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Unveiling aberrancies and neurotoxic properties of ALS astrocytes using unspecific and microglia-specific activation models

Isabel Maria dos Santos Maurício

Dissertation supervised by Professor Dora Maria Tuna de Oliveira Brites and
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The studies presented in this thesis were performed in the Neuroinflammation, Signaling and Neuroregeneration headed by Doctor Dora Brites, at the Research Institute for Medicines (iMed ULisboa), Faculty of Pharmacy, Universidade de Lisboa, under the supervision of Professor Dora Brites and Doctor Ana Rita Vaz.

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Em memória do meu pai

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Resumo

A Esclerose Lateral Amiotrófica (ELA) é uma doença neurodegenerativa caraterizada pela perda progressiva dos neurónios motores (NMs) superiores e inferiores no Sistema Nervoso Central, nomeadamente, no cérebro, tronco cerebral e medula espinal. A perda de NMs resulta numa paralisia extensa que começa localmente na musculatura dos membros superiores, inferiores ou bulbar. A ELA é uma doença fatal, com uma sobrevida normalmente de 2 e 5 anos após o diagnóstico. Os doentes sucumbem à doença devido a atrofia muscular grave, paralisia e por fim a desenervação dos músculos respiratórios. É uma doença extremamente heterogénea na sua sintomatologia, e com uma patogénese pouco clara. Atualmente, existem apenas duas abordagens terapêuticas muito limitadas quanto à sua eficácia, tornando-se premente compreender as causas subjacentes e os fatores de risco, de forma a encontrar novos alvos terapêuticos para a ELA.

Apesar do envolvimento dos NMs, é bem conhecida a importância da disfuncionalidade das células gliais na doença. Na ELA, os astrócitos alteram a sua forma e padrões de expressão molecular e são referidos como astrócitos reativos ou ativados, perdendo as suas funções benéficas e adquirindo papéis prejudiciais. No entanto, não se sabe qual a causa de tal reatividade e do fenótipo aberrante dos astrócitos na ELA, ou seja, se são intrínsecos à patologia, se são potenciados pela libertação de citocinas da microglia ativada, ou se pelo ambiente neuroinflamatório e seus mediadores.

Esta tese teve a possibilidade única de explorar a morfologia e assinatura inflamatória de astrócitos diretamente convertidos de fibroblastos (iAstrócitos) de uma doente com forma esporádica da ELA, em comparação com uma amostra controlo obtida da mesma forma, de uma pessoa não doente, do mesmo sexo e idade (controlo). Para tornar mais evidente as reações de patogenicidade dos astrócitos ELA, foram usados dois estímulos inflamatórios diferentes, ambos descritos na literatura e envolvendo a ação de citoquinas pró-inflamatórias, para atestar qual o mais eficaz na indução do fenótipo aberrante dos astrócitos na ELA.

Usámos células induzidas percursoras neurais geradas de fibroblastos da doente e respetivo controlo, cedidas por Kathrin Meyer no âmbito da nossa colaboração, que foram diferenciados em astrócitos induzidos (iAstrócitos), durante 7 dias *in vitro*. Os astrócitos foram de seguida incubados com as citocinas da microglia ativada (TNF-α/IL-1α/C1q) e as associadas a qualquer processo inflamatório (TNF-α/IL-1β) durante 48 horas. Após este período, recolheram-se as células e o seu secretoma.

Os astrócitos da doente com ELA exibiram menos células polarizadas, maior aparência de fibroblastos e menor soma, quando comparados com a amostra controlo, se bem que sem evidência estatística. Esta tendência manteve-se após estimulação com TNF-

α/IL-1α/C1q, mas não totalmente com TNF-α/IL-1β, dado que neste caso apenas se verificou a presença de menor soma. A avaliação das propriedades dinâmicas da mitocôndria nos astrócitos, permitiu evidenciar uma diminuição marcada nos astrócitos ELA que se tornaram menos evidentes com TNF-α/IL-1α/C1q e ausentes com TNF-α/IL-1β. Contrariamente ao descrito para os genes associados ao fenótipo aberrante dos astrócitos na ELA, não observámos nos astrócitos ELA o aumento de Cx43, nem de S100B, os quais se apresentaram diminuídos ($p<0.01$ para ambos), nem qualquer alteração para o transcrito GFAP, habitualmente reduzido. Contudo, identificámos um aumento do marcador de proliferação celular, o Ki-67 ($p<0.05$). Na presença de TNF-α/IL-1α/C1q, tanto o GFAP como o S100B foram encontrados diminuídos ($p<0.05$) nos astrócitos ELA. O S100B foi igualmente encontrado diminuído na presença de TNF-α/IL-1β que levou a um aumento marcado da proliferação nos astrócitos ELA. A avaliação de S100B por imunocitoquímica, confirmou a diminuição de S100B nos astrócitos ELA na presença tanto de TNF-α/IL-1α/C1q, como de TNF-α/IL-1β ($p<0.05$), mas não na ausência destes estímulos. Quanto aos marcadores inflamatórios associados à ativação do inflamassoma, NLRP3, IL1R1 e IL-1β, há a salientar a diminuição dos genes IL-1β e NLRP3 nos astrócitos ELA não estimulados. A redução do NLRP3 foi sustentada na presença de qualquer um dos modelos de estimulação.

Os resultados obtidos para os astrócitos da doente com a forma esporádica da ELA apresentam alterações fenotípicas relativamente à amostra controlo, sugestivas de latência e menor capacidade reativa a estímulos, mas mantendo um aumento da capacidade proliferativa, na ausência e presença de TNF-α/IL-1β. Quanto aos efeitos dos dois estímulos testados, nem sempre foram comparáveis. Este trabalho foi pioneiro em mostrar déficits de uma amostra de astrócitos obtidos de doente com ELA, os quais necessitam, contudo, de ser reforçados com um maior número de amostras, incluindo formas familiares. Em suma, o processo inflamatório deverá ser tido em conta na avaliação da patologia associada à ELA e os iAstrócitos sobressaem como uma ferramenta importante na medicina personalizada para esta doença.

Palavras-chave: Forma esporádica da ELA, iAstrócitos, Marcadores inflamatórios, Morfologia aberrante, Neuroinflamação, Reatividade astrocítica.

Abstract

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterised by the progressive loss of upper and lower motor neurons (MNs) in the central nervous system, namely in the brain, brainstem and spinal cord.

The loss of MNs results in extensive paralysis starting locally in the muscles of the upper, lower or bulbar limbs. ALS is a fatal disease, with survival typically between 2 and 5 years after diagnosis. Patients succumb to the disease due to severe muscle atrophy, paralysis, and ultimately the denervation of the respiratory muscles. It is an extremely heterogeneous disease in its symptomatology, and with an unclear pathogenesis. Currently, there are only two therapeutic approaches that are very limited in their efficacy, making it urgent to understand the underlying causes and risk factors in order to find new therapeutic targets for ALS.

Despite the involvement of MNs, the importance of glial cell dysfunctionality in the disease is well known. In ALS, astrocytes change their shape and molecular expression patterns and are referred to as reactive or activated astrocytes, losing their beneficial functions and acquiring detrimental roles. However, it is not known what causes such reactivity and the aberrant phenotype of astrocytes in ALS, i.e., whether they are intrinsic to the pathology, whether they are potentiated by cytokine release from activated microglia, or whether by the neuroinflammatory environment and its mediators.

This thesis had the unique possibility to explore the morphology and inflammatory signature of astrocytes directly converted from fibroblasts (iAstrocytes) from a patient with the sporadic form of ALS, compared to a control sample obtained in the same way, from a non-diseased person of the same gender and age (control). To make the pathogenicity reactions of ALS astrocytes more evident, two different inflammatory stimuli were used, both described in the literature and involving the action of pro-inflammatory cytokines, to test which was more effective in inducing the aberrant phenotype of astrocytes in ALS.

