficial functions and acquire detrimental roles (Yamanaka et al., 2018). Effectively, dysfunctional astrocytes with a reactive and neurotoxic phenotype in both brain cortex and SC have been extensively described as key players in disease onset and progression, strongly contributing to neuroinflammation and MN degeneration (Filipi et al., 2020). Indeed, cortical and SC astrocytes from mSOD1 mice are neurotoxic, develop early deficits, and lose their physiological, homeostatic, and neurosupportive properties before disease onset, releasing several soluble toxic factors and pro-inflammatory mediators that directly induce MN cell death (Vaz et al., 2021a). In fact, patient-derived mSOD1 astrocytes were toxic to cocultured MNs *in vitro*. Knockdown of SOD1 in the astrocytes alleviated this neurotoxicity, indicating that the mSOD1 in astrocytes exerts a toxic effect on MNs (Hayashi et al., 2016). In addition to astrogliosis, the aberrant accumulation of mSOD1 protein in astrocytes in the SC of mSOD1 mice provided evidence about the role of astrocytes in ALS models (Yamanaka et al., 2018).

In ALS, astrocytes express different aberrant and reactive markers, such as GFAP, S100B, Cx43, and vimentin. They are phenotypically different depending on their location/region in the CNS (cortical or spinal astrocytes) and on the different stages of the disease (presymptomatic or symptomatic) (Gomes et al., 2019; Gomes et al., 2020), thus indicating high regional diversity and heterogeneity of astrocytes (Vaz et al., 2021a). Indeed, induced pluripotent stem cell (iPSC)-based approaches have provided a growing recognition that there are heterogeneous populations of astrocytes, emphasizing the existence of subtypes and the need for patient stratification to better understand their phenotypic-associated specific roles (Gomes et al., 2022).

In addition to the differential expression of astrocytic reactivity markers, a differential expression of inflamma-miRNAs is also observed in $SOD1^{G93A}$ mice models. For instance, miR-146a and miR-21 are downregulated in mature mSOD1 cortical astrocytes, although upregulated in mature spinal astrocytes (Gomes et al., 2019; Gomes et al., 2020). Using the trans-differentiation process of converting fibroblasts from sALS and SOD1 (mSOD1-ALS) patients into induced astrocytes (iAstrocytes), Gomes and colleagues (2022) also demonstrate that some of the markers mentioned above, including astrocyte-associated GFAP and Cx43, as well as inflamma-miR-155 and miR-146a, are not similarly expressed by all ALS patient iAstrocytes, and that the latter ones show distinct cell subtype signatures, providing new insights into patient stratification.

The secretome from ALS aberrant astrocytes was shown to potentiate MN death in both ALS-patient-derived astrocytes (Gomes et al., 2022; Meyer et al., 2014) and in *in vitro/in vivo* models (Diaz-Amarilla et al., 2011; Gomes et al., 2019), and to lead to activated microglia (Filipi et al., 2020). One of the mechanisms may be associated with the secretion and transfer of sEVs containing miRNAs and misfolded proteins from mSOD1 astrocytes. These molecules will then enter neighboring cells, leading to morphological, functional, and even pathological alterations in MNs and microglia, consequently leading to disease propagation (Barbosa et al., 2021; Gomes et al., 2020). For instance, reactive astrocytes secrete high levels of pro-inflammatory cytokines, glutamate, ROS, and NO, associated with insufficient production of neurotrophic factors and excitotoxicity caused by impaired glutamate clearance (Parisi et al., 2016), leading to microglial activation and MN death (Filipi et al., 2020; Mishra et al., 2016).

All these observations strongly imply that astrocytes have multidimensional roles in disease and show that targeting astrocytes for therapy would have great potential to prevent or rescue ALS disease progression. For functional recovery, astrocyte intervention strategies may include the modulation of astrocytic activity with the use of medicines or engineered exosomes, targeting the inhibition of inflammatory mechanisms and oxidative stress (Vaz et al., 2021a).

1.5.3. Oligodendrocyte Dysfunction

There is growing evidence supporting the contribution of Ols, the CNS myelinating cells, to ALS pathogenesis (Mejzini et al., 2019). Myelin is composed of lipids and proteins, being proteolipid protein (PLP) and myelin basic protein (MBP), the most abundant protein components of the myelin sheath. This myelin sheath allows fast and efficient propagation of action potentials over long distances along the axons through saltatory conduction (Nonneman et al., 2014). Although Ols are generally responsible for producing the myelin sheath that isolates the neuronal axons, gray matter Ols are not involved in myelin sheath formation and are thought to provide trophic metabolic support to neurons (de Faria et al., 2019; Mejzini et al., 2019). In recent years, the impact of Ol loss and myelin dysfunction **(Figure I.4)** in various neurodegenerative disorders, including ALS, has become increasingly clear. Indeed, pathological alterations of the white matter have been detected in ALS patients and animal models, suggesting that Ols and myelin structures are also affected during disease progression (Raffaele et al., 2021).

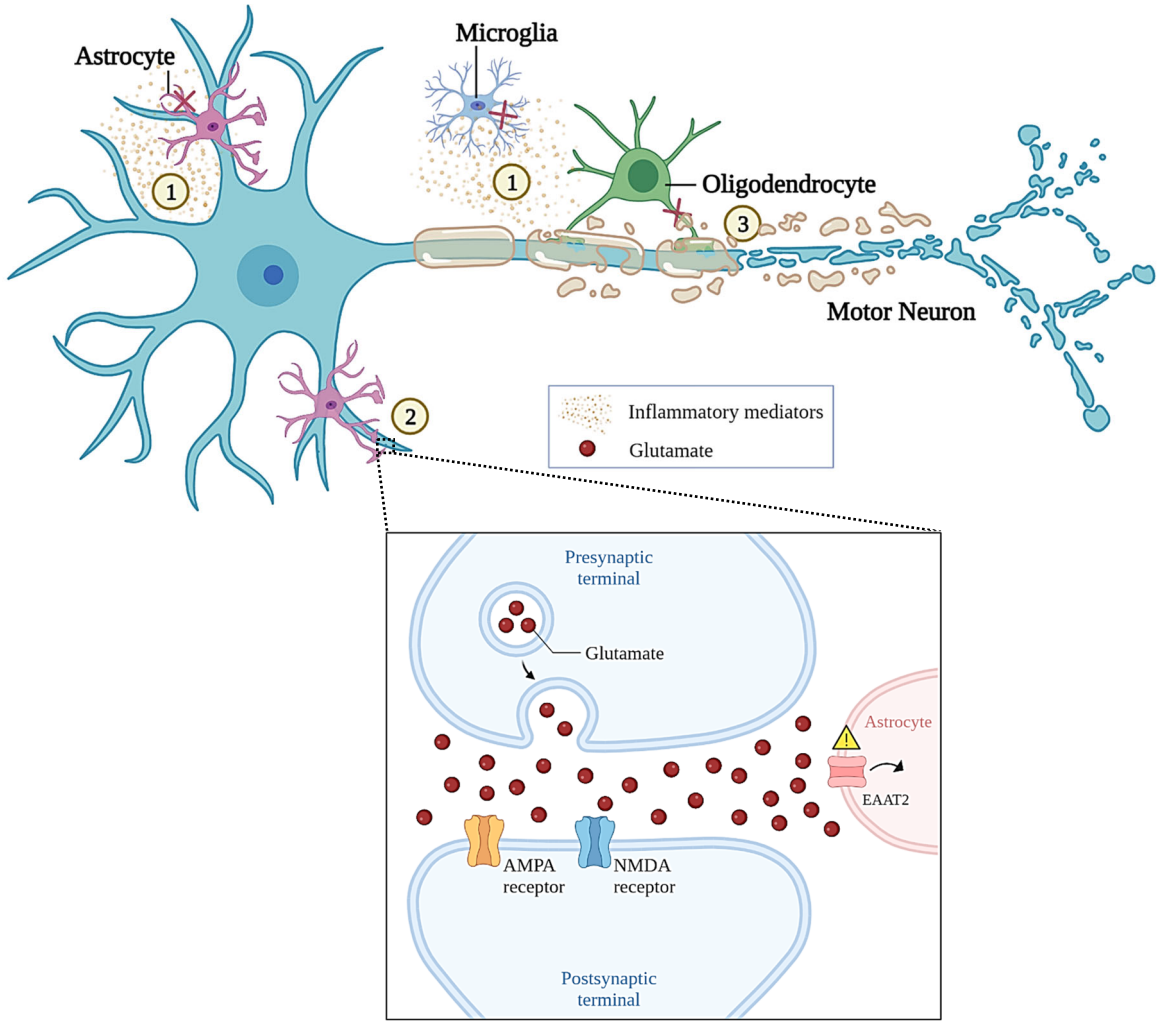

Figure I.4 — Loss of the physiological role of glial cells and/or gain of toxic functions are critical in inducing motor neuron (MN) degeneration in amyotrophic lateral sclerosis (ALS), contributing to disease progression. Although several pathways and mechanisms are associated with the MN itself, the glial cells strongly contribute to MN degeneration in ALS. In fact, there is an intricate crosstalk between altered glial cells and MNs that end up triggering ALS: **(1)** Neuroinflammation characterized by reactive and dysfunctional astrocytes and microglia associated with the release of cytotoxic and pro-inflammatory mediators and deficits in the production of neurotrophic factors **(2)** Excitotoxicity associated with astrocytic glutamate transport deficits **(3)** Myelination impairment. (Created with BioRender.com). **AMPA**, α-Amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid; **NMDA**, N-methyl-D-aspartate.

Pathological protein aggregates of mSOD1 in periaxonal OL extensions in the $SOD1^{G93A}$ mice and signs of myelin damage have been described at a late disease stage in the SC anterior horn, as well as in upper structures of the brain. Additionally, in mSOD1 mice, the number of degenerating Ols increase dramatically with disease progression. Defects in myelin structures and decreased levels of MBP have been reported in the SC of $SOD1^{G93A}$ mice and ALS patients' motor cortex and SC. Intriguingly, degeneration of mature Ols and myelin abnormalities start at the presymptomatic stage in $SOD1^{G93A}$ mice SC, suggesting that myelin disruption anticipates MN degeneration and directly contributes to disease aggravation (Raffaele et al., 2021). Kang and colleagues (2013) demonstrated that $SOD1^{G93A}$ mice also have impaired function and extensive degeneration of gray matter (GM) Ols in the SC, with Ol precursors failing to fully differentiate. Additionally, this degeneration was accompanied by microglial activation and microglial localization to apoptotic Ols.

Furthermore, Ols provide vital metabolic support to axons through the transport of lactate via monocarboxylate transporter 2; accordingly, Ols' dysfunction contributes to the MN axonopathy in ALS (Hardiman et al., 2017).

As referred in section 1.3, the dysregulation of these three types of non-neuronal cells also contributes to MN degeneration.

Neuroinflammation, a pathological event that results from harmful effects of diverse stimuli, is characterized by activated microglia, reactive astrocytes, infiltrating T lymphocytes and macrophages, and elevated levels of pro-inflammatory mediators (Liu et al., 2017) **(Figure I.4)**. Furthermore, several studies have indicated that the aberrant activation of the complement system in the CNS may also be involved in neuronal injury and ALS progression, which in turn will contribute to neurodegeneration (Chio et al., 2020; Mancuso et al., 2015). Neuroinflammation, a fundamental component of ALS pathophysiology, can be observed in imaging studies in ALS patients, human *post-mortem* samples, and rodent models of ALS (Hardiman et al., 2017).

Glutamate is the primary excitatory neurotransmitter in the CNS and exerts its effects through an array of ionotropic and metabotropic postsynaptic glutamate receptors (Ferraiuolo et al., 2011). Excitotoxicity **(Figure I.4)** is a pathological process that involves the excessive stimulation of glutamate receptors resulting in neuronal injury or degeneration. When the extracellular glutamate level is elevated, neurons are damaged through overstimulation of glutamate receptors (Mejzini et al., 2019; Prasad et al., 2019). Unrestrained release or insufficient reuptake of glutamate from the synaptic cleft overstimulates the MNs receiving the signals, thereby triggering Ca^{2+} overload in postsynaptic MNs by the overactivation of Ca^{2+}-permeable glutamate receptors. As they have a low Ca^{2+} buffering capacity, these augmented levels of intracellular Ca^{2+} lead to peroxidation of membrane lipids, damage to RNA/DNA, and disruption of mitochondria, contributing to MN degeneration (Jaiswal, 2019; Mejzini et al., 2019).

Astrocytes are the central cells that control the extracellular concentrations at the synaptic junctions, mainly through the glutamate transporter EAAT2, the main synaptic glutamate reuptake transporter. In mSOD1 mice, it was reported that defects in glutamate clearance by astrocytes are due to the downregulation of the EAAT2 in both sALS and fALS (Rosenblum et al., 2017). The loss of GLT-1/EAAT2 has already been observed in both rodent models and ALS patients, respectively (Hardiman et al., 2017), and increased levels of glutamate have been detected in the CSF of the latter ones (Chio et al., 2020). As an additional effect, the astrocytes that express mSOD1 lose the ability to regulate the composition of MN ionotropic AMPA receptors, thus resulting in the reduction of their important subunit glutamate receptor 2. The lack of this subunit enhances the Ca^{2+} permeability of AMPA receptors, which increases the vulnerability of MNs to glutamate-mediated neurotoxicity

(Hayashi et al., 2016). Furthermore, hyperexcitation of NMDA glutamate receptors has also been demonstrated in ALS (Chio et al., 2020). Whether as a primary or a propagating process, it seems that glutamate toxicity contributes to the MN injury in ALS. This is supported by the finding that, even though modest, anti-glutamate treatment with Riluzole has some effect in prolonging survival in ALS patients and mSOD1 mouse models (Shaw, 2005).

2. miRNA Dysregulation in ALS

miRNAs are regulatory single-stranded small noncoding RNA molecules with 21-23 nucleotides long (Guay et al., 2013). They are endogenously produced and have been identified in various organisms, including humans and other animals. These molecules negatively regulate gene expression at the post-transcriptional level through binding to complementary sites in the 3' untranslated region of target mRNAs (Brites, 2020; Wahid et al., 2010). There are many potential target sites for each miRNA, even within the same gene, with several factors influencing miRNA-mRNA interactions. Functional analysis revealed that hundreds of target mRNAs could be repressed and destabilized by a single miRNA (Landgraf et al., 2007).

miRNA-dependent gene expression regulation is fundamental in almost all biological processes. Evidence suggests that miRNAs are involved in critical developmental and physiological events, including cell proliferation and differentiation, cell death, metabolism, stress responses, neuronal patterning, and immunity (Ambros et al., 2018; He et al., 2004). For instance, these molecules present an important modulating function of the immune system, whose dysregulation plays a relevant role in the development and progression of several neuroinflammatory diseases (Parisi et al., 2013).

Several studies have explored miRNAs dysregulation, thus pointing out that the miRNA signature could be useful for discovering ALS biomarkers and therapeutic targets (Akbari Dilmaghani et al., 2021). As miRNAs take part in the microglia-astrocyte-neuronal crosstalk through the secretome of these cells, it is necessary to understand the intricate communication between these cell types since they all went through a series of changes in ALS, negatively influencing each other and contributing to neurodegeneration.

Among the wide range of miRNAs, several important and well-studied miRNAs in the context of neuroinflammation were shown to be involved in ALS pathogenesis (Slota et al., 2019). In particular, some miRNAs have been defined as inflamma-miRNAs since they are associated with multiple inflammatory pathways (Gomes et al., 2022; Tahamtan et al., 2018).

2.1. miRNA Biogenesis, Mechanisms of Action, and Circulation

miRNAs biogenesis can be divided into canonical and noncanonical pathways, being the first one the predominant pathway by which miRNAs are processed (O'Brien et al., 2018).

In the canonical pathway **(Figure I.5)**, miRNA-coding genes are transcribed from genomic DNA into primary miRNA (pri-miRNA) transcripts by RNA polymerase II (Pol II). Inside the nucleus, pri-miRNAs are cleaved by the complex composed of ribonuclease III enzyme Drosha and DiGeorge syndrome critical region 8 (DGCR8) to produce precursor miRNAs (pre-miRNAs) hairpins of approximately 70 nucleotides (Han et al., 2004). After nuclear processing, pre-miRNAs are exported into the cytoplasm by the nuclear export factor Exportin-5 (Yi et al., 2005). These molecules are further excised by a complex composed of the endoribonuclease Dicer, the cofactor TAR RNA-binding protein 2 (TRBP), and Argonaute, thus producing duplexes of ~ 22 nucleotides consisting of a mature guide strand (miRNA) and a complementary passenger strand (miRNA*) (Fareh et al., 2016; Guay et al., 2013). Posteriorly, the passenger miRNA* strand is degraded, whereas the mature and functional miRNA is subsequently loaded into the effector RNA-induced silencing complex (RISC). Depending

on the level of complementarity between the RISC-incorporated miRNA and target mRNA sequences, the miRNA will act on its target gene transcript by translational repression or transcript degradation (He et al., 2004; Wahid et al., 2010).

The mature miRNAs that do not interact with their target mRNA may be sorted from the cell as cargo of small and large extracellular vesicles (EVs) and can also be released by the cells, either attached to proteins or loaded in vesicles such as exosomes and microvesicles that are secreted into the extracellular milieu/fluids (Brites, 2020; Gallo et al., 2012; Iftikhar et al., 2016).

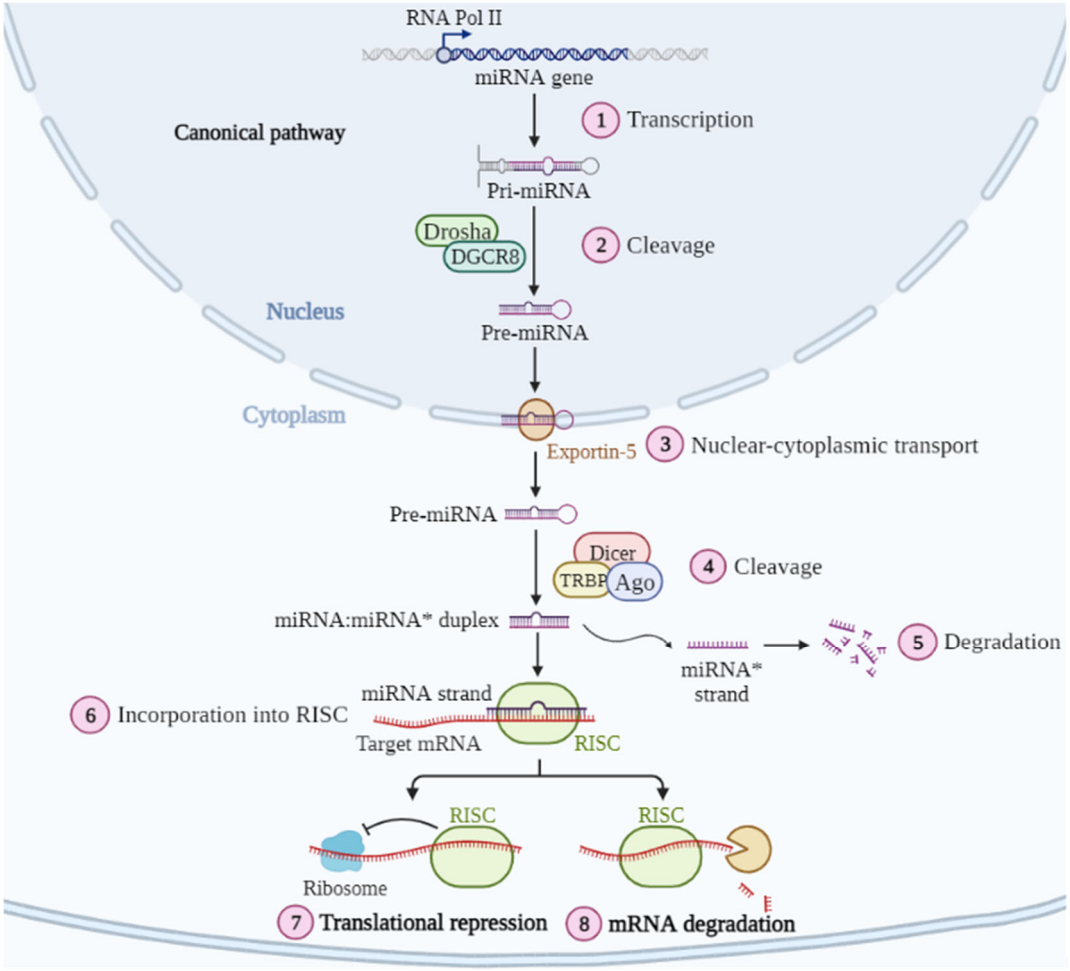

Figure I.5 — The canonical pathway of miRNA biogenesis. In animals, miRNAs are synthesized endogenously. miRNAs coding genes are transcribed into pri-miRNA transcripts by RNA Pol II **(1)**. Inside the nucleus, pri-miRNAs are cleaved into pre-miRNAs by the complex Drosha/DGCR8 **(2)**. Next, pre-miRNAs are transported to the cytoplasm through Exportin-5 **(3)** and are processed into miRNA:miRNA* duplexes by a complex of Dicer, the cofactor TRBP and Argonaute **(4)**. The passenger miRNA* strand is degraded **(5)** while the mature miRNA strand is incorporated into the RISC complex **(6)**, subsequently silencing gene expression of its target mRNA by translational repression **(7)** or cleavage **(8)**. *Adapted from "miRNA Processing Mechanisms in the Brain", by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates*. **DGCR8**, DiGeorge syndrome critical region 8; **miRNA**, microRNA; **pre-miRNA**, precursor miRNA; **pri-miRNA**, primary miRNA; **RISC**, RNA-induced silencing complex; **RNA Pol II**, RNA polymerase II; **TRBP**, TAR RNA-binding protein 2.

Precise control of miRNA biogenesis is crucial to maintain normal cellular functions since there is a strong correlation between miRNA imbalance and several human diseases (Jiang et al., 2009). For instance, several miRNAs are dysregulated in ALS, compromising neurogenesis, synaptic signaling, NMJ function, and protein/RNA metabolism (Nguyen et al., 2022).

miRNAs are very stable in various biofluids since they are often associated with Argonaute proteins, microvesicles, or exosomes which protect them from RNases, and represent potentially informative biomarkers for a range of diseases, including CNS disorders (Sun et al., 2018). As a result of being highly stable, extracellular/circulating miRNAs have been detected in biological fluids, such as plasma, serum, CSF, saliva, and urine. The transfer of miRNAs between distantly located cells constitutes a striking novel cell-to-cell communication mode, making them crucial intercellular signaling molecules (Guay et al., 2013; O'Brien et al., 2018). Actually, extracellular miRNAs can be delivered to target cells where they may act as autocrine, paracrine, and/or endocrine regulators, exerting key biological functions in recipient cells to modulate a variety of cellular events (Iftikhar et al., 2016). Circulating miRNAs can be uptaken through distinct mechanisms, including receptor-mediated capture, endocytosis, or direct fusion of exosomes with the plasma membrane of recipient cells (Guay et al., 2013).

In ALS, as miRNAs are secreted in the CSF, their analysis in this biofluid could be used for clinical diagnosis (Nguyen et al., 2022). miRNAs are also muscle-specific and, consequently, they may have a broad application as biomarkers in ALS (Akbari Dilmaghani et al., 2021; Wang, 2021).

One of the major necessities of modern research is to discover useful non-invasive biomarkers as tools for early diagnosis, prognostic purposes, and as indicators of treatment response, contributing to precision medicine. Extracellular miRNAs are found at detectable and stable levels in the blood and other biofluids and might be used as potential biomarkers for a variety of diseases. Numerous studies have shown that miRNAs are differentially expressed in ALS patients compared to non-ALS controls without the disease in body fluids such as CSF and in the blood-derived components, including plasma and serum (Joilin et al., 2019; O'Brien et al., 2018).

Considering this, it is essential to study these miRNAs and understand how they may be involved in the specific pathogenic mechanisms of ALS to modulate and interfere with them, using them as possible therapeutic targets for the treatment of ALS. Actually, miRNAs are emerging as novel therapeutic targets for various human diseases, including those that affect the CNS (Gaudet et al., 2018).

2.2. Inflamma-miRNAs

miRNAs may either promote or inhibit inflammatory signaling, exacerbating or ameliorating the pathological consequences of excessive neuroinflammation (Slota et al., 2019). Indeed, several important miRNAs have been considered as inflamma-miRNAs in the context of neuroinflammation, and their dysregulation is implicated in ALS pathogenesis. miR-155 and miR-125b are believed to preferentially drive a pro-inflammatory response, while miR-124, miR-146a, and miR-21 are more associated with anti-inflammatory effects (Yelick et al., 2020).

Among all the miRNAs, miR-155 is a central mediator of the CNS, usually upregulated in inflammatory and neurological disorders. This miRNA required for normal B cell function and cytokine production, and is critical for modulating inflammatory signaling and effective responses of macrophages and T cells. It is known to have broad pro-inflammatory effects in an array of immune cell types, including microglia and astrocytes (Gaudet et al., 2018). Koval and colleagues (2013) showed an upregulation of miR-155 in the SC of both fALS and sALS patients and end-stage $SOD1^{G93A}$ mice. Interestingly, inhibition of miR-155 with anti-miR-155 in the $SOD1^{G93A}$ mouse model of ALS considerably prolonged survival, likely by diminishing the inflammatory potential of microglia (Butovsky et al., 2015; Koval et al., 2013). Cunha and colleagues (2018) demonstrated that miR-155 is upregulated in the mSOD1 mice SC at both presymptomatic and symptomatic stages of the disease. This finding indicates that miR-155 upregulation occurs before disease onset, highlighting the critical role of

miR-155 during disease progression and as an early biomarker for neuroinflammation and perhaps as a potential therapeutic target based on its inflammatory role.

miR-21 is one of the most abundant miRNAs in mammalian cells (Sheedy, 2015). Even though frequently seen as anti-inflammatory, miR-21 can also have harmful effects in some inflammatory conditions, making it essential to understand how miRNAs affect a specific phenotype in each neuroinflammatory disorder and disease stage (Brites, 2020; Gaudet et al., 2018). In the context of ALS, increased levels of miR-21 were observed in the SC of SOD1^{G93A} mice (Cunha et al., 2018; Zhou et al., 2018), while reduced levels were found in the respective cerebral cortex at the symptomatic stage (Gomes et al., 2019), supporting different roles according to the CNS region.

Another important neuroimmune miRNA to consider is miR-146a, which acts as a negative regulator of inflammation (Gaudet et al., 2018), and is expressed in microglia, neurons, and astrocytes (Slota et al., 2019). It plays a crucial role in the innate immune system, controlling astrocyte-mediated inflammatory responses (Iyer et al., 2012). In addition, the target genes of miR-146a are implicated in regulating pathophysiological processes in neurological diseases, particularly in neuroinflammation (Fan et al., 2020). miR-146a is upregulated in the muscle of sALS patients and the SC of symptomatic mSOD1 mice (Campos-Melo et al., 2013; Cunha et al., 2018), suggesting that miR-146a overexpression exerts a protective effect in ALS, with a regulatory role in the inhibition of neuroinflammation (Fan et al., 2020). In contrast, downregulated miR-146a was found in the cerebral cortex at both presymptomatic and symptomatic stages (Gomes et al., 2019). Interestingly, Barbosa and colleagues (2021) showed that the pre-miR-146a transfection in the mSOD1 cortical astrocytes abrogated aberrant markers and prevented paracrine pathogenicity on microglia and MNs. In addition, the modulation with miR-146a mimic in ALS patient-iAstrocytes counteracted their reactive/inflammatory profile and restored miR-146a levels in sEVs (Gomes et al., 2022).

miR-125b is a highly conserved miRNA that shows different expression patterns and effects depending on the cellular context. It has multiple targets, including proteins that regulate apoptosis, innate immunity, inflammation, and differentiation (Duroux-Richard et al., 2016). Regarding ALS, miR-125b was found upregulated in the brain of ALS mice at late disease stages (Marcuzzo et al., 2015) and in mSOD1 MNs (Vaz et al., 2021b). Since miR-125b was neurotoxic and upregulated in the lumbar SC of symptomatic SOD1^{G93A} mice, miR-125b enrichment accounted for decreased MN survival in ALS (Cunha et al., 2018). miR-125b also modulates microglial nuclear factor kappa B signaling, contributing to elevated inflammation in SOD1^{G93A} mice (Yelick et al., 2020). *TARDBP* and *FUS*, two ALS causative genes, are particularly involved in miR-125b processing, supporting the correlation of this specific miRNA to ALS disease (Parisi et al., 2016).

2.3. The Impact of miR-124 on ALS Pathogenesis

miR-124 is among the most abundantly expressed miRNAs in the brain and has an incredibly massive number of targets in the CNS. miR-124 biogenesis indicates that this molecule shows specific temporal and spatial profiles in numerous cells and tissue types, which in turn affects an extensive spectrum of biological functions in the CNS.

Although preferentially expressed in neurons, miR-124 is also highly expressed in microglia, where it takes part in the regulation of their immune homeostasis. It has been reported to participate in several neuronal functions, such as neuronal development, differentiation and survival, neurogenesis, neurite outgrowth, and regulation of synaptic plasticity (Han et al., 2019; Hou et al., 2015; Sun et al., 2015). Besides its neuronal functions, miR-124 was described as a regulator of microglia activation (Brites, 2020). Curiously, miR-124 is considered a potent immune suppressor of microglia by repressing pro-

inflammatory and inducing anti-inflammatory microglial gene expression and controlling the choice between neuronal and astrocyte differentiation during neurodevelopment (Neo et al., 2014). Besides their numerous nervous functions, miR-124 is also a critical modulator of immunity and inflammation, performing a crucial role in the development of the immune system, regulation of immune responses, and inflammatory disorders (Qin et al., 2016).

Along with the other inflamma-miRNAs, miR-124 has been associated with different phenotypes and functions of microglia, which may differentially account for microglia overactivation and persistent neuroinflammation in ALS (Brites et al., 2014). For instance, increased miR-124 levels caused upregulation of *TNF-α*, *IL-1β*, and *Inos* expression (Zhao et al., 2021). In addition, microglia-associated inflammatory biomarkers, and pro-inflammatory cytokines, were increased during the symptomatic stage of ALS, accompanied by upregulated miR-124 expression.

Increased levels of miR-124 were detected in the CSF of sALS patients (Waller et al., 2017). Although miR-124 is the most abundantly expressed miRNA in neuronal cells and essential for their function and survival, previous studies from our group in *ex vivo* models associated the increased levels of miR-124 with MN dysfunction in $SOD1^{G93A}$ NSC-34 MN-like cells and in their derived secretome, suggesting that elevation of miR-124 may be more closely related with the existence of neurodegeneration (Vaz et al., 2021b). Pinto and colleagues (2017) also showed that exosomes derived from mSOD1 MNs are enriched in miR-124, which in turn can activate microglia to promote the release of several cytokines, such as IL-1β and TNF-α, together with iNOS. Moreover, increased levels of miR-124 were found in the brain of ALS mice at the late disease stage and in the SC of ALS symptomatic mice (Cunha et al., 2018; Marcuzzo et al., 2015).

Recently, our group showed that the normalization of miR-124 levels after transfection with anti-miR-124 had benefits in mSOD1 MNs, while the modulation of wild type (WT) MNs with pre-miR-124 led to their early apoptosis and dysfunction. Furthermore, the secretome from anti-miR-124-modulated ALS MNs prevented glial reactivity and the pathological inflamma-miRNA profile when incubated in organotypic cultures (OCs) from the SC of early symptomatic ALS mice (Vaz et al., 2021b). This issue was never explored in the ALS mSOD1 mouse model, though recent evidence sustains that cell secretomes may have therapeutic effects (Kim et al., 2022; Sandona et al., 2021).

2.4. Potential Therapeutic Benefits of miRNAs: miR-124 Modulation

As previously referred, miR-124 has been shown to potentially contribute to ALS pathogenesis. To attain the desiderates of personalized medicine, new and more accurate biomarkers need to be discovered. Recently, miRNAs have been proposed as a new class of potential biomarkers for diagnosis, prognosis, and treatment monitoring and as therapeutic targets for neurodegenerative diseases. In fact, these molecules meet most of the required criteria for being an ideal biomarker, such as high specificity and sensitivity, and have the facility to be easily accessible, this is, they are discovered and measured through minimally invasive procedures (Condrat et al., 2020).

miRNAs combine some unique features that make them desirable molecules for drug development: (i) a single miRNA is capable of downregulating hundreds of targets genes; (ii) their small length for which miRNA drugs can easily be designed and facilitates their vehiculation; (iii) they have known sequences; (iv) they are highly conserved among multiple animal species and (v) can be delivered *in vivo* through various drug delivery systems (Christopher et al., 2016; Rupaimoole et al., 2017; Sun et al., 2018). Effectively, the pharmacological treatment based on miRNAs as a novel therapeutic approach in ALS has been exploited in several preclinical studies by stimulating or inhibiting miRNAs production through diverse delivering techniques, such as adeno-associated virus vectors, EVs, or antisense oligonucleotides (ASOs) (Wang, 2021).

A global dysregulation of the miRNA profile has been associated with ALS, contributing to disease onset and progression. Thus, some ALS therapeutic approaches are related to the restoration of physiological levels of some miRNAs. miRNA-based therapeutics include miRNA mimics (pre-miRNAs) and miRNA inhibitors (anti-miRs or antagomiRs) **(Figure I.6)** (Rupaimoole et al., 2017).

In the first case, the main goal of miRNA mimics is to increase the expression of the downregulated miRNA or bring them back to its physiological levels through a small synthetic double-stranded oligonucleotide miRNA that has the same sequence and functions as the dysregulated endogenous miRNA. Thus, this miRNA mimic will bind to and decrease the expression of target mRNAs.

By contrast, anti-miRs are used against upregulated miRNAs. They are single-stranded complementary ASOs that inhibit the function of the miRNAs of interest, downregulating its levels and preventing the binding of the miRNA to its target, therefore increasing the expression of target genes (Angelucci et al., 2019; Rinchetti et al., 2018; Sun et al., 2018; Wang, 2021).