We used induced neural precursor cells generated from patient and control fibroblasts, provided by Kathrin Meyer within our collaboration, which were differentiated into induced astrocytes (iAstrocytes) for 7 days *in vitro*. The astrocytes were then incubated with the cytokines of activated microglia (TNF-α/IL-1α/C1q) and those associated with any inflammatory process (TNF-α/IL-1β) for 48 hours. After this period, the cells and their secretome were collected.

Astrocytes from the ALS patient exhibited fewer polarised cells, greater fibroblast appearance and lower soma, when compared to the control sample, although without statistical evidence. This trend was maintained after stimulation with TNF-α/IL-1α/C1q, but not entirely with TNF-α/IL-1β, as only lower soma was present in this case.

Assessment of the dynamic properties of mitochondria in astrocytes, provided evidence of a marked decrease in ALS astrocytes that became less evident with TNF-α/IL-1α/C1q and absent with TNF-α/IL-1β. Contrary to what has been described for genes associated with the aberrant astrocyte phenotype in ALS, we did not observe in ALS astrocytes increased Cx43, nor S100B, which were decreased ($p<0.01$ for both), nor any change for the usually reduced GFAP transcript. However, we identified an increase in the cell proliferation marker, Ki-67 ($p<0.05$). In the presence of TNF-α/IL-1α/C1q, both GFAP and S100B were found decreased ($p<0.05$) in ALS astrocytes. S100B was also found decreased in the presence of TNF-α/IL-1β which led to a marked increase in proliferation in ALS astrocytes. Evaluation of S100B by immunocytochemistry, confirmed the decrease of S100B in ALS astrocytes in the presence of both TNF-α/IL-1α/C1q and TNF-α/IL-1β ($p<0.05$), but not in the absence of these stimuli. As for the inflammatory markers associated with inflammasome activation, NLRP3, IL1R1 and IL-1β, one should note the decrease in IL-1β and NLRP3 genes in unstimulated ALS astrocytes. The reduction in NLRP3 was sustained in the presence of either stimulation model.

The results obtained for astrocytes from the patient with the sporadic form of ALS show phenotypic changes in relation to the control sample, suggestive of latency and lower reactive capacity to stimuli, but maintaining an increased proliferative capacity, in the absence and presence of TNF-α/IL-1β. As for the effects of the two stimuli tested, they were not always comparable. This work was pioneering in showing deficits in a sample of astrocytes obtained from an ALS patient, which need, however, to be reinforced with a larger number of samples, including familial forms. In summary, the inflammatory process should be considered in the assessment of ALS-associated pathology and iAstrocytes stand out as an important tool in personalised medicine for this disease.

Keywords: Sporadic ALS, iAstrocytes, Inflammatory markers, Aberrant morphology, Neuroinflammation, Astrocytic reactivity.

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Abbreviations

ACM	Astrocytes conditioned medium
ALS	Amyotrophic lateral sclerosis
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
CCL5	Chemokine (C-C motif) ligand 5
cDNA	Complementary DNA
CNS	Central nervous system
Cx43	Connexin 43
C1q	Complementary component
DIV	Days *in vitro*
EAAT	Excitatory amino acid transporter
FAD	Familial Alzheimer's disease
fALS	Familial amyotrophic lateral sclerosis
GFAP	Glial fibrillary acid protein
IL	Interleukin
iNPC	Induced neuronal progenitor cell
iPSCs	Induced pluripotent stem cells
KI-67	Proliferation marker Ki-67
LMN	Lower motor neuron
miRNAs	MicroRNAs
MMP	Matrix metalloproteinase
MN	Motor neuron
mSOD1	Mutant SOD1
NLRP3	Nucleotide binding oligomerization domain (NOD)-like receptor protein
ON	Over night
RT-qPCR	Real-Time Quantitative PCR
ROS	Reactive oxygen species
S100B	S100 calcium-binding protein B
SAD	Sporadic Alzheimer's disease
sALS	Sporadic amyotrophic lateral sclerosis
SOD1	Superoxide dismutase 1
TNF-α	Tumor necrosis factor-α
UMN	Upper motor neuron
WT	Wild-type

Chapter I

Introduction

1. Amyotrophic lateral sclerosis: disease overview

Amyotrophic lateral sclerosis (ALS) also known as Lou Gehrig´s disease, is an adult-onset fatal neurodegenerative disorder characterized by progressive loss of upper motor neuron (UMN) and lower motor neuron (LMN) (Zinman and Cudkowicz, 2011; Ragagnin *et al.*, 2019), in the motor cortex of the brain, brainstem motor nuclei and anterior horn of the spinal cord (Cirulli *et al.*, 2015;Taylor, *et al*., 2016). The neurologist Jean-Martin Charcot as classified this disease about is specificity in 1869, based on several years of detailed observation of *post-mortem* tissues (Kumar *et al.,* 2011). The name "Amyotrophic Lateral Sclerosis" reflects the selective degeneration of motor neuron (MN) being a combination of "Lateral" referring to the lateral spinal cord, "Amyotrophic" from de Greek meaning lacking muscle nourishment and the demise of spinal MN, and "Sclerosis" (or fibrosis) referring to gliosis of the crossed corticospinal trat in the dorsolateral quadrant of the spinal cord (Frey *et al.*, 2000; Pun *et al.*, 2006).

The ALS disease is characterised by the variability of phenotype cases, beginning with weakness but rapidly progressive paralysis, muscular atrophy, respiratory failure and malnutrition status leading to death within 1 to 5 years after symptom onset (Figure I.1)(Cirulli *et al.,* 2015).

In terms of prevalence, the onset of the disease occurs during adulthood, between 58-63 years for sporadic disease and 47-52 years for familial disease (Kiernan *et al.*, 2011; Logroscino and Piccininni, 2019). The incidence of ALS in Europe is 2-16 per 100 000 person-year, and men have higher incidence of disease (3 per 100 000 person-year; 95% CI 2.8-3.3)(Kiernan *et al.*, 2011). The prevalence in Portugal has been increasing in the last years. Data from 2016 estimate ALS/MN disease prevalence was higher in men (12.08 per 100 000; 95% CI 9.66-17.15), than in women (8.56 per 100 000; 95% CI 6.84-12.32). In women, the highest age prevalence was 28.77 per 100 000 (95% CI 22.02-41.31) for 71-80 years; for men, the highest age prevalence was 48.85 (95% CI 38.72-71.40) for 71-80 years. The distribution of the cases per region reveals high prevalence in Centre region, 11.15 (8.9-15.34) followed by Lisbon and Alentejo, 10.74 (8.6-14.82)(Conde *et al*., 2019).

Even knowing the symptoms of ALS, its diagnosis proves to be a clinical challenge even the efforts because of the existing of several mimic syndromes which present similar clinical features (Campanari *et al*., 2019). At present, no definitive diagnostic test or biomarker for ALS exist, which complicate de determination of a specific date of disease onset and the hypothetic duration between pathological changes and manifestations of clinical disease (Kiernan *et al.*, 2011).

Approximately 5-10% of ALS patients have a clear familial history indicating an autosomal dominant genetic inheritance, corresponding to familial ALS (fALS) cases in which more than one family member is affected. In the remaining 90% of ALS cases there is no indication of

genetic inheritance and are designed sporadic ALS (sALS) (Van Blitterswijk *et al.*, 2012; Roggenbuck *et al.,* 2017).