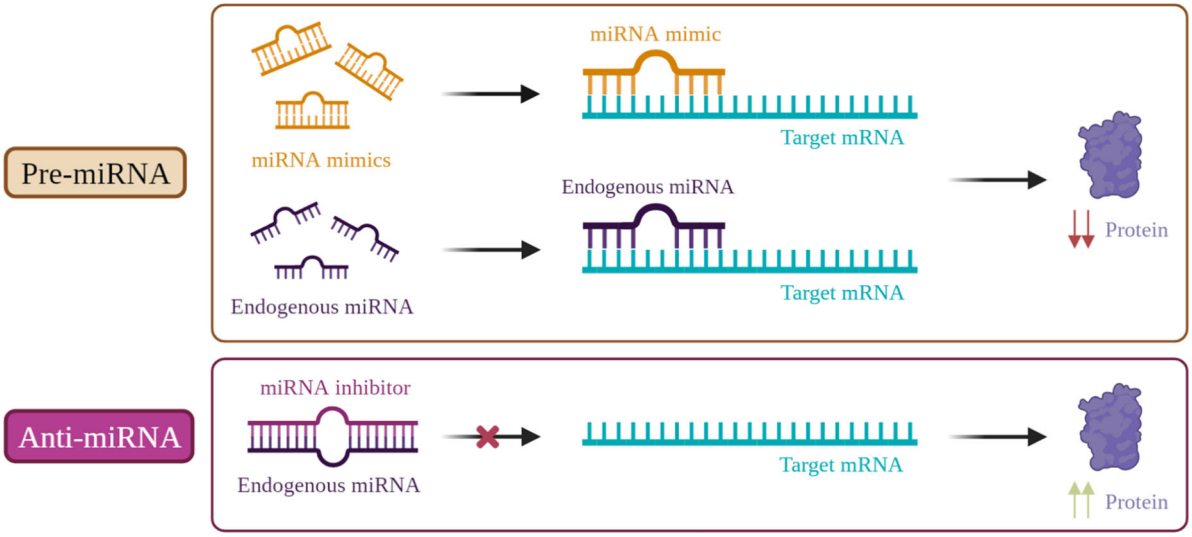

Figure I.6 — miRNA modulation as a novel therapeutic approach. In ALS, several miRNAs are dysfunctionally expressed and seem to contribute to disease pathogenesis. Therefore, some ALS therapeutic approaches are associated with the restoration of physiological levels of those miRNAs and, consequently, target proteins. miRNA-based strategies consist mainly of **(a)** pre-miRNAs (miRNA mimics) that are chemically synthesized double-stranded molecules used to increase the levels of downregulated miRNAs. A miRNA mimic (orange) has the same sequence as the endogenous one and will bind to the same target mRNAs (blue). This will allow to compensate for the loss of miRNA expression. As it can behave similarly to endogenous miRNA, it will ultimately block target genes, thus reducing protein expression; and **(b)** anti-miRNAs (miRNA inhibitors) are artificial single-stranded molecules used to reduce the levels of endogenous upregulated miRNAs (purple). miRNA inhibitors specifically bind to their miRNA targets, inhibiting their action and decreasing their levels, preventing them to reach mRNAs. That way, mRNAs can be translated with consequent increased protein levels (Created with BioRender.com).

Since increased levels of miR-124 have been detected and its contribution to ALS pathogenesis depicted, a therapy using miR-124 inhibitors, such as anti-miRs, seems to be a pertinent approach to recover the deleterious effects caused by the disease.

3. Dysregulation of Cell-to-Cell Communication in ALS

Besides the involvement of MNs and glial cells, many other extracellular components are implicated in ALS pathogenesis, namely in disease spreading and progression. Extracellular elements can be divided into two distinct fractions: the soluble fraction, containing growth factors, cytokines, enzymes,

and nucleic acids; and the vesicular fraction, which includes several subtypes of vesicles specialized in carrying signaling molecules (Pinho et al., 2020).

3.1. Secretome

The term secretome is defined by the set of biologically active molecules secreted by a cell, tissue, or organism into the extracellular space under a defined time. It includes cytokines, chemokines, growth factors, proteins, and nucleic acids. Beyond soluble factors, the secretome also has the presence of lipids and EVs, which carry important molecules, including RNAs, miRNAs, and proteins. The components of cellular origin present in the secretome are excellent paracrine signaling mediators, regulating several biological events in the neighboring and distant cells, tissues, and organs.

Recently, different secretome fractions have been thoroughly characterized and analyzed by their immunomodulatory and regenerative/protective properties. For instance, several authors reported the presence of many proteins in the secretome that benefit neuronal survival, differentiation, neurite outgrowth, and immunomodulation (Pinho et al., 2020). Globally, the secretome participates in cell-to-microenvironment and cell-to-cell communication, namely in neuron-microglia communication. The cell secretome is an essential component of paracrine and autocrine cell signaling mechanisms, with an essential role in regulating many physiological processes (Mendes-Pinheiro et al., 2020; Panaro et al., 2020). Each cell or tissue has a specific secretome-associated profile, and this secretome signature changes as a response to variations in physiologic states or pathologic conditions (Zullo et al., 2015).

Despite not being much explored in the scope of ALS, developing secretome-based therapies for CNS disorders is an emerging field. Recent evidence sustains that cell secretomes may have therapeutic effects, including in ALS (Kim et al., 2022; Sandona et al., 2021). For instance, the secretome from mesenchymal stem cells has shown to be an effective tool for the neuroprotection and survival of neural cells through the secretion of trophic factors and vesicles (Pinho et al., 2020). Walker and colleagues (2018) demonstrated that stem cell secretomes contain various beneficial factors and cytokines and that the administration of the secretome of adipose-derived stem cells in the mSOD1 mouse model during early NMJ denervation ameliorated NMJ disruption, prevented MN loss, and extended lifespan.

Compared to neurons and astrocytes, microglia are classified as better recipients of secretome/EVs, being successfully activated by these. In addition, previous studies revealed that EVs mediate phenotypic changes in microglia and their surrounding cells (Li et al., 2018; Pinto et al., 2017).

Since secretomes can carry diverse cargos, secretome-based approaches are considered potential therapeutic agents in ALS and promising vehicles for drug delivery since their molecules can reach inaccessible regions of the CNS. miR-124 is released into the secretome and carried by exosomes, which may have harmful effects on recipient cells. For instance, reduced levels of miR-124 in the secretome of MNs and microglial cells can result in beneficial paracrine influence toward functional cell recovery by diminishing the pathological-miRNA propagation. Our group demonstrated that when added to SC OCs (SCOCs) from early symptomatic mSOD1 mice (10-12-week-old), the secretome derived from anti-miR-124-modulated MNs counteracted the pathology associated with GFAP decrease, postsynaptic density protein 95 (PSD-95) and CX3CL1-CX3CR1 signaling impairment and neuroimmune homeostatic imbalance (Vaz et al., 2021b).

3.2. Extracellular Vesicles (EVs)

EVs is the collective term for cell-secreted phospholipid bilayer-bound structures present in cell culture media and several biological fluids such as blood, urine, saliva, amniotic fluid, and CSF. These vesicles are generated by an evolutionarily conserved process and are released by all human cell types tested so far, and they can interact with the recipient cell by direct binding or ligand-receptor binding

(Sandona et al., 2021). For instance, EVs are released from neurons, astrocytes, Ols, and microglia and can transmit molecular cargo between cells (Brites, 2020). In ALS, EVs secreted by neurons and glia play a key role in intercellular communication and neuroinflammation (Pegtel et al., 2014). Evidence indicates that besides their potential as biomarkers and novel therapeutic vehicles, EVs are recognized as having a role in the pathogenesis and dissemination of various human diseases (Brites et al., 2015).

These vesicles are a heterogeneous group classified according to morphological, biochemical, and biogenic parameters. They can be divided in microvesicles, sEVs/exosomes, and apoptotic bodies. Microvesicles are EVs with around 200–1.000 nm generated by plasma membrane outward budding and fission. Diversely, apoptotic bodies (1–5 μm) are secreted by apoptotic cells upon their membrane disintegration after apoptosis (Loch-Neckel et al., 2022).

Exosomes are the best characterized species of EVs. They are endogenous, cell-secreted small extracellular membrane vesicles with approximately 50–200 nm in diameter produced by the exocytosis of multivesicular bodies and released upon fusion with the plasma membrane. Exosomes freely cross the BBB and carry cargos associated with signaling functions and intercellular communication, even at long distances, by direct transfer of mRNA, proteins, lipids, and miRNAs, the last being essential for regulating gene expression in the recipient cells (Cunha et al., 2016; Loch-Neckel et al., 2022; Sun et al., 2018). The transfer of exosomes to neurons was shown to be mediated by Ols, microglia, and astrocytes that may be either supportive to neurons, or instead disseminate the disease, contributing to the pathogenesis of neurodegenerative diseases (Brites et al., 2015). In ALS, the secretion of exosomes derived from NSC-34 cells overexpressing mutant human $SOD1^{G93A}$ ($hSOD1^{G93A}$) was proposed as a mechanism of cell-to-cell transfer of mSOD1 toxicity (Gomes et al., 2007).

The small size of EVs compared to the one of the whole cells also offers therapeutic benefits. Some studies have shown that EVs offer high physical-chemical stability, cell-selective fusion, and long-distance communication. In addition, sEVs are naturally biocompatible, with superior targeting capability, safety profile, nanometric size, and immune compatibility, and can be loaded with both lipophilic and hydrophilic agents. Furthermore, the cargo transference between different cells allows a fast alteration in gene expression and control of cell functions and critical processes. All these properties provide potential advantages of sEVs over conventional drug delivery nanocarrier systems. A variety of therapeutic materials, including drugs, short interfering-RNAs, anti-miRs, pre-miRs, and recombinant proteins, can be loaded into sEVs during their biosynthesis or after isolation. These approaches may result in different loading efficiencies and stabilities of the drugs in the sEVs (Loch-Neckel et al., 2022).

Additionally, EVs could be chemically and biologically modified to broaden, alter, and/or enhance their therapeutic capability, the engineering of EVs becoming an emerging focus of research, including in the field of CNS pathologies (Pinho et al., 2020).

4. Experimental Models in ALS Research

To identify novel therapeutic targets and biomarkers for ALS, it is essential to have a much better understanding of the cellular and molecular mechanisms of the disease and pathological events that lead to MN degeneration in both sALS and fALS. Over the years, the advances in identifying genetic mutations linked to ALS have led to the development of multiple *in vivo* and *in vitro* models of the disease. For that, a vast spectrum of models carrying different mutations in *Sod1*, *C9orf72*, *Tardbp,* and *Fus*, have been established to elucidate ALS pathological mechanisms. Such models vary from *in vitro* models, including cell lines, primary cell cultures, and OCs, and extend to *in vivo* models and, more recently, to human patient-derived iPSCs models, this last providing equilibrium between the advantages of a cellular model and the approach to the human disease (Van Damme et al., 2017).

4.1 *In vitro* Models

The *in vitro* models enable the analysis of specific intracellular mechanisms and dysfunctionalities of an isolated cell type, allowing the comparison between cells derived from healthy and TG animals. Therefore, with these less complex systems, it is possible to better understand the specific molecular pathways involved in each cell type and disease stage.

As the main disadvantage, it can be pointed out that *in vitro* models do not recapitulate three-dimensional cytoarchitecture found *in vivo* since it does not replicate the conditions of the organism, and there is no interaction with other systems (Gois et al., 2020).

4.1.1. Cell Lines

The most common cell line used to study MNs in ALS research is the NSC-34 (Madji Hounoum et al., 2016). NSC-34 is a mouse neural hybrid cell line produced by the fusion of MNs from the SC of mouse embryos with mouse neuroblastoma cells N18TG2 (Cashman et al., 1992), allowing to study the molecular and cellular mechanisms involved in ALS neurodegeneration. Indeed, NSC-34 mimics several features of MNs, including action potential generation, neurofilament and synaptic proteins expression, synapse formation and acetylcholine synthesis, storage, and release (Gomes et al., 2008; Tovar et al., 2009).

NSC-34 MN-like cell line overexpressing the $hSOD1^{G93A}$ is commonly used to study morphological and physiological properties of MNs in ALS. Previous results from our group showed that $hSOD1^{G93A}$ NSC-34 cells evidence cellular and molecular alterations of MNs, including mSOD1 aggregates in the cell body, diminished proliferation rate, apoptotic nuclei, mitochondrial impairment, Golgi apparatus fragmentation, ATP depletion, and increased release of nitrites to the extracellular media (Vaz et al., 2015), thus mimicking ALS endogenous MNs.

The microglial N9 cell line results from the immortalization of primary microglia cells isolated from the cortex of CD1 mouse embryos with oncogenes of the MH2 retrovirus (Righi et al., 1989; Stansley et al., 2012). They present diverse features similar to microglia in primary cell cultures, such as phagocytosis, migration, and inflammation-related features (Mejzini et al., 2019). $hSOD1^{G93A}$ N9 cells can release exosomes that recapitulate miRNA cargo of the origin cells and show the capacity to uptake exosomes from NSC-34 MNs (Vaz et al., 2019).

NSC-34 and N9 cell lines can even be used in co-cultures, being an excellent model to study the mechanisms involved in MNs/microglia communication in ALS pathogenesis. The main advantages of these cell lines are that they are less time-consuming, and many cells can be obtained quickly.

4.1.2. Primary Cultures

Primary cell cultures, useful *in vitro* models to study specific intracellular mechanisms of an isolated cell type, consist of cells directly taken from tissues for processing. These cultures are obtained from animal models, using cortex and SC at different stages of the disease, and allow the extraction, purification, and maintenance of neurons, astrocytes, and microglia under optimized culture conditions. In ALS pathology, glial cell cultures are relevant models that have helped to elucidate the role and to show new insights of non-neuronal cells (Gomes et al., 2019). Particularly, primary SC cultures, the most commonly used, have been established to study the morphological, biochemical, and electrophysiological characteristics found in each cell type, enabling a more focused understanding of specific cell biology features (Tovar et al., 2009).

Compared to cell lines, primary cultures have the advantage of resembling more closely the properties of *in vivo* cells (Stansley et al., 2012). The factors that constitute a major drawback of these

experimental models are that they are time-consuming, show limited growth potential, the absence of interaction with neighboring cells and the need of more expertise and optimized culture conditions.

4.1.3. Organotypic Cultures

OCs or 3D cultures constitute an *ex vivo* cellular model that combines the benefits of *in vivo* and *in vitro* models and maintains a specific tissue's basic structure and connective organization. It involves the preservation of an entire tissue slice and provides the best way to preserve all the cellular content, the three-dimensional cytoarchitecture and the complex electrophysiological and biochemical organization of the cells. Organotypic slices can be obtained from a variety of tissues, including the brain and SC, from both embryos and postnatal animals, allowing the analysis of different stages of the disease (Berthod et al., 2012) and the study of cell-to-cell interactions under normal and pathological conditions in their biochemical and morphological environment.

The most used technique is the membrane interface method, where thin slices of a sectioned tissue are placed on a porous membrane filter at the interface between an upper air surface that allows sufficient oxygenation of the cells, and a culture medium, that provides nutrients through capillary action (Li et al., 2016; Tan et al., 2018).

Specifically, SCOCs have been used as *ex vivo* models of ALS to study cellular interactions under ALS pathological-like conditions (Berthod et al., 2012). They preserve an *in vivo* horizontal architecture, leaving MNs *in situ*, with all the other cells and connections in place. Moreover, the MNs in an OC system can survive for more than three months (Li et al., 2008). Generally, after dissection of the lumbar SC and removal of the meninges, transversal sections are sectioned and transferred into membrane inserts fitting in 6-12-well culture plates (Calderó et al., 2010). Our group characterized the SCOCs derived from 10-12-week-old mSOD1 mice (early symptomatic stage of the disease) and observed decreased levels of GFAP and PSD-95, impairment of the CX3CL1-CX3CR1 axis, and an imbalance of neuroimmune homeostasis (Vaz et al., 2021b). Moreover, increased levels of miR-124 were noticed, as previously demonstrated in the mSOD1 NSC-34 MNs and their respective secretome/sEVs (Pinto et al., 2017; Vaz et al., 2021b). Interestingly, modulation of mSOD1 NSC-34 MNs with anti-miR-124 produced a secretome able to prevent the pathogenesis observed in ALS SC slices, emphasizing the organotypic SC as a proper model to assess neuroprotection (Vaz et al., 2021b). Therefore, examining MN degeneration and glial cell reactivity in SC slices derived from $SOD1^{G93A}$ mice may open new avenues for discovering novel pathophysiological mechanisms in ALS.

4.1.4. Human-derived iPSCs

The discovery of iPSCs constituted a major scientific breakthrough and a new world of possibilities to study several diseases and has recently emerged in ALS research (Mejzini et al., 2019).

Reprogramming techniques to generate iPSCs from human-differentiated somatic cells (e.g., skin, fibroblasts) that can be differentiated into various cell types of interest has opened new paths and revolutionized ALS research. These cells carry endogenous gene variants within the context of an individual's genetic background, including the genetic and epigenetic makeup of patients, making them particularly valuable to study the sALS cases in which the causative genetic factors are unknown (Mejzini et al., 2019). Therefore, understanding the cause of the disease through the study of MNs and glial cells derived from sALS patients would be challenging but extremely promising (Berthod et al., 2012). Moreover, they may eradicate the chances of immune rejection during transplantation by using the patient's cells (Singh et al., 2015).

By introducing a set of four specific transcription factors (Kruppel-like factor 4, POU transcription factor Oct-3/4, SRY-related HMG-Box Gene 2, and cellular myelocytomatosis) into somatic patient-derived cells, it is now feasible to generate iPSCs from ALS patients (Berthod et al., 2012; Van Damme

et al., 2017). The pluripotent cells can then be guided to differentiate into the desired cell lineage, including MNs, astrocytes, and microglia (Mejzini et al., 2019).

Recent advances have led to more direct approaches to convert fibroblasts into specific cell types of interest. Through the bypass of the pluripotency stage, it is possible to induce direct conversion of adult human patient fibroblasts into tripotent induced neural progenitor cells (iNPCs) with the four reprogramming factors mentioned above and subsequent exposure to a specific NPC medium (Meyer et al., 2014). For instance, our group has already used this approach using iNPCs-derived astrocytes from patients with sALS and SOD1 mutations (mSOD1-ALS) to verify their neurotoxic nature and to assess differences in the cell line signatures for astrocyte reactive markers and inflamma-miRNAs (Gomes et al., 2022).

4.2 *In vivo* Models: SOD1^{G93A} (mSOD1) Mice Model

Although multiple ALS models exist for different genes in different species, TG mouse models represent the gold standard of preclinical ALS modeling and have provided important insights into MN degeneration pathways and disease mechanisms (Tan et al., 2017; van Es et al., 2017). Indeed, TG animal studies have yielded the most significant wealth of information to date.

In 1994, the SOD1^{G93A} mouse was generated and had since dominated clinical model studies in the field. This model develops the main clinical, electrophysiological, and histopathological features of both fALS and sALS cases, being the one that closely mimics the ALS symptoms and pathophysiology found in SOD1-related ALS patients. Histopathological findings observed in these TG animals include progressive accumulation of aggregates containing SOD1 and ubiquitin and aberrant neurofilament accumulations in degenerating MNs. The overexpression of mSOD1 protein in mice leads to Golgi fragmentation, neuroinflammation, massive degeneration, and death of MNs in the ventral horn of the SC, and loss of myelinated axons in ventral motor roots (Berthod et al., 2012). Regarding cellular processes, mSOD1 mice evidenced astrogliosis and microgliosis at early onset, axonal transport impairments, excitotoxicity mediated by glutamate, mitochondrial vacuolization, reduced metabolic support to MNs by their surrounding glial cells, and substantial denervation of the NMJ (Gurney, 1994; Philips et al., 2015).

SOD1^{G93A} mice express large amounts of mSOD1 and develop adult-onset neurodegeneration of spinal MNs and progressive motor deficits leading to paralysis (Gurney et al., 1994). It recapitulates many features of clinical ALS, including adult-onset, rapidly progressive motor symptoms and muscle wasting, different patterns of symptom progression, and comparable loss of MNs that replicate the disease progression observed in ALS patients (Gaja-Capdevila et al., 2021; Tan et al., 2017).

Despite all the advantages however, mSOD1 models have as their main limitations the absence of TDP-43 inclusions, the most common neuropathological feature of ALS. Furthermore, although different TG mice have been extremely useful in studying fALS, they shows limited success in investigating sALS. Moreover, translational success from most promising therapies developed in mSOD1 mice failed in clinical trials, with reduced efficacy in identifying drugs alleviating the disease symptoms (Berthod et al., 2012; Tan et al., 2017). Nevertheless, and despite the limitations mentioned above, this human mSOD1 murine model continues to be the most accurate experimental model of clinical ALS and the most widely characterized and used in the assessment of molecular targets, biomarkers, and novel drugs/treatments for ALS, providing more translatable results to clinical ALS (Brites et al., 2014; Van Damme et al., 2017).

To evaluate mice symptoms, there are numerous behavioral tests frequently performed to assess motor function. The rotarod test determines the animal's motor coordination, strength, and balance by measuring the maximum maintenance time in a rotating cylinder at a constant speed. The hanging-wire evaluates the neuromuscular strength by measuring the time the mouse can hold on in suspension in an

upside-down wire lid. In the treadmill test, the animals are encouraged to run on a treadmill equipped with an electrical shock grid at the rear. Performance is measured based on the time each mouse stays on the shock grid, and the animal will spend more time on the shock grid as the disease progresses. The footprint test is used to evaluate the gait quality by measuring the stride length of the mice, which is the distance between two pawprints. A shorter stride length means abnormalities in the gait. These tests demonstrated deterioration of motor performance of $SOD1^{G93A}$ mice at the symptomatic stage of the disease, reflecting the development of an ALS-related phenotype (Correia et al., 2021; Gurney et al., 1994; Knippenberg et al., 2010; Oliván et al., 2015; Zhao et al., 2019).

Distinct routes of administration can be applied to access the CNS, such as intravenous (IV), intranasal (IN), intracerebroventricular (ICV), and intrathecal (IT) administration.

The ICV and IT injections allow a direct delivery to the brain and the space surrounding the SC, respectively, without the need to cross the BBB. For instance, some studies showed the therapeutic ability of the ICV injection of engineered miRNAs in mSOD1 mice since these approaches resulted in the extension of animal survival (Koval et al., 2013; Nolan et al., 2014). Nevertheless, these are more invasive therapeutic approaches since they require surgical intervention, which presents a possible drawback for the translation into the clinics (Turner et al., 2011). The IN administration emerged as a non-invasive route to deliver therapeutics to the brain and SC. For instance, IN administration has successfully provided growth factors, proteins, and stem cells-derived EVs to the CNS in ALS mice (Bonafede et al., 2020; Martinez et al., 2008). The IV injection is also a non-invasive, clinically applicable strategy with reduced risk that allows the administration of large volumes of therapeutics. In these cases, and contrary to what happens with the ICV and IT injections, the therapeutic drug has to cross longer distances and the BBB, being necessary to consider their short half-life in the blood and the use of massive concentrations that could lead to systemic exposure and toxicity (Bellettato et al., 2018). Despite the advantages and disadvantages presented above for the different administration routes, the potential of IT delivery for ALS has been well demonstrated by several therapeutic trials using gene therapy in animal models of ALS. For instance, the intrathecal route proved to be the most relevant for ALS therapy because of its targeted delivery of recombinant adeno-associated virus (rAAV) to the disease afflicted site, which reduces the required rAAV dose and the undesired periphery transduction. Moreover, due to feasibility, safety, and high efficiency, IT injection has served as one of the most attractive ways to deliver drugs in preclinical and clinical trials (Li et al., 2017).

The concept of precision medicine, which combines genetic, environmental, and clinical diagnostic testing to stratify the patients, emerged once ALS patients are characterized by substantial genetic and phenotypic heterogeneity (Picher-Martel et al., 2016). With this personalized medicine approach, it will be possible to apply a single or combined therapeutic strategies to a specific subgroup of patients.

II

AIMS OF THE STUDY

ALS is a progressive neurodegenerative disease with a short life expectancy and no effective therapy, characterized by MN loss and glial activation. Inflamma-miRNAs were found dysregulated in neural cells and recapitulated in their secretome when using several ALS models, including in SOD1^{G93A} (mSOD1) mouse models, and contributing to disease propagation. Following what was explained in the Introduction section, our group identified upregulated levels of the neuronal miR-124 in mSOD1 NSC-34 MNs, that is sorted into exosomes and lead to microglial activation. We also demonstrated that these increased levels of miR-124 were responsible for MN degeneration, mitochondrial dysfunction, and axonal/synaptic impairment. The same study showed that cell transfection with miR-124 inhibitor (anti-miR-124) in mSOD1 MNs prevented neuronal function. Moreover, their secretome counteracted pathogenicity and prevented glial reactivity and inflammatory-miRNA instability in the SCOCs from early symptomatic ALS mice (12-week-old). Therapeutic benefits of the secretome from pathological MNs after miR-124 modulation was never explored in the ALS mSOD1 mouse model, though recent evidence sustains that secretomes from different cell types may have therapeutic effects.

Thus, we aimed to:

- **(i)** Characterize the mSOD1 mouse model at the symptomatic stage of the disease in comparison with the aged-matched WT mice;
- **(ii)** Investigate the potential neuroprotective effects of the preconditioned secretome when intrathecally injected in the mSOD1 mice SC at the early symptomatic stage (12-weeks-old) by characterizing the lumbar SC from those mice at 15-week-old mice (symptomatic stage of the disease) in comparison with aged-matched mSOD1 mice injected with vehicle (basal neuronal media).

For that, our group performed the intrathecal injection of the secretome derived from anti-miR-124-modulated SOD1^{G93A} NSC-34 MNs (preconditioned secretome) into the lumbar SC of 12-week-old (early symptomatic stage of the disease) mSOD1 mice, and here we assess the inflammatory-miRNA signature, glial reactivity, and neurodegeneration in the 15-week-old mice (symptomatic phase).

If the data obtained in this study suggest that the pathological secretome derived from ALS MNs engineered with anti-miR-124 might have a therapeutic interest in preventing neuroinflammation, we will be able to propose a promising cell-free based therapeutic strategy that may be translated to ALS patients that can halt/delay disease progression. For this proposed approach, it will be necessary to stratify patients with upregulated levels of miR-124 before its translation into clinics. As an autologous treatment, this modulatory strategy may contribute to precision medicine in ALS by using iNPCs/iPSC derived from patient fibroblasts obtained by different ALS patients, either familial or sporadic.

III

MATERIALS AND METHODS

1. Material

1.1 Animals and Ethics Statement

Mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA), namely the TG B6SJL-TgN mSOD1Gur/J males (no. 002726) hSOD1^{G93A} (mSOD1) and non-TG B6SJLF1/J WT females. The animals were housed at the animal facility of the Life and Health Science Research Institute *(ICVS)*, University of Minho, where the colony was also established. They were maintained under standard conditions [12 h light / 12 h dark cycles, room temperature (RT) at 22–24°C and 55% humidity] and provided with food and water *ad libitum.* The colony was maintained on a background B6SJL by breeding mSOD1 TG males with non-TG females. WT and mSOD1 mice were compared to the respective aged-matched control mice. Experiments were conducted in both mSOD1, and WT mice and both male and female mice were used. For mice genotyping, a tail section was collected 6 days after birth and analyzed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) (Cunha et al., 2018).

The SOD1^{G93A} mice on B6SJL background develop normally until about 12 weeks of age when they start to exhibit functional deficits in locomotion and muscle strength, and weakness progresses until death occurs at an average of 20 weeks. From 8 weeks, electrophysiological tests identify clear abnormalities in these mice. Therefore, the following timepoints for the SOD1^{G93A} mice can be considered: 4 weeks (normal juvenile), 8 weeks (presymptomatic stage), 12-14 weeks (early symptomatic stage), and 16 weeks (advanced symptomatic stage) (Rubio et al., 2022).

All the procedures performed in the present study were in accordance with the European Community guidelines (Directives 86/609/EU and 2010/63/EU, Recommendation 2007/526/CE, European Convention for the Protection of Vertebrate Animals used for Experimental or Other Scientific Purposes ETS 123/Appendix A) and Portuguese Laws on Animal Care (Decreto-Lei 129/92, Portaria 1005/92, Portaria 466/95, Decreto-Lei 197/96, Portaria 1131/97). All the protocols used in this study were approved by the Portuguese National Authority (General Direction of Veterinary) and Ethical Subcommittee in Life and Health Sciences (SECVS; ID: 018/2019). According to the 3R's principle, every effort was made to minimize the number of animals used and their suffering.

1.2 Biological Samples

The samples used in this thesis were obtained previously by our research group, as detailed and recently published (Barbosa et al., 2022).

Briefly, Mice NSC-34 MN cell line, stably transfected with human SOD1 (hSOD1), either WT [NSC-34 hSOD1 WT (WT MNs)] or mutated in G93A [NSC-34 hSOD1^{G93A} (mSOD1 MNs)], was grown in proliferation media and then differentiation was induced. Then, mSOD1 MNs were transfected with 15 nM anti-miR-124 (Ambion® Anti-miR™ miRNA inhibitor, #AM10691), followed by the collection of the medium containing the factors secreted by mSOD1 MNs (secretome). Since intrathecal injection requires very low volumes of secretome (Hu et al., 2018), it was concentrated 100x and then stored at −80°C until being used for the intrathecal injection experiments **(Figure III.1)**.

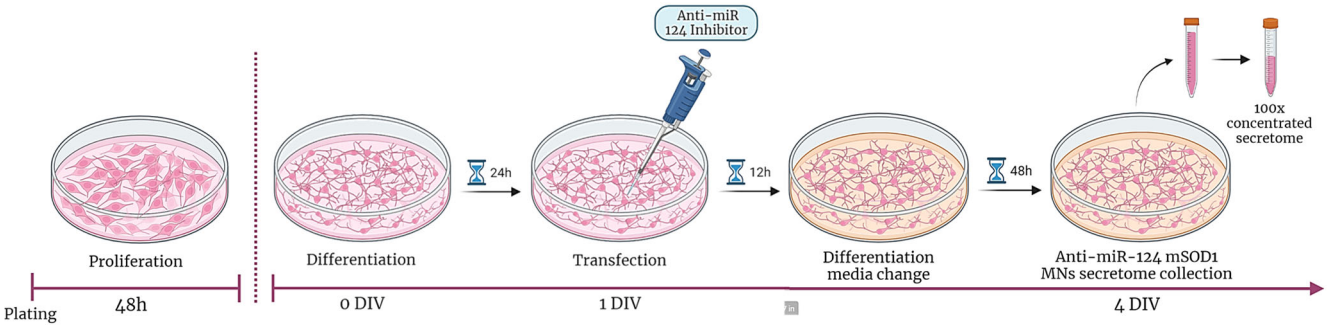

Figure III.1 — Schematic representation of the experimental procedures performed in the mSOD1 NSC-34 motor neuron (MN) cell line. After 48 h of proliferation, differentiation was induced in the time considered as 0 days in vitro (0 DIV). After 1 DIV, mSOD1 MNs were transfected with 15 nM of anti-miR-124 (Ambion® Anti-miR™ miRNA inhibitor, #AM10691) and mixed with X-tremeGENE™ HP DNA Transfection Reagent (Sigma-Aldrich) in a proportion 2:1. Cell transfection occurred during 12 h. Fresh differentiation medium was added 12 h after and the transfected cells were maintained for more 48 h. Lastly, the secretome derived from mSOD1 MNs was collected at 4 DIV and concentrated 100x. (Created with BioRender.com).

The control groups used in the experiment were the WT and mSOD1 mice injected with the vehicle [differentiated NSC-34 cell media with 1% of fetal bovine serum (FBS) without sEVs] in a total of 8 and 7 animals, respectively. The treated group was mSOD1 mice injected with the concentrated secretome from anti-miR-124-treated mSOD1 MNs (mSOD1 + sec, n = 8). A single intrathecal injection was performed in the WT and mSOD1 mice with 12-week-old (early symptomatic stage of the disease).

To determine the impact of the secretome from anti-miR-124-treated mSOD1 MNs on motor behavior, the motor performance was assessed in the animals two weeks after the injection with the following behavioral tests: footprint, the hanging wire, the cylinder, and the limb clasping and grasping tests, as described (Barbosa et al., 2022). The behavioral alterations in the mSOD1 mice were assessed at the symptomatic stage (14-week-old mice), as the deterioration of motor performance was previously observed in an ALS mice model at this stage of the disease (Alves et al., 2011; Gurney, 1994; Knippenberg et al., 2010; Oliván et al., 2015; Trabjerg et al., 2021).

After dissection, the lumbar SC was rapidly frozen at −80 °C for RT-qPCR and Western blot (WB) assays. The lumbar SC was fixed with paraformaldehyde (PFA) for histological and immunohistochemistry studies. For sectioning, SC was previously embedded in Tissue-Tek® O.C.T. Compound (Sakura Finetek, USA, CA), and transversal 20-µm thick sections were serially cut using the cryostat Leica CM1900 (UV Leica, Wetzlar, Germany). The sections were collected on Superfrost Plus glass slides (Thermo Scientific) and preserved at −80ºC.

The sequence of the experiments developed in this study is graphically summarized in **Figure III.2**.

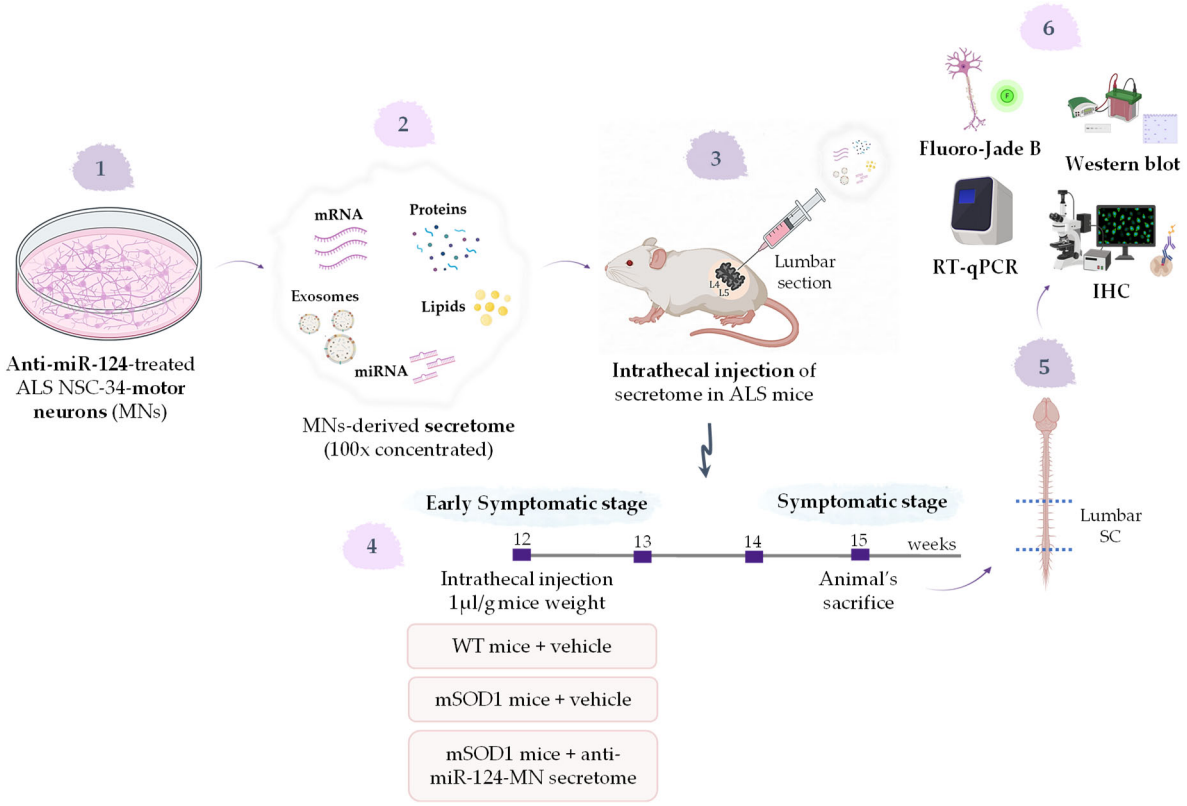

Figure III.2 — Experimental design. NSC-34 motor neuron (MN) cell line stably overexpressing human SOD1, either wild type (WT) or with G93A mutation (mSOD1), was differentiated for 4 days. In parallel, mSOD1 cells were transfected with Anti-miR™ 124 Inhibitor **[1]**. Then, the medium containing the factors secreted by mSOD1 MNs (secretome) was collected and concentrated 100x **[2]**. Afterwards, the WT and $SOD1^{G93A}$ (mSOD1) mice were injected into the lumbar spinal cord (SC) at the early symptomatic stage (12-week-old), either with the vehicle (control group; differentiated NSC-34 cell media) or with anti-miR-124-mSOD1 MN secretome (only the mSOD1 mice) **[3, 4]**. At the 15th week, the animals were sacrificed and the lumbar SC isolated **[5]** to perform reverse transcription-quantitative polymerase chain reaction (RT-qPCR), Western blot (WB), immunohistochemistry (IHC) and Fluoro-Jade B assessments **[6].** (Created with BioRender.com).