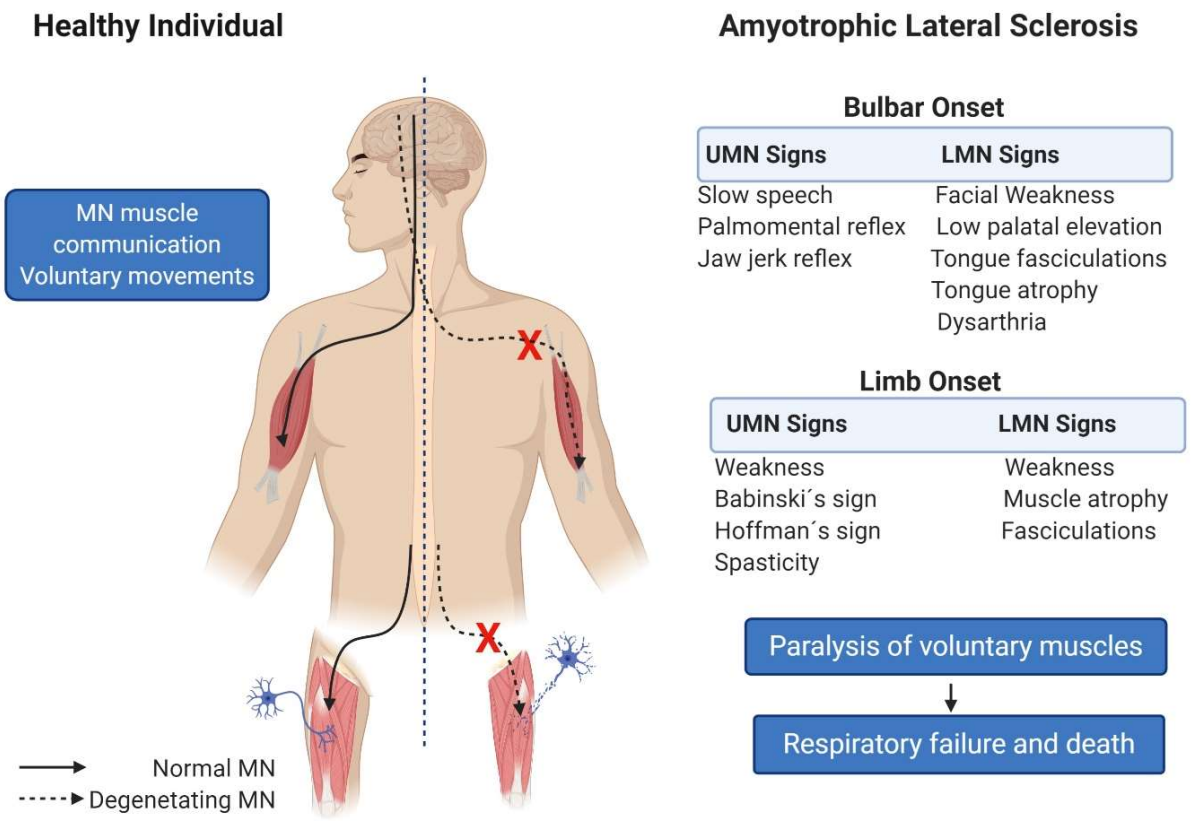

Figure I.1. ALS is a prototypical neurodegenerative disease that is characterized by progressive degeneration of MN in the brain and spinal cord. Bulbar onset symptoms disease is related with muscles controlling speech, mastication and swallowing, losing their functions, and causing to the patient tongue atrophy with thickness of speech and difficulty swallowing or slow and highly dysfunctional speech, often accompanied by the accentuation of emotional reflexes; Limb onset disease described symptoms present initially in the upper arm or and or lower limbs, leg, or foot, developing weakness with flaccidity and atrophy, prominent hyperreflexia, and spasticity. At disease end stage, only support and palliation are available, and patients usually die from respiratory failure, 3 to 5 years after diagnosis. ALS, amyotrophic lateral sclerosis; LMN, lower motor neuron; MN, motor neuron; UMN, upper motor neuron. (Artwork by Isabel Maurício)

Currently, there are no effective treatments for ALS. Only two FDA-approved drugs for the treatment of ALS, Riluzole® and Edaravone®, have so far been approved. In Portugal, only Riluzole is approved by the INFARMED, and its dispensing is exclusive of hospital pharmacy. Riluzole is an inhibitor of glutamate release with neuroprotective effect, but only increases the survival of patients by 3 a 6 months (Miller *et al.*, 2007; Kiernan *et al.*, 2011; Petrov *et al.*, 2017). This benefit seems more suitable for patients with moderate functional impairment (Bensimon *et al.,* 2002). The Edaravone®, has an intravenous injection presentation, is a free radical scavenger which inhibits matrix metalloproteinase (MMP-9) expression, has shown to

slows down the disease progression in a subpopulation of ALS patients (Yoshino and Kimura, 2006; Ito *et al.*, 2008; Hardiman and van den Berg, 2017).

1.1 Genetics and pathophysiology of ALS

The genetic etiology of sALS and approximately 30% of fALS (European descent) is still unknown (Laferrièrea and Polymenidou, 2015). fALS patients are associated with mutations in chromosome 9 open reading frame 72 (*C9orf72)*, superoxide dismutase 1 (*SOD1*), transactive response (TAR) DNA-binding protein 43kDa (*TDP-43*), fused in sarcoma (*FUS*), valosin-containing protein (*VCP*), sequestosome 1(SQSTM1), optineurin (*OPTN*), and TANK binding kinase 1 (*TBK1*) (Maruyama *et al.*, 2010; He *et al.*, 2015).

At present, new ALS causing genes have been discovered and play an important role for understanding of the pathogenesis of fALS and sALS. Given the rapid discovery of new mutations, please consult *alsod.iop.kcl.ac.uk/*.

When the medical community began to look at ALS, it was thought that it would be a sporadic disease. Even after the *SOD1* gene mutation discovery, only about 10% of patients could definitively be pointed to have a familial history of the disease (Ajroud-Driss and Siddique 2015). However, this concept was altered with the discovery of new genes and genetic processes that could trigger the disease. In 2014, of all cases, two thirds of fALS and 10% of sALS had a genetic explanation. fALS can have great variability in age of onset and disease duration. This suggests that disease-modifying factors play a role. The distinction between fALS and sALS based on family history may be artificial as new genes are being discovered.

SOD1 was the first gene to be discovered (1993) for ALS. This gene has a predisposition for leading to the death of the MN, likely by protein accumulating in this MN and astrocytes, causing a toxic gain of function. Families with *SOD1* mutations have strong autosomal dominant penetrance, an earlier age of onset, and death or respiratory failure within 1 to 2 years of onset (Rosen *et al.,* 1993).

A major feature of ALS is the accumulation of a protein, TDP-43. This protein plays multiple roles in RNA processing. The gene for this protein is known as *TARDBP*, and mutations of *TARDBP* account for 5% of fALS cases (Neumann *et al.,* 2006). TDP-43 aggregates can even be found in ALS patients without the mutated form of the gene, suggesting that this protein may play a role in sALS.

FUS is a protein with two RNA binding domains and a terminal nuclear localization signal (Warraich *et al.,* 2010). Despite being a nuclear protein, it accumulates in the cytoplasm in ALS associated with FUS mutations. This similarity to TDP-43 suggests the possibility of a common mechanism of disease (Kwiatkowski *et al.,* 2009). FUS also appears to interact with histones and play a role in the repair of DNA damage. This role may be important for the integrity of the MN. FUS mutations account for about 5% of fALS.

A hexanucleotide repeat expansion (GGGGCC) in the noncoding region *C9orf72* can cause ALS and may account for the largest proportion of inherited ALS. When copies reach as few as 30, this expansion appears to be capable of producing the disease. No protein aggregate is associated with this mutation, and much is still being discovered about this gene and protein product in ALS pathogenesis (Vance *et al.*, 2009; Renton *et al.*, 2011; DeJesus-Hernandez *et al.*, 2011).

From the other genes that have been associated with fALS, each account for a small proportion of cases. It is estimated that 60% of individuals with fALS have an identified genetic mutation (DeJesus-Hernandez *et al.,* 2011). The genetics of ALS are of research interest because of the potential to help uncover the mechanism of MN death in ALS. Neuronal cytoplasmic protein aggregation and defective RNA metabolism appear to be common pathogenic mechanisms involved in ALS. All of these genes are clinically not distinguishable, and the disease-phenotype are similar.

Therefore, ALS is one of the diseases most requiring further investigation for the underlying mechanisms of pathogenesis, which is a condition for the discovery of new therapeutic approaches.

1.2 Glial cells in ALS

Although MN diseases establish the motor clinical commitment, its pathophysiological mechanisms are wider and involve a complex network of cell interactions, non-neuronal cells of the central nervous system (CNS), glial cells, whose dysfunctions lead to a cascade of events culminating in the death of the MN (Valori *et al.,* 2014). These glial cells are the astrocytes, which represents the major glial component in the CNS, microglia as responsible for immunocompetent and specialized brain macrophages, oligodendrocytes and Schwann cells, which form sheets of myelin around neuronal axons in the central and peripheral nervous system, NG2 cells or polydendrocytes that express proteoglycan and receive synaptic input from neurons (Valori *et al.,* 2014).

In the following sections, the importance of microglia, astrocytes and oligodendrocytes in ALS pathology will be described, however, astrogliosis will be described in a separate chapter.

1.2.1 Microglia

Microglial cells derived from the hematopoietic cell lineage, are the main immune-competent cells of CNS and play an important role in the maintenance of CNS function, as protection of neurons from infection or injury and in synaptic regulation. (Lasiene and Yamanaka, 2011; Philips and Rothstein, 2014; Filipi *et al.*, 2020; Clarke and Patani, 2021). This characteristic of

the microglia is due to the fact that it does not originate in the ectoderm but in the macrophages primitives of the yolk sac which reach the CNS during development (Ginhoux *et al.,* 2013).
At a pathological event (injury, trauma, or other stimuli) or a change in the homeostasis of the CNS, these cells undergo graded and temporal changes in their morphology and gene expression in the release of pro-inflammatory (toxic phenotype), like tumor necrosis factor alpha (TNF-α), interferon 1 beta (IL-1β), nitric oxide (NO), O_2, interferon gamma (IFN-γ) factors which limit biological hazards (Liao *et al* 2012; Philips and Rothstein, 2014; Szalay *et al.*, 2016; Ajami *et al.*, 2018; Haruwaka *et al.*, 2019; Hammond *et al.*, 2020). Microglia can also became protective followed by the release of anti-inflammatory (neuroprotective phenotype) which release interleukin 4 (IL-4), IL-10, insulin growth factor 1 (IGF-1) factors which repair and mediate restoration (Chiu *et al.*, 2011; Liao *et al.*, 2012; Philips and Rothstein, 2014). This inflammatory response is in a close interaction between T-cells and astrocytes.

1.2.1.1 Microglia in ALS

Microglial activation has been observed from the onset of disease in mutant SOD1 (m*SOD1*) mouse models and ALS patients in all stages, near at the site of MN degeneration (Chiu *et al.*, 2008; Corcia *et al.*, 2012). The inhibition of CSFR1, a protein responsible for microglial proliferation, survival, and maturation, reduce the activity of microglia and extended survival of mSOD1 mice, as well as attenuated MN cell death (Martínez-Muriana *et al.,* 2016). However, the expression of mSOD1 in microglia alone is insufficient to cause MN degeneration. In studies that have been carried out much earlier, it has been suggested that microglia played an active role in the late phase of the disease. A recent study contradicts this assumption, noting that recent spinal cord spatial transcriptomics of mSOD1 mouse showed that significant changes in gene expression in microglia preceded changes in MNs (Maniatis *et al.,* 2019).
In relation to the number of microglia cells, it is known that they were decreased before the onset disease in mSOD1 mice. This early functional deficits coincide with alterations of neuromuscular junctions (Gerber *et al.,* 2012).
Studies performed with co-cultures in which primary cultures from neonatal mice exposure to exogenous mSOD1 and adult mSOD1 microglia reveal to be toxic to MN (Zhao *et al.*, 2010; Roberts *et al.*, 2013; Frakes *et al.*, 2014). In primary mouse model microglia, both wild-type (WT) and mutant TDP-43 have been reported to stimulate free radicals and cytokine production toxic to MNs, such as TNF-α and IL-1β, itself can activate both microglia and astrocytes by an autocrine pathway, further promoting MN death and ALS disease progression (Zhao *et al.,* 2015). A study performed in a reversible mouse model of doxycycline suppressible neuronal human TDP-43 containing a defective nuclear localization signal, minor changes occur in microglia activation in spinal cord despite progressive MN loss, but when TDP-43

expression was switched off during disease, mice recovered motor function and microglia become activated and cleared neuronal TDP-43 (Spiller e*t al.,* 2018).

Mislocalization of FUS has been found in MN of sALS spinal cord tissue from multiple cases and mouse transgenic models, suggesting a role in the majority of ALS cases (Tyzack *et al.,* 2019). In primary microglia cultures which in turn were incubated with astrocyte conditioned media from astrocyte cultures overexpressing WT FUS, promoted cell death and pro-inflammatory microglia activation (Ajmone-Cat *et al.,* 2019). Pro- and anti-inflammatory microglial activation in spinal cord cells and malfunction of acetylcholine synapse transmission in MNs has been reported in mice expressing a truncated FUS protein lacking a nuclear localization signal, both before and after disease onset, suggesting that mislocalization of FUS is sufficient to result in microglial activation (Funikov *et al.,* 2018).

In a study performed in mouse model, C9orf72 is highly expressed in microglia with age-related neuroinflammation similar to C9orf72 ALS but not sALS patient tissue, suggesting that altered microglial function may contribute to neurodegeneration in C9orf72 expansion carriers (O'Rourke *et al.,* 2016).

In a recent study, reduction of C9orf72 expression in a mouse model with a repeat expansion resulted in hippocampal neuron loss, microglial activation, implicating both gain and loss of C9orf72 function in modulating microglial activation (Zhu *et al.,* 2020).

1.2.2 Astrocytes

Astrocytes are responsible for homeostatic functions in the CNS, regulation of plasticity of synapses and synthesis of neurotransmitters, functional and structural role in maintaining the blood-brain barrier (BBB), and they provide neurotrophic support to MNs by releasing glial-derived neurotrophic factor (GDNF) (Vasile *et al*., 2017), transforming growth factor b1 (GF-b1) and vascular endothelial growth factor (VEGF) (Volterra and Meldolesi, 2005; Allaman *et al*., 2011; Sloan *et al*., 2014). In response to brain damage, astrocytes take part in brain protection, as reactive astrogliosis, scar formation and secretion of proinflammatory factors (Liddelow *et al.,* 2017).

1.2.2.1 Astrocytes in ALS

In neurodegeneration, and similarly to the behaviour of microglia, astrocytes present themselves in two distinct states, either reactive or activated (Yamanaka *et al.,* 2008). When activated, is neuroprotective functions become neurotoxic during disease, however, they are

implicated in the progression rather than onset of ALS (Ilieva *et al.,* 2009; Valori *et al.*, 2014; Das and Svendsen, 2015). This neurotoxic state gene expression is influenced by microglia, converging in both loss homeostatic functions and a transformation to a neurotoxic state (Liddelow *et al.*, 2017; Jha *et al.*, 2019). Remarkably similar to the microglia, astrocytes dysfunction has been implicated in non-cell autonomous MN death, since loss of homeostatic functions and a toxic release factors been implicated in astrocytic non-cell autonomous mechanisms of MN death in ALS (Serio and Patani, 2018).