1.3 Supplements and Chemicals used for Molecular Biology Analysis

Hank's balanced salt solution (HBSS), FBS, Heat-inactivated horse serum, and TRIzol® reagent were purchased from Life Technologies (Waltham, Massachusetts, USA). Bovine serum albumin (BSA), Tris-base, β-mercaptoethanol, Hoechst 33258 dye, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), and Fluoromount-G were purchased from Sigma-Aldrich (St. Louis, MO, USA). N, N, N', N'-Tetramethyl ethylenediamine, Triton X-100, glycerol, acetic acid, glycine, acrylamide, bis-acrylamide, Tween-20, and absolute ethanol were acquired from Merck (Darmstadt, Germany). The set of primers **(indicated in Table III.1)** used to amplify protein-coding genes was acquired from Thermo Fisher Scientific (Waltham, MA, USA), and the set of primers used to amplify miRNAs **(in Table III.2)** and SNORD110 was acquired from Qiagen (Valencia, CA, USA). Sodium dodecyl sulfate (SDS) was acquired from VWR-Prolabo. All the other commonly used reagents were purchased either from Sigma-Aldrich or Merck.

1.4 General Equipment

Eppendorf 5810R (Eppendorf, Hamburg, Germany) centrifuge, Stuart Gyro-rocker SSL3 (Cole-Parmer, Stone, Staffordshire, ST15 OSA, UK), Jenway Model 3510 pH Meter (Cole-Parmer), MSH-300 Magnetic Stirrer with a hot plate (Biosan SIA, Riga, LV-1067, Latvia) and Varioskan™ LUX multimode microplate reader (Thermo Fisher Scientific) were used for several experimental procedures.

2. Methods

2.1 Protein and Gene Expression

2.1.1 RT-qPCR

For the lumbar SC portions, a Pellet Mixer (VWR Life Science, Radnor PA, USA) was used for sample homogenization and the TRIzol® Reagent (Life Technologies) for total RNA isolation (Cunha et al., 2018). Then, the total RNA extracted was quantified on NanoDrop ND100 Spectrophotometer (NanoDrop Technologies, Wilmington, USA), following the manufacturer's instructions.

For gene expression, the total RNA was reverse transcribed into complementary DNA (cDNA) using the Xpert cDNA Synthesis Supermix Kit (GRiSP, Porto, Portugal) under the manufacturer's instructions at optimized conditions: 37ºC for 15 min, 60ºC for 10 min, and 95ºC for 3 min. Subsequently, cDNA was amplified by quantitative RT-qPCR using Xpert Fast SYBR Mastermix BLUE Kit (GRiSP) and the designed primer sequences **(listed in Table III.1)**. Amplification products were obtained under the following optimized conditions: 50°C for 2 min, 95°C for 2 min, followed by 40 amplification cycles at 95°C for 5 s and 62°C for 30 s. The 60S ribosomal L19 (*Rpl19*) was used as an endogenous control to normalize each gene's expression levels. After the amplification protocol, a melting curve analysis was performed to verify the amplified product's specificity.

For miRNA expression, cDNA synthesis was performed using miRCURY LNA™ RT Kit (Qiagen), using 5 ng of total RNA according to the following protocol: 60 min at 42°C succeeded by heat-inactivation of the reverse transcriptase for 5 min at 95°C. For miRNA quantification, the PowerUp™ SYBR™ Green Master Mix (Life Technologies) was used in combination with the pre-designed primers **(listed in Table III.2)**. SNORD110, a reference gene, was used as an endogenous control to normalize each miRNA expression levels. The reaction conditions consisted of polymerase activation/denaturation at 95°C for 10 min, followed by 50 amplification cycles at 95°C for 10 s and 60°C for 1 min (ramp-rate of 1.6 °/s). In the end, a melting curve analysis was done to verify the specificity of the amplified product.

Relative mRNA/miRNA concentrations were measured via the $2^{-\Delta\Delta CT}$ equation relative to that of the endogenous control, as previously performed in studies of our laboratory (Cunha et al., 2018). Results were expressed as fold change *vs.* the respective control, depending on the experiment.

This technique was performed in 384-well plates, with each sample measured in duplicate and including non-template controls for each amplification product. The cDNA synthesis was performed in a thermocycler (Biometra®, Göttingen, Germany) and the RT-qPCR was run on QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems).

Table III.1 — List of primer sequences used in RT-qPCR to amplify protein-coding genes.

Mouse gene	Forward primer (5'-3')	Reverse primer (5'-3')
Arg1	CTTGGCTTGCTTCGGAACTC	GGAGAAGGCGTTTGCTTAGTTC
Cx3cl1	CTCACGAATCCCAGTGGCTT	TTTCTCCTTCGGGTCAGCAC
Cx3cr1	ATGGGGTCTCTGTCTGCTCT	TACTGGCAATGGGTGGCATT
Cx43	ACAGCGGTTGAGTCAGCTTG	GAGAGATGGGGAAGGACTTGT
Dlg4	GAGGCTGGCGGCCAGTACACCAG	ACAGAGCAGGCGGTCAG
Dyn	GCCTCAGTCTCTGTCCCATC	AAGTCCTGGGGTAAGGTGCT
Gfap	CAAACTGGCTGATGTCTACC	GCTTCATCTGCCTCCTGTCTA
Gpr17	AGCTACGAGGAGTCCACCTG	AGACCGTTCATCTTGTGGCTCT
Il-10	ATGCTGCTTGCTCTTACTGA	GCAGCTCTAGGAGCATGTGG
Il-1β	CAGGCTCCGAGATGAACAAC	GGTGGAGAGCTTTCAGCTCATA
Inos	ACCCACATCTGGCAGAATGAG	AGCCATGACCTTTCGCATTAG
Kif5b	GGTCCTACAGTTGCCACCTA	ATTGAAATACGCCAGGCCCA
Mbp	CCATCCAAGAAGACCCCACA	CCCCTGTCACCGCTAAAGAA
Mfg-e8	AGCCTGAATGGTAGGGTTGG	GAGACTGCATCCTGCAACCA
NeuN	CCAGGCACTGAGGCCAGCACACAGC	CTCCGTGGGGTCGGAAGGGTGG
P2ry12	CACCTCAGCCAATACCACCT	CAGGACGGTGTACAGCAATG
Plp	TGGCGACTACAAGACCACCA	GACACACCCGCTCCAAAGAA
Rpl19	ATGAGTATGCTCAGGCTACAGA	GCATTGGCGATTTCATTGGTC
S100b	GAGAGAGGGTGACAAGCACAA	GGCCATAAACTCCTGGAAGTC
Synaptophysin	GACGTTGGTAGTGCCTGTGA	GCACAGGAAAGTAGGGGGTC
Timp2	AGCCAAAGCAGTGAGCGAGAAG	GCCGTGTAGATAAACTCGATGTC
Tmem119	CCCAGAGCTGGTTCCATAGC	GGGAGTGACACAGAGTAGGC
Tnf-α	TACTGAACTTCGGGGTGATTGGTCC	CAGCCTTGTCCCTTGAAGAGAACC
Trem2	AACTTCAGATCCTCACTGGACC	CCTGGCTGGACTTAAGCTGT

Arg1, arginase 1; ***Cx3cl1***, c-x3-c motif chemokine ligand 1/fractalkine; ***Cx3cr1***, c-x3-c chemokine receptor 1; ***Cx43***, connexin 43; ***Dlg4***, discs large MAGUK scaffold protein 4 (encodes for PSD-95); ***Dyn*****,** dynein; ***Gfap,*** glial fibrillary acidic protein; ***Gpr17***, G protein-coupled receptor 17; ***Il-10*****,** interleukin 10; ***Il-1β*****,** interleukin 1β; ***Inos***, inducible nitric oxide synthase; ***Kif5b***, kinesin family member 5B (encodes for kinesin); ***Mbp***, myelin basic protein; ***Mfg-e8***, milk fat globule-epidermal growth factor 8; ***NeuN***, hexaribonucleotide binding protein 3; ***P2ry12***, purinergic receptor p2y12; ***Plp***, proteolipid protein; ***Rpl19***, 60S ribosomal L19; **RT-qPCR**, reverse transcription-quantitative polymerase chain reaction; ***S100b***, S100 calcium-binding protein B; ***Timp2***, tissue inhibitor of metalloproteinases 2; ***Tmem119***, transmembrane protein 119; ***Tnf-α***, tumor necrosis factor-alpha; ***Trem2***, triggering receptor expressed on myeloid cells *2*.

Table III.2 — List of miRNA sequences used in RT-qPCR to amplify miRNAs.

miRNA	Target sequence (5'-3')
hsa-miR-146a-5p	UGAGAACUGAAUUCCAUGGGUU
mmu-miR-155-5p	CTCAGAGAGGTGGAAGACCATGT
hsa-miR-21-5p	UAGCUUAUCAGACUGAUGUUGA
hsa-miR-124-3p	UAAGGCACGCGGUGAAUGCC
hsa-miR-125b-5p	UCCCUGAGACCCUAACUUGUGA
SNORD110	Reference gene

miRNA, microRNA; **mmu**, *mus musculus*; **hsa**, *homo sapiens* (with homology to *mmu* species).

2.1.2 Western Blot

Total protein isolation from organic phases of Trizol-chloroform lumbar SC was performed by using TRIzol® Reagent (Life Technologies), as previously described (Simões et al., 2013) and usually in our lab (Cunha et al., 2018). Briefly, 150 µl of this solution was added to each lumbar SC portion, and then the samples were homogenized using a Pellet Mixer motor homogenizer (VWR Life Science). At the end of the protocol, all protein samples were sonicated in 10 cycles of 15 seconds each using Ultrasonic Processor UP100H (Hielscher-Ultrasound Technology, Teltow, Germany). After that, samples were centrifuged at 3200g, 4ºC for 10 minutes. Lastly, the supernatant was collected, and pellets were discarded. Following protein extraction, total protein concentrations were determined using the Coomassie (Bradford) Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer's specifications.

WB Protocol was then performed as usual in our laboratory (Pinto et al., 2017). The system employed in Western-Blot assays was Mini-PROTEAN Tetra cell with PowerPac™ Basic Power Supply (Bio-Rad Laboratories, Hercules, CA, USA). According to total protein concentration measurements, calculations were performed to obtain equivalent amounts of protein (50 µg) to be loaded into the gel. Loading buffer [0.25 M Tris-HCl (pH 6,8); 4% (w/v) SDS; 40% (v/v) glycerol; 0,2% (w/v) bromophenol blue; 1% (v/v) β-mercaptoethanol] was added to each sample before its denaturation at 100ºC for 5 minutes. Then, samples were separated on dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with the following conditions: 200 volts (V), 40 milliamperes (mA) per gel, at least 1 h 30 min.

Subsequently, proteins were transferred (200 V, 300 mA) into a nitrocellulose membrane (Amersham™ Protran™ 0,45 µm NC, GE Healthcare Life science, Germany) for at least 1 h 30 min. Afterwards, proteins present in the membranes were identified by Amido black (Sigma-Aldrich) staining. The membranes were washed 3 times, for 5 min, with Tris-Buffered Saline (TBS) and then blocked for 1 h with blocking buffer [5% (w/v) non-fat dried milk in TBS-0.1% Tween 20 (TBS-T)] at RT with mild agitation. After this, membranes were washed 3 times, for 5 min, with TBS-T, followed by overnight incubation with specific primary antibodies **(indicated in Table III.3)** diluted in TBS-T with 5% of BSA at 4°C with mild agitation.

On the following day, membranes were washed 3 times with TBS-T for 5 min and incubated with the respective secondary antibody conjugated with horseradish peroxidase **(listed in Table III.3)** diluted in blocking buffer (1:5000) for 1 h at RT with gentle agitation. Membranes were washed 3 more times with TBS-T in the same conditions.

Posteriorly, immunoreactive bands were detected after the incubation with the Western Bright™ Sirius western blotting detection kit (K-12043-D10, Advansta, Menlo Park, CA, USA) and using

Invitrogen iBright™ FL1500 Imaging System (ThermoFisher Scientific). Bands were quantified using the iBright™ Analysis Software (Thermo Fisher Scientific). Results were normalized to the expression of β-actin and indicated as fold change.

Membranes were reused to perform further immunoblotting. Consequently, a mild stripping was conducted in the presence of sodium hydroxide (NaOH) at 0.2 M for 5 min, then washed with Milli Q water for 5 min, and washed twice with TBS-T for 5 min. After this, the membranes were ready to start a novel immunoblotting procedure.

Table III.3 — List of primary and secondary antibodies used for Western blot (WB).

	Antibodies	Source	Species	Dilution
Primary	Anti-β-actin	Sigma, A5441	Mouse	1:2500
	Anti-GFAP	Sigma-Aldrich, G9269	Rabbit	1:500
	Anti-Iba-1	Wako, 019-19741	Rabbit	1:500
	Anti-NeuN	Millipore, MAB377	Mouse	1:100
	Anti-S100B	AbCam, ab52642	Rabbit	1:1000
	Anti-Vimentin	Santa Cruz Biotechnology, sc-32322	Mouse	1:200
Secondary	HRP anti-rabbit	Santa Cruz Biotechnology, sc2357	Mouse	1:5000
	Mouse-IgGκ BP-HRP	Santa Cruz Biotechnology, sc516102	-	1:5000

GFAP, glial fibrillary acidic protein; **HRP**, horseradish peroxidase; **Iba-1**, ionized calcium-binding adapter molecule 1; **NeuN**, hexaribonucleotide binding protein 3; **S100B**, S100 calcium-binding protein B.

2.2 Histological Analysis

2.2.1 Fluoro-Jade B Staining

Following lumbar SC sectioning, Fluoro-Jade B (Chemicon International, California, USA), a fluorescein derivative, was used for staining degenerating neurons to evaluate neurodegeneration. Required solutions (Potassium Permanganate ($KMnO_4$) 0.06%; Acetic Acid (CH_3COOH) 0.1%; Fluoro-Jade B 0.001%) were prepared and adequately protected from the light. The samples were defrosted at RT for 10 minutes. Then, the lumbar SC slices in glass slides were incubated in 0.06% $KMnO_4$ for 30 minutes with mild agitation. After removing $KMnO_4$, lumbar SC sections were washed with Milli-Q water for 5 minutes to clean excessive staining. Then, the samples were stained with a 0.001% solution of Fluoro-Jade B diluted in 0.1% CH_3COOH for 30 min with gentle agitation.

Afterwards, slices were washed 3 times with phosphate-buffered saline (PBS) for 5 min each with mild agitation and mounted in Fluoromount-G (Sigma-Aldrich) with a glass coverslip on the top for confocal microscopy. Slides were protected from light during the whole procedure.

The fluorescence images were acquired in a Leica DMi8-CS inverted microscope (Leica Microsystems CMS GmbH, Mannheim, Germany) with Leica LAS X software, and the different z-stacks were merged and analyzed with Fiji software (1.50i, National Institutes of Health, USA). The mean fluorescence of the ventral horn of one lumbar SC transversal section per animal (n = 3 per group) stained with Fluoro-Jade B was measured and obtained with 20x magnification.

2.2.2 Immunohistochemistry Assay

The glial content in the lumbar SC was evaluated by immunohistochemistry by quantifying the expression of cell-specific markers, including Iba-1 for microglia and GFAP for astrocytes. The protocol was followed as previously described with minor modifications (Vaz et al., 2021b).

After defrosting, the permeabilization/blocking of the sections was performed using a blocking solution [HBSS with 2% heat-inactivated horse serum, 10% FBS, 1% BSA, 0.25% Triton X-100), and 1 nM HEPES] for 3 h at RT. Posteriorly, the slices were incubated with the primary antibodies **(listed in Table III.4)** diluted in blocking solution on a moist bed at 4°C for 48 h. Following 3 washes with 0.01% Triton X-100 in PBS for 20 min, the sections were incubated for 24 h at 4ºC with secondary antibodies **(indicated in Table III.4)** diluted in blocking solution:PBS (1:1). Slices were washed 3 more times for 20 min with 0.01% Triton X-100 in PBS. The cell nucleus was stained with Hoechst dye diluted in PBS (0.1 µg/ml) for 10 min. Slices were additionally washed 3 times with PBS. Lastly, the slices were mounted with one drop of Fluoromount-G (Sigma-Aldrich), and one glass coverslip was applied to be used in the confocal microscopy.

The fluorescence images were obtained in a Leica DMi8-CS inverted microscope with Leica LAS X software, and the different z-stacks were merged and analyzed with Fiji software. Images stained with Iba-1 and GFAP were obtained with 40x magnification and analyzed from 5 ventral horn fields per animal (from 3 animals per group), where the fraction of the area occupied by the GFAP and Iba-1 positive cells was measured.

Table III.4 — List of antibodies used for immunohistochemistry (IHC).

	Antibodies	Source	Species	Dilution
Primary	Anti-GFAP	NovoCastra, GFAP-GA5-6035278	Mouse	1:100
	Anti-Iba-1	Wako, 019-19741	Rabbit	1:250
Secondary	AlexaFluor 488 anti-mouse	Invitrogen, A-10680	Goat	1:500
	AlexaFluor 594 anti-rabbit	Invitrogen, A-11012	Goat	1:500

GFAP, glial fibrillary acidic protein; **Iba-1**, ionized calcium-binding adapter molecule 1.

2.3 Statistical analysis

The unpaired and parametric Student's *t*-test was applied to the data since they followed a normal distribution (Shapiro–Wilk test $p > 0.05$). Results of at least 3 different experiments were expressed as mean ± SEM. Results were represented as fold *vs.* WT + vehicle or *vs.* mSOD1 + vehicle. For every experiment, it was always compared the difference between WT + vehicle and mSOD1 + vehicle, as well as mSOD1 + vehicle and mSOD1 + secretome. Welch's *t*-test correction was applied when variances were different between the two groups. Values of $p < 0.05$ were considered statistically significant. Data were expressed as mean ± SEM values. Results were analyzed using GraphPad Prism 8.0.1 (GraphPad Software, San Diego, CA, USA).

IV
RESULTS

Former data from our research group demonstrated that miR-124 is upregulated in mSOD1 NSC-34 MNs in comparison to the non-mutated ones overexpressing $hSOD1^{WT}$ (WT MNs) and that such mutated cells produced a pathological secretome and sEVs with the same elevation of miR-124, causing alterations in the microglia phenotype (Pinto et al., 2017; Vaz et al., 2021b). The overexpression of miR-124 in mSOD1 MNs is linked to some pathological features in ALS MNs, while also exerts harmful effects on their neighboring cells, namely in astrocytes and microglia, through paracrine signaling (Vaz et al., 2021b). Increased miR-124 levels were also observed in the SC of mSOD1 mice at the symptomatic stage (Cunha et al., 2018), as well as in sEVs from spinal MNs isolated from such mice at the presymptomatic stage and sEVs from ALS patients (Yelick et al., 2020). Considering the potential pathological role of increased miR-124 in ALS and that its regulation in MNs could have neuroprotective effects, our group regulated its levels in such mSOD1 MNs with its inhibitor (anti-miR-124) and demonstrated that it had beneficial effects in preventing neurodegeneration and the dysregulation of the mechanisms involved in mSOD1 MN dysfunction including mitochondrial dynamics, axonal transport, and synaptic signaling (Vaz et al., 2021b). The same publication reveals that the secretome from anti-miR-124-modulated mSOD1 MNs prevented microglia activation and SC pathogenicity relatively to the secretome from the non-treated mSOD1 MNs in OCs derived from the SC of 12-weeks old mSOD1 mice.

Although the downregulation of miR-124 in ALS MNs showed to be neuroprotective and to regulate neuroinflammation in *in vitro* and *ex vivo* experimental ALS models (Vaz et al., 2021b), therapeutic benefits of the secretome from pathological MNs after miR-124 modulation was never explored in ALS *in vivo* models, such as mSOD1 mice. Previous evidence from our group also showed that the modulation of miR-124 in cells was recapitulated in their sEVs and secretome (Garcia et al., 2021), pointing to the secretome as a driver of miR-124 regulation in recipient cells (Garcia et al., 2022). Based on such findings, the present study aimed to address whether this secretome from anti-miR-124-treated mSOD1 MNs no longer has cytotoxicity or even its potential therapeutic benefits against mSOD1 mouse pathogenicity when injected intrathecally at the early symptomatic stage (12-week-old mice), though recent evidence sustains that cell secretome from different cell types may have therapeutic effects (Kim et al., 2022; Sandona et al., 2021).

It is important to note that, considering its advantages for ALS therapeutics, it was decided to use the whole secretome and not only the injection of sEVs because: (i) the secretome storage and administration are easier than that of sEVs and facilitate their preservation; (ii) the secretome also contains additional bioactive molecules that may enhance its neuroprotective potential, as a consequence of the effects of anti-miR-124 on MN survival (Vaz et al., 2021b). Concerning ALS patients, the present

research may also open new opportunities for manipulating and engineering their own cells toward the collection of the secretome for autologous transplantation.

In the following sections, to further investigate the potential restorative effects of the secretome from pathological MNs modulated with the miR-124 inhibitor (preconditioned secretome) after its injection into the SC of the mSOD1 mice, we assessed the inflamma-miRNA profile, astrocyte/microglia phenotypes, and neuronal dynamics by Fluoro-Jade B staining, RT-qPCR, WB, and immunohistochemistry assays. Thus, in the present study, we tested whether the injection of the preconditioned secretome could halt or delay disease progression in the mSOD1 mouse model.

1. *Expression of miR-124 in the SC of mSOD1 Mice Is Downregulated after 3 Weeks of the Intrathecal Injection of Secretome derived from Anti-miR-124-Modulated mSOD1 MNs*

Previous data from our group have found that mSOD1 MNs and their derived secretome, including sEVs, are enriched in miR-124 (Pinto et al., 2017; Vaz et al., 2021b), as well as in the SC of 12-14-week-old mSOD1 mice (Cunha et al., 2018). Beneficial effects of the transfection with the miR-124 inhibitor in mSOD1 MNs, including in cell viability, synaptic dynamics, and anterograde axonal transport, were also demonstrated. Furthermore, their secretome prevented microglia activation and inflammatory-miRNA instability and counteracted mSOD1 pathogenicity in SCOCs from early symptomatic ALS mice (12-week-old) (Vaz et al., 2021b). These findings suggest that the regulation of miR-124 could be a promising therapy to be applied in ALS patients revealing upregulated levels of miR-124, contributing to precision medicine.

Here, we intended to assess whether ALS MNs can be transfected with miR-124 inhibitor and their secretome used to rescue neurodegeneration. For that, we used the model of anti-miR-124-treated mSOD1 MNs previously established in our laboratory and evaluated molecular alterations induced in 12-week-old mSOD1 animals after secretome injection in the lumbar SC. The first aim was to determine whether the intrathecal injection of the secretome derived from anti-miR-124-treated mSOD1 MNs in presymptomatic mSOD1 mice decreased the expression of miR-124 in the lumbar SC of ALS mice when assessed at 15-week-old (symptomatic stage). This will be crucial to infer the potential benefits that our approach may have.

The expression of miR-124 was found upregulated in the mSOD1 mice treated with the vehicle (differentiated NSC-34 cell media with 1% of FBS without sEVs) when compared with the matched WT mice (**Figure IV.1.A**, $p < 0.01$), thus proving what our group had already determined in other ALS experimental models. We can clearly see that the injection of the secretome from anti-miR-124-modulated MNs, under the same conditions, was efficacious in downregulating the expression of miR-124 (**Fig. IV.1.B**, $p < 0.05$), validating the basis for the subsequent studies.

As such, in the following sections, we evaluated the efficacy of this novel strategy in avoiding the progression of the disease in the ALS mice from the early symptomatic stage to the symptomatic stage by preventing neurodegeneration, demyelination, neuroinflammation, and inflammatory instability.

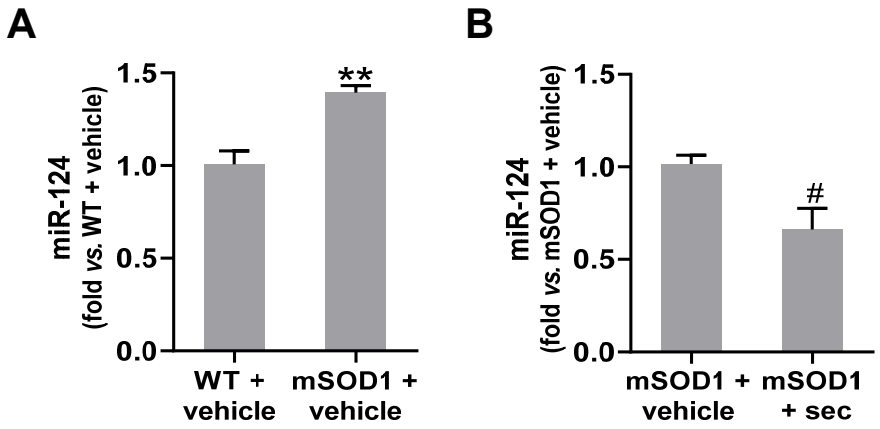

Figure IV.1 — Intrathecal injection of the secretome from anti-miR-124-treated mSOD1 motor neurons (MNs) in the lumbar spinal cord (SC) of mSOD1 mice at 12-week-old abolishes the upregulation of miR-124 after 3 weeks of its administration. miR-124 expression in the lumbar SC of **(A)** $SOD1^{G93A}$ (mSOD1) mice injected at 12-week-old with the vehicle (basal media of NSC-34 MNs) comparatively with the respective wild type (WT) animals, and **(B)** mSOD1 mice injected with the secretome from anti-miR-124-modulated mSOD1 MNs (mSOD1 + sec) relatively to mSOD1 mice injected only with the vehicle (mSOD1 + vehicle). The results were obtained at 15-week-old and were normalized to SNORD110. Data are expressed as fold change *vs.* **(A)** WT + vehicle and **(B)** mSOD1 + vehicle (mean ± SEM) from at least 5 animals per group. ** $p < 0.01$ *vs.* WT + vehicle; $^{\#}$ $p < 0.05$ *vs.* mSOD1 + vehicle, unpaired and parametric *t*-test (with Welch's correction when needed).

2. *Neurodegeneration is Averted in the Lumbar SC of ALS mice After the Administration of Secretome Derived from Anti-miR-124-Modulated mSOD1 MNs*

Previous studies from our group noticed the existence of neurodegeneration in the SC of mSOD1 mice (Cunha et al., 2018) and showed that miR-124 downregulation restored the mSOD1 MN viability toward basal levels by preventing early apoptosis (Vaz et al., 2021b). Based on the findings presented above, we wanted to investigate the preconditioned secretome's capability in preventing neurodegeneration when injected into mSOD1 mice at 12 weeks of age. For that, the lumbar SC was collected 3 weeks after the intrathecal injection, and the obtained transversal sections were stained with Fluoro-Jade B.

We noticed a higher fluorescence intensity in the SC from 15-week-old mSOD1 mice compared to the matched WT animals ($p < 0.01$, **Figure IV.2.B, C**), which goes in line with an augmented number of degenerating neurons. Conversely, in the samples obtained from the mSOD1 mice treated at 12-week-old with the secretome from anti-miR-124-modulated mSOD1 MNs, we found a decrease of Fluoro-Jade B-positive staining when compared to the mSOD1 mice injected with the vehicle ($p < 0.01$, **Figure IV.2.D, E**), supporting secretome preventive influence against neurodegeneration in the mSOD1 animals.

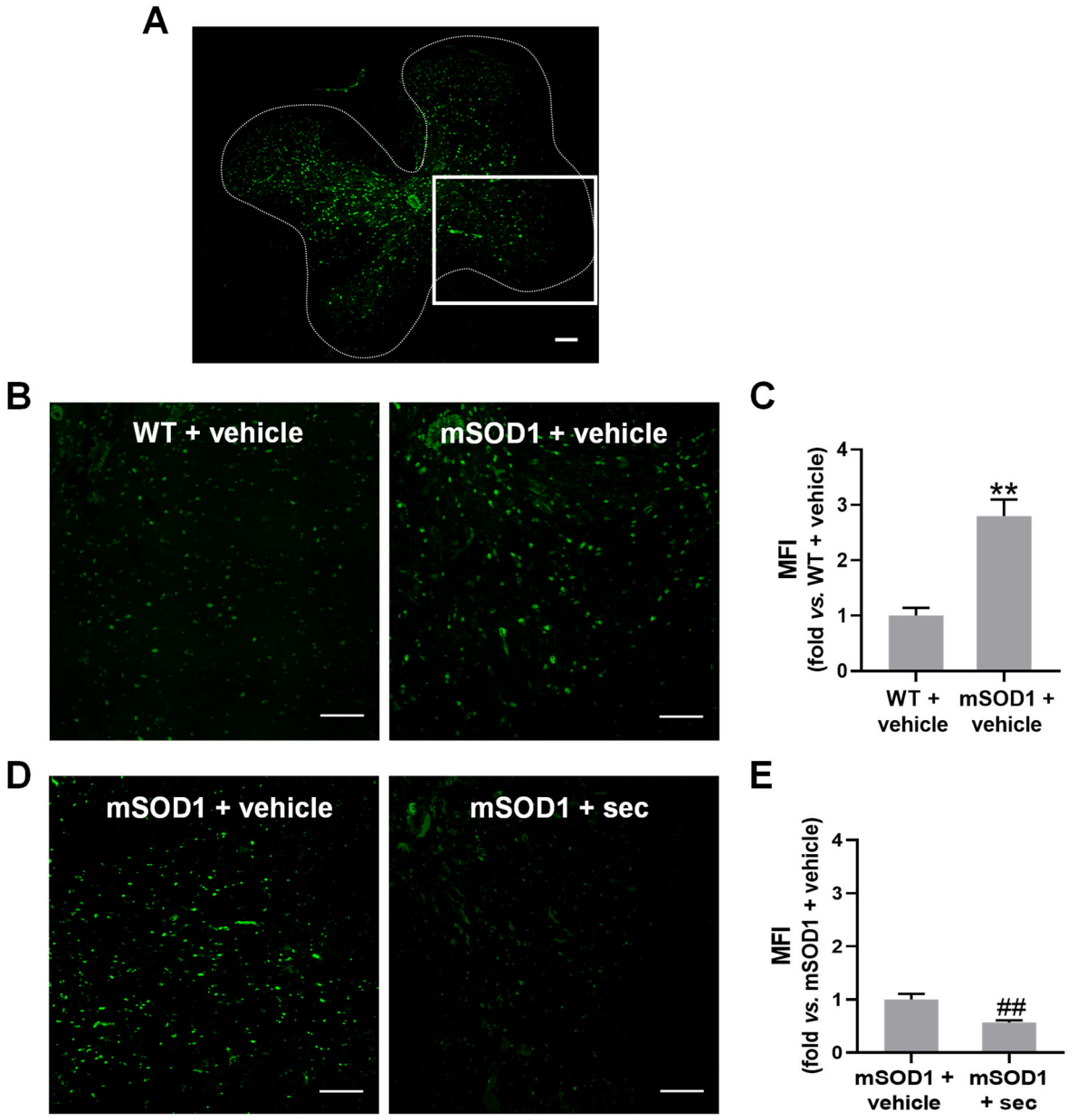

Figure IV.2 — The secretome derived from mSOD1 motor neurons (MNs) transfected with anti-miR-124 averts neurodegeneration in the spinal cord (SC) of ALS mice after 3 weeks of intrathecal injection. **(A)** Representative image of the gray matter (GM) section from transversal SC slice of mSOD1 mice stained with Fluoro-Jade B. The section outlined by the thin white line corresponds to the quantified mean fluorescence. The white square represents the ventral horn portion of the lumbar SC GM represented in the figures **(B, D).** Representative images of the ventral horn of the lumbar section GM stained by Fluoro-Jade B fluorescence from 15-week-old **(B)** wild type (WT)/SOD1^{G93A} (mSOD1) mice injected with the vehicle (basal media of NSC-34 MNs) and **(D)** mSOD1 mice injected with the vehicle in comparison with the mSOD1 mice injected with the secretome derived from anti-miR-124-modulated mSOD1 MNs (mSOD1 + sec) with the respective quantifications of mean fluorescence intensity **(C, E)**. Scale bars: **(A)** 300 µm and **(B, D)** 100 µm. Data from 3 animals per group are expressed as fold change (mean ± SEM) *vs.* WT + vehicle **(C)**, or *vs.* mSOD1 + vehicle **(E)**. ** $p < 0.01$ *vs.* WT + vehicle; $^{\#\#}$ $p < 0.01$ *vs.* mSOD1 + vehicle, unpaired and parametric *t*-test. **MFI**, mean fluorescence intensity.

3. *MN loss and Dysfunction Are Prevented in the Lumbar SC of ALS mice Injected with the Secretome Derived from Anti-miR-124-Transfected mSOD1 MNs*

Once we observed a neuroprotective ability of the secretome from anti-miR-124-treated mSOD1 MNs, our next aim was to investigate markers associated with MN functionality.