The interaction between microglia-astrocyte has been partially addressed but well succeeded in the mSOD1 mouse model. Studies reveal that deletion of *SOD1* in mice model astrocytes delayed disease progression, but not onset of the disease, and resulted in a decrease in microglial activation (Yamanaka *et al*., 2008; Wang *et al*., 2011), whereas deletion of mSOD1 from familial MNs delay onset disease (Wang *et al.,* 2009). This observation is corroborated by the study of gene expression changes in MNs, astrocytes and oligodendrocytes reveal changes before disease onset in SOD^{G37R}mice, although these alterations are first observed in MNs (Sun *et al.,* 2015).

An elegant study performed by Liddelow *et al.*, described two different subsets of reactive astrocytes in adult CNS, A1 and A2, in turn A1 reactive astrocytes were associated with the death of both neurons and oligodendrocytes (Liddelow *et al.*, 2017; Miller, 2018). However, recent studies show a more subsets of reactive astrocytes.

Evidence has shown that astrocytes mediate MN degeneration via the release of neurotoxic factors. Conditioned medium from primary astrocyte cultures of SOD1G86R and TPD-43A315T mice also induces MN death through activation of sodium channels and nitro-oxidative stress (Rojas *et al.,* 2014), in two different ALS models through a common pathogenic pathway.

Astrocytes expressing mFUSR521G trigger MN death, which is preceded by progressing degeneration of neurites, by secreting pro-inflammatory TNF-α (Kia *et al*., 2018). Preeminent studies reveal that SOD1G93A aggregates in astrocytes appear in late disease stages, in regions with extensive neuronal degeneration and featured astrogliosis, suggesting that astroglial aggregate formation is triggered by MN degeneration, and disease possible progresses from neuron to glia (Jaarsma *et al.*, 2008; Sun *et al.*, 2015).

The analysis of all studies presented above suggests a cycle in the involvement of astrocytes in ALS MN degeneration, in which they are able to cope with small levels of misfolded proteins but in the face of MN degeneration, they become vulnerable with the release of toxic factors to the MN; the neuronal resistance to astrocyte reactivity will call for cell-autonomous autonomic factors such genetic background and transcriptional profiles (Sun *et al.*, 2015).

1.2.3 Oligodendrocytes

Oligodendrocytes are responsible for providing metabolic support to neurons and maintaining the myelin sheath required for neuronal saltatory conduction of action potentials, essential for their provision with trophic and metabolic support (Philips *et al.*, 2013; Clarke and Patani, 2021). Oligodendrocytes and myelinated axons are metabolically coupled.

1.2.3.1 Oligodendrocytes in ALS

Given the neurotrophic function, oligodendrocytes are thought to contribute to the pathogenesis of neurodegenerative disease, neuronal atrophy and death, including ALS (Ilieva *et al*., 2009). The potential involvement of oligodendrocytes in ALS had not been as explored as other glia cells, although several pathological studies have reported oligodendrocyte anomalies in both human and rodent models of ALS spinal cord (Niebroj-Dobosz *et al.,* 2007). It was also found that the aberrant location from TDP-43 to cytoplasm instead of the nucleus, which is a morphological mark of sALS patients, is not limited to MN, but also in oligodendrocytes (Neumann *et al.,* 2007).

Over the past 10 years, studies have already suggested that oligodendrocytes are key pieces in neurodegeneration. Oligodendrocytes express the monocarboxylate transporter 1 (MCT1, known as SLC16A1), which provides MNs with trophic support through the release of lactate. In patients with ALS, ALS rodent models as well *in vitro*, it was found that MCT1 expression levels are decreased, thus contributing to neurodegeneration and death of the MNs (Lee *et al.*, 2012; Philips *et al.*, 2013).

Degeneration of oligodendrocytes in mSOD1 mice was observed, accompanied by activation of the microglia and microglial localization to apoptotic oligodendrocytes. When mSOD1 is removed from the oligodendrocytes there is a delay in the development of the disease, concomitant with a delay in activation of the microglia (S. H. Kang *et al.,* 2013). In the same study, a proliferation of oligodendrocyte progenitor cells in the mSOD1 model was observed to replace non-functional oligodendrocytes. *In vitro* studies have shown that mSOD1 mouse oligodendrocytes induced excitability and death in WT MNs.

In sALS and fALS oligodendrocytes derived from humans, where two different methods of reprogramming were used induced the death of the NM both with the medium conditioned and in co-culture, where a decrease in lactate production and release was associated (Ferraiuolo *et al.,* 2016).

However, further studies are needed to establish the relationship between oligodendrocytes and ALS.

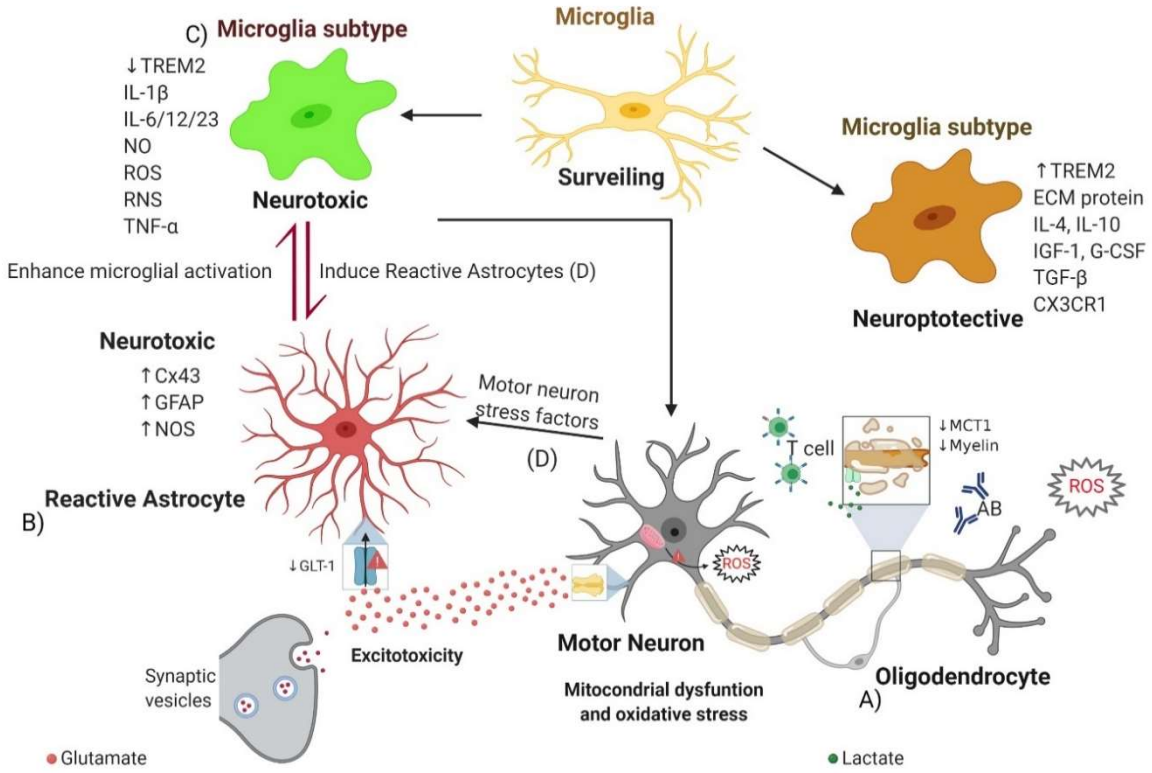

Figure I.2 Mechanism impairments of glial cells and motor neuron implicated in ALS disease. A) reduction in the levels of lactate transporter MCT1 diminishes energy supplied by oligodendrocytes to motor neurons, myelin sheath disruption. B) failure of astrocytes to clear synaptic glutamate via the transporter GLT-1 repetitive firing of motor neuron and excitotoxicity. C) hyperactivation of microglia produces extracellular superoxidase, which triggers inflammation and degeneration in motor neurons. (D) Release of toxic cytokines by reactive microglia leads to a state of motor neuron toxicity, which in turn leads to a neurotoxic state of the astrocyte. TREM, triggering receptor expressed on myeloid cell; IL-1β, interleukin; NO, nitric oxide; ROS, reactive oxygen species; RNS, reactive nitrogen species; TNF-α, tumor necrosis factor; Cx-43, connexin 43; GFAP, glial fibrillary acid protein; GLT, glutamate transporter; MCT, monocarboxylate transporter 1; IGF, insulin-like growth factor 1; G-CSF, granulocyte colony stimulating factor; TGF-β, transforming growth factor; CX3CR1, CX3C chemokine receptor 1. (Artwork by Isabel Maurício)

1.3 The motor neuron

MNs are the primary cell type affected in ALS. In a mice mSOD1 model, transcriptional alterations occur in astrocytes and oligodendrocytes after changes in MN even at symptomatic disease stages, suggesting for this vulnerability divergent levels of endoplasmic reticulum chaperones, much higher in astrocytes and oligodendrocytes than in MN, and a recent spatial transcriptomics suggests that microglial dysfunctions precedes changes in MN (Sun *et al.*, 2015; Maniatis *et al.*, 2019). We could suggest that the microglia are the cell type who leads to MN death, but a previous study reveals that the expression of mSOD1 in microglia alone does not conduct to disease (Beers *et al.*, 2006) and there is a lack of microglial-mediated MN death in C9orf72 mice, even though widespread microglial activation occurs, but not sALS patient tissue (O'Rourke *et al.*, 2016).

Even though it is not the main trigger cell of glia in the degeneration of the MN, microglia may play an important modulatory role in MN death in ALS. Studies have consistently revealed that mSOD1 in MN is not sufficient to cause disease and glial cells including microglia have been shown to affect disease progression in mSOD1 mice (Pramatarova *et al.*, 2001).

1.4 The signature of astrocytes in ALS

Astrocytes are the major cell population in CNS and play an important signature in homeostasis, functional and structural role. Malfunction and reactive phenotype were implicated in neurodegenerative disease, including ALS. In this chapter, we expose the involvement of astrocytes in MNs death through several key molecules.

1.4.1 The astrocyte-glutamate cycle

Glutamate is the main excitatory neurotransmitter in the CNS and during axonal depolarization, glutamate is released from presynaptic neurons and it activates specific metabotropic and ionotropic glutamate receptors located on the postsynaptic neuron membrane that regulate the influx of positive ions, specially calcium (Heath and Shaw, 2002; Ferraiuolo and Meyer, 2014; Yuan *et al*., 2017). The excitatory signal is terminated by the removal of glutamate from de synaptic cleft by glutamate re-uptake transporters located at the cell membranes of astrocytes and postsynaptic neurons, the EAAT2 also called GLT1 (Ferraiuolo and Meyer, 2014). Accumulation of glutamate in the synaptic cleft leads to disruption of intracellular calcium homeostasis causing excitotoxicity (Geevasinga *et al.,* 2016). Calcium regulates several intracellular mechanisms, such as proteolytic and reactive oxygen species (ROS), production of enzymes, mitochondrial function and ATP, which in turn are intrinsic to excitotoxicity (Ferraiuolo and Meyer, 2014). Recovery of excess of glutamate by astrocyte, via EAAT2, is subsequently catalysed by enzyme glutamine synthetase (GS) in glutamine conversion through ATP-dependent amidation. Glutamine can be released to extracellular fluids for subsequent uptake by neurons or transport to the blood vessel. Glutamine deamidation by mitochondrial glutaminase (GA) regenerates glutamate, closing the cycle (Yuan *et al.,* 2017). In this disease, the glutamate release to synaptic cleft is not all recovered, and excitotoxicity is one of the mechanisms involve in ALS.

Studies in *post mortem* biopsies of ALS patients reveals high levels of cerebrospinal fluid glutamate, decrease in the expression and activity of astrocytic EAAT2 in both sporadic and familial patients, especially in the ventral horn were MN are found, resulting in accumulation of glutamate in synaptic cleft (Shaw *et al.*, 1995; Lin *et al.*, 1998). These findings suggest that excitotoxicity in such active way contributes to MN death, given its vulnerability to such toxicity (Kuner *et al.*, 2005; Rosenblum *et al*., 2017).

A study carried out with cultures of induced pluripotent stem-derived astrocytes from patients with ALS-causing mutations SOD1-A4V and C9orf72 demonstrated similar ou greater basal expression levels of EAAT2 and glutamate uptake when compared to control. Also exhibited an increase in uptake when co-culture with neurons. Such results suggest that EAAT2 expression in astrocytes of human patients not be decreased (Zhang *et al.*, 2016). The results are controversial when we analyse previous studies where the decrease protein level in EAAT2 were observed in spinal cord homogenates of SOD1-G85R mice (Bruijn *et al.*, 1997), spinal cord homogenate and the ventral horn of the lumbar spinal cord of SOD1G93A mice (Bendotti *et al.*, 2001). Studies performed in ALS patients shows a decreased of EAAT2 in motor cortex and spinal cord, with a pronounced decrease in motor cortex (Rothstein *et al.,* 1995), EAAT2-specific antibody show decreased in the gray matter of the lumbar spinal cord and slightly increased in the middle laminae of the motor cortex (Fray *et al.* 1998). Such a discrepancy could be due to the fact by differences in the disease progression of patients. However, most recently has proposed that the discrepancies could be due to the existence of multiple isoforms of EAAT2 (Lauriat and McInnes, 2007). Dumont and colleagues (Dumont *et al.*, 2013), discovered that the mRNA levels of EAAT2a and EAAT2b isoforms were differently regulated during disease course. In young SOD1G93A rats, EAAT2a mRNA were higher than EAAT2b in the cortex; in adult and end-stage rats, EAAT2a mRNA decreased more than half and EAAT2ab increased. These changes in proportions coincided with a decrease in excitatory amino acid uptake by EAAT2 in cortical synaptosomes. In the lumbar spinal cord, EAAT2b was elevated in young SOD1G93A rats but gradually decreased in both ventral and dorsal horns with disease progression. In the motor cortex of ALS patients, levels of EAAT2 decreased accompanied by a loss in function, while EAAT2b increased more than twofold (Maragakis *et al*., 2004).

Despite these studies with different results developed in different models, the use of induced pluripotent stem cells (iPSCs) of ALS patients, and subsequently induced neural progenitor cells (iNPCs), as a humanized disease model better allows the examination of changes in the EAAT2 expression, function, and excitotoxicity across the spectrum of sALS cases.

1.4.2 Altered metabolic homeostasis in ALS

Metabolic dysregulation in ALS and mechanisms leading to disrupted energy supply is an area of extreme importance, though it is challenging to assess this condition due to difficulty of the patients in swallowing, leading to nutritional deficiency (Ioannides *et al.*, 2016). Individuals with ALS develop defects in energy metabolism (decreased production of ATP and glucose metabolism in neurons) leading to low body mass index (BMI), hypermetabolism, and hyperlipidemia (Dupuis *et al.*, 2011; Ngo and Steyn, 2015; Steyn *et al.*, 2018). (Fig I.5). Weight

loss and hypermetabolism are associated with faster disease progression and shorter survival in ALS (Jawaid *et al.*, 2010; Peter *et al.*, 2017; Steyn *et al.*, 2018).