As depicted in **Figure IV.3.A**, we first noted the reduced gene expression of hexaribonucleotide binding protein 3 (*NeuN*), discs large MAGUK scaffold protein 4 [*Dlg4*; which encodes for PSD-95], *Dyn*, *Kif5b* (encodes for kinesin), and *Cx3cl1* (at least $p < 0.05$ *vs.* WT + vehicle) in the lumbar SC of the mSOD1 mice at 15 weeks of age. These data pointed to an impairment in neuronal function, postsynaptic signaling, retrograde/anterograde axonal transport, and the CX3CL1/CX3CR1 axis, which may compromise paracrine signaling between neurons and microglia (CX3CR1, microglial receptor). Neuronal demise was further corroborated by the reduction in the protein NeuN ($p < 0.01$ *vs.* WT + vehicle, **Figure IV.3.B**). To note that despite the dysregulation of *Dlg4*, we did not identify any significant changes in the gene expression of presynaptic synaptophysin in the mSOD1 mice SC, neither with nor without treatment with the secretome **(Figure IV.3.A, C)**.

Notably, 3 weeks after the intrathecal injection of the preconditioning secretome from anti-miR-124-treated mSOD1 MNs in the ALS animals, we noticed some protective effects. Actually, and although not statistically significant, we observed a regularization of the gene expression of *Dyn*, *Kif5b*, and *Cx3cl1*, simultaneously with a substantial upregulation of *Dlg4* and NeuN (including the protein expression) ($p < 0.05$ *vs.* mSOD1 + vehicle, **Figure IV.3.C, D**).

These results sustain the efficacy of the preconditioned secretome in halting disease progression in the early symptomatic ALS mice.

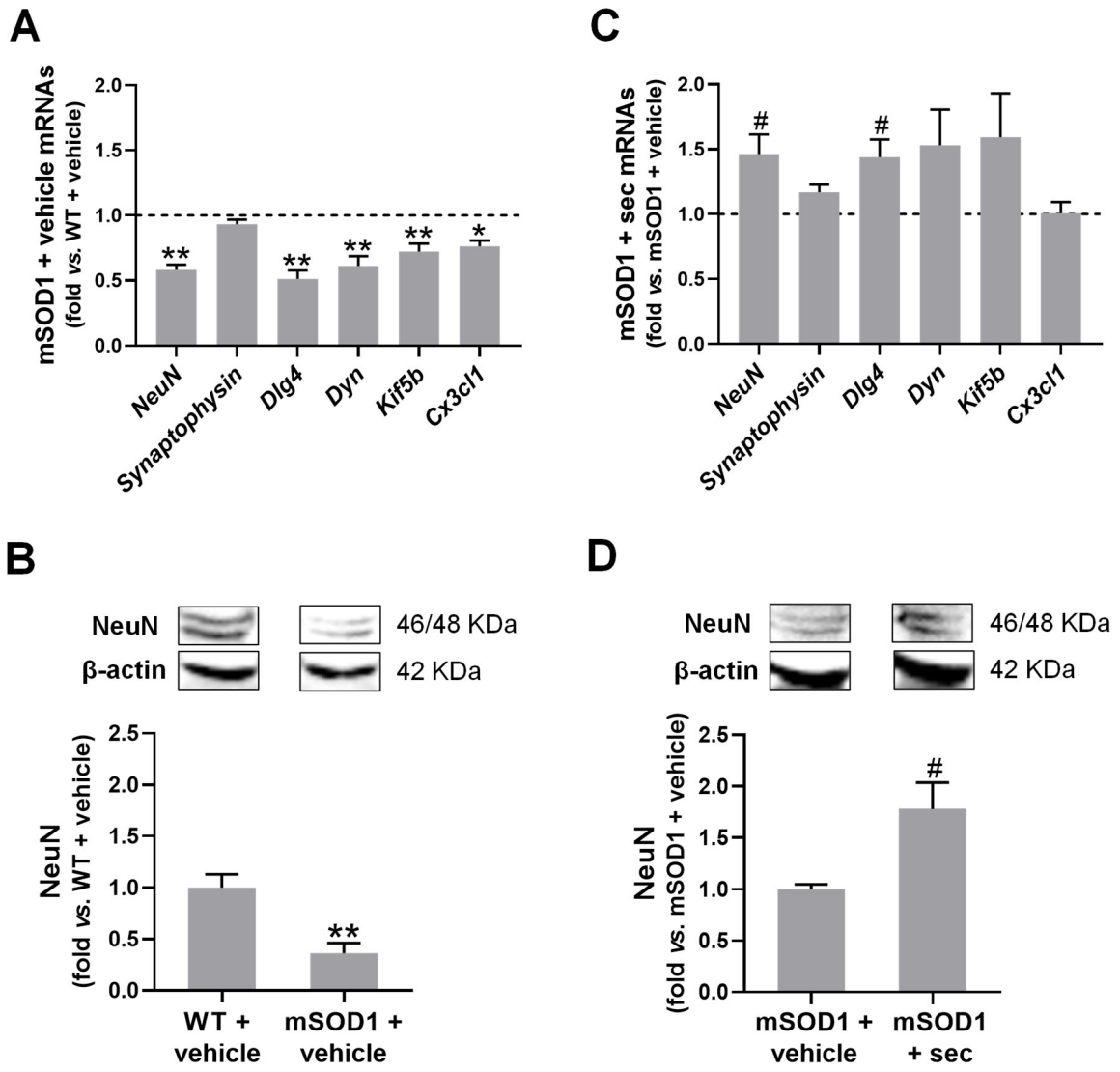

Figure IV.3 — Intrathecal injection of anti-miR-124-treated ALS motor neuron (MN)-derived secretome in 12-week-old mSOD1 mice prevents neuronal demise and dysregulation of synaptic signaling, axonal transport, and CX3CL1-CX3CR1 axis observed at the symptomatic stage. (A) Gene expression of neuronal-associated *NeuN*, S*ynaptophysin*, *Dlg4*, *Dyn*, *Kif5b*, and *Cx3cl1* and **(B)** protein expression of NeuN in the SC of 15-week-old $SOD1^{G93A}$ (mSOD1) mice treated with the vehicle (basal media of NSC-34 MNs) at 12-week-old, relatively to the respective wild type (WT) values (dashed line). **(C, D)** Data from matched experiments performed in the mSOD1 mice treated with the secretome (mSOD1 + sec) in comparison with those injected with the vehicle (dashed line). **(B, D)** Representative bands from one blot are shown. The results were normalized to *Rpl19* for RT-qPCR and β-actin for Western blot. Data are expressed as fold change *vs.* **(A, B)** WT + vehicle and **(C, D)** mSOD1 + vehicle (mean ± SEM) from at least 5 animals per group. ** $p < 0.01$ and * $p < 0.05$ *vs.* WT + vehicle; $^{\#}$ $p < 0.05$ *vs.* mSOD1 + vehicle, unpaired and parametric *t*-test (with Welch's correction when needed). ***Cx3cl1***, c-x3-c motif chemokine ligand 1/fractalkine; ***Dlg4***, discs large MAGUK scaffold protein 4; ***Dyn,*** dynein; ***Kif5b***, kinesin family member 5B (encodes for kinesin); ***NeuN***, hexaribonucleotide binding protein 3; ***Rpl19***, 60S ribosomal L19; **RT-qPCR**, reverse transcription-quantitative polymerase chain reaction.

4. *Myelination Deficits Are Prevented by Intrathecal Injection of the Secretome from Anti-miR-124-mSOD1 MNs in the Lumbar SC of ALS Mice*

Neuronal survival and axonal maintenance depend on myelin integrity since it allows the fast and efficient processing of complex information and provides trophic and metabolic support to the axons it ensheaths (de Faria et al., 2019).

Here, we found downregulated mRNA levels of *Mbp* and *Plp* in the lumbar SC of symptomatic mSOD1 mice injected with the vehicle (at least $p < 0.05$ *vs.* WT + vehicle, **Figure IV.4.A**). P2Y-like G protein-coupled receptor 17 (*Gpr17*), a pivotal regulator of oligodendrocyte precursor cells (OPCs) maturation, was already found abnormally upregulated in the SOD1^{G93A} mice when compared to age-matched WT animals (Bonfanti et al., 2020). We corroborated these findings by observing a 2.5-fold increase in its gene expression in the mSOD1 animals injected with the vehicle ($p < 0.01$ *vs.* WT + vehicle, **Figure IV.4.A**).

Notoriously, 3 weeks after the intrathecal injection of the preconditioned secretome, we detected improvements in the myelination capacity by the increase of *Mbp* and *Plp* mRNA levels (at least $p < 0.05$ *vs.* mSOD1 + vehicle, **Figure IV.4.B**), emphasizing the benefits of this secretome-based approach in preventing demyelination. No significant changes were observed for *Gpr17* levels (**Figure IV.4.B**).

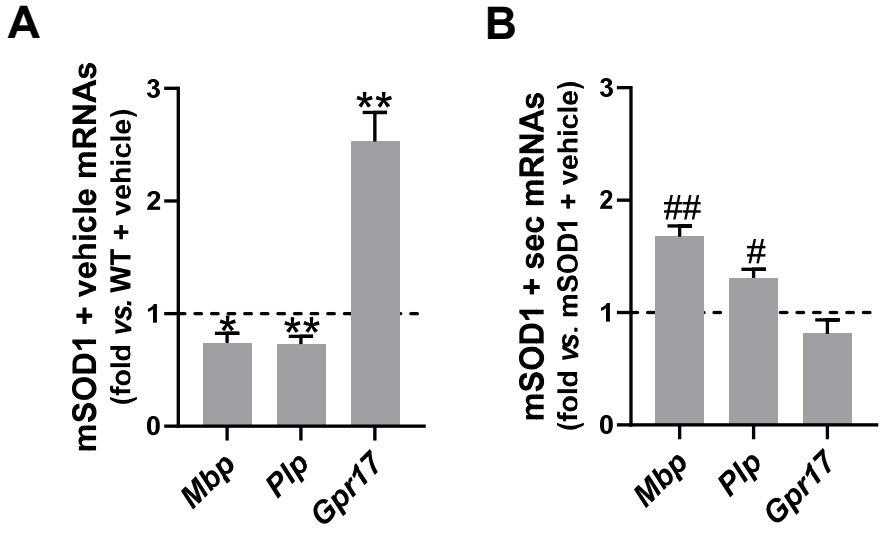

Figure IV.4 — Deficits in myelination in the lumbar spinal cord (SC) of 15-week-old mSOD1 mice are prevented by the intrathecal injection of the secretome derived from modulated mSOD1 motor neurons (MNs) in 12-week-old animals. (A) Gene expression of myelin-related genes (*Mbp*, *Plp* and *Gpr17*) in the SC of 15-week-old SOD1^{G93A} (mSOD1) mice treated with the vehicle (basal media of NSC-34 MNs) at 12-week-old in comparison with the respective wild type (WT) ones (dashed line). **(B)** Data from matched experiments realized in the mSOD1 mice injected with the secretome derived from anti-miR-124-treated mSOD1 MNs (mSOD1 + sec) in comparison with those treated with the vehicle (dashed line). **(A, B)** The results were normalized to *Rpl19* for RT-qPCR. Data are expressed as fold change *vs.* **(A)** WT + vehicle and **(B)** mSOD1 + vehicle (mean ± SEM) from at least 5 animals per group. ** $p < 0.01$ and * $p < 0.05$ *vs.* WT + vehicle; $^{\#\#}$ $p < 0.01$ and $^{\#}$ $p < 0.05$ *vs.* mSOD1 + vehicle, unpaired and parametric *t*-test (with Welch's correction when needed). ***Gpr17***, G protein-coupled receptor 17; ***Mbp***, myelin basic protein; ***Plp***, proteolipid protein; ***Rpl19***, 60S ribosomal L19; **RT-qPCR**, reverse transcription-quantitative polymerase chain reaction.

5. *Intrathecal Injection of the Secretome from Anti-miR-124-Transfected mSOD1 MNs in 12-Week-Old mSOD1 Mice Counteracts Glial Reactivity in the Lumbar SC at the 15-Week-Old Symptomatic Stage*

Microglia and astrocytes play crucial roles in neuroinflammatory responses in several neurodegenerative diseases, including ALS. The dysregulation of these glial cells is severely correlated with the initiation and propagation of neurodegeneration (Garland et al., 2022; Kwon et al., 2020).

Based on the promising results obtained for neuronal function with the intrathecal administration of the preconditioned secretome in 12-week-old mSOD1 mice, we questioned whether such beneficial effects could be reinforced by the prevention of the homeostatic instability of microglia and astrocyte activation.

One of the microglia's functions known to be impaired in ALS is their phagocytic ability. As such, we started by determining the gene expression of some phagocytosis-associated markers. We identified a reduction in the levels of *Trem2* ($p < 0.001$ *vs*. WT + vehicle, **Figure IV.5.A**) and the milk fat globule-epidermal growth factor 8 (*Mfg-e8*) ($p < 0.05$ *vs*. WT + vehicle, **Figure IV.5.A**), suggesting an impairment in microglial phagocytic ability (Fuller et al., 2008; Xie et al., 2022). We also evaluated gene expression regarding the categorization of microglia immunoreactivity. We found decreased levels of *Arg1* in the lumbar SC of symptomatic mSOD1 mice 3 weeks after vehicle injection in the early symptomatic mice ($p < 0.05$ *vs*. WT + vehicle, **Figure IV.5.A**), which are known to exacerbate neuroinflammation by activating microglia and impairing phagocytosis (Ma et al., 2020). Additionally, we observed a downregulation of purinergic receptor P2Y12 (*P2ry12*) ($p < 0.01$ *vs*. WT + vehicle, **Figure IV.5.A**), which may suppress microglial motility and migration toward local sites of CNS injury (Lou et al., 2016). The regulation of microglial activation may have been determined by the upregulated levels of tissue inhibitor of metalloproteinases 2 (*Timp2*), the specific microglia marker *Tmem119,* and of *Cx3cr1* (at least $p < 0.05$ *vs*. WT + vehicle, **Figure IV.5.A**), that are shown to be crucial regulators of neuroinflammation (Garland et al., 2022; González-Prieto et al., 2021; Hunter et al., 2021; Lee et al., 2014; Zhang et al., 2018).

In 3 weeks after the intrathecal injection of the modulated secretome, we detected an inverted signature in some SC parameters, including those related to microglial phagocytosis (*Trem2*, $p < 0.05$ *vs.* mSOD1 + vehicle, **Figure IV.5.C**) and *Arg1* ($p < 0.05$ *vs.* mSOD1 + vehicle, **Figure IV.5.C**), which are associated to a more reparative phenotype. Furthermore, although not statistically significant, we also detected a regularization in the expression of *Mfg-e8* and *P2ry12* (**Figure IV.5.C**).

Concerning the reactive profile of astrocytes, we observed augmented levels of *Gfap* ($p < 0.05$ *vs.* WT + vehicle, **Figure IV.5.B**) and *Cx43* ($p < 0.001$ *vs.* WT + vehicle, **Figure IV.5.B**) in the lumbar SC of mSOD1 mice, suggesting the presence of reactive astrocytes. Remarkably, the preconditioned secretome led to a substantial decrease of such parameters (*Gfap*, $p < 0.01$ and *Cx43*, $p < 0.05$ *vs.* mSOD1 + vehicle, **Figure IV.5.D**). To additionally note the non-existence of variations in the gene expression of *S100b* both with and without the administration of the preconditioned secretome (**Figure IV.5.B, D)**.

Furthermore, we also evaluated the microglial and astrocytic prevalence in the lumbar SC slices by assessing the area stained for Iba-1 and GFAP, respectively. Data revealed an elevation in the area occupied by GFAP- and Iba-1-positive cells in the mSOD1 SC ($p < 0.01$ *vs.* WT + vehicle, **Figure IV.6.B, C**) compared to the matched WT samples. The expression of proteins as assessed by WB analysis also indicated an increase for GFAP and S100B (though not significant) and for vimentin and Iba-1 in mSOD1 SC, in comparison with the WT samples ($p < 0.05$ *vs.* WT + vehicle, **Figure IV.6.F,**

G), supporting astrocyte reactivity and microglial activation. Importantly, the preconditioned secretome not only decreased the area occupied by GFAP- and Iba-1-positive cells (at least $p < 0.001$ *vs.* mSOD1 + vehicle, **Figure IV.6.D, E**) but also the protein levels of GFAP, S100B and Iba-1 ($p < 0.05$ *vs.* mSOD1 + vehicle, **Figure IV.6.H, I**) in comparison with the SC from mSOD1 mice injected with the vehicle, suggesting a calming effect on microglia activation and astrocyte reactivity. Taken together, these findings support the immune remodeling capacity of the modulated secretome.

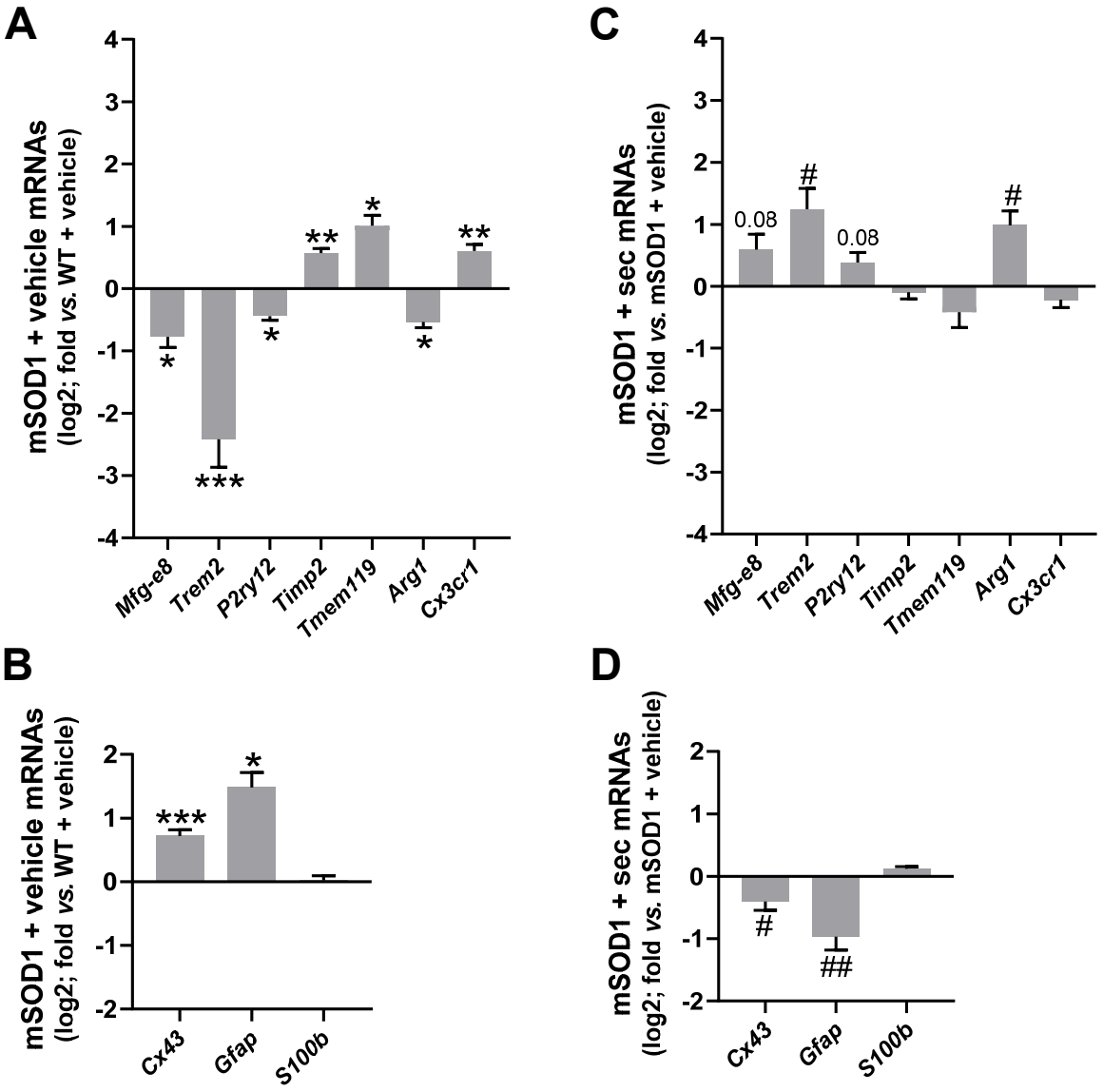

Figure IV.5 — Microglia activation and astrocyte reactivity in the lumbar spinal cord (SC) of mSOD1 mice at the symptomatic stage are averted by intrathecal administration of the secretome from anti-miR-124-modulated mSOD1 motor neurons (MNs) in the 12-week-old animals. Gene expression of **(A)** microglia-related markers (*Mfg-e8*, *Trem2*, *P2ry12*, *Timp2*, *Tmem119*, *Arg1*, *Cx3cr1*) and **(B)** astrocyte-associated markers (*Cx43*, *Gfap*, *S100b*) in the SC of $SOD1^{G93A}$ (mSOD1) mice injected with the vehicle (basal media of NSC-34 MNs) in comparison with those in wild type (WT) animals and **(C, D)** after the injection of the preconditioned secretome (mSOD1 + sec) in comparison with mSOD1 mice treated with the vehicle (mSOD1 + vehicle). The results were normalized to *Rpl19*. Data are expressed as fold change *vs.* **(A, B)** WT + vehicle and **(C, D)** mSOD1 + vehicle (mean ± SEM) from at least 5 animals per group. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ *vs.* WT + vehicle; $^{\#\#}$ $p < 0.01$ and $^{\#}$ $p < 0.05$ *vs.* mSOD1 + vehicle, unpaired and parametric *t*-test (with Welch's correction when needed). ***Arg1***, arginase 1; ***Cx3cr1***, c-x3-c chemokine receptor 1; ***Cx43***, connexin 43; ***Gfap***, glial fibrillary acidic protein; ***Mfg-e8***, milk fat globule-epidermal growth factor 8; ***P2ry12***, purinergic receptor p2y12; ***Rpl19***, 60S ribosomal L19; ***S100b***, S100 calcium-binding protein B; ***Timp2***, tissue inhibitor of metalloproteinases 2; ***Tmem119***, transmembrane protein 119; ***Trem2***, triggering receptor expressed on myeloid cells 2.

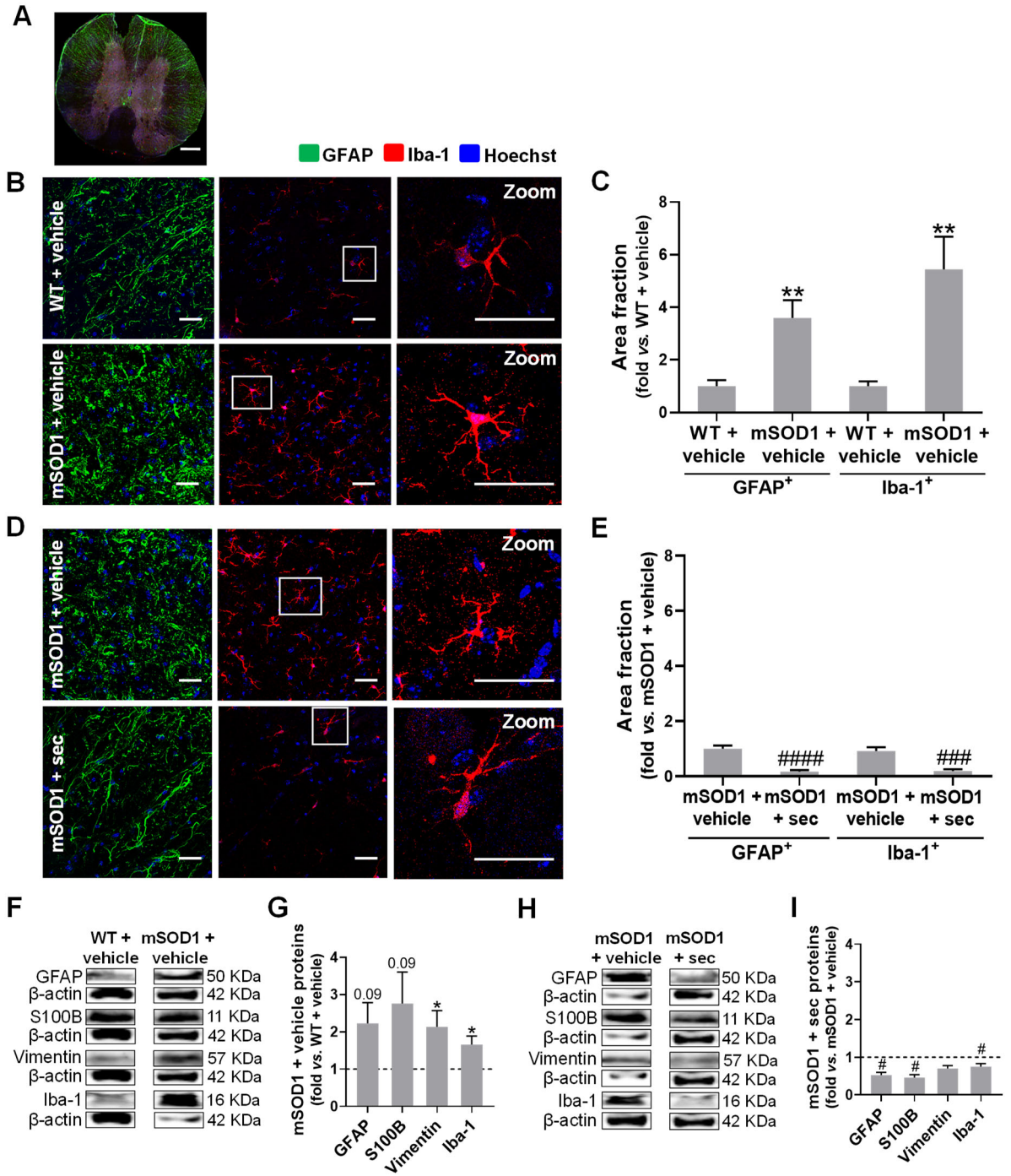

Figure IV.6 — Dysregulated glia-driven immunoreactivity processes in the spinal cord (SC) of symptomatic mSOD1 mice are counteracted 3 weeks after intrathecal administration of the secretome from anti-miR-124-treated mSOD1 motor neurons (MNs). **(A)** Representative image of a transversal lumbar SC slice stained with Iba-1 (microglia-related marker) and GFAP (astrocyte-associated marker). **(B, D)** Representative images for GFAP (green)- and Iba-1- (red) positive cells from the lumbar SC of wild type (WT) and SOD1^{G93A} (mSOD1) mice injected with the vehicle, and mSOD1 mice injected with the secretome from anti-miR-124-modulated mSOD1 MNs (mSOD1 + sec). Iba-1-positive zoomed cells are shown. Nuclei were stained with Hoechst (blue). **(C, E)** Area fraction occupied by GFAP- and Iba-1-positive cells, respectively. **(F, H)** Representative bands obtained by Western blot (WB) are shown. **(G, I)** Data obtained from WB analysis of reactive astrocytic markers (GFAP, S100B, and vimentin) and microglial marker Iba-1. The results were normalized to β-actin for WB. Scale bars: **(A)** 300 µm and **(B, D)** 30 µm. Data are expressed as fold change *vs.* **(C, G)** WT + vehicle (dashed line) and **(E, I)** mSOD1 + vehicle (dashed line) (mean ± SEM) from images of 5 ventral horn fields per animal (from 3 animals per group) for immunohistochemistry and 5 animals per group for WB analysis. ** $p < 0.01$ and * $p < 0.05$ *vs.* WT + vehicle; $^{\#\#\#\#}p < 0.0001$, $^{\#\#\#}p < 0.001$, and $^{\#}p < 0.05$ *vs.* mSOD1 + vehicle, unpaired and parametric *t*-test (with Welch's correction when required). **GFAP**, glial fibrillary acidic protein; **Iba-1**, ionized calcium-binding adaptor molecule 1; **S100B**, S100 calcium-binding protein B.

6. *The Secretome from Anti-miR-124-Modulated mSOD1 MNs Counteracts the Dysregulated Inflammatory Status Observed in the Lumbar SC of mSOD1 Mice at 3 Weeks After Administration*

Several miRNAs have been found dysregulated in ALS patients (Ricci et al., 2018) and in cortical brain and SC from mSOD1 mice (Cunha et al., 2018), namely miR-146a, miR-155, miR-21 and miR-125b. Thus, and based on the obtained results, we wondered whether the preconditioned secretome would be able to control such miRNAs instability considering the normalized levels of miR-124 obtained by its inhibitor in the SC of mSOD1 mice treated with the engineered secretome **(Fig. IV.1)**. Initially, we wanted to confirm that such miRNAs were upregulated in the mSOD1 mice injected with the vehicle at 12 weeks of age and analyzed 3 weeks thereafter, the design we used to test the potential therapeutic benefits of the intrathecal injection of the modulated secretome derived from the anti-miR-124-transfected mSOD1 MNs.

All the analyzed miRNAs, except miR-125b, were upregulated in the lumbar SC tissue isolated from the symptomatic mSOD1 mice at 15-week-old ($p < 0.05$ *vs.* WT + vehicle, **Figure IV.7.A**), which is in line with what we were expecting. Noteworthy, the preconditioned secretome counteracted the upregulation of 2 main inflamma-miRNAs, this is, miR-146a ($p < 0.05$ *vs.* mSOD1 + vehicle, **Fig. IV.7.B**) and miR-155 ($p < 0.01$ *vs.* mSOD1 + vehicle, **Figure IV.7.B**), in comparison with the mSOD1 mice injected with the vehicle. These observations confirm the regulatory potential of this preconditioned secretome in preventing inflamma-miRNA instability.

When we assessed the inflammation-associated genes, we noticed an imbalance with downregulation of *Inos* and *Il-10*, together with increased levels of *Tnf-α* (at least $p < 0.01$ *vs.* WT + vehicle, **Figure IV.7.C**), while no significant differences were observed for *Il-1β*. The modulated secretome caused an elevation of *Il-10* and *Inos* ($p < 0.05$ *vs.* mSOD1 + vehicle, **Figure IV.7.D**), which may compensate each other towards a steady state. Once again, these data highlight the preconditioned secretome's efficiency in regulating the immunoreactivity observed in the lumbar SC of mSOD1 mice.

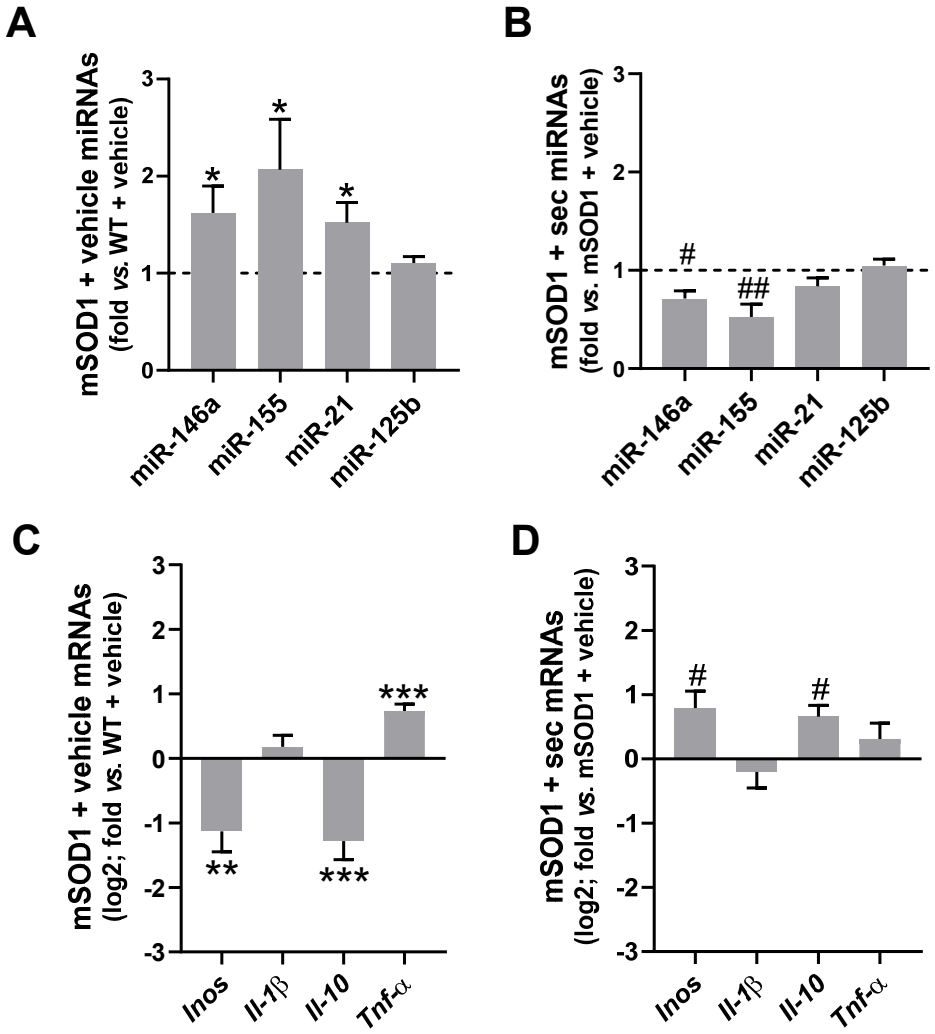

Figure IV.7 — The secretome derived from the mSOD1 motor neurons (MNs) engineered with anti-miR-124 prevents the upregulation of inflammatory-associated miRNAs and the dysregulated inflammatory status observed in the lumbar spinal cord (SC) of symptomatic ALS mice. (A) Expression of inflammatory-associated miRNAs (miR-146, miR-155, miR-21, and miR-125b) in the SC of $SOD1^{G93A}$ (mSOD1) mice injected with the vehicle (basal media of NSC-34 MNs) when compared with those in wild type (WT) animals (dashed line), and **(B)** of mSOD1 mice injected with the secretome derived from anti-miR-124-treated mSOD1 MNs (mSOD1 + sec) in comparison with mSOD1 animals treated with the vehicle (dashed line). The results were normalized to SNORD110. Gene expression of **(C)** inflammatory-associated markers (*Inos*, *Il-1β*, *Il-10*, and *Tnf-α*) in the SC of mSOD1 mice injected with the vehicle in comparison with those in WT animals and **(D)** after the administration of the preconditioned secretome (mSOD1 + sec) when compared with mSOD1 mice treated with the vehicle. The results were normalized to *Rpl19*. Data is expressed as fold change *vs.* **(A, C)** WT + vehicle and **(B, D)** mSOD1 + vehicle (mean ± SEM) from at least 5 animals per group. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ *vs.* WT + vehicle; $^{\#\#}$ $p < 0.01$ and $^{\#}$ $p < 0.05$ *vs.* mSOD1 + vehicle, unpaired and parametric *t*-test (with Welch's correction when needed). ***Il-10***, interleukin 10; ***Il-1β***, interleukin 1β; ***Inos***, inducible nitric oxide synthase; ***Rpl19***, 60S ribosomal L19; ***Tnf-α***, tumor necrosis factor-alpha.