The cause of these metabolic changes is unknown but may result from hypothalamic atrophy. Gorges *et al* (Gorges *et al.*, 2017) have shown that the hypothalamus is atrophied in ALS patients and in pre symptomatic ALS mutation carriers, mainly in those carrying the *C9orf72* mutation. Furthermore, they found a modest but significant correlation between hypothalamic volume and BMI, especially in patients with fALS, and observed that anterior hypothalamic volumes correlate with age of disease onset (Gorges *et al.,* 2017). While these findings are not specific to *C9orf72* carriers, they do suggest that hypothalamic atrophy, BMI, and disturbances in energy homeostasis could provide prognostic insight. In conformity, targeting of adenosine triphosphate-sensitive potassium (KATP) channels may be a promising approach toward neuroprotection in ALS (Ngo and Steyn, 2015). In addition, energy dysfunction was also suggested to negatively regulate RNA homeostasis and many ALS genes encode RNA-binding proteins, contributing to impaired mitochondrial function (Liu *et al.*, 2017), reason why further studies should investigate its contribution in ALS pathogenesis.

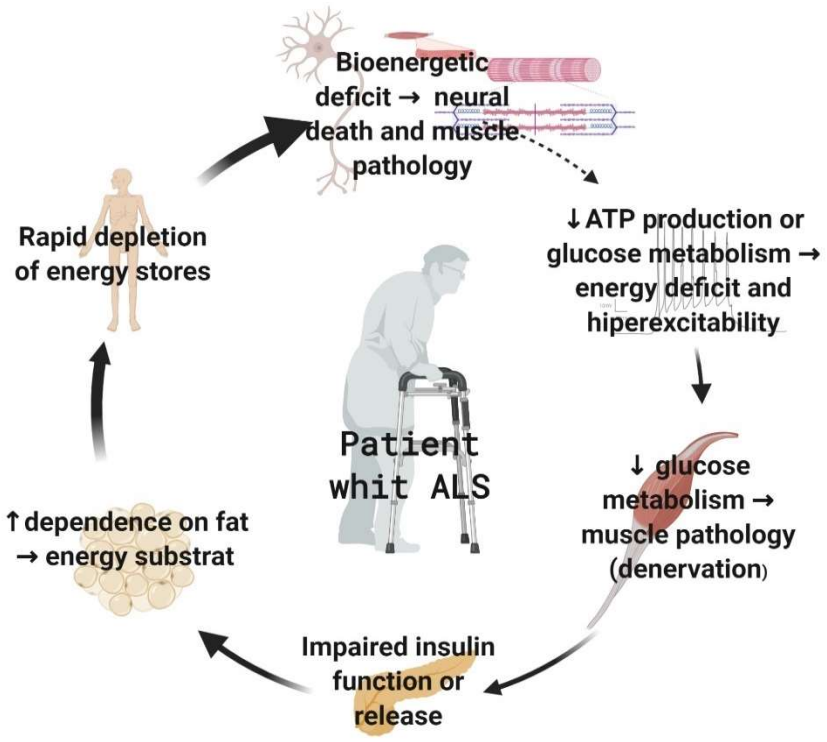

Figure I.3: Potential mechanisms of altered energy balance in ALS. Decreased production of ATP or decreased glucose metabolism in neurons and in the skeletal muscle may contribute to the hyperexcitability and selective degeneration of upper and lower motor neurons and muscle pathology/ denervation in ALS, respectively. Insulin resistance and glucose intolerance may underpin an inability to efficiently use glucose as an energy substrate. Overall, an inability to use glucose in the periphery, in neurons and in skeletal muscle will result in an increased dependence on the use of fat as an energy substrate to offset energy deficit. With escalating metabolic pressure, the rapid depletion of endogenous energy stores will result in a catastrophic failure to meet increased metabolic demand. Thus, a vicious cycle of bioenergetic deficit may underpin or exacerbate disease pathogenesis in ALS.

From: Ngo, S.T. & Steyn, F.J. (2015).The interplay between metabolic homeostasis and neurodegeneration: insights into the neurometabolic nature of amyotrophic lateral sclerosis. ALS, amyotrophic lateral sclerosis; ATP, adenosine triphosphate. (Artwork by Isabel Maurício)

1.4.3 Neuroinflammation and inflammasome

Neuroinflammation plays an important role in the pathogenesis of ALS, involves the activation of astrocytes, microglia, infiltrated T cells and overproduction of pro-inflammatory cytokines and other neurotoxic or neuroprotective molecules, can induce MN death (Glass *et al.*, 2010; Philips and Robberecht, 2011; Endo *et al.*, 2016). In neuroinflammation, astrocytes and microglia switch from a neuroprotective phenotype to a pro inflammatory phenotype.

Neuroinflammation is mediated by protein complexes, inflammasomes, which have a role during brain aging and in pathological conditions, such as ALS (Singhal *et al.*, 2014). Inflammasomes containing a nucleotide binding oligomerization domain (NOD)-like receptor protein (NLRP) including NLRP1, NLRP3, NLRC4, NLRC5, NLRP6, NLRP7 and NLRP12 and the interferon inducible HIN-200 family member absent in melanoma 2 (AIM2) (Johann *et al.*, 2015; Zhou *et al.*, 2016). Of this family, the NLRP3 is the best characterised and most clinically relevant inflammasome. The NLRP3 inflammasome is composed of NLRP3, the adaptor molecule apoptosis associated speck-like protein (ASC) containing a caspase activating and recruitment domain (CARD) and procaspase-1 (Johann *et al.*, 2015). The activation of NLRP3 inflammasome appears to occur by two signals, the activation of toll-like receptor (TLR) or mediated by pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) stimulation (Zhou *et al.*, 2016).

The initial priming signal, induced by TLR/nuclear factor nuclear kappa-light-chain-enhancer of activated B cells (NK-kB) pathway induced a transcriptional regulation and also triggers posttranslational modifications of inflammasome components, increasing the transcription of NLRP3, pro-interleukin (IL)-1β, and pro-IL-18 (Hornung and Latz, 2010; Sutterwala *et al.*, 2014; Zhou *et al.*, 2016).

The second signal triggers assembly of the NLRP3 inflammasome complex, which can be activated by exogenous (infection, tissue damage, metabolic dysregulation) and endogenous molecules (extracellular ATP, hyaluronan, amyloid fibrils, uric acid crystals) (Lamkanfi and Dixit, 2009; Koizumi *et al.*, 2012). The mainly mechanisms regarding activation of the NLRP3 inflammasome is the generation of ROS, the efflux of potassium and rupture of lysosomal or mitochondrial dysfunction (Abais *et al.*, 2015; He *et al.*, 2016; Jo *et al.*, 2016; Zhou *et al.*, 2016).

Regarding to astrocytes and their role in ALS neuroinflammatory, previous studies suggests that gene expression profiling of astrocytes are early activated in ALS, indicating that inflammation in the pre-symptomatic phase of the disease may precede MN degeneration (Vargas *et al.,* 2008). Studies performed with spinal cord astrocytes in pre and early phase of

G9A3-SOD1 mice models suggest that the inflammasome is increased with high levels of IL1β protein. In human spinal cord tissue samples from sALS patients, the expression of the inflammasome revels active caspase 1 and IL18 into astrocytes (Johann *et al.*, 2015). The main bulk, caspase 1, ASC, IL18 and NLRP3 protein levels are elevated in human sALS (Gugliandolo *et al.,* 2018) tissue and also increase levels of IL18 in serum patients (Italiani *et al.,* 2014). Once more, astrocytes were the major cell type expressing NLRP3 and ASC. When comparing with microglia expressions and SOD1 mouse model, levels of ASC but not NLRP3 are found in human sALS samples, perhaps due to the fact that other inflammasome sensor may play a role in microglia-driven neuroinflammation (Johann *et al.,* 2015). Other studies demonstrated that caspase 1 and caspase 3 are activated in spinal motor neurons of SOD1 G93A mice and their inhibition delayed disease progression (Bisseling *et al.*, 2013). Further studies demonstrated that activation of caspase 1 precedes the activation of caspase 3 in MN disease (Pasinelli *et al.*, 2000). Studies in anterodorsal thalamic nucleus neurodegeneration in SOD1 G9A3 mice, observed an increase in NLRP3, ASC and IL1β levels in neurons (Debye *et al.,* 2018).