Collectively, our data highlight the therapeutic interest of intrathecal injection of the secretome derived from mSOD1 MNs engineered with anti-miR-124 regarding its ability to prevent most of the pathological events observed in the mSOD1 mice, including MN degeneration/demise/dysfunctionality, glial reactivity, impaired phagocytosis, and inflammatory imbalance. In the future, we expect to translate this therapeutic strategy into clinics as an autologous treatment, mainly for ALS patients with upregulated miR-124 levels, to slow down disease progression and prolong their lifetime, thus using an approach based on stratification and precision medicine in ALS.

V
DISCUSSION

ALS is a progressive, fatal neurodegenerative disease with an unknown etiology characterized by the loss of MNs and glial activation and reactivity, which contribute to disease onset and progression. miRNAs have gained a lot of interest in the diagnosis and potential treatment of the disease as their dysfunction has a key role in the occurrence and development of neurodegenerative diseases (Wang et al., 2020). As such, several studies with inflamma-miRNA expression and their modulation on different cells from the CNS have recently emerged. As such, the main interest of this project was to validate if the benefits of regulating miR-124 levels in mSOD1 MNs using a miR-124 inhibitor were translated to mSOD1 mice concerning the prevention of disease progression. Previous data from our group demonstrated that upregulated levels of miR-124 in mSOD1 MNs were linked to neurodegeneration (Pinto et al., 2017) and that its secretome promoted glial activation, findings that were prevented when the mSOD1 MNs were transfected with anti-miR-124 (Vaz et al., 2021b).

miR-124 is the most abundant miRNA in the adult brain. It is mainly expressed in neuronal cells and participates in diverse biological processes, including neuronal development and differentiation, synaptic plasticity, neurite outgrowth regulation, and even neuronal identity acquisition and preservation (Han et al., 2019; Sun et al., 2015). Although the overexpression of miR-124 in Alzheimer's disease (AD) has shown benefits in some experimental models and patients (An et al., 2017; Garcia et al., 2021), in ALS, upregulation of miR-124 in mSOD1 MNs was demonstrated to compromise neurite network, synaptic signaling, axonal transport and mitochondrial dynamics (Vaz et al., 2021b). Indeed, miR-124 is one of the most commonly dysregulated miRNAs in ALS (Foggin et al., 2019). Noteworthy, enhanced levels of miR-124 were found in the SC and brainstem of mSOD1 mice and have been associated with neurodegenerative sites (Zhou et al., 2018). Furthermore, the secretome derived from mSOD1 MNs also revealed increased levels of miR-124 and induced pathogenicity in the SCOCs derived from early symptomatic ALS mice (12-week-old) (Pinto et al., 2017; Vaz et al., 2021b). Previous studies from our group in mSOD1 MNs evidenced that when the miR-124 was restored to physiological levels by transfecting mSOD1 MNs with anti-miR-124, the secretome was able to recapitulate the levels of miR-124 in neuronal cells, and became neuroprotective in this disease model (Vaz et al., 2021b). These observations suggest that the differential beneficial/detrimental roles of miR-124 upregulation in AD and ALS, respectively, might depend on the neuronal type (neurons or MNs) and the region of the CNS affected by each specific pathological condition.

Recently, miRNA mimics and inhibitors have been used to modify the endogenous levels of specific miRNAs and regulate cell function (He et al., 2019; Zhong et al., 2017), as in the case of miR-124 (Ohnuma et al., 2019; Zhou et al., 2016). However, no previous approach on the modulation of the

miR-124 in the SOD1^{G93A} mice, the best characterized model, was performed before. With this in mind, and since cell secretome-based therapies have emerged as novel therapeutics to tackle diverse pathologies, including CNS disorders (Pinho et al., 2020), we intended to explore this opportunity in the mSOD1 mouse model. Thus, the main interest in this study was to investigate whether the benefits that our group previously observed in the *in vitro* and *ex vivo* ALS models with the preconditioned secretome derived from mSOD1 MNs treated with the miR-124 inhibitor could be recapitulated in the mSOD1 mouse model. For that, this preconditioned secretome was tested for its ability to halt MN degeneration and death and neuron/glia deregulation in the mSOD1 mice. To achieve this goal, the intrathecal injection of the modulated secretome in the lumbar section of the SC of mSOD1 mice at the early symptomatic stage (12-week-old) was performed. Then, the data were compared with matched mSOD1 and WT controls injected with the vehicle (differentiated NSC-34 cell media with 1% of FBS depleted in sEVs) when animals achieved 15-week-old. These experimental controls allowed us to evaluate the preventive effects of the preconditioned secretome injection on the pathological findings of mSOD1 mice at the symptomatic stage and to compare them with the physiological molecular status of the WT mice. By only injecting the vehicle, we were sure not to alter the course of the disease in the TG mice and to not cause any alteration in the WT animals besides the eventual effect caused by the injection (Barbosa et al., 2022).

Molecular assessments were performed in the lumbar SC collected after the sacrifice of 15-week-old mice, which precedes the advanced symptomatic stage of the disease (16-week-old) (Rubio et al., 2022).

As previously mentioned, miR-124 was upregulated in the SC of symptomatic mSOD1 mice (Cunha et al., 2018) and in the mSOD1 MNs (Pinto et al., 2017; Vaz et al., 2021b), an observation that was also confirmed in the present study. Therefore, the next step was to validate that the preconditioned secretome derived from anti-miR-124-transfected mSOD1 MNs was successful in downregulating the expression of miR-124 toward basal levels in the SC. In fact, here, we show that the preconditioned secretome could re-establish the miR-124 levels observed in the 15-week-old WT mice. This finding is in concordance with previous results from our group demonstrating that such secretome regulates miR-124 levels in SC organotypic slices derived from mSOD1 mice (Vaz et al., 2021b). This finding was essential to proceed in the experimental design toward establishing the preconditioned secretome as a novel and promising therapeutic strategy against ALS.

Firstly, we observed a high neurodegeneration and MN demise in the lumbar SC of symptomatic mSOD1 mice, which is in line with what the group had previously published (Cunha et al., 2018). To contribute to these pathological features, a decreased expression of *Dlg4* marked the disruption of postsynaptic signaling, as already described in the SOD1^{G93A} mouse SC tissue (Broadhead et al., 2022), observed in SCOCs obtained from early symptomatic SOD1^{G93A} mice (Vaz et al., 2021b) and found in *post-mortem* samples of ALS patients (Genç et al., 2017). Furthermore, the retrograde and anterograde axonal transport also seemed to be defective in the mSOD1 SC, as we observed a reduced expression of *Dyn* and *Kif5b*, respectively. Actually, such deficits in the axonal transport of various organelles were already shown in some ALS models and ALS patients (De Vos, 2017 #57). A reduction of kinesin and dynein, together with a significant loss of spinal MNs, had also been previously reported in the mSOD1 mouse model (Warita et al., 1999).

Neuron-microglia communication plays a crucial role in the MNs death in ALS. Neurons can express the chemokine ligand CX3CL1, which mediates microglial activation via interacting with its sole receptor CX3CR1 in microglia (Liu et al., 2019). Indeed, the axis between the neuronal fractalkine CX3CL1 and its microglial receptor CX3CR1 is known to stimulate the production of soluble factors linked to neuronal survival and microglial phagocytosis, therefore exerting neuroprotective and

immunoregulatory effects in the mSOD1 mice (Rogers et al., 2011). However, it is also well established that the disruption of the CX3CL1/CX3CR1 axis could result in microglial activation and overexpression of IL-1β and TNF-α (Rogers et al., 2011). Our data showed an increase of *Cx3cr1* and a downregulation in *Cx3cl1*, as already noticed in previous studies by our group (Cunha et al., 2018; Vaz et al., 2021b), suggesting a disrupted neuronal-microglial communication that contributes to the progression of MN degeneration and pathophysiology in ALS (Zhang et al., 2018).

In sum, and although the injection of the preconditioned secretome showed not to be able to significantly prevent such defects in neuron-microglia communication, the secretome from anti-miR-124-modulated ALS MNs prevented neurodegeneration occurring from the 12 weeks to the 15 weeks possibly by sustaining mSOD1 MN viability by inhibiting early apoptosis (Vaz et al., 2021b), as we can observe by the increase of NeuN both at gene and protein level. Moreover, it also favored postsynaptic signaling and showed a slight benefit in axonal transport systems, reinforcing the significance of keeping homeostatic miR-124 levels to support synaptic plasticity, as already stated by (Hou et al., 2015).

The impact of Ol dysfunction and myelin damage has been reported in the SC of ALS mouse models and patients (Raffaele et al., 2021). Accordingly with published data that demonstrate the Ol degeneration in SC of ALS patients and $SOD1^{G93A}$ mice (Philips, 2013 #6)(Raffaele et al., 2021), the decreased expression of *Mbp* that we found in the SC of mSOD1 mice may have been implicated in the dysfunction of newly differentiated Ols, and also suggesting that the myelin formation could be impaired. In addition, the upregulation of *Gpr17*, a key regulator in Ol differentiation, also impacts OPCs maturation. Though necessary for the transition from OPCs to immature Ols, *Gpr17* downregulation is mandatory to allow maturation of OPCs, a process that is compromised in ALS (Bonfanti et al., 2020). Studies have showed that toxic aggregates of mSOD1 induce Ol demyelination (Kang et al., 2013; Kim et al., 2019). In order to compensate, OPCs increase their proliferation rate and differentiate into new mature Ols. From this point of view, upregulation of *Gpr17* will be crucial for the OPC maturation. Nevertheless, an aberrant upregulation of this receptor in the SC of mSOD1 mice at the symptomatic stage inhibit the OPC terminal maturation (Bonfanti et al., 2020). As such, the upregulated *Grp17* levels might restrain Ol maturation, further supported by the reduced gene expression of *Mbp* and *Plp* that we observed.

The injection of the secretome from engineered mSOD1 MNs with miR-124-inhibitor favored myelin production by upregulating *Mbp* and *Plp* levels, suggesting that the restoration of homeostatic miR-124 levels is essential for the prevention of demyelination. Indeed, augmented levels of miR-124 were shown to be correlated with hippocampal demyelination and memory dysfunction, highlighting that its regulation is critical for controlling myelin processes (Dutta et al., 2013).

Microglia and astrocytes play vital roles in the CNS, exerting close communication to influence and coordinate each other and their effects on the brain homeostasis and influencing neuronal function (Garland et al., 2022). Microglia activation and astrocyte reactivity contribute to the propagation of neurodegeneration (Brites et al., 2015; Kwon et al., 2020). Previous studies from our group demonstrated an immune imbalance in the SCOCs derived from the mSOD1 mice (Cunha et al., 2018; Vaz et al., 2021b).

The reduced expression of *Mfg-e8*, *Trem2,* and *P2ry12* that we observed in the SC of mSOD1 mice indicates an impairment in the phagocytic capabilities of microglia. Decreased levels of MFG-E8 had already been identified in this model at both presymptomatic and symptomatic stages of the disease (Cunha et al., 2018). The surface receptor TREM2, which, within the CNS, is exclusively expressed by microglia, plays an essential role in microglial proliferation, migration, activation, metabolism, and phagocytosis. Although its function in ALS pathogenesis is still barely explored, TREM2 is suggested to be important in ALS (Xie et al., 2022). Moreover, the downregulated purinergic receptor P2RY12 is

also associated with the loss of microglia phagocytic abilities, motility (chemotaxis), and cell migration (Butovsky et al., 2015; Moore et al., 2015). In addition, diminished expression of P2RY12 in microglia has been reported in most neuropathologies related to neuroinflammation (Gomez Morillas et al., 2021). Such results could be connected with the increased expression of miR-155 that we found since they showed to interfere with the phagocytic function of microglia. In fact, genetic ablation of miR-155 restores the phagocytic ability of microglial cells (Butovsky et al., 2015). Downregulated expression of *Arg1*, as we identified here in the mSOD1 mice SC, was shown to be linked to the existence of neuropathological mechanisms, microglia activation, and neuroinflammation in an AD mouse model (Ma et al., 2020) and to dysfunctional microglia in the SC of $SOD1^{G93A}$ rats at disease end-stage (Nikodemova et al., 2014). To note that the preconditioned secretome sustained a complete opposite fingerprint of these parameters, indicating that they are characteristic of the mSOD1 mice symptomatic stage and can be prevented. Such effect may be related to the reduction we observed for miR-155 and the upregulation of *Arg1*, supporting the existence of a more functional microglia after the intrathecal administration with the anti-miR-124-modulated secretome. The increased expression of Iba-1 in the mSOD1 mice is also associated with an activated state of microglia and was already found in prior data from our group (Cunha et al., 2018). Once again, the preconditioned secretome effectively prevented such alterations from occurring in the mSOD1 mice with 15-week-old, thus supporting a more functional and reparative microglia phenotype.

The development of the single-cell RNA sequencing (RNAseq) method has allowed the identification of distinct microglial populations based on their genetic expression (Garland et al., 2022). Lately, RNAseq analysis of microglia isolated from the SC of mSOD1 mice revealed the existence of various activated phenotypes, highlighting the heterogeneity of the microglial populations. Single-cell RNAseq analysis of CNS immune cells in ALS revealed a novel subset of microglia with unique transcriptional signatures, known as DAM, characterized by the downregulation of several microglia homeostatic genes, including *P2ry12*, *Cx3cr1,* and *Tmem119,* and by the upregulation of *Trem2* and *Timp2* (Keren-Shaul et al., 2017; Xie et al., 2022). Collectively, the results we obtained for the dysregulation of *P2ry12*, *Cx3cr1*, *Tmem119,* and *Timp2* genes suggest the presence of activated microglia and neuroinflammation (González-Prieto et al., 2021; Hunter et al., 2021; Lee et al., 2014), though we cannot discard that if evaluated for subtypes we could also identify the DAM microglia. However, with so many definitions of microglia phenotypes in several diseases, it became critical to better look for microglia functions instead of subtypes based on the gene signature. Summarily, we detected microglia activation with possible impairments in their phagocytic and migration abilities, which we believe to promote neurodegeneration. Noteworthy, the preconditioned secretome revealed to be effective in preventing such pathological alterations in the microglia if injected at 12-week-old presymptomatic mice, with lasting effects considering that the benefic effects are still notorious in 15-week-old animals.

The results we obtained also support an increased astrocyte reactivity as well as their potential neurotoxic effects in the SC of the mSOD1 mice, based on the augmented levels of GFAP, Cx43, S100B, and vimentin, which are in accordance with other studies performed in our group (Cunha et al., 2018; Gomes et al., 2020). The preconditioned secretome completely avoided the appearance of such pathological astrocytic phenotype, highlighting its immune remodeling ability if we also consider its preventive effects on the *Tnf-α* upregulation and *Il-10* downregulation, known to be inflammatory markers (Cunha et al., 2018; Vaz et al., 2021b).

Regarding the inflammatory-related markers, we observed an imbalance in the mSOD1 mice with a decrease in *Inos*, which is responsible for the production of NO that is often associated with oxidative stress and induced inflammation in microglial cells, and in *Il-10*, an anti-inflammatory cytokine. Previous work from our group already showed that the symptomatic mSOD1 mice exhibit upregulated

levels of miR-155 and miR-146a in the SC, which dysregulation is known to contribute to neuroinflammation (Cunha et al., 2018). Likewise, miR-21 was upregulated in the mSOD1 mice (Parisi et al., 2013). Interestingly, the same pattern was observed here, with all these inflammatory miRNAs showing increased levels in the SC of the symptomatic mSOD1 mice. Once more, we could observe that the secretome, regulated for miR-124, has prevented the dysregulated inflammatory status, clearly highlighting the anti-inflammatory action of its intrathecal administration in the ALS mouse model. In fact, the secretome was able to re-establish the levels of *Inos*, increase *Il-10* and downregulate the inflamma-miRNAs.

CONCLUDING REMARKS

In conclusion, the present thesis validates the therapeutic potential of the secretome derived from anti-miR-124-engineered mSOD1 MNs in preventing pathological progression of neurodegeneration and inflammatory signatures at the symptomatic stage when intrathecally injected in the early symptomatic mSOD1 mice (12-week-old). Impressively, one single intrathecal injection of the preconditioned secretome carrying basal levels of miR-124 was effective in normalizing miR-124 toward physiological levels and showed to have therapeutic benefits at least during 3 weeks after injection. With this promising approach, we sustained anti-inflammatory/phagocytic microglia, as well as prevented MN demise, postsignaling impairment, demyelination, astrocyte reactivity, dysregulated inflammatory status, and neuroimmune imbalance at 3 weeks after injection, this is, at the time of the symptomatic stage where all those mechanisms become pathological. Overall, this preconditioned secretome, based on the strict regulation of miR-124 levels, showed potential neuroregenerative, immunoregulatory and anti-inflammatory properties and broad neuroprotection. The main findings of this study are summarized and represented in **Figure 1**. In this way, it opens a new possibility to develop a novel effective therapeutic strategy that can be translated into the clinics, hopefully in the near future. For that, it will be crucial to stratify patients showing upregulated levels of miR-124, using MNs differentiated from iPSCs or iNPCs generated from their own somatic cells.

In this sense, our data demonstrate that it will be possible to use patient MNs to modulate intracellular levels of upregulated miR-124 with its inhibitor and collect their preconditioned secretome as a therapeutic tool in personalized medicine. We predict that this preconditioned secretome might be used as an autologous treatment in stratified ALS patients for miR-124 to halt or at least delay disease progression.

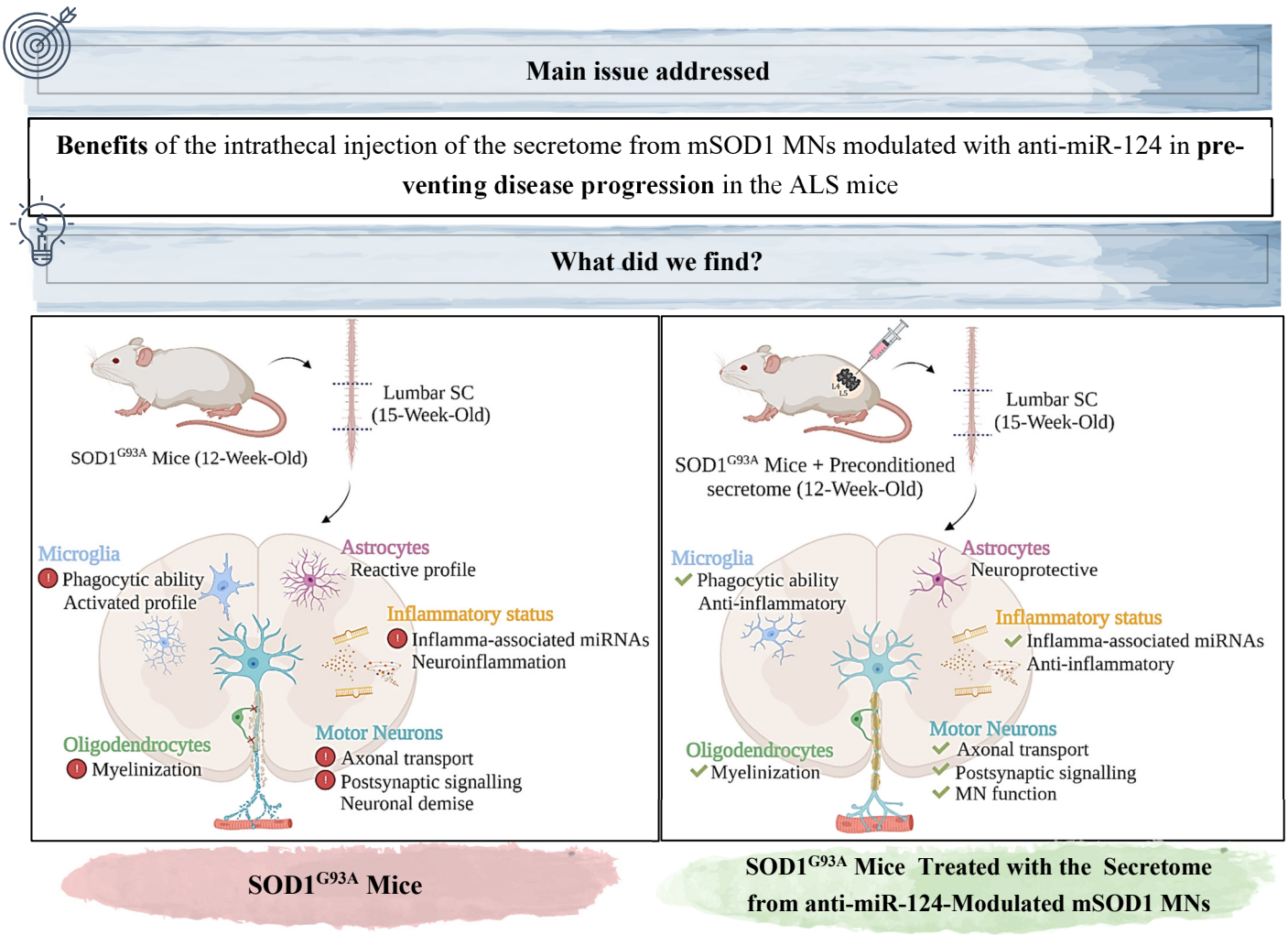

Figure 1 — Schematic representation of the main findings ensuing from the experimental data obtained in this thesis on the therapeutic potential of the administration of the preconditioned secretome in the spinal cord (SC) of the SOD1^{G93A} mice, a model of amyotrophic lateral sclerosis (ALS). Data was obtained with the secretome derived from mSOD1 motor neurons (MNs) treated with anti-miR-124. Through cellular and molecular assessments, we have shown that **(A)** the mSOD1 mice display high levels of neurodegeneration and MN loss, the disruption of postsynaptic signaling and axonal transport systems, myelination impairments, glial reactivity/activation, together with an inflammatory imbalance. In contrast, **(B)** the intrathecal injection of the preconditioned secretome at the early ALS symptomatic stage (12-week-old) was able to regulate miR-124 levels toward basal ones when assessed at the symptomatic stage of the disease (15-week-old). Furthermore, this approach also prevented alterations on the axonal transport and phagocytic/anti-inflammatory microglia, as well as avoided neurodegeneration, MN demise, demyelination, astrocyte reactivity, immune deregulation, and inflammatory instability in the SC of symptomatic ALS mice. Therefore, this secretome reveals to be a promising therapeutic tool that deserves to be tested in selected patients with upregulated miR-124 levels at symptom onset, contributing to the advance of precision medicine (Created with BioRender.com).

FUTURE PERSPECTIVES

Based on the findings of this thesis, where the ability of the preconditioned secretome from ALS MNs engineered with anti-miR-124 to prevent spinal ALS MN degeneration was shown, we are getting closer to the development and implantation of an autologous transplantation for ALS patients. As such, and with an ambitious perspective in mind, we can say that the used preconditioned secretome, if validated in future studies with patient MNs revealing upregulated levels of miR-124, may be a step forward to be accepted as a promising therapeutic option for personalized medicine in ALS patients. In fact, before the translation into clinics, it will be critical to study this specific modulation in iPSCs or iNPCs derived from the patients since most of the therapies that showed efficacy in mice failed when translated into humans.

However, despite the promising results obtained, there are clearly aspects that need to be explored. For instance, it would be interesting to better characterize the secretome for their constituents to understand if some other beneficial factors [e.g., proteins or growth factors, such as the brain-derived neurotrophic factor (BDNF) or glial-cell derived neurotrophic factor (GDNF)], beyond the regulated levels of miR-124, are contributing to its beneficial outcome. It would also be particularly interesting to perform these evaluations in other ALS *in vivo* models besides the mSOD1 model since several other mutations are associated with ALS. Furthermore, modulations with other inflamma-miRNAs could also be explored and even combined to test the efficacy of multiple modulations at the same time.

REFERENCES

Abrahams, Newton, Niven, Foley, & Bak.(2014).Screening for cognition and behaviour changes in ALS. *Amyotroph Lateral Scler Frontotemporal Degener,15*(1-2),9-14.doi:10.3109/21678421.2013.805784

Akbari Dilmaghani, Hussen, Nateghinia, Taheri, & Ghafouri-Fard.(2021).Emerging role of microRNAs in the pathogenesis of amyotrophic lateral sclerosis. *Metab Brain Dis,36*(5),737-749.doi:10.1007/s11011-021-00697-5

Al-Chalabi, & Hardiman.(2013).The epidemiology of ALS: a conspiracy of genes, environment and time. *Nat Rev Neurol,9*(11),617-628.doi:10.1038/nrneurol.2013.203

Al-Chalabi, van den Berg, & Veldink.(2017).Gene discovery in amyotrophic lateral sclerosis: implications for clinical management. *Nat Rev Neurol,13*(2),96-104.doi:10.1038/nrneurol.2016.182

Almer, Vukosavic, Romero, & Przedborski.(1999).Inducible nitric oxide synthase up-regulation in a transgenic mouse model of familial amyotrophic lateral sclerosis. *J Neurochem,72*(6),2415-2425.doi:10.1046/j.1471-4159.1999.0722415.x

Alves, de Santana, Santos, de Oliveira, Duobles, et al.(2011).Early motor and electrophysiological changes in transgenic mouse model of amyotrophic lateral sclerosis and gender differences on clinical outcome. *Brain research,1394*,90-104.doi.org/10.1016/j.brainres.2011.02.060

Ambros, & Ruvkun.(2018).Recent Molecular Genetic Explorations of Caenorhabditis elegans MicroRNAs. *Genetics, 209*(3),651-673.doi:10.1534/genetics.118.300291

Amin, Perera, Beart, Turner, & Shabanpoor.(2020).Amyotrophic Lateral Sclerosis and Autophagy: Dysfunction and Therapeutic Targeting. *Cells, 9*(11).doi:10.3390/cells9112413

An, Gong, Wang, Bian, Yu, et al.(2017).MiR-124 acts as a target for Alzheimer's disease by regulating BACE1. *Oncotarget, 8*(69), 114065-114071.doi:10.18632/oncotarget.23119

Angelucci, Cechova, Valis, Kuca, Zhang, et al.(2019).MicroRNAs in Alzheimer's Disease: Diagnostic Markers or Therapeutic Agents? *Front Pharmacol, 10*, 665.doi:10.3389/fphar.2019.00665

Azadmanesh, & Borgstahl.(2018).A Review of the Catalytic Mechanism of Human Manganese Superoxide Dismutase. *Antioxidants (Basel),7*(2).doi:10.3390/antiox7020025

Balendra, & Isaacs.(2018).C9orf72-mediated ALS and FTD: multiple pathways to disease. *Nat Rev Neurol,14*(9),544-558.doi:10.1038/s41582-018-0047-2

Barbosa, Gomes, Sequeira, Goncalves-Ribeiro, Pina, et al.(2021).Recovery of Depleted miR-146a in ALS Cortical Astrocytes Reverts Cell Aberrancies and Prevents Paracrine Pathogenicity on Microglia and Motor Neurons. *Front Cell Dev Biol,9*,634355.doi:10.3389/fcell.2021.634355

Barbosa, Santos, de Sousa, Duarte-Silva, Vaz, et al.(2022).Intrathecal Injection of the Secretome from ALS Motor Neurons Regulated for miR-124 Expression Prevents Disease Outcomes in SOD1-G93A Mice.*10*(9),2120.

Batra, Jain, Singh, Sharma, Singh, et al.(2019).Novel therapeutic targets for amyotrophic lateral sclerosis. *Indian journal of pharmacology,51*(6),418-425.doi:10.4103/ijp.IJP_823_19

Bellettato, & Scarpa.(2018).Possible strategies to cross the blood-brain barrier. *Italian journal of pediatrics,44*(Suppl 2),131.doi:10.1186/s13052-018-0563-0

Bennett, Tanaz, Cobos, & Torrente.(2019).Epigenetics in amyotrophic lateral sclerosis: a role for histone post-translational modifications in neurodegenerative disease. *Transl Res,204*,19-30.doi:10.1016/j.trsl.2018.10.002

Berthod, & Gros-Louis.(2012).In Vivo and In Vitro Models to Study Amyotrophic Lateral Sclerosis. In (pp. 81-124).

Bonafede, Turano, Scambi, Busato, Bontempi, et al.(2020).ASC-Exosomes Ameliorate the Disease Progression in SOD1(G93A) Murine Model Underlining Their Potential Therapeutic Use in Human ALS. *Int J Mol Sci, 21*(10).doi:10.3390/ijms21103651

Bonfanti, Bonifacino, Raffaele, Milanese, Morgante, et al.(2020).Abnormal Upregulation of GPR17 Receptor Contributes to Oligodendrocyte Dysfunction in SOD1 G93A Mice. *Int J Mol Sci, 21*(7).doi:10.3390/ijms21072395

Brenner, & Weishaupt.(2019).Update on amyotrophic lateral sclerosis genetics. *Curr Opin Neurol, 32*(5), 735-739.doi:10.1097/WCO.0000000000000737

Brites.(2020).Regulatory function of microRNAs in microglia. *Glia,68*(8),1631-1642.doi:10.1002/glia.23846

Brites, & Fernandes.(2015).Neuroinflammation and Depression: Microglia Activation, Extracellular Microvesicles and microRNA Dysregulation. *Front Cell Neurosci, 9*,476.doi:10.3389/fncel.2015.00476

Brites, & Vaz.(2014).Microglia centered pathogenesis in ALS: insights in cell interconnectivity. *Front Cell Neurosci, 8*,117.doi:10.3389/fncel.2014.00117

Broadhead, Bonthron, Waddington, Smith, Lopez, et al.(2022).Selective vulnerability of tripartite synapses in amyotrophic lateral sclerosis. *Acta Neuropathol,143*(4),471-486.doi:10.1007/s00401-022-02412-9

Brown, & Al-Chalabi.(2017).Amyotrophic Lateral Sclerosis. *N Engl J Med,377*(2),162-172.doi:10.1056/NEJMra1603471

Buratti.(2015).Functional Significance of TDP-43 Mutations in Disease. *Adv Genet,91*,1-53.doi:10.1016/bs.adgen.2015.07.001

Buratti, & Baralle.(2012).TDP-43: gumming up neurons through protein-protein and protein-RNA interactions. *Trends Biochem Sci, 37*(6),237-247.doi:10.1016/j.tibs.2012.03.003

Butovsky, Jedrychowski, Cialic, Krasemann, Murugaiyan, et al.(2015). Targeting miR-155 restores abnormal microglia and attenuates disease in SOD1 mice. *Ann Neurol,77*(1),75-99.doi:10.1002/ana.24304

Butovsky, Siddiqui, Gabriely, Lanser, Dake, et al.(2012).Modulating inflammatory monocytes with a unique microRNA gene signature ameliorates murine ALS. *J Clin Invest,122*(9),3063-3087.doi:10.1172/JCI62636

Caldeira, Oliveira, Cunha, Vaz, Falcão, et al.(2014).Microglia change from a reactive to an age-like phenotype with the time in culture.*8*.doi:10.3389/fncel.2014.00152

Calderó, Brunet, Tarabal, Piedrafita, Hereu, et al.(2010).Lithium prevents excitotoxic cell death of motoneurons in organotypic slice cultures of spinal cord. *Neuroscience,165*(4),1353-1369.doi:10.1016/j.neuroscience.2009.11.034

Campos-Melo, Droppelmann, He, Volkening, & Strong.(2013).Altered microRNA expression profile in Amyotrophic Lateral Sclerosis: a role in the regulation of NFL mRNA levels. *Mol Brain,6*, 26.doi:10.1186/1756-6606-6-26

Cashman, Durham, Blusztajn, Oda, Tabira, et al.(1992).Neuroblastoma x spinal cord (NSC) hybrid cell lines resemble developing motor neurons. *Developmental dynamics : an official publication of the American Association of Anatomists,194*(3),209-221.doi:10.1002/aja.1001940306

Chio, Mazzini, & Mora.(2020).Disease-modifying therapies in amyotrophic lateral sclerosis. *Neuropharmacology, 167*, 107986. doi:10.1016/j.neuropharm.2020.107986

Christopher, Kaur, Kaur, Kaur, Gupta, et al.(2016).MicroRNA therapeutics: Discovering novel targets and developing specific therapy. *Perspect Clin Res,7*(2),68-74.doi:10.4103/2229-3485.179431

Clarke, & Patani.(2020).The microglial component of amyotrophic lateral sclerosis. *Brain,143*(12),3526-3539.doi:10.1093/brain/awaa309

Condrat, Thompson, Barbu, Bugnar, Boboc, et al.(2020).miRNAs as Biomarkers in Disease: Latest Findings Regarding Their Role in Diagnosis and Prognosis. *Cells,9*(2).doi:10.3390/cells9020276

Correia, Neves-Carvalho, Mendes-Pinheiro, Pires, Teixeira, et al.(2021).Preclinical Assessment of Mesenchymal-Stem-Cell-Based Therapies in Spinocerebellar Ataxia Type 3. *Biomedicines, 9*(12).doi:10.3390/biomedicines9121754

Cunha, Gomes, Vaz, & Brites.(2016).Exploring New Inflammatory Biomarkers and Pathways during LPS-Induced M1 Polarization. *Mediators Inflamm,2016*,6986175.doi:10.1155/2016/6986175

Cunha, Santos, Gomes, Fernandes, Correia, et al.(2018).Downregulated Glia Interplay and Increased miRNA-155 as Promising Markers to Track ALS at an Early Stage. *Mol Neurobiol,55*(5),4207-4224.doi:10.1007/s12035-017-0631-2

de Faria, Gonsalvez, Nicholson, & Xiao.(2019).Activity-dependent central nervous system myelination throughout life. *J Neurochem,148*(4),447-461.doi:10.1111/jnc.14592

De Vos, & Hafezparast.(2017).Neurobiology of axonal transport defects in motor neuron diseases:Opportunities for translational research? *Neurobiol Dis,105*,283-299.doi:10.1016/j.nbd.2017.02.004

Deczkowska, Keren-Shaul, Weiner, Colonna, Schwartz, et al.(2018).Disease-Associated Microglia: A Universal Immune Sensor of Neurodegeneration. *Cell,173*(5),1073-1081.doi:10.1016/j.cell.2018.05.003

Diaz-Amarilla, Olivera-Bravo, Trias, Cragnolini, Martinez-Palma, et al.(2011).Phenotypically aberrant astrocytes that promote motoneuron damage in a model of inherited amyotrophic lateral sclerosis. *Proc Natl Acad Sci USA,108*(44),18126-18131.doi:10.1073/pnas.1110689108

Dorst, Ludolph, & Huebers.(2018).Disease-modifying and symptomatic treatment of amyotrophic lateral sclerosis. *Ther Adv Neurol Disord,11*, 1756285617734734.doi:10.1177/1756285617734734

Doucette, Whitson, Cao, Schirf, Demeler, et al.(2004).Dissociation of human copper-zinc superoxide dismutase dimers using chaotrope and reductant. Insights into the molecular basis for dimer stability. *J Biol Chem,279*(52),54558-54566.doi:10.1074/jbc.M409744200

Duroux-Richard, Roubert, Ammari, Presumey, Grun, et al.(2016).miR-125b controls monocyte adaptation to inflammation through mitochondrial metabolism and dynamics. *Blood,128*(26),3125-3136.doi:10.1182/blood-2016-02-697003

Dutta, Chomyk, Chang, Ribaudo, Deckard, et al.(2013).Hippocampal demyelination and memory dysfunction are associated with increased levels of the neuronal microRNA miR-124 and reduced AMPA receptors. *Ann Neurol,73*(5),637-645.doi:10.1002/ana.23860

Fan, Liang, Ou, Zou, Sun, et al.(2020).MicroRNA-146a Is a Wide-Reaching Neuroinflammatory Regulator and Potential Treatment Target in Neurological Diseases. *Front Mol Neurosci,13*, 90.doi:10.3389/fnmol.2020.00090

Fareh, Yeom, Haagsma, Chauhan, Heo, et al.(2016).TRBP ensures efficient Dicer processing of precursor microRNA in RNA-crowded environments. *Nat Commun,7*,13694.doi:10.1038/ncomms13694

Ferraiuolo, Kirby, Grierson, Sendtner, & Shaw.(2011).Molecular pathways of motor neuron injury in amyotrophic lateral sclerosis. *Nat Rev Neurol, 7*(11), 616-630. doi:10.1038/nrneurol.2011.152

Figlioli, Bogliolo, Catucci, Caleca, Lasheras, et al. (2019).The FANCM:p.Arg658* truncating variant is associated with risk of triple-negative breast cancer. *NPJ Breast Cancer,5*, 38.doi:10.1038/s41523-019-0127-5

Filipi, Hermanova, Tureckova, Vanatko, & Anderova.(2020).Glial Cells-The Strategic Targets in Amyotrophic Lateral Sclerosis Treatment. *J Clin Med,9*(1).doi:10.3390/jcm9010261

Flores-Barrantes, Mavrogianni, Iglesia, Mahmood, Willems, et al. (2022).Can food parenting practices explain the association between socioeconomic status and children's food intake? The Feel4Diabetes-study. *Public Health Nutr*,1-35.doi:10.1017/S1368980022000891

Foggin, Mesquita-Ribeiro, Dajas-Bailador, & Layfield.(2019).Biological Significance of microRNA Biomarkers in ALS-Innocent Bystanders or Disease Culprits? *Front Neurol,10*, 578.doi:10.3389/fneur.2019.00578

Fuller, & Van Eldik.(2008).MFG-E8 regulates microglial phagocytosis of apoptotic neurons. *J Neuroimmune Pharmacol,3*(4),246-256.doi:10.1007/s11481-008-9118-2

Gaja-Capdevila, Hernandez, Navarro, & Herrando-Grabulosa.(2021).Sigma-1 Receptor is a Pharmacological Target to Promote Neuroprotection in the SOD1(G93A) ALS Mice. *Front Pharmacol,12*,780588.doi:10.3389/fphar.2021.780588

Gallo, Tandon, Alevizos, & Illei.(2012).The majority of microRNAs detectable in serum and saliva is concentrated in exosomes. *PLoS One,7*(3),e30679.doi:10.1371/journal.pone.0030679

Garcia, Fernandes, Stein, & Brites.(2022).Protective Signature of IFN γ -Stimulated Microglia Relies on miR-124-3p Regulation From the Secretome Released by Mutant APP Swedish Neuronal Cells. *Front Pharmacol,13*,833066.doi:10.3389/fphar.2022.833066

Garcia, Pinto, Cunha, Fernandes, Koistinaho, et al.(2021).Neuronal Dynamics and miRNA Signaling Differ between SH-SY5Y APPSwe and PSEN1 Mutant iPSC-Derived AD Models upon Modulation with miR-124 Mimic and Inhibitor. *Cells,10*(9).doi:10.3390/cells10092424

Garland, Hartnell, & Boche.(2022).Microglia and Astrocyte Function and Communication: What Do We Know in Humans? *Front Neurosci,16*,824888.doi:10.3389/fnins.2022.824888

Gaudet, Fonken, Watkins, Nelson, & Popovich. (2018).MicroRNAs: Roles in Regulating Neuroinflammation. *Neuroscientist,24*(3),221-245.doi:10.1177/1073858417721150

Geloso, Corvino, Marchese, Serrano, Michetti, et al.(2017).The Dual Role of Microglia in ALS: Mechanisms and Therapeutic Approaches. *Front Aging Neurosci,9*,242.doi:10.3389/fnagi.2017.00242

Genç, Jara, Lagrimas, Pytel, Roos, et al.(2017).Apical dendrite degeneration, a novel cellular pathology for Betz cells in ALS. *Sci Rep,7*,41765.doi:10.1038/srep41765

Gois, Mendonca, Freire, & Santos.(2020).In Vitro and in Vivo Models of Amyotrophic Lateral Sclerosis: An Updated Overview. *Brain Res Bull,159*,32-43.doi:10.1016/j.brainresbull.2020.03.012

Gomes, Cunha, Nascimento, Ribeiro, Vaz, et al.(2019).Cortical Neurotoxic Astrocytes with Early ALS Pathology and miR-146a Deficit Replicate Gliosis Markers of Symptomatic SOD1G93A Mouse Model. *Mol Neurobiol,56*(3),2137-2158.doi:10.1007/s12035-018-1220-8

Gomes, Keller, Altevogt, & Costa.(2007).Evidence for secretion of Cu,Zn superoxide dismutase via exosomes from a cell model of amyotrophic lateral sclerosis. *Neurosci Lett,428*(1),43-46.doi:10.1016/j.neulet.2007.09.024

Gomes, Palma, Almeida, Regalla, McCluskey, et al.(2008).Establishment of a cell model of ALS disease: Golgi apparatus disruption occurs independently from apoptosis. *Biotechnol Lett, 30*(4),603-610.doi:10.1007/s10529-007-9595-z

Gomes, Sequeira, Barbosa, Cunha, Vaz, et al.(2020).Astrocyte regional diversity in ALS includes distinct aberrant phenotypes with common and causal pathological processes. *Exp Cell Res, 395*(2),112209.doi:10.1016/j.yexcr.2020.112209

Gomes, Sequeira, Likhite, Dennys, Kolb, et al.(2022).Neurotoxic Astrocytes Directly Converted from Sporadic and Familial ALS Patient Fibroblasts Reveal Signature Diversities and miR-146a Theragnostic Potential in Specific Subtypes. *Cells,11*(7).doi:10.3390/cells11071186

Gomez Morillas, Besson, & Lerouet.(2021).Microglia and Neuroinflammation: What Place for P2RY12? *Int J Mol Sci,22*(4).doi:10.3390/ijms22041636

González-Prieto, Gutiérrez, García-Bueno, Caso, Leza, et al.(2021).Microglial CX3CR1 production increases in Alzheimer's disease and is regulated by noradrenaline. *Glia,69*(1),73-90.doi:10.1002/glia.23885

Guay, & Regazzi.(2013).Circulating microRNAs as novel biomarkers for diabetes mellitus. *Nat Rev Endocrinol,9*(9),513-521.doi:10.1038/nrendo.2013.86

Guo, Hong, Wang, Zhang, Korner, et al.(2019).MicroRNAs in Microglia: How do MicroRNAs Affect Activation, Inflammation, Polarization of Microglia and Mediate the Interaction Between Microglia and Glioma? *Front Mol Neurosci,12*,125.doi:10.3389/fnmol.2019.00125

Gurney. (1994).Transgenic-mouse model of amyotrophic lateral sclerosis. *N Engl J Med,331*(25),1721-1722.doi:10.1056/nejm199412223312516

Gurney, Pu, Chiu, Dal Canto, Polchow, et al.(1994).Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. *Science (New York, N.Y.),264*(5166),1772-1775.doi:10.1126/science.8209258

Han, Dong, Zheng, & Nao.(2019).MiR-124 and the Underlying Therapeutic Promise of Neurodegenerative Disorders. *Front Pharmacol,10*,1555.doi:10.3389/fphar.2019.01555

Han, Lee, Yeom, Kim, Jin, et al.(2004).The Drosha-DGCR8 complex in primary microRNA processing. *Genes Dev,18*(24),3016-3027.doi:10.1101/gad.1262504

Hardiman, Al-Chalabi, Chio, Corr, Logroscino, et al.(2017).Amyotrophic lateral sclerosis. *Nat Rev Dis Primers,3*,17071.doi:10.1038/nrdp.2017.71
Hardiman, van den Berg, & Kiernan.(2011).Clinical diagnosis and management of amyotrophic lateral sclerosis. *Nat Rev Neurol,7*(11),639-649.doi:10.1038/nrneurol.2011.153
Hayashi, Homma, & Ichijo.(2016).SOD1 in neurotoxicity and its controversial roles in SOD1 mutation-negative ALS. *Adv Biol Regul,60*,95-104.doi:10.1016/j.jbior.2015.10.006
He, & Hannon. (2004).MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet,5*(7),522-531.doi:10.1038/nrg1379
He, Yip, Jabar, Mohtarrudin, Dusa, et al.(2019).Effect of the miR-96-5p inhibitor and mimic on the migration and invasion of the SW480-7 colorectal cancer cell line. *Oncology letters,18*(2),1949-1960.doi:10.3892/ol.2019.10492
Hickman, Izzy, Sen, Morsett, & El Khoury.(2018).Microglia in neurodegeneration. *Nat Neurosci,21*(10),1359-1369.doi:10.1038/s41593-018-0242-x
Hirokawa, Niwa, & Tanaka.(2010).Molecular motors in neurons: transport mechanisms and roles in brain function, development, and disease. *Neuron,68*(4),610-638.doi:10.1016/j.neuron.2010.09.039
Hogden, Foley, Henderson, James, & Aoun.(2017).Amyotrophic lateral sclerosis: improving care with a multidisciplinary approach. *J Multidiscip Healthc,10*,205-215.doi:10.2147/jmdh.S134992
Hou, Ruan, Gilbert, Wang, Ma, et al.(2015).MicroRNA miR124 is required for the expression of homeostatic synaptic plasticity. *Nat Commun,6*,10045.doi:10.1038/ncomms10045
Hu, Lin, Duan, Cui, Li, et al.(2018).Intrathecal Injection of scAAV9-hIGF1 Prolongs the Survival of ALS Model Mice by Inhibiting the NF-kB Pathway. *Neuroscience,381*,1-10.doi:10.1016/j.neuroscience.2018.02.004
Hulisz.(2018).Amyotrophic lateral sclerosis: disease state overview. *Am J Manag Care,24*(15 Suppl), S320-S326.
Hunter, Spiller, Dominique, Xu, Hunter, et al.(2021).Microglial transcriptome analysis in the rNLS8 mouse model of TDP-43 proteinopathy reveals discrete expression profiles associated with neurodegenerative progression and recovery. *Acta Neuropathol Commun,9*(1),140.doi:10.1186/s40478-021-01239-x
Iftikhar, & Carney.(2016).Evidence and potential in vivo functions for biofluid miRNAs: From expression profiling to functional testing: Potential roles of extracellular miRNAs as indicators of physiological change and as agents of intercellular information exchange. *Bioessays,38*(4),367-378.doi:10.1002/bies.201500130
Irwin, McMillan, Brettschneider, Libon, Powers, et al.(2013).Cognitive decline and reduced survival in C9orf72 expansion frontotemporal degeneration and amyotrophic lateral sclerosis. *J Neurol Neurosurg Psychiatry,84*(2),163-169.doi:10.1136/jnnp-2012-303507
Iyer, Zurolo, Prabowo, Fluiter, Spliet, et al.(2012).MicroRNA-146a: a key regulator of astrocyte-mediated inflammatory response. *PLoS One,7*(9),e44789.doi:10.1371/journal.pone.0044789
Jaiswal.(2019). Riluzole and edaravone: A tale of two amyotrophic lateral sclerosis drugs. *Med Res Rev,39*(2),733-748.doi:10.1002/med.21528
Jiang, Wang, Hao, Juan, Teng, et al.(2009).miR2Disease: a manually curated database for microRNA deregulation in human disease. *Nucleic Acids Res, 37*,D98-104.doi:10.1093/nar/gkn714
Jo, Lee, Jeon, Kim, Kwon, et al.(2020).The role of TDP-43 propagation in neurodegenerative diseases: integrating insights from clinical and experimental studies. *Exp Mol Med,52*(10),1652-1662.doi:10.1038/s12276-020-00513-7
Joilin, Leigh, Newbury, & Hafezparast.(2019).An Overview of MicroRNAs as Biomarkers of ALS. *Front Neurol,10*,186.doi:10.3389/fneur.2019.00186
Kang, Li, Fukaya, Lorenzini, Cleveland, et al.(2013).Degeneration and impaired regeneration of gray matter oligodendrocytes in amyotrophic lateral sclerosis. *Nat Neurosci,16*(5),571-579.doi:10.1038/nn.3357
Keren-Shaul, Spinrad, Weiner, Matcovitch-Natan, Dvir-Szternfeld, et al.(2017).A Unique Microglia Type Associated with Restricting Development of Alzheimer's Disease. *Cell,169*(7),1276-1290.e1217.doi:10.1016/j.cell.2017.05.018

Kim, Chung, Na, Lee, Jeong, et al.(2019).Myelin degeneration induced by mutant superoxide dismutase 1 accumulation promotes amyotrophic lateral sclerosis. *Glia,67*(10),1910-1921.doi:10.1002/glia.23669

Kim, Gautier, Tassoni-Tsuchida, Ma, & Gitler.(2020).ALS Genetics: Gains, Losses, and Implications for Future Therapies. *Neuron,108*(5),822-842.doi:10.1016/j.neuron.2020.08.022

Kim, Kim, Sung, Patel, Wong, et al.(2022).Autologous treatment for ALS with implication for broad neuroprotection. *Transl Neurodegener, 11*(1), 16. doi:10.1186/s40035-022-00290-5

Klionsky, Abdel-Aziz, Abdelfatah, Abdellatif, Abdoli, et al.(2021).Guidelines for the use and interpretation of assays for monitoring autophagy (4th edition). *Autophagy,17*(1),1-382.doi:10.1080/15548627.2020.1797280

Knippenberg, Thau, Dengler, & Petri.(2010).Significance of behavioural tests in a transgenic mouse model of amyotrophic lateral sclerosis (ALS). *Behavioural Brain Research,213*(1),82-87.doi.org/10.1016/j.bbr.2010.04.042

Korzhevskii, & Kirik.(2016).Brain Microglia and Microglial Markers. *Neuroscience and Behavioral Physiology,46*(3),284-290.doi:10.1007/s11055-016-0231-z

Koval, Shaner, Zhang, du Maine, Fischer, et al.(2013).Method for widespread microRNA-155 inhibition prolongs survival in ALS-model mice. *Hum Mol Genet,22*(20),4127-4135.doi:10.1093/hmg/ddt261

Kwon, & Koh.(2020).Neuroinflammation in neurodegenerative disorders: the roles of microglia and astrocytes. *Transl Neurodegener,9*(1),42.doi:10.1186/s40035-020-00221-2

Lall, & Baloh.(2017).Microglia and C9orf72 in neuroinflammation and ALS and frontotemporal dementia. *J Clin Invest,127*(9),3250-3258.doi:10.1172/JCI90607

Landgraf, Rusu, Sheridan, Sewer, Iovino, et al. (2007).A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell,129*(7),1401-1414.doi:10.1016/j.cell.2007.04.040

Layalle, They, Ourghani, Raoul, & Soustelle.(2021).Amyotrophic Lateral Sclerosis Genes in Drosophila melanogaster. *Int J Mol Sci,22*(2).doi:10.3390/ijms22020904

Lee, & Kim.(2014).The anti-inflammatory role of tissue inhibitor of metalloproteinase-2 in lipopolysaccharide-stimulated microglia. *J Neuroinflammation,11*,116.doi:10.1186/1742-2094-11-116

Li, Han, & Wang.(2016).Organotypic Hippocampal Slices as Models for Stroke and Traumatic Brain Injury. *Mol Neurobiol,53*(6),4226-4237.doi:10.1007/s12035-015-9362-4

Li, Liu, Li, Bu, Sun, et al.(2008).Effect of ALS IgG on motor neurons in organotypic spinal cord cultures. *The Canadian journal of neurological sciences. Le journal canadien des sciences neurologiques,35*(2),220-225.doi:10.1017/s0317167100008672

Li, Liu, Yang, Wang, Wu, et al.(2017).Slow Intrathecal Injection of rAAVrh10 Enhances its Transduction of Spinal Cord and Therapeutic Efficacy in a Mutant SOD1 Model of ALS. *Neuroscience,365*,192-205.doi:10.1016/j.neuroscience.2017.10.001

Li, Wang, Kodali, Chen, Kim, et al.(2018).In vivo evidence for the contribution of peripheral circulating inflammatory exosomes to neuroinflammation. *J Neuroinflammation,15*(1),8.doi:10.1186/s12974-017-1038-8

Lin, Kumar, Ramesh, Anderson, Nguyen, et al.(2021).Interactions between ALS-linked FUS and nucleoporins are associated with defects in the nucleocytoplasmic transport pathway. *Nat Neurosci,24*(8),1077-1088.doi:10.1038/s41593-021-00859-9

Liu, Hong, Chen, Niu, Duan, et al. (2019).Evidence for a protective role of the CX3CL1/CX3CR1 axis in a model of amyotrophic lateral sclerosis.*400*(5),651-661.doi:doi:10.1515/hsz-2018-0204

Liu, & Wang.(2017).Role of Neuroinflammation in Amyotrophic Lateral Sclerosis: Cellular Mechanisms and Therapeutic Implications. *Front Immunol, 8*, 1005.doi:10.3389/fimmu.2017.01005

Loch-Neckel, Matos, Vaz, & Brites.(2022).Challenges in the Development of Drug Delivery Systems Based on Small Extracellular Vesicles for Therapy of Brain Diseases. *Front Pharmacol, 13*, 839790.doi:10.3389/fphar.2022.839790

Longinetti, & Fang. (2019).Epidemiology of amyotrophic lateral sclerosis: an update of recent literature. *Curr Opin Neurol,32*(5),771-776.doi:10.1097/WCO.0000000000000730

Lossi, Alasia, Salio, & Merighi.(2009).Cell death and proliferation in acute slices and organotypic cultures of mammalian CNS. *Progress in neurobiology,88*(4),221-245.doi:10.1016/j.pneurobio.2009.01.002

Lou, Takano, Pei, Xavier, Goldman, et al.(2016).Purinergic receptor P2RY12-dependent microglial closure of the injured blood-brain barrier. *Proc Natl Acad Sci USA,113*(4),1074-1079.doi:10.1073/pnas.1520398113

Ma, Hunt, Selenica, Sanneh, Sandusky-Beltran, et al.(2020).Arginase 1 Insufficiency Precipitates Amyloid-beta Deposition and Hastens Behavioral Impairment in a Mouse Model of Amyloidosis. *Front Immunol,11*,582998.doi:10.3389/fimmu.2020.582998

Madji Hounoum, Vourc'h, Felix, Corcia, Patin, et al.(2016).NSC-34 Motor Neuron-Like Cells Are Unsuitable as Experimental Model for Glutamate-Mediated Excitotoxicity. *Front Cell Neurosci,10*,118.doi:10.3389/fncel.2016.00118

Mancuso, & Navarro. (2015).Amyotrophic lateral sclerosis: Current perspectives from basic research to the clinic. *Progress in neurobiology,133*,1-26.doi:10.1016/j.pneurobio.2015.07.004

Marcuzzo, Bonanno, Kapetis, Barzago, Cavalcante, et al.(2015). Up-regulation of neural and cell cycle-related microRNAs in brain of amyotrophic lateral sclerosis mice at late disease stage. *Mol Brain,8*,5.doi:10.1186/s13041-015-0095-0

Martinez, Francis, Liu, Pradzinsky, Fine, et al.(2008).Intranasal delivery of insulin and a nitric oxide synthase inhibitor in an experimental model of amyotrophic lateral sclerosis. *Neuroscience,157*(4),908-925.doi:10.1016/j.neuroscience.2008.08.073

Masrori, & Van Damme.(2020).Amyotrophic lateral sclerosis: a clinical review. *Eur J Neurol,27*(10),1918-1929.doi:10.1111/ene.14393

Mathis, Goizet, Soulages, Vallat, & Masson.(2019).Genetics of amyotrophic lateral sclerosis: A review. *J Neurol Sci,399*,217-226.doi:10.1016/j.jns.2019.02.030

Matias, Morgado, & Gomes.(2019).Astrocyte Heterogeneity: Impact to Brain Aging and Disease. *Front Aging Neurosci,11*,59.doi:10.3389/fnagi.2019.00059

Mejzini, Flynn, Pitout, Fletcher, Wilton, et al.(2019).ALS Genetics, Mechanisms, and Therapeutics: Where Are We Now? *Front Neurosci,13*,1310.doi:10.3389/fnins.2019.01310

Mendes-Pinheiro, Marote, Marques, Teixeira, Ribeiro, et al.(2020).Applications of the stem cell secretome in regenerative medicine. In *Mesenchymal Stem Cells in Human Health and Diseases* (pp. 79-114).

Meyer, Ferraiuolo, Miranda, Likhite, McElroy, et al.(2014).Direct conversion of patient fibroblasts demonstrates non-cell autonomous toxicity of astrocytes to motor neurons in familial and sporadic ALS. *Proc Natl Acad Sci USA,111*(2),829-832.doi:10.1073/pnas.1314085111

Michalska, & Leon.(2020).When It Comes to an End: Oxidative Stress Crosstalk with Protein Aggregation and Neuroinflammation Induce Neurodegeneration. *Antioxidants (Basel), 9*(8).doi:10.3390/antiox9080740

Mishra, Dhull, Nalini, Vijayalakshmi, Sathyaprabha, et al.(2016).Astroglia acquires a toxic neuroinflammatory role in response to the cerebrospinal fluid from amyotrophic lateral sclerosis patients. *J Neuroinflammation,13*(1),212.doi:10.1186/s12974-016-0698-0

Moore, Ase, Kinsara, Rao, Michell-Robinson, et al.(2015).P2Y12 expression and function in alternatively activated human microglia. *Neurology(R) neuroimmunology & neuroinflammation,2*(2),e80.doi:10.1212/nxi.0000000000000080

Neo, Yap, Lee, Looi, Khandelia, et al.(2014).MicroRNA miR-124 controls the choice between neuronal and astrocyte differentiation by fine-tuning Ezh2 expression. *J Biol Chem,289*(30),20788-20801.doi:10.1074/jbc.M113.525493

Ng, Khan, Young, & Galea.(2017).Symptomatic treatments for amyotrophic lateral sclerosis/motor neuron disease. *Cochrane Database Syst Rev, 1*(1), Cd011776.doi:10.1002/14651858.CD011776.pub2

Nguyen, Kumar, Fedele, Bonanno, & Bonifacino.(2022).MicroRNA Alteration, Application as Biomarkers, and Therapeutic Approaches in Neurodegenerative Diseases. *Int J Mol Sci, 23*(9).doi:10.3390/ijms23094718

Nikodemova, Small, Smith, Mitchell, & Watters.(2014).Spinal but not cortical microglia acquire an atypical phenotype with high VEGF, galectin-3 and osteopontin, and blunted inflammatory responses in ALS rats. *Neurobiol Dis,69*,43-53.doi:10.1016/j.nbd.2013.11.009

Nolan, Mitchem, Jimenez-Mateos, Henshall, Concannon, et al.(2014).Increased expression of microRNA-29a in ALS mice: functional analysis of its inhibition. *Journal of molecular neuroscience: MN,53*(2),231-241.doi:10.1007/s12031-014-0290-y

Nonneman, Robberecht, & Van Den Bosch.(2014).The role of oligodendroglial dysfunction in amyotrophic lateral sclerosis. *Neurodegenerative disease management,4*(3),223-239.doi:10.2217/nmt.14.21

O'Brien, Hayder, Zayed, & Peng.(2018).Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. *Front Endocrinol (Lausanne),9*,402.doi:10.3389/fendo.2018.00402

Ohnuma, Kasagi, Uto, Noguchi, Nakamachi, et al.(2019).MicroRNA-124 inhibits TNF-α- and IL-6-induced osteoclastogenesis. *Rheumatology international,39*(4),689-695.doi:10.1007/s00296-018-4218-7

Oliván, Calvo, Rando, Muñoz, Zaragoza, et al.(2015).Comparative study of behavioural tests in the SOD1G93A mouse model of amyotrophic lateral sclerosis. *Experimental animals,64*(2),147-153.doi:10.1538/expanim.14-0077

Oskarsson, Gendron, & Staff.(2018).Amyotrophic Lateral Sclerosis: An Update for 2018. *Mayo Clin Proc,93*(11),1617-1628.doi:10.1016/j.mayocp.2018.04.007

Panaro, Benameur, & Porro.(2020).Extracellular Vesicles miRNA Cargo for Microglia Polarization in Traumatic Brain Injury. *Biomolecules,10*(6).doi:10.3390/biom10060901

Pang, & Hu. (2021).Cellular and physiological functions of C9ORF72 and implications for ALS/FTD. *J Neurochem,157*(3),334-350.doi:10.1111/jnc.15255

Pansarasa, Bordoni, Diamanti, Sproviero, Gagliardi, et al. (2018).SOD1 in Amyotrophic Lateral Sclerosis: "Ambivalent" Behavior Connected to the Disease. *Int J Mol Sci, 19*(5).doi:10.3390/ijms19051345

Parisi, Arisi, D'Ambrosi, Storti, Brandi, et al.(2013).Dysregulated microRNAs in amyotrophic lateral sclerosis microglia modulate genes linked to neuroinflammation. *Cell Death Dis,4*, e959.doi:10.1038/cddis.2013.491

Parisi, Napoli, Amadio, Spalloni, Apolloni, et al.(2016).MicroRNA-125b regulates microglia activation and motor neuron death in ALS. *Cell Death Differ,23*(3),531-541.doi:10.1038/cdd.2015.153

Pegtel, Peferoen, & Amor.(2014).Extracellular vesicles as modulators of cell-to-cell communication in the healthy and diseased brain. *Philos Trans R Soc Lond B Biol Sci, 369*(1652).doi:10.1098/rstb.2013.0516

Petrov, Mansfield, Moussy, & Hermine.(2017).ALS Clinical Trials Review: 20 Years of Failure. Are We Any Closer to Registering a New Treatment? *Front Aging Neurosci,9*,68.doi:10.3389/fnagi.2017.00068

Philips, & Rothstein.(2015).Rodent Models of Amyotrophic Lateral Sclerosis. *Current protocols in pharmacology,69*,5.67.61-65.67.21.doi:10.1002/0471141755.ph0567s69

Picher-Martel, Valdmanis, Gould, Julien, & Dupré. (2016).From animal models to human disease: a genetic approach for personalized medicine in ALS. *Acta Neuropathol Commun,4*(1),70.doi:10.1186/s40478-016-0340-5

Pinho, Cibrao, Silva, Monteiro, & Salgado.(2020).Cell Secretome: Basic Insights and Therapeutic Opportunities for CNS Disorders. *Pharmaceuticals (Basel),13*(2).doi:10.3390/ph13020031

Pinto, Cunha, Barbosa, Vaz, & Brites.(2017).Exosomes from NSC-34 Cells Transfected with hSOD1-G93A Are Enriched in miR-124 and Drive Alterations in Microglia Phenotype. *Front Neurosci, 11*,273.doi:10.3389/fnins.2017.00273

Prasad, Bharathi, Sivalingam, Girdhar, & Patel.(2019).Molecular Mechanisms of TDP-43 Misfolding and Pathology in Amyotrophic Lateral Sclerosis. *Front Mol Neurosci,12*,25.doi:10.3389/fnmol.2019.00025

Prinz, Jung, & Priller.(2019).Microglia Biology: One Century of Evolving Concepts. *Cell,179*(2),292-311.doi:10.1016/j.cell.2019.08.053

Qin, Wang, Su, & Liu.(2016).miRNA-124 in Immune System and Immune Disorders. *Front Immunol,7*,406.doi:10.3389/fimmu.2016.00406

Qin, Wu, Wen, Jia, Zhang, et al.(2022).Comparative assessment of blood Metal/metalloid levels, clinical heterogeneity, and disease severity in amyotrophic lateral sclerosis patients. *Neurotoxicology,89*,12-19.doi:10.1016/j.neuro.2022.01.003

Raffaele, Boccazzi, & Fumagalli.(2021).Oligodendrocyte Dysfunction in Amyotrophic Lateral Sclerosis: Mechanisms and Therapeutic Perspectives. *Cells,10*(3).doi:10.3390/cells10030565
Ratti, & Buratti. (2016).Physiological functions and pathobiology of TDP-43 and FUS/TLS proteins. *J Neurochem, 138 Suppl 1*,95-111.doi:10.1111/jnc.13625
Ricci, Marzocchi, & Battistini.(2018).MicroRNAs as Biomarkers in Amyotrophic Lateral Sclerosis. *Cells,7*(11).doi:10.3390/cells7110219
Righi, Mori, De Libero, Sironi, Biondi, et al.(1989).Monokine production by microglial cell clones. *European journal of immunology,19*(8),1443-1448.doi:10.1002/eji.1830190815
Rinchetti, Rizzuti, Faravelli, & Corti.(2018).MicroRNA Metabolism and Dysregulation in Amyotrophic Lateral Sclerosis. *Mol Neurobiol,55*(3),2617-2630.doi:10.1007/s12035-017-0537-z
Rogers, Morganti, Bachstetter, Hudson, Peters, et al.(2011).CX3CR1 deficiency leads to impairment of hippocampal cognitive function and synaptic plasticity. *The Journal of neuroscience : the official journal of the Society for Neuroscience,31*(45),16241-16250.doi:10.1523/jneurosci.3667-11.2011
Roggenbuck, Quick, & Kolb.(2017).Genetic testing and genetic counseling for amyotrophic lateral sclerosis: an update for clinicians. *Genet Med,19*(3),267-274.doi:10.1038/gim.2016.107
Rosenblum, & Trotti.(2017).EAAT2 and the Molecular Signature of Amyotrophic Lateral Sclerosis. *Advances in neurobiology,16*,117-136.doi:10.1007/978-3-319-55769-4_6
Rowland, & Shneider.(2001).Amyotrophic lateral sclerosis. *N Engl J Med,344*(22),1688-1700.doi:10.1056/nejm200105313442207
Rubio, Herrando-Grabulosa, Gaja-Capdevila, Vilches, & Navarro.(2022).Characterization of somatosensory neuron involvement in the SOD1(G93A) mouse model. *Sci Rep,12*(1),7600.doi:10.1038/s41598-022-11767-8
Rupaimoole, & Slack.(2017).MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. *Nat Rev Drug Discov,16*(3),203-222.doi:10.1038/nrd.2016.246
Sama, Ward, & Bosco.(2014).Functions of FUS/TLS from DNA repair to stress response: implications for ALS. *ASN Neuro,6*(4).doi:10.1177/1759091414544472
Sandona, Di Pietro, Esposito, Ventura, Silini, et al.(2021).Mesenchymal Stromal Cells and Their Secretome: New Therapeutic Perspectives for Skeletal Muscle Regeneration. *Front Bioeng Biotechnol,9*,652970.doi:10.3389/fbioe.2021.652970
Scarrott, Herranz-Martín, Alrafiah, Shaw, & Azzouz.(2015).Current developments in gene therapy for amyotrophic lateral sclerosis. *Expert opinion on biological therapy,15*(7),935-947.doi:10.1517/14712598.2015.1044894
Shaw.(2005).Molecular and cellular pathways of neurodegeneration in motor neurone disease. *J Neurol Neurosurg Psychiatry,76*(8),1046-1057.doi:10.1136/jnnp.2004.048652
Sheedy. (2015).Turning 21: Induction of miR-21 as a Key Switch in the Inflammatory Response. *Front Immunol,6*,19.doi:10.3389/fimmu.2015.00019
Shibata, Hirano, Yamamoto, Kato, & Kobayashi.(2000).Superoxide dismutase-1 mutation-related neurotoxicity in familial amyotrophic lateral sclerosis. *Amyotrophic lateral sclerosis and other motor neuron disorders : official publication of the World Federation of Neurology, Research Group on Motor Neuron Diseases,1*(3),143-161.doi:10.1080/14660820050515151
Simões, Pereira, Amaral, Nunes, Gomes, et al.(2013).Efficient recovery of proteins from multiple source samples after trizol® or trizol®LS RNA extraction and long-term storage. *BMC Genomics,14*(1),181.doi:10.1186/1471-2164-14-181
Singh, Kalsan, Kumar, Saini, & Chandra. (2015).Induced pluripotent stem cells: applications in regenerative medicine, disease modeling, and drug discovery. *Front Cell Dev Biol,3*,2.doi:10.3389/fcell.2015.00002
Slota, & Booth.(2019).MicroRNAs in Neuroinflammation: Implications in Disease Pathogenesis, Biomarker Discovery and Therapeutic Applications. *Noncoding RNA,5*(2).doi:10.3390/ncrna5020035
Stansley, Post, & Hensley.(2012).A comparative review of cell culture systems for the study of microglial biology in Alzheimer's disease. *J Neuroinflammation,9*,115.doi:10.1186/1742-2094-9-115

Subhramanyam, Wang, Hu, & Dheen.(2019).Microglia-mediated neuroinflammation in neurodegenerative diseases. *Semin Cell Dev Biol,94*,112-120.doi:10.1016/j.semcdb.2019.05.004

Suk, & Rousseaux.(2020).The role of TDP-43 mislocalization in amyotrophic lateral sclerosis. *Mol Neurodegener,15*(1),45.doi:10.1186/s13024-020-00397-1

Sun, Liu, Jickling, Sharp, & Yin.(2018).MicroRNA-based therapeutics in central nervous system injuries. *J Cereb Blood Flow Metab,38*(7),1125-1148.doi:10.1177/0271678X18773871

Sun, Luo, Guo, Su, & Liu.(2015).An updated role of microRNA-124 in central nervous system disorders: a review. *Front Cell Neurosci,9*,193.doi:10.3389/fncel.2015.00193

Tahamtan, Teymoori-Rad, Nakstad, & Salimi.(2018).Anti-Inflammatory MicroRNAs and Their Potential for Inflammatory Diseases Treatment. *9*.doi:10.3389/fimmu.2018.01377

Takei, Watanabe, Yuki, Akimoto, Sakata, et al.(2017).Edaravone and its clinical development for amyotrophic lateral sclerosis. *Amyotroph Lateral Scler Frontotemporal Degener,18*,5-10.doi:10.1080/21678421.2017.1353101

Talbott, Malek, & Lacomis.(2016).The epidemiology of amyotrophic lateral sclerosis. *Handb Clin Neurol,138*,225-238.doi:10.1016/B978-0-12-802973-2.00013-6

Tan, Furber, Thangaraj, Sobchishin, Doucette, et al.(2018).Organotypic Cultures from the Adult CNS: A Novel Model to Study Demyelination and Remyelination Ex Vivo. *Cell Mol Neurobiol,38*(1),317-328.doi:10.1007/s10571-017-0529-6

Tan, Ke, Ittner, & Halliday.(2017).ALS/FTLD: experimental models and reality. *Acta Neuropathol,133*(2),177-196.doi:10.1007/s00401-016-1666-6

Tovar, Santa-Cruz, & Tapia.(2009).Experimental models for the study of neurodegeneration in amyotrophic lateral sclerosis. *Mol Neurodegener,4*,31.doi:10.1186/1750-1326-4-31

Trabjerg, Andersen, Huntjens, Oklinski, Bolther, et al.(2021).Downregulating carnitine palmitoyl transferase 1 affects disease progression in the SOD1G93A mouse model of ALS. *Commun Biol,4*(1),509.doi:10.1038/s42003-021-02034-z

Tripathi, Rodriguez-Muela, Klim, de Boer, Agrawal, et al.(2017).Reactive Astrocytes Promote ALS-like Degeneration and Intracellular Protein Aggregation in Human Motor Neurons by Disrupting Autophagy through TGF-β1. *Stem Cell Reports,9*(2),667-680.doi.org/10.1016/j.stemcr.2017.06.008

Turner, Brabb, Pekow, & Vasbinder.(2011).Administration of substances to laboratory animals: routes of administration and factors to consider. *Journal of the American Association for Laboratory Animal Science: JAALAS,50*(5),600-613.

Van Damme, Robberecht, & Van Den Bosch.(2017).Modelling amyotrophic lateral sclerosis: progress and possibilities. *Dis Model Mech,10*(5),537-549.doi:10.1242/dmm.029058

van Es, Hardiman, Chio, Al-Chalabi, Pasterkamp, et al.(2017).Amyotrophic lateral sclerosis. *The Lancet,390*(10107),2084-2098.doi:10.1016/s0140-6736(17)31287-4

Vaz, Cunha, Gomes, Schmucki, Barbosa, et al.(2015).Glycoursodeoxycholic acid reduces matrix metalloproteinase-9 and caspase-9 activation in a cellular model of superoxide dismutase-1 neurodegeneration.*Mol Neurobiol,51*(3),864-877.doi:10.1007/s12035-014-8731-8

Vaz, Pinto, Ezequiel, Cunha, Carvalho, et al. (2019).Phenotypic Effects of Wild-Type and Mutant SOD1 Expression in N9 Murine Microglia at Steady State, Inflammatory and Immunomodulatory Conditions. *Front Cell Neurosci,13*,109.doi:10.3389/fncel.2019.00109

Vaz, Pinto, Sebastião, & Brites.(2021a).Astrocytes in Amyotrophic Lateral Sclerosis. In T. Araki (Ed.), *Amyotrophic Lateral Sclerosis*. Brisbane (AU): Exon Publications

Vaz, Vizinha, Morais, Colaco, Loch-Neckel, et al.(2021b).Overexpression of miR-124 in Motor Neurons Plays a Key Role in ALS Pathological Processes. *Int J Mol Sci,22*(11).doi:10.3390/ijms22116128

Wahid, Shehzad, Khan, & Kim.(2010).MicroRNAs: synthesis, mechanism, function, and recent clinical trials. *Biochim Biophys Acta,1803*(11),1231-1243.doi:10.1016/j.bbamcr.2010.06.013

Walker, Meadows, Merfeld-Clauss, Du, March, et al.(2018).Adipose-derived stem cell conditioned medium impacts asymptomatic peripheral neuromuscular denervation in the mutant superoxide dismutase (G93A) transgenic mouse model of amyotrophic lateral sclerosis. *Restor Neurol Neurosci,36*(5),621-627.doi:10.3233/RNN-180820

Waller, Wyles, Heath, Kazoka, Wollff, et al.(2017).Small RNA Sequencing of Sporadic Amyotrophic Lateral Sclerosis Cerebrospinal Fluid Reveals Differentially Expressed miRNAs Related to Neural and Glial Activity. *Front Neurosci,11*,731.doi:10.3389/fnins.2017.00731

Wang.(2021). Phylogenetic analysis of microRNA biomarkers for amyotrophic lateral sclerosis. *Biocell,45*(3),547-561.doi:10.32604/biocell.2021.014343

Wang, & Zhang.(2020).MicroRNAs in amyotrophic lateral sclerosis: from pathogenetic involvement to diagnostic biomarker and therapeutic agent development. *Neurol Sci,41*(12),3569-3577.doi:10.1007/s10072-020-04773-z

Warita, Itoyama, & Abe.(1999).Selective impairment of fast anterograde axonal transport in the peripheral nerves of asymptomatic transgenic mice with a G93A mutant SOD1 gene. *Brain research,819*(1-2),120-131.doi:10.1016/s0006-8993(98)01351-1

Xie, Zhao, Bosco, Nguyen, & Wu.(2022).Microglial TREM2 in amyotrophic lateral sclerosis. *Dev Neurobiol,82*(1),125-137.doi:10.1002/dneu.22864

Yamanaka, & Komine.(2018).The multi-dimensional roles of astrocytes in ALS. *Neurosci Res,126*, 31-38.doi:10.1016/j.neures.2017.09.011

Yelick, Men, Jin, Seo, Espejo-Porras, et al.(2020).Elevated exosomal secretion of miR-124-3p from spinal neurons positively associates with disease severity in ALS. *Exp Neurol,333*, 113414.doi:10.1016/j.expneurol.2020.113414

Yi, Doehle, Qin, Macara, & Cullen.(2005).Overexpression of exportin 5 enhances RNA interference mediated by short hairpin RNAs and microRNAs. *RNA,11*(2),220-226.doi:10.1261/rna.7233305

Yun, & Ha.(2020).CRISPR/Cas9-Mediated Gene Correction to Understand ALS. *Int J Mol Sci,21*(11).doi:10.3390/ijms21113801

Zhang, Liu, Liu, Li, Cheng, et al.(2018).Dynamic changes of CX3CL1/CX3CR1 axis during microglial activation and motor neuron loss in the spinal cord of ALS mouse model. *Transl Neurodegener,7*,35.doi:10.1186/s40035-018-0138-4

Zhao, Beers, & Appel.(2013).Immune-mediated mechanisms in the pathoprogression of amyotrophic lateral sclerosis. *J Neuroimmune Pharmacol, 8*(4), 888-899. doi:10.1007/s11481-013-9489-x

Zhao, Fu, Li, & Li.(2019).Neuroprotective Effects of Genistein in a SOD1-G93A Transgenic Mouse Model of Amyotrophic Lateral Sclerosis. *J Neuroimmune Pharmacol,14*(4),688-696.doi:10.1007/s11481-019-09866-x

Zhao, He, & Wang.(2021).MicroRNA-124: A Key Player in Microglia-Mediated Inflammation in Neurological Diseases. *Front Cell Neurosci,15*,771898.doi:10.3389/fncel.2021.771898

Zhong, Guo, Zhang, Zhang, Wang, et al.(2017).Bioinformatics prediction of miR-30a targets and its inhibition of cell proliferation of osteosarcoma by up-regulating the expression of PTEN. *BMC medical genomics,10*(1),64.doi:10.1186/s12920-017-0300-3

Zhou, Xu, Ren, Chen, & Xin.(2016).MicroRNA-124 (MiR-124) Inhibits Cell Proliferation, Metastasis and Invasion in Colorectal Cancer by Downregulating Rho-Associated Protein Kinase 1(ROCK1). *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology,38*(5),1785-1795.doi:10.1159/000443117

Zhou, Zhang, Guan, Chen, Lu, et al.(2018).Screening the expression characteristics of several miRNAs in G93A-SOD1 transgenic mouse: altered expression of miRNA-124 is associated with astrocyte differentiation by targeting Sox2 and Sox9. *J Neurochem,145*(1),51-67.doi:10.1111/jnc.14229

Zullo, Matsumoto, Xavier, Ratliff, & Goligorsky.(2015).The cell secretome, a mediator of cell-to-cell communication. *Prostaglandins Other Lipid Mediat,120*,17-20.doi:10.1016/j.prostaglandins.2015.03.012

ANNEX 1

Intrathecal injection of secretome from anti-miR-124-treated ALS motor neurons in mSOD1 mice shows promise as a therapeutic strategy

Barbosa M (1), Santos M (1), de Sousa N (2,3), Vaz A R (1,4), Salgado A J (2,3), Brites D (1,4)

(1) University of Lisbon, Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Lisbon, Portugal; (2) University of Minho, Life and Health Sciences Research Institute (ICVS), School of Medicine, Braga, Portugal; (3) ICVS/3B's Associate Lab, PT Government Associated Lab, Braga/Guimarães, Portugal; (4) University of Lisbon, Department of Pharmaceutic Sciences and Medicine, Faculty of Pharmacy, Lisbon, Portugal

Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease with a fast progression and without an effective treatment. Modulation of deregulated inflammatory miRNAs in ALS[1,2,3] might be a very promising therapy. We observed that these miRNAs are part of the secretome, either as free species or cargo in small extracellular vesicles[1,4,5]. Lately, we showed that the secretome from SOD1-G93A (mSOD1) cortical astrocytes with downregulated miRNA(miR)-146a treated with its mimic recovered motor neuron (MN) and microglia steady-state functions[2]. In the same way, neurodegeneration was prevented in mSOD1 MNs with upregulated miR-124 after transfection with anti-miR-124, and their secretome counteracted microglia activation and cell spinal pathogenicity[3].

Here, we assessed the therapeutic potential of the secretome from anti-miR-124 treated mSOD1 MNs in recovering disabilities in the mSOD1 mice at early disease onset by assessing motor performance, inflamma-miRNA profile, astrocyte/microglia phenotypes and synaptic dynamics.

The modulated and concentrated secretome was injected intrathecally in the 12-week-old mSOD1 mice. Control groups were WT and mSOD1 mice, injected with the vehicle (NSC-34 basal media). We performed the limb clasping and grasping tests to evaluate the corticospinal function and footprint test to assess gait quality. The lumbar spinal cord (SC) was isolated one week after motor tests to evaluate inflammatory miRNAs (miR-124, miR-146a, miR-155), glial reactivity gene markers (GFAP, iNOS, arginase 1, IL-10 and TREM2) and synaptic-related genes (synaptophysin and PSD-95) by RT-qPCR.

The non-treated mSOD1 mice exhibited changes in hindlimb clasping and grasping and performed shorter strides. In addition, an upregulation of GFAP, miR-146a and miR-155 and downregulation of iNOS, arginase 1, IL-10, TREM2 and miR-124 were detected in mSOD1 lumbar SC. Moreover, a *reduction of the post-synaptic PSD-95 with no changes in the pre-synaptic synaptophysin were also observed. However, the injected modulated secretome in mSOD1 mice* recovered behaviour alterations, namely motor performance and corticospinal function, while also *prevented the dysregulated miRNA profile and gene signature of neuronal/glial markers.*

In sum, the secretome from the anti-miR-124 treated mSOD1 MNs prevented many disabilities associated to MN and glial pathological mechanisms in the mSOD1 mice showing promise as a novel therapeutic tool to be translated into ALS clinics if validated in future studies.

[1]Pinto, S et al. Front Neurosci. 2017,11:273
[2]Barbosa, M et al. Front Cell Dev Biol. 2021, 9:634355
[3]Vaz AR et al. Int J Mol Sci. 2021, 22:6128
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Funding: SCML ELA-2015-002 and FCT (PTDC/MED-NEU/31395/2017, LISBOA-01-0145-FEDER-031395, UIDB/UIDP/04138/2020, UID/DTP/04138/2019, SFRH/BD/129586/2017).

Intrathecal injection of secretome from anti-miR-124-treated ALS motor neurons in mSOD1 mice shows promise as a therapeutic strategy

Marta Barbosa (1), Marta Santos (1), Nídia de Sousa (2,3), Ana Rita Vaz (1,4), António Salgado (2,3), Dora Brites (1,4)

´1) Neuroinflammation, Signaling and Neuroregeneration Lab, iMed.ULisboa, Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal
2) Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Braga, Portugal
3) ICVS/3B's Associate Lab, PT Government Associated Lab, Braga/Guimarães, Portugal
4) Department of Pharmaceutic Sciences and Medicine, Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal

Background/Aims

Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease with a fast progression and without an effective treatment. Modulation of deregulated inflammatory miRNAs in ALS[1,2,3] might be a very promising therapy. We observed that these miRNAs are part of the secretome, either as free species or cargo in small extracellular vesicles[1,4,5]. Lately, we showed that the secretome from SOD1-G93A (mSOD1) cortical astrocytes with downregulated miRNA(miR)-146a treated with its mimic recovered motor neuron (MN) and microglia steady-state functions[2]. In the same way, neurodegeneration was prevented in mSOD1 MNs with upregulated miR-124 after transfection with anti-miR-124, and their secretome counteracted microglia activation and cell spinal pathogenicity[3].

Here, we assessed the therapeutic potential of the secretome from anti-miR-124 treated mSOD1 MNs in recovering disabilities in the mSOD1 mice at early disease onset by assessing motor performance, inflamma-miRNA profile, astrocyte/microglia phenotypes and synaptic dynamics.

Methods

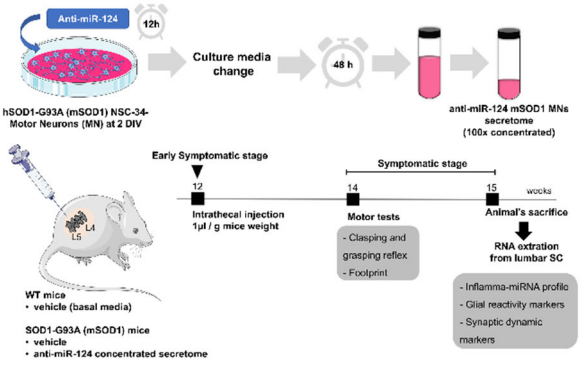

Fig. 1 – Schematic representation of the experimental procedure to obtain anti-miR-124 concentrated secretome, as well as, to perform the intrathecal injection of secretome and further motor behavior tests and molecular analysis in mice.

Results

ALS mice show deficits in corticospinal function, which is improved after intrathecal injection with anti-miR-124 neuronal secretome

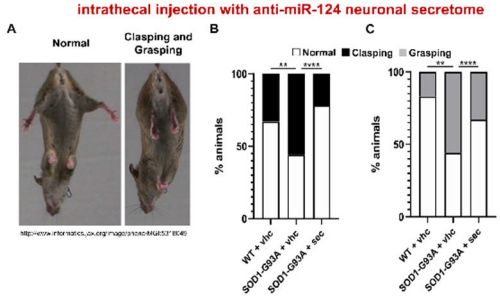

Fig. 2 - (A) Representative illustration of clasping and grasping reflex tests. The mice were suspended by holding their tails and their hindlimbs and toes position were observed for some seconds. An abnormal phenotype consists in retracting the hindlimbs toward the abdomen (clasping reflex) and/or curling the toes (grasping reflex) during the suspended time, meaning the presence of motor dysfunctionality. The percentage (%) of animals positive for **(B)** clasping and **(C)** grasping reflex. The group controls were both the WT (WT + vhc) and SOD1-G93A (mSOD1 + vhc) mice injected with NSC-34 basal media (vehicle). The anti-miR-124-derived secretome was injected in SOD1-G93A mice (SOD1-G93A + sec) to evaluate their therapeutic potential. Two weeks after the injection, the motor tests were performed. Results are mean (± SEM) from at least six experiments per group. $^{\wedge\wedge\wedge}$p<0.0001 and $^{\wedge\wedge}$p<0.01 *vs* WT or mSOD1, Chi-square (and Fisher's exact) test was used.

References:
[1]Pinto, S et al. Front Neurosci. 2017,11:273; [2]Barbosa, M et al. Front Cell Dev Biol. 2021, 9:634355; [3]Vaz AR et al. Int J Mol Sci. 2021, 22:6128; [4]Fernandes, A et al. Biochimie 2018, 155:67; [5]Garcia, G et al. Cells 2021, 10:2424

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Results

The anti-miR-124-MN derived secretome prevents abnormalities on the gait in ALS mice

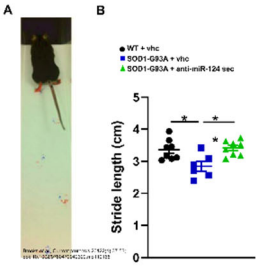


Fig. 3 - (A) Representative illustration of footprint test. The fore and hind paws of mice were painted with food dyes of different colours and placed over an absorbent paper to walk in a straight line. We measured the stride length (in centimetre, cm), which is the distance between the center of the plantar of the fore-foot and the center of the hind-foot plantar on the same side of the body, within the same stride. **(B)** The stride length (in centimetre, cm) performed by the mice. The group controls were both the WT (WT + vhc) and SOD1-G93A (mSOD1 + vhc) mice injected with NSC-34 basal media (vehicle). The anti-miR-124-derived secretome was injected in SOD1-G93A mice (SOD1-G93A + sec) to evaluate their therapeutic potential. Two weeks after the injection, the motor tests were performed. Results are mean (± SEM) from at least six experiments per group. **p<0.01 and *p<0.05 *vs* WT or mSOD1, unpaired and non-parametric Mann-Whitney test was used.

The lumbar SC of ALS mice shows a deregulation of inflamma-miRNAs and genes related with glial reactivity and synaptic signaling

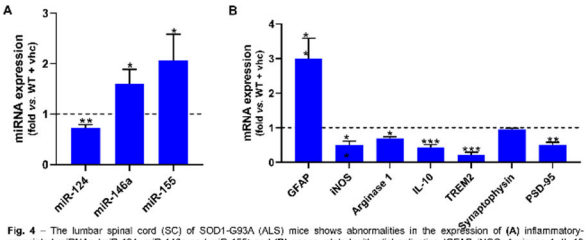


Fig. 4 – The lumbar spinal cord (SC) of SOD1-G93A (ALS) mice shows abnormalities in the expression of **(A)** inflammatory-associated miRNAs (miR-124, miR-146a and miR-155) and **(B)** genes related with glial activation (GFAP, iNOS, Arginase 1, IL-10 and triggering receptor expressed on myeloid cells 2 (TREM2)) and synaptic dynamics (synaptophysin and postsynaptic density protein 95 (PSD-95)). Results are mean (± SEM) from at least six experiments per group. ***p<0.001, **p<0.01and *p<0.05 *vs* WT + vehicle (vhc), unpaired t-student test was used.

The anti-miR-124 neuronal secretome prevents the dysregulated miRNA profile and gene signature of neuronal/glial markers in the lumbar SC of ALS mice

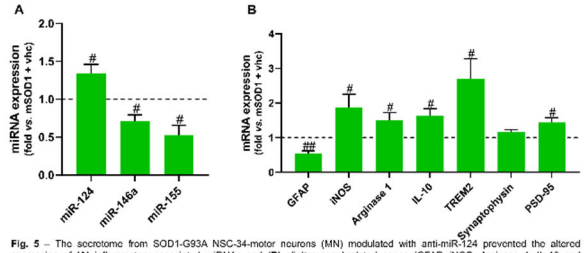
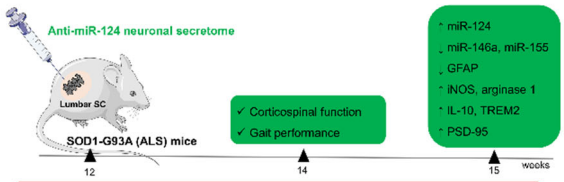


Fig. 5 – The secretome from SOD1-G93A NSC-34-motor neurons (MN) modulated with anti-miR-124 prevented the altered expression of **(A)** inflammatory-associated miRNAs and **(B)** glial/neuronal-related genes (GFAP, iNOS, Arginase 1, IL-10 and triggering receptor expressed on myeloid cells 2 (TREM2), synaptophysin and postsynaptic density protein 95 (PSD-95)) in the lumbar spinal cord (SC) of SOD1-G93A (ALS) mice. Results are mean (± SEM) from at least six experiments per group. $^{\#\#}$p<0.01and $^{\#}$p<0.05 *vs* mSOD1 + vehicle (vhc), unpaired t-student test was used.

Take Home message



The secretome from the anti-miR-124 treated SOD1-G93A MNs prevented some motor disabilities and pathological mechanisms associated to MNs and glia in the ALS mice, showing promise as a novel therapeutic tool to be translated into ALS clinics if validated in future studies.

ANNEX 2

NOVA SCHOOL OF SCIENCE & TECHNOLOGY

Jornadas Intercalares das Dissertações Anuais dos Mestrados 2022
Departamento de Ciências da Vida
Departamento de Química

CERTIFICADO DE PARTICIPAÇÃO

Certifica-se que

Marta Alexandra Marques dos Santos

participou nas **Jornadas Intercalares das Dissertações Anuais dos Mestrados dos Departamentos de Química e de Ciências da Vida 2022** da FCT/UNL, tendo realizado uma comunicação oral intitulada

Therapeutic potential of intrathecal aplication of miR-124 based secretome in the SOD1G93A mouse model of ALS

Estas Jornadas realizaram-se via ZOOM nos dias 10 e 11 de fevereiro de 2022.

Caparica, 10 de fevereiro de 2022

A Coordenação do Mestrado em Genética Molecular e Biomedicina

AlexandraRFernandes

(Professora Doutora Maria Alexandra Núncio de Carvalho Ramos Fernandes)

ANNEX 3

Intrathecal application of secretome from anti-miR-124-mSOD1 motor neurons as a therapeutic strategy for autologous transplantation in ALS disease

Santos M. (1), Barbosa M. (1), de Sousa N (2,3), Salgado A J (2,3), Vaz A. R. (1,4), Brites D. (1,4)

(1) University of Lisbon, Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Lisbon, Portugal

(2) University of Minho, Life and Health Sciences Research Institute (ICVS), School of Medicine, Braga, Portugal

(3) ICVS/3B's Associate Lab, PT Government Associated Lab, Braga/Guimarães, Portugal

(4) University of Lisbon, Department of Pharmaceutical Sciences and Medicines, Faculty of Pharmacy, Lisbon, Portugal

Keywords: ALS mouse model; anti-microRNA-124; motor neurons; cell-modulated secretome; glial cell activation/deactivation; intraspinal delivery

Introduction: Amyotrophic Lateral Sclerosis (ALS) is an incurable neurodegenerative disease characterized by motor neuron (MN) loss and glial activation. We showed that inflammatory-associated microRNAs (miRNAs) were dysregulated in neural cells and recapitulated in their secretome when using several ALS models [1,2,3]. We demonstrated that cell transfection with miRNA mimics/inhibitors recovered the MN/astrocyte homeostasis and that their secretome produced benefits in neighboring neural cells, including microglia, when using *in vitro* systems [4,5]. Among the evaluated miRNAs, the neuronal miRNA(miR)-124 was the most upregulated in SOD1G93A (mSOD1) MN-like cells (NSC-34) and responsible for MN degeneration, mitochondrial dysfunction, and axonal/synaptic dysregulation. Treatment with anti-miR-124 in mSOD1 MNs recovered neuronal function. Moreover, their secretome prevented glial reactivity and inflammatory-miRNA instability in spinal organotypic cultures from early symptomatic ALS mice [6]. [7,8].
Thus, we aimed to investigate the restorative effects of the secretome from pathological MNs modulated with the miR-124 inhibitor, after its injection into the spinal cord (SC) of the mSOD1 mice, by assessing inflamma-miRNA profile, astrocyte/microglia phenotypes, and neuronal synaptic dynamics, and later comparison with data from animal behavioral tests.

Materials and Methods: mSOD1 MNs were transfected with anti-miR-124, as usual in our lab [6]. The secretome from these treated cells was collected, concentrated, and intrathecally injected in early symptomatic mSOD1 mice (12-week-old) [9]. Control groups were WT and mSOD1 mice injected with the naïve MN secretome. At 15-week-old, the animals were sacrificed, and the lumbar SC was collected. Gene profile associated with MN degeneration, glial cell reactivity and hit inflammatory-associated miRNAs was assessed by RT-qPCR. GraphPad Prism software was used for data analysis and comparison with behavioral repertoire.

Results and Discussion: Pathological findings in the mSOD1 mice SC included the decrease of neuronal nuclei (NeuN), compromised post-synaptic signaling (PSD-95), impaired retrograde/anterograde axonal transport (dynein, kinesin) and reduced mitochondrial fission (Drp1). For glial cells, we observed: (i) reduction of microglial phagocytosis (MFGE8, TREM2) and enhanced immunoreactivity (CX3CR1, P2RY12, TIMP2, TMEM119, GPR17); (ii) increased astrocyte reactivity (GFAP, CX43); and (iii) myelination deficits (MBP, PLP). Downregulated IL-10, iNOS and arginase-1, and overexpressed TNFα, miR-124, miR-155, miR-146a and miR-21 reinforced the deregulated immune balance in the

ALS mice. Most of these pathological events were prevented by the secretome from anti-miR-124-treated ALS MNs. Indeed, we succeeded in restoring the secretome neuroprotective properties by pushing NeuN and PSD-95 toward physiological levels, as well as upregulating microglial phagocytic-associated TREM2, oligodendrocyte PLP, and parenchymal IL-10, iNOS, and arginase-1 levels after its administration in the mSOD1 mice. Finally, the reestablishment of the inflammatory dynamic balance was supported by the reduction of GFAP, Cx43, miR-155 and miR-146a levels.

Conclusion: Collectively, these data suggests that the secretome derived from ALS MNs engineered with anti-miR-124 might be a promising cell-free based therapeutic strategy in ALS patients to halt/delay disease progression. Preclinical studies should validate the proposed approach in stratified patients with upregulated miR-124 before its translation into clinics. As an autologous treatment it would contribute to precision medicine in ALS.

Funded by Fundação para a Ciência e a Tecnologia (FCT): PTDC/MED-NEU/31395/2017, LISBOA-01-0145-FEDER-031395 and La Caixa Foundation HR21-00931, (to D.B), and partially UIDB/UIDP/04138/2020 and UID/DTP/04138/2019-2020 (to iMed.ULisboa).

1. Pinto S, Cunha C, Barbosa M, Vaz AR, Brites D. Exosomes from NSC-34 cells transfected with hSOD1-G93A are enriched in mir-124 and drive alterations in microglia phenotype. Front Neurosci. 2017;11:273.

2. Gomes C, Cunha C, Nascimento F, Ribeiro JA, Vaz AR, Brites D. Cortical Neurotoxic Astrocytes with Early ALS Pathology and miR-146a Deficit Replicate Gliosis Markers of Symptomatic SOD1G93A Mouse Model. Mol Neurobiol. 2019;56(3):2137–58.

3. Gomes C, Sequeira C, Barbosa M, Cunha C, Vaz AR, Brites D. Astrocyte regional diversity in ALS includes distinct aberrant phenotypes with common and causal pathological processes. Exp Cell Res. 2020;395(2).

4. Barbosa M, Gomes C, Sequeira C, Gonçalves-Ribeiro J, Pina CC, Carvalho LA, et al. Recovery of Depleted miR-146a in ALS Cortical Astrocytes Reverts Cell Aberrancies and Prevents Paracrine Pathogenicity on Microglia and Motor Neurons. Front Cell Dev Biol. 2021;9:1–28.

5. Gomes C, Sequeira C, Likhite S, Dennys CN, Kolb SJ, Shaw PJ, et al. Neurotoxic Astrocytes Directly Converted from Sporadic and Familial ALS Patient Fibroblasts Reveal Signature Diversities and miR-146a Theragnostic Potential in Specific Subtypes. 2022;

6. Vaz AR, Vizinha D, Morais H, Colaço AR, Loch-Neckel G, Barbosa M, et al. Overexpression of mir-124 in motor neurons plays a key role in als pathological processes. Int J Mol Sci. 2021;22(11).

7. Sandonà M, Di Pietro L, Esposito F, Ventura A, Silini AR, Parolini O, et al. Mesenchymal Stromal Cells and Their Secretome: New Therapeutic Perspectives for Skeletal Muscle Regeneration. Front Bioeng Biotechnol. 2021;9:1–16.

8. Kim D, Kim S, Sung A, Patel N, Wong N, Conboy MJ, et al. Autologous treatment for ALS with implication for broad neuroprotection. Transl Neurodegener. 2022;11(1):1–19.

9. Hu HJ, Lin HQ, Duan WS, Cui C, Li ZY, Liu YK, et al. Intrathecal Injection of scAAV9–hIGF1 Prolongs the Survival of ALS Model Mice by Inhibiting the NF-kB Pathway. Neuroscience. 2018;381:1–10.

ANNEX 4

Intrathecal injection of the secretome from anti-miR-124 treated pathological MNs in ALS mice provides neuroprotection

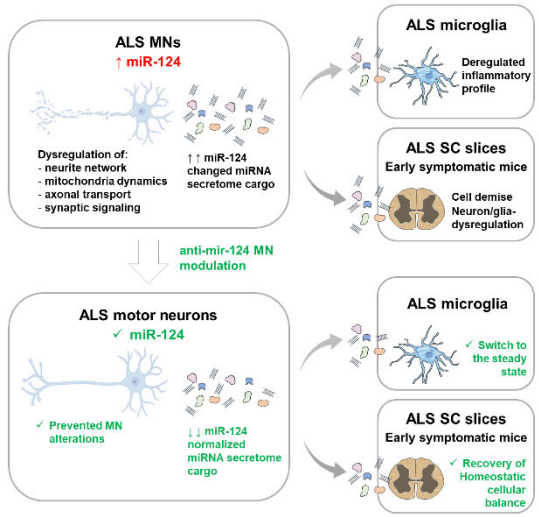
Marta Barbosa (1), Marta Santos (1), Nídia de Sousa (2,3), Ana Rita Vaz (1,4), António Salgado (2,3), Dora Brites (1,4)

(1) Neuroinflammation, Signaling and Neuroregeneration Lab, iMed.ULisboa, Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal
(2) Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Braga, Portugal
(3) ICVS/3B's Associate Lab, PT Government Associated Lab, Braga/Guimarães, Portugal
(4) Department of Pharmaceutic Sciences and Medicine, Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal

Background / Aims

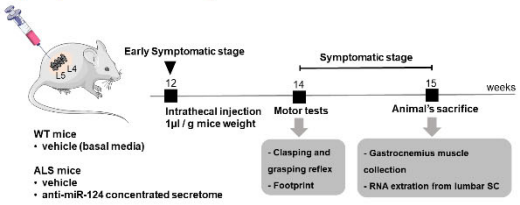
Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease with a fast progression and without an effective treatment. Modulation of deregulated inflammatory miRNAs in ALS[1,2,3] might be a very promising therapy. We observed that these miRNAs are part of the secretome either as free species or cargo in small extracellular vesicles[1,4,5]. In particular, modulation of miR-124 in ALS motor neurons (MNs) prevented neurodegeneration and their secretome counteracted microglia activation and cell spinal pathogenicity[3]. Here, we assessed the therapeutic potential of the secretome from anti-miR-124 treated ALS MNs in recovering disabilities in the ALS mice at early disease onset by assessing motor performance, muscle integrity, inflamma-miRNA profile and markers associated with MN functionality, immune cell phenotypes and myelinization.

Targeting upregulated miR-124 in ALS MNs prevents cell dysregulation and paracrine deleterious effects



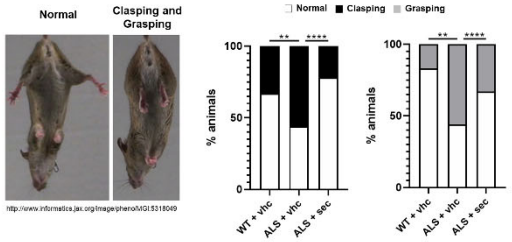
[3]Vaz AR et al. Int J Mol Sci. 2021

Experimental design



Results

Deficits in corticospinal function are counteracted by intrathecal injection of the secretome from anti-miR-124 treated pathological MNs in the ALS mice



The group controls were both the WT (WT + vhc) and ALS (ALS + vhc) mice injected with NSC-34 basal media (vehicle). The anti-miR-124-derived secretome was injected in ALS mice (ALS + sec) to evaluate their therapeutic potential. Two weeks after the injection, the clasping and grasping tests were performed. Results are mean (± SEM) from at least six experiments per group. ****p<0.0001 and **p<0.01 *vs* WT or ALS, Chi-square (and Fisher's exact) test was used.

References:
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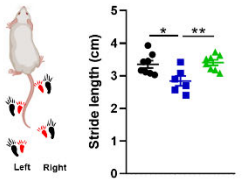
Intrathecal injection of the secretome from anti-miR-124 treated pathological MNs in the ALS mice prevents gait abnormalities



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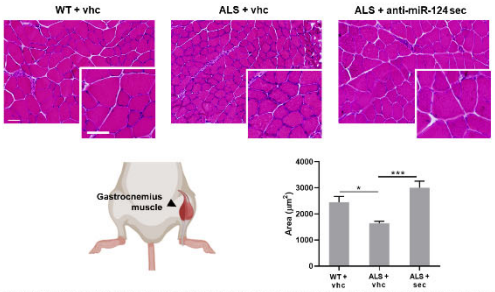
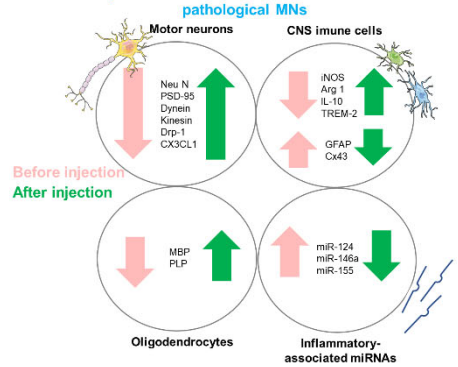
Deficits in the muscle fiber area are recovered by intrathecal injection of the secretome from anti-miR-124 treated pathological MNs in the ALS mice



Gastrocnemius muscle transversal sections from control animals injected with the vehicle (WT + vhc and ALS + vhc) and treated ALS mice with the secretome derived from anti-miR-124-treated ALS motor neurons (ALS + sec), both stained with hematoxylin–eosin. Scale bar: 50 µm. The average of muscle fibers area was measured. Results are mean ± SEM from at least 3 independent experiments * p < 0.05 and *** p < 0.001, one-way ANOVA followed by Bonferroni post hoc test was used.

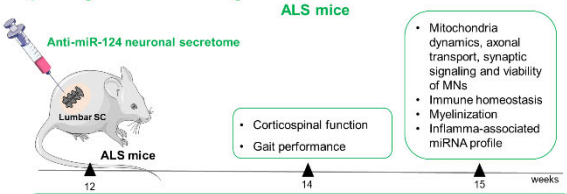
Dysregulated gene and miRNA signatures in ALS mice are prevented by intrathecal injection of the secretome from anti-miR-124 treated pathological MNs



Postsynaptic density protein 95 (PSD-95), Dynamin-related protein 1 (Drp-1), C-X3-C Motif Chemokine Ligand 1 (CX3CL1), Inducible Nitric Oxide Synthase (iNOS), Arginase 1 (Arg 1), Interleukin 10 (IL-10), Triggering receptor expressed on myeloid cells 2 (TREM-2), Glial fibrillary acidic protein (GFAP), Connexin 43 (Cx-43), Myelin basic protein (MBP), Myelin proteolipid protein 1 (PLP)

Take Home Message

The secretome from the anti-miR-124 treated ALS MNs prevents MN and glial pathological mechanisms together with amelioration of motor disabilities in the ALS mice



The secretome from the anti-miR-124 treated ALS MNs shows a promise therapeutic option to be translated in ALS clinics if validated in future studies.

ANNEX 5

Potencial regenerativo de secretoma derivado de neurónios motores modulados com microRNAs em modelos animais de Esclerose Lateral Amiotrófica

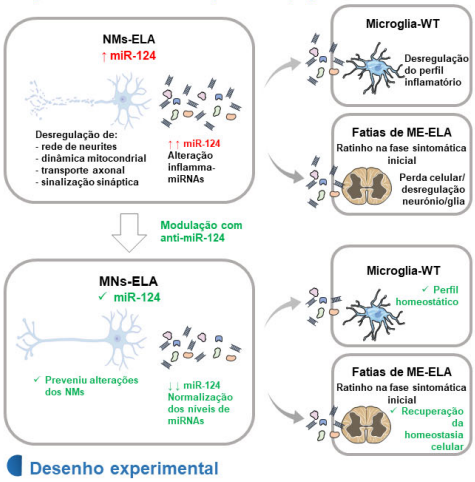
Marta Barbosa (1), Marta Santos (1), Nídia de Sousa (2,3), Ana Rita Vaz (1,4), António Salgado (2,3), Dora Brites (1,4)

(1) Grupo Neuroinflamação, Sinalização e Neuroregeneração, iMed.ULisboa, Faculdade de Farmácia, Universidade de Lisboa, Lisboa, Portugal
(2) Instituto de Investigação em Ciências da Vida e Saúde, Escola de Medicina, Universidade do Minho, Braga, Portugal
(3) Laboratório associado ao ICVS/3B's, Universidade do Minho, Braga/Guimarães, Portugal
(4) Departamento de Ciências Farmacêuticas e do Medicamento, Faculdade de Farmácia, Universidade de Lisboa, Lisboa, Portugal

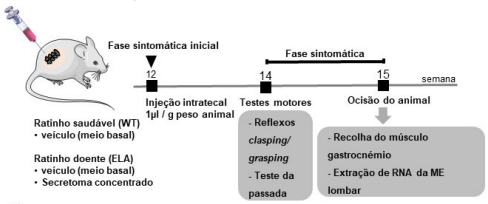
Introdução / Objectivos

A Esclerose Lateral Amiotrófica (ELA) é uma doença fatal, com uma progressão rápida e sem uma terapia eficaz. A modulação de micro(mi)RNAs associados à inflamação poderá constituir um avanço promissor para o tratamento da ELA[A,B,C]. Nós observámos que estes miRNAs circulam no secretoma livremente ou encapsulados em pequenas vesículas extracelulares[A,D,E]. Em particular, a modulação do miR-124 nos neurónios motores mutados (NMs-ELA) preveniu a neurodegeneração; para além disso, o secretoma dessas células preveniu a ativação microglial e a patogenicidade observada em culturas organotípicas da medula espinhal (ME)[C]. Neste trabalho, avaliámos o potencial do secretoma derivado dos NMs-ELA na recuperação da incapacidade motora de ratinhos ELA na fase inicial da doença. Para tal, analisámos a função motora, integridade muscular, perfil de miRNAs associados à inflamação e marcadores associados à funcionalidade dos NMs, bem como aqueles associados aos fenótipos das células gliais (astrócitos/microglia), função imune e capacidade de mielinização.

A reversão dos níveis elevados de miR-124 nos NMs-ELA previne a desregulação celular e os efeitos parácrinos prejudiciais dos NMs-ELA[C]

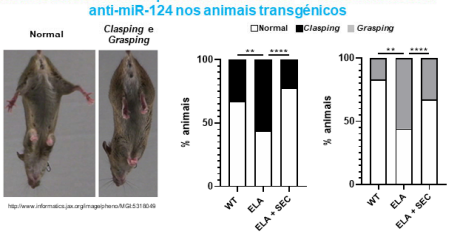


Desenho experimental



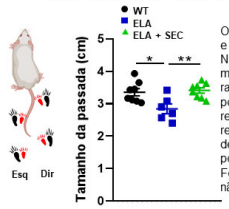
Resultados

Prevenção do défice da função corticoespinhal após injeção intratecal do secretoma proveniente dos NMs-ELA modulados com anti-miR-124 nos animais transgénicos



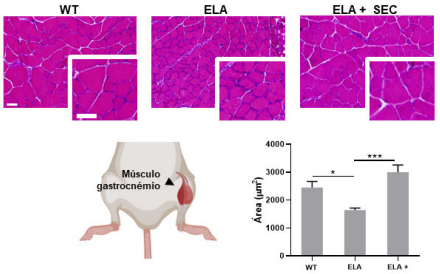
Os grupos controlo são os animais saudáveis (WT) e transgénicos (ELA) injetados com o meio basal dos NMs (veículo). O secretoma derivado dos NMs-ELA modulados com o anti-miR-124 foi injetado no ratinho ELA (ELA + SEC). Duas semanas após a injeção, realizaram-se os testes *clasping* e *grasping*. Os resultados resultam da média (± desvio padrão da média) de pelo menos 6 animais por grupo, sendo apresentados em percentagem (%). ***p<0.0001 e **p<0.01 *vs* WT ou ELA. Foi utilizado o teste do qui-quadrado (e exato de Fisher).

A injeção intratecal do secretoma proveniente dos NMs ELA tratados com anti-miR-124 previne anomalias na passada dos animais ELA



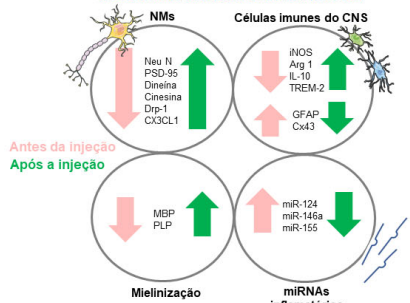
Os grupos controlo são os animais saudáveis (WT) e doentes (ELA) injetados com o meio basal dos NMs (veículo). O secretoma derivado dos NMs-ELA modulados com o anti-miR-124 foi injetado no ratinho doente (ELA + SEC) para avaliar o seu potencial terapêutico. O teste da passada foi realizado duas semanas após a injeção. Os resultados estão representados pela média (± desvio padrão da média) de pelo menos 6 animais por condição. **p<0.01 e *p<0.05 *vs.* WT ou ELA. Foi usado o teste *Mann-Whitney* não-paramétrico e não-emparelhado.

Recuperação da área da fibra muscular dos animais transgénicos após a injeção do secretoma obtido dos NMs ELA modulados com anti-miR-124



Secções transversais do músculo gastrocnémio de animais saudáveis injetados com o veículo (WT), e animais doentes injetados com veículo (ELA) ou com o secretoma (ELA + SEC), corados com hematoxilina-eosina. Barra de escala: 50 µm. Foi obtida a média da área das fibras musculares. (± desvio padrão da média), de pelo menos 3 animais por condição. * p < 0.05 e *** p < 0.001. Foi feita uma Análise de Variância simples *(One way ANOVA)* e aplicada a correção de *Bonferroni*.

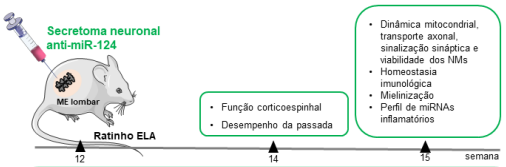
A desregulação do perfil génico e de miRNAs na ME do modelo animal de ELA é prevenida pela injeção intratecal do secretoma proveniente dos NMs-ELA tratados com anti-miR-124



Postsynaptic density protein 95 (PSD-95), Dynamin-related protein 1 (Drp-1), C-X3-C Motif Chemokine Ligand 1 (CX3CL1), Inducible Nitric Oxide Synthase (iNOS), Arginase 1 (Arg 1), Interleukin 10 (IL-10), Triggering receptor expressed on myeloid cells 2 (TREM-2), Glial fibrillary acidic protein (GFAP), Connexin 43 (Cx-43), Myelin basic protein (MBP), Myelin proteolipid protein 1 (PLP)

Conclusão

O secretoma proveniente de NMs modulados com anti-miR-124 previne os mecanismos patológicos associados aos NMs/glia e melhora os défices motores observados no modelo animal de ELA



O secretoma proveniente de NMs mutados e modulados com anti-miR-124 mostrou ser uma opção terapêutica promissora para ser aplicada em medicina regenerativa, se validada em estudos futuros com modelos animais e células de doentes.

Referências:
[A]Pinto, S et al. Front Neurosci. 2017,11:273; [B]Barbosa, M et al. Front Cell Dev Biol. 2021, 9:634355; [C]Vaz AR et al. Int J Mol Sci. 2021, 22:6128; [D]Fernandes, A et al. Biochimie 2018, 155:67; [E]Garcia, G et al. Cells 2021, 10:2424

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SCML ELA-2015-002 (to DB)
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ANNEX 6

P107: Secretome from anti-miR-124-treated ALS motor neurons prevents microglia and inflammatory miRNA dysfunction after intrathecal injection in mSOD1 mice

Santos M. (1), Barbosa M. (1), de Sousa N. (2,3), Salgado A.J. (2,3), Vaz A.R. (1,4), Brites D. (1,4)

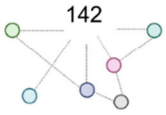
(1) Neuroinflammation, Signaling and Neuroregeneration Lab, Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa, Lisbon, Portugal; (2) University of Minho, Life and Health Sciences Research Institute (ICVS), School of Medicine, Braga, Portugal; (3) ICVS/3B's Associate Lab, PT Government Associated Lab, Braga/Guimarães, Portugal; (4) Department of Pharmaceutical Sciences and Medicines, Faculty of Pharmacy, Universidade de Lisboa, Lisbon, Portugal

Amyotrophic Lateral Sclerosis (ALS) is an incurable neurodegenerative disease characterized by motor neuron (MN) loss and microglia activation. Inflammatory(inflamma)-associated microRNAs (miRNAs) were found dysregulated in SOD1G93A (mSOD1) mouse models, as well as in MNs and microglia and their secretome [1-3]. We demonstrated that treatment with miR-124 inhibitor (anti-miR-124) in mSOD1 MNs recovered cell function, while their secretome prevented microglia activation and inflammatory-miRNA instability in spinal cord (SC) organotypic cultures from early symptomatic ALS mice [3]. Therapeutic benefits of the secretome from pathological MNs after miR-124 modulation was never explored in the ALS mSOD1 mouse model, though recent evidence sustain the existence of therapeutic effects for the secretome from different cell types. Thus, we aimed to explore the benefits of the secretome from pathological MNs treated with anti-miR-124 in the ALS mice. For that we assessed gene and protein profile associated with microglia reactivity and inflammatory-miRNA signature in the SC of mSOD1 mice, after intrathecal injection of such modulated ALS MN-derived secretome. This secretome was concentrated and administered in early symptomatic mSOD1 mice (12-week-old). Controls were WT and mSOD1 mice injected with the MN culture media. At 15-week-old, the animals were sacrificed, and the SC collected to assess gene (RT-qPCR) and protein (western blot and immunohistochemistry) profiles. The mSOD1 mouse SC showed a reduction of genes associated with microglial phagocytosis (MFGE8/TREM2) and immunoreactivity (CX3CR1/P2RY12/ TIMP2/TMEM119/GPR17), validating the existence of microglia activation in the ALS mouse model. We also observed an altered inflammatory-dynamic balance supported by downregulated iNOS/arginase-1, and overexpressed inflamma-miR-124/miR-155/miR-146a/miR-21. At the protein level, we noticed increased levels of the microglia-specific marker Iba1 and elevated levels of Iba1+ cells in the lumbar SC slices. Administration of the secretome from anti-miR-124-treated ALS MNs prevented most of these pathological events, namely the microglia-inflammatory markers iNOS/arginase-1, the inflamma-miR-155 and miR-146a, while enhanced TREM2 and IL-10. Moreover, Iba1 protein levels and Iba+ cells were restored towards physiological levels, suggesting a calming effect on microglia activation. Together, these data point to the pathological secretome from ALS MNs engineered with anti-miR-124 as having therapeutic interest in preventing neuroinflammation after autologous transplantation, supporting its potential translation into clinics.

Keywords: ALS mouse model; anti-microRNA-124; microglia activation/deactivation; secretome-based therapy; miRNA inflammatory profile

Acknowledgements: Funded by Santa Casa da Misericórdia de Lisboa: ELA-2015-002 (DB); Fundação para a Ciência e a Tecnologia (FCT): PTDC/MED-NEU/31395/2017, LISBOA-01-0145-FEDER-031395 (to DB), UIDB/UIDP/04138/2020 and UID/DTP/04138/2019-2020
(iMed.ULisboa) and by La Caixa Foundation-Luzón Foundation through project HR21-00931 (DB)

References: [1] Cunha, C. et al. 2018, D. Mol Neurobiol., 55(5), 4207–24. [2] Butovsky, O. et al. 2015, Ann Neurol., 77(1), 75–99; [3] Vaz, A.R. et al. 2021, Int J Mol Sci., 22(11), 6128.

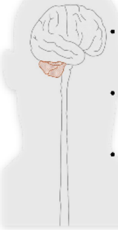


Secretome from anti-miR-124-treated ALS motor neurons prevents microglia and inflammatory miRNA dysfunction after intrathecal injection in mSOD1 mice

Marta Santos (1), Marta Barbosa (1), Nídia de Sousa (2,3), António Salgado (2,3), Ana Rita Vaz (1,4), Dora Brites (1,4)

(1) Neuroinflammation, Signalling and Neuroregeneration Lab, Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa, Lisbon, Portugal;
(2) University of Minho, Life and Health Sciences Research Institute (ICVS), School of Medicine, Braga, Portugal;
(3) ICVS/3B's Associate Lab, PT Government Associated Lab, Braga/Guimarães, Portugal;
(4) Department of Pharmaceutical Sciences and Medicines, Faculty of Pharmacy, Universidade de Lisboa, Lisbon, Portugal

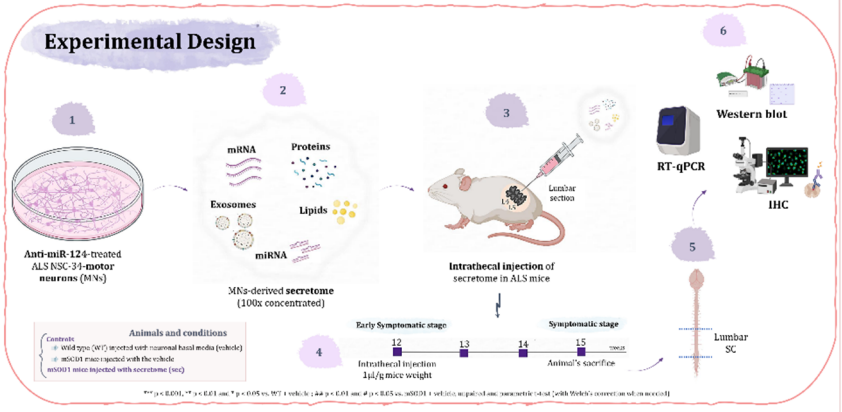
Background



- Amyotrophic Lateral Sclerosis (ALS) → degeneration of motor neurons (MNs) in the motor cortex, brainstem and spinal cord (SC) and **glial activation**
- **Inflammatory-miRNA profile** is dysregulated in SOD1G93A (mSOD1) mouse model, as well as in MNs and microglia and their secretome [1-3]
- Treatment with **anti-miR-124** in mSOD1 MNs prevented cell dysfunction, while their secretome prevented microglial activation and inflammatory-miRNA instability in SC organotypic cultures from early symptomatic ALS mice [3]

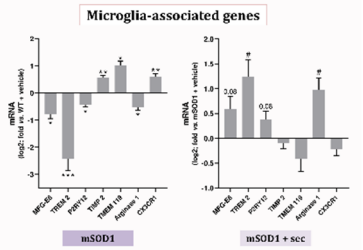
What are the **benefits** of the **secretome** derived from mSOD1 MNs modulated with anti-miR-124 to **microglia reactivity** and **inflammatory-miRNA signature** in the ALS mice?

Experimental Design

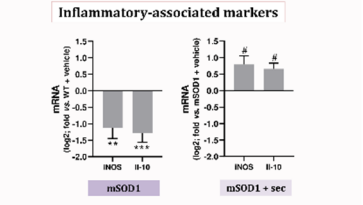


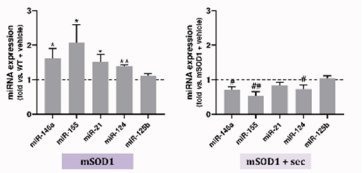
Findings

(A) mRNA expression



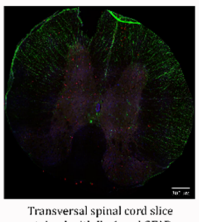
The secretome was able to prevent the compromised microglial phagocytosis and immunoreactivity



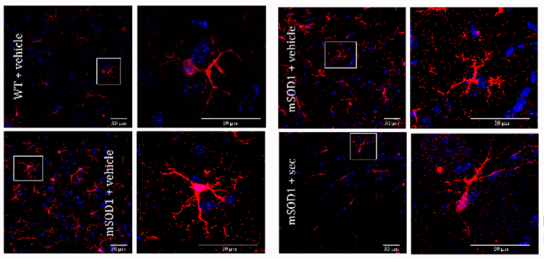


The secretome prevented the deregulated inflammatory status by re-establishing the levels of iNOS, increasing IL-10 and downregulating inflamma-miRNAs

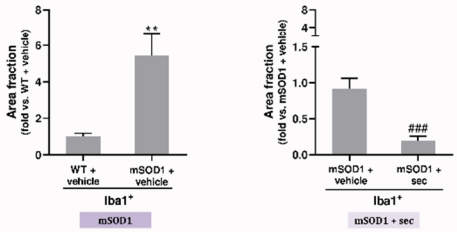
(C) Immunohistochemistry



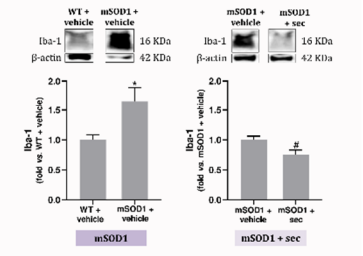
Transversal spinal cord slice stained with Iba1 and GFAP



The secretome induced Iba$^+$ cells towards physiological levels, suggesting a calming effect on microglial activation



(B) Protein expression



The secretome was able to prevent microglia activation by the reduction of microglia-specific marker Iba1

Conclusions

The secretome from ALS MNs engineered with anti-miR-124 prevented most of the pathological events observed in mSOD1 mice, namely the microglia activation and the altered inflammatory status. Thus, it shows therapeutic interest in preventing neuroinflammation after transplantation, supporting its potential translation into clinics.

Funding

SCML ELA-2015-002

FCT (PTDC/MED-NEU/31395/2017, LISBOA-01-0145-FEDER-031395, UIDB/UIDP/04138/2020, UID/DTP/04138/2019, SFRH/BD/129586/2017), La Caixa Foundation HR21-00931 and Fundación Luzón to DB

References

[1] Cunha C, Santos C, Gomes C, Fernandes A, Correia AM, Sebastião AM, Vaz, A.R, Brites, D. Mol Neurobiol. 2018;55(5):4207–24.
[2] Butovsky O, Jedrychowski MP, Cialic R, Krasemann S, Fanek Z, Greco DJ, et al. Ann Neurol. 2015;77(1):75–99.
[3] Vaz AR, Vizinha D, Morais H, Colaço AR, Loch-Neckel G, Barbosa M., Brites D. Int J Mol Sci. 2021;22(11). 6128.

13th iMed.ULisboa Postgraduate Students Meeting

July 4-5th 2022

Poster Pitch Certificate

The ipSC hereby acknowledges that:

Marta Alexandra Marques dos Santos

presented a poster pitch on the 13th iMed.Ulisboa Meeting, which took place on the 4th and 5th of July 2022.

ipSC
iMed.ULisboa Postgraduate Students Commission

imed
Instituto de Investigação do Medicamento

João Gonçalves
Coordinator of iMed

ANNEX 7

Neuroinflammation, Signaling and Neuroregeneration

PI: Dora Brites

OC18: Secretome from anti-miR-124-treated ALS motor neurons manifests therapeutic potential after intrathecal injection in the transgenic mice

Barbosa M. (1), Santos M. (1), de Sousa N. (2,3), Vaz A. R. (1,4), Salgado A. J. (2,3), Brites D. (1,4)

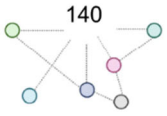
(1) Neuroinflammation, Signaling and Neuroregeneration Lab, Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal; (2) Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Braga, Portugal; (3) ICVS/3B's Associate Lab, PT Government Associated Lab, Braga/Guimarães, Portugal; (4) Department of Pharmaceutical Sciences and Medicines, Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal

Amyotrophic Lateral Sclerosis (ALS) is a fast progressive and fatal neurodegenerative disease without an effective treatment. We showed that changes in inflammatory-associated microRNAs in motor neurons (MNs) and glial cells contribute to ALS pathophysiology and that their cell modulation may constitute a promising therapy [1-3]. Such studies also demonstrated that the recovery of homeostatic balance derived from either the miRNAs released as free species or as cargo in exosomes after being collected by recipient cells. Moreover, the normalization of upregulated miR-124 in SOD1G93A (ALS) MNs repaired their function and the released secretome protected microglia activation and spinal cord pathogenicity in the ALS mice [3]. Here, we aimed to test the intrathecal injection of such secretome in the 12-week-old ALS mice, for benefits in the improvement of their motor disabilities and prevention of neurodegeneration and astrocyte reactivity, when compared to wild type and ALS mice injected with the vehicle (MN basal media). Changes in hindlimb clasping and grasping and shorter strides were observed in ALS mice. Atrophied muscle fibers, together with increased neurodegeneration and astrocyte reactivity in lumbar spinal cord (LSC) were also noticed. At the molecular level, we noticed a downregulation of genes associated with neuronal loss (Neu N) and compromised function (dynein/kinesin/PSD-95). After validating such impairments in the ALS mice, we assessed the reparative potential of the intrathecal injection of the secretome from anti-miR-124-treated ALS MNs. We obtained improvements in the limb clasping and grasping, together with the footprint test two weeks after injection. The histological analysis (H&E staining) of the gastrocnemius muscle revealed a recovery of the muscle fibers' area. When assessing LSC using immunohistochemistry, western blot, and RT-qPCR we verified a decrease of MN degeneration (Fluoro-Jade staining) and demise (Neu N), together with the repair of the postsynaptic activity (PSD-95) and axonal dynamics (dynein/kinesin). In addition, the tested strategy also regulated Cx43-mRNA and GFAP-protein/gene expression levels demonstrating to further prevent astrocyte reactivity. Overall, the secretome from anti-miR-124-treated ALS MNs showed promise to prevent neuronal- and astrocyte-associated pathological mechanisms, as well as motor disabilities in the ALS mice, supporting its translation into clinics and autologous application in patients.

Keywords: miR-124 inhibitor to stop ALS progression; secretome-based therapy; astrocyte repair; neuroregeneration; motor recovery

Acknowledgements: Funded by Santa Casa da Misericórdia de Lisboa: ELA-2015-002 (DB); Fundação para a Ciência e a Tecnologia (FCT): PTDC/MED-NEU/31395/2017, LISBOA-01-0145-FEDER-031395 (DB), UIDB/UIDP/04138/2020 and UID/DTP/04138/2019-2020 (iMed.ULisboa) and by La Caixa Foundation-Luzón Foundation through project HR21-00931 (DB).

References: [1] Gomes C. et al. 2022, D. Neurotoxic. Cells, 11, 1186; [2] Barbosa M. et al. 2021, D. Front Cell Dev Biol., 9, 634355; [3] Vaz A.R. et al. 2021, D. Int J Mol Sci., 22(11), 6128.



ANNEX 8

Secretome from anti-miR-124-treated ALS motor neurons shows therapeutic potential after intrathecal injection in the early symptomatic ALS mouse model

M. Barbosa (1), M. Santos (1), N. de Sousa (2,3), A. R. Vaz (1,4), A. J. Salgado (2,3), D. Brites (1)

(1) Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal;
(2) Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Braga, Portugal;
(3) ICVS/3B's Associate Lab, PT Government Associated Lab, Braga/Guimarães, Portugal;
(4) Department of Pharmaceutical Sciences and Medicines, Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal

Dysregulation of inflammatory (inflamma)-miRNAs in cells and their dissemination via secretome contribute to Amyotrophic Lateral Sclerosis (ALS) pathophysiology and their regulation may constitute a therapeutic approach. We showed that transfection of mutant SOD1G93A (mSOD1) motor neurons (MNs) with anti-miR-124 prevented neurodegeneration and its secretome counteracted spinal pathogenicity in organotypic cultures from early symptomatic (12-week-old) mSOD1 mice (Vaz AR et al. (2021) Int J Mol Sci 22:6128). Therefore, we aimed to test the potential benefit properties of the modulated concentrated secretome after intrathecal injection in mSOD1 mice with 12-week-old. We evaluated motor performance, inflamma-miRNA profile and glial/synaptic markers. WT and mSOD1 mice were injected with the MN basal media as control. Two weeks later, we performed the limb clasping/grasping tests to evaluate the corticospinal function and footprint test to assess gait quality. The lumbar spinal cord (SC) was isolated from 15-week-old mice and molecular evaluation was performed by RT-qPCR. The mSOD1 mice developed hind-limb clasping and grasping and performed shorter strides. Homogenates of their lumbar SC showed overexpressed GFAP and miR-146a/-155, together with iNOS, arginase 1, IL-10, TREM2, post-synaptic PSD-95 and PLP downregulation, evidencing neuro-immune dysregulation and myelination deficits. The injection of the treated secretome not only recovered the motor performance and corticospinal function in the mSOD1 mouse model, but also normalized the expression of inflamma-miRNAs and genes related with glial activation, synaptic function and myelination. Overall, this study validates the potential of anti-miR-124 MN secretome in recovering early motor disabilities associated with MN/glia pathogenicity, opening new promises for the treatment of patients with mutations in the SOD1 gene.

FCT (PTDC/MED-NEU/31395/2017, LISBOA-01-0145-FEDER-031395, UIDB/UIDP/04138/2019-20, SFRH/BD/129586)

Secretome from anti-miR-124-treated ALS motor neurons shows therapeutic potential after intrathecal injection in the early symptomatic ALS mouse model

Marta Barbosa (1), Marta Santos (1), Nídia de Sousa (2,3), Ana Rita Vaz (1,4), António Salgado (2,3), Dora Brites (1,4)

(1) Neuroinflammation, Signaling and Neuroregeneration Lab, iMed.ULisboa, Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal

(2) Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Braga, Portugal

(3) ICVS/3B's Associate Lab, PT Government Associated Lab, Braga/Guimarães, Portugal

(4) Department of Pharmaceutic Sciences and Medicine, Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal

Background/Aims

Dysregulation of inflammatory (inflamma)-miRNAs in cells and their dissemination via secretome contribute to Amyotrophic Lateral Sclerosis (ALS) pathophysiology and their regulation may constitute a therapeutic approach. We showed that transfection of mutant SOD1G93A (mSOD1) motor neurons (MNs) with anti-miR-124 prevented neurodegeneration and its secretome counteracted spinal pathogenicity in organotypic cultures from early symptomatic (12-week-old) mSOD1 mice (Vaz AR et al. (2021) Int J Mol Sci 22:6128).

Therefore, we aimed to test the potential benefit properties of the modulated concentrated secretome after intrathecal injection in mSOD1 mice with 12-week-old. We evaluated motor performance, inflamma-miRNA profile and glial/synaptic markers.

Methods

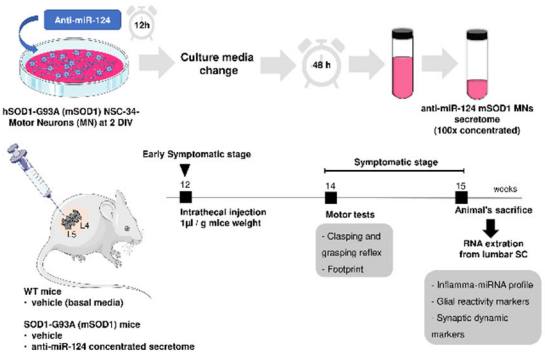


Fig. 1 – Schematic representation of the experimental procedure to obtain anti-miR-124 concentrated secretome, as well as, to perform the intrathecal injection of secretome and further motor behavior tests and molecular analysis in mice.

Results

ALS mice show deficits in corticospinal function, which is improved after intrathecal injection with anti-miR-124 neuronal secretome

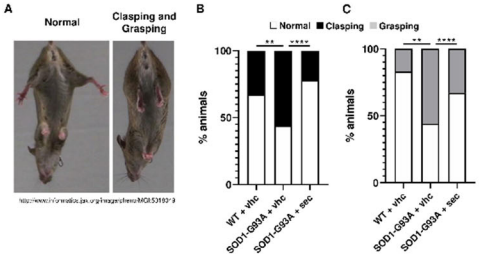


Fig. 2 - **(A)** Representative illustration of clasping and grasping reflex tests. The mice were suspended by holding their tails and their hindlimbs and toes position were observed for some seconds. An abnormal phenotype consists in retracting the hindlimbs toward the abdomen (clasping reflex) and/or curling the toes (grasping reflex) during the suspended time, meaning the presence of motor dysfunctionality. The percentage (%) of animals positive for **(B)** clasping and **(C)** grasping reflex. The group controls were both the WT (WT + vhc) and SOD1-G93A (mSOD1 + vhc) mice injected with NSC-34 basal media (vehicle). The anti-miR-124-derived secretome was injected in SOD1-G93A mice (SOD1-G93A + sec) to evaluate their therapeutic potential. Two weeks after the injection, the motor tests were performed. Results are mean (± SEM) from at least six experiments per group. ****p<0.0001 and **p<0.01 *vs* WT or mSOD1, Chi-square (and Fisher's exact) test was used.

The anti-miR-124-MN derived secretome prevents abnormalities on the gait in ALS mice

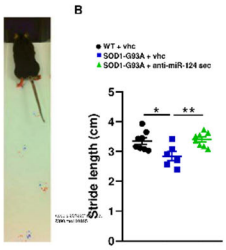


Fig. 3 - **(A)** Representative illustration of footprint test. The fore and hind paws of mice were painted with food dyes of different colours and placed over an absorbent paper to walk in a straight line. We measured the stride length (in centimetre, cm), which is the distance between the center of the plantar of the fore-foot and the center of the hind-foot plantar on the same side of the body, within the same stride. **(B)** The stride length (in centimetre, cm) performed by the mice. The group controls were both the WT (WT + vhc) and SOD1-G93A (mSOD1 + vhc) mice injected with NSC-34 basal media (vehicle). The anti-miR-124-derived secretome was injected in SOD1-G93A mice (SOD1-G93A + sec) to evaluate their therapeutic potential. Two weeks after the injection, the motor tests were performed. Results are mean (± SEM) from at least six experiments per group. **p<0.01 and *p<0,05 *vs* WT or mSOD1, unpaired and non-parametric Mann-Whitney test was used.

The lumbar SC of ALS mice shows a deregulation of inflamma-miRNAs and genes related with glial reactivity and synaptic signaling

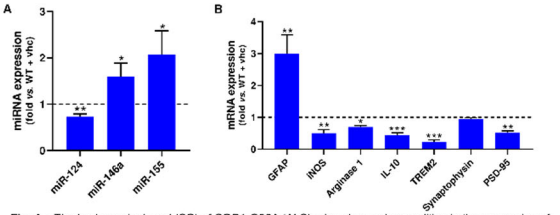


Fig. 4 – The lumbar spinal cord (SC) of SOD1-G93A (ALS) mice shows abnormalities in the expression of **(A)** inflammatory-associated miRNAs (miR-124, miR-146a and miR-155) and **(B)** genes related with glial activation (GFAP, iNOS, Arginase 1, IL-10 and triggering receptor expressed on myeloid cells 2 (TREM2)) and synaptic dynamics (synaptophysin and postsynaptic density protein 95 (PSD-95)). Results are mean (± SEM) from at least six experiments per group. ***p<0.001, **p<0.01and *p<0.05 *vs* WT + vehicle (vhc), unpaired t-student test was used.

The anti-miR-124 neuronal secretome prevents the dysregulated miRNA profile and gene signature of neuronal/glial markers in the lumbar SC of ALS mice

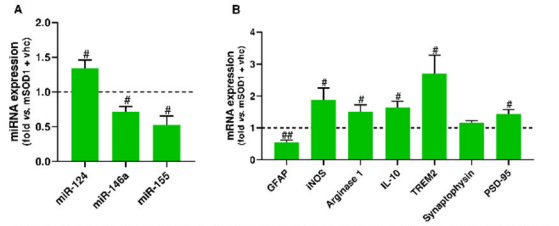
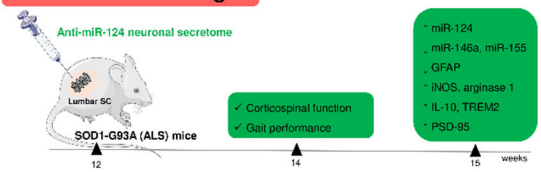


Fig. 5 – The secretome from SOD1-G93A NSC-34-motor neurons (MN) modulated with anti-miR-124 prevented the altered expression of **(A)** inflammatory-associated miRNAs and **(B)** glial/neuronal-related genes (GFAP, iNOS, Arginase 1, IL-10 and triggering receptor expressed on myeloid cells 2 (TREM2), synaptophysin and postsynaptic density protein 95 (PSD-95)) in the lumbar spinal cord (SC) of SOD1-G93A (ALS) mice. Results are mean (± SEM) from at least six experiments per group. ##p<0.01and #p<0.05 *vs* mSOD1 + vehicle (vhc), unpaired t-student test was used.

Take Home message



The secretome from the anti-miR-124 treated SOD1-G93A MNs prevented some motor disabilities and pathological mechanisms associated to MNs and glia in the ALS mice, showing promise as a novel therapeutic tool to be translated into ALS clinics if validated in future studies.

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MARTA ALEXANDRA MARQUES DOS SAMTOS

THERAPEUTIC POTENTIAL OF INTRATHECAL APPLICATION OF miR-124-BASED SECRETOME IN THE SOD1^{G93A} MOUSE MODEL OF AMYOTROPHIC LATERAL SCLEROSIS

NOVA SCHOOL OF SCIENCE & TECHNOLOGY