In conclusion, NLRP3 inflammasome activation highlight the role of neuroinflammation in ALS disease.

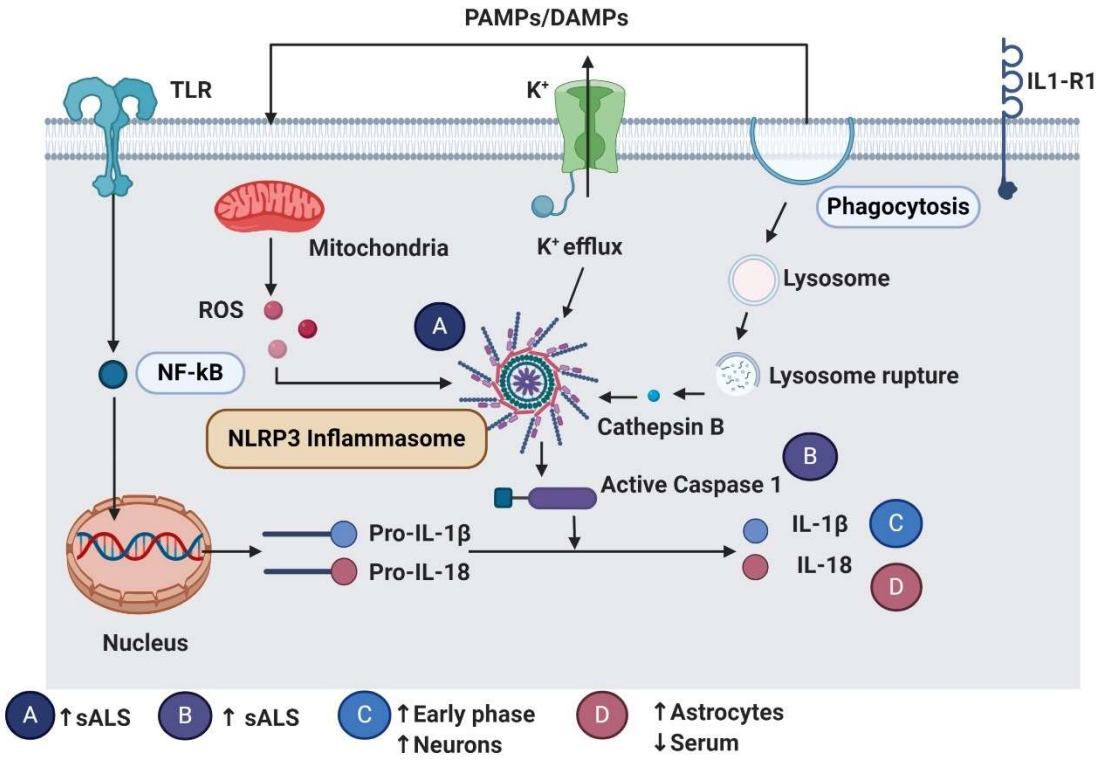

Figure I.4 NLRP3 Inflammasome activation and signaling in ALS. Activation of the NLRP3 inflammasome involves a two-step mechanism. The primary signal comes from the activation of TLRs receptors and is responsible for the upregulation of NLRP3 and pro-IL-1β in an NF-kB-dependent manner. Secondary signals come from multiple pathways: K^+ efflux via P2X7 receptor activation, mitochondrial dysfunction, NADPH oxidase, frustrated phagocytosis and lysosomal rupture, pathways which all appear to converge in the production of ROS. These

primary and secondary signals activate the NLRP3 inflammasome, resulting in proteolytic cleavage of caspase-1 and the maturation of IL-1β. Studies had demonstrated that in sALS NLRP3 inflammasome and active caspase 1 were increased. In early phase of disease and motor neurons, IL-1β were increased, while in serum IL-18 is decreased and elevated in astrocytes. ALS, amyotrophic lateral sclerosis; DAMP, damage-associated molecular pattern; IL-1R, interleukin receptor; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; NLRP, nucleotide binding oligomerization domain (NOD)-like receptor protein; PAMP, pathogen-associated molecular pattern; ROS, reactive oxygen species; sALS, sporadic amyotrophic lateral sclerosis; TLR, toll-like receptor. (Artwork by Isabel Maurício)

1.4.4 Oxidative stress in ALS

Oxidative stress arises from an imbalance between the generation of ROS and the ability of the system to remove or repair the damage caused and restore the prevailing reducing environment (Barber and Shaw, 2010). ROS are a normal product of oxygen metabolism and regularly removed by *SOD1* and *SOD2*, peroxiredoxin, glutathione and catalases. Accumulation of ROS leads to cellular damage through oxidation of nucleic acids and lipids (Ferraiuolo and Meyer, 2014).

The identification of SOD1 mutations, as the first genetic cause associated with the ALS, revealed the association between oxidative stress and disease onset. Such signs of oxidative damage are found in ALS mouse model tissues (Mitsumoto *et al.,* 2008), biologic samples (referring to cerebrospinal fluid, plasma and urine) from sALS patients (Smith *et al.*, 1998; Bogdanov *et al.*, 2000; Simpson *et al.*, 2004; Mitsumoto *et al.*, 2008), as well as in post-mortem tissue (Shaw *et al.*, 1995; Shibata *et al.*, 2001; Chang *et al.*, 2008). Oxidative stress damage in MN were identified was as a potential cell-autonomous death mechanism. Studies have already shown that astrocytes are also affected by oxidative stress, which in turn causes death to the MN (Cassina *et al.*, 2008).

A study performed with SOD1 G93A transgenic mice revels the vulnerability of DNA and RNA to oxidation, and mRNA oxidation precede MN degeneration and reduce the expression of the encoded proteins in such model (Chang *et al.,* 2008).

Mitochondria from mSOD1 rat astrocytes display decrease oxygen consumption and membrane potential, in association with mitochondrial nitroxidative damage and superoxide radical formation in astrocytes both *in vitro* and *in vivo*. Human primary astrocytes expressing mSOD1 were cocultured with human embryonic stem cell (hESC)-derived MN and were detected a selective MN toxicity correlated with inflammatory response in SOD1 mutated astrocytes. Astrocytes can activate NOX2 in order to increased superoxide production and that effect can be reverse with NOX2 inhibitor, apocynin, preventing the loss of MN and death (Marchetto *et al.*, 2008).

SOD1 is a target of oxidative damage and linked to misfolding and aggregation (Rakhit *et al.*, 2002), has been show to disrupt mitochondrial function and the increase of superoxide production, a cycle in which mitochondrial damage and oxidative stress caused bay misfolded

SOD1 leads to exacerbation of SOD1 misfolding and downstream mitochondrial damage (Israelson *et al.*, 2010; Pickles *et al.*, 2013; Pickles *et al.*, 2016).
There is evidence that oxidative stress activation in astrocytes influences MN survival and accelerate the development of ALS.

1.4.4.1 Impaired mitochondrial dynamics

Mitochondria are dynamic organelles that play a fundamental role in remodelling processes according to the energetic requirements of the cell. Mitochondria can fuse to or fission from the mitochondrial network in response to signals (Westrate *et al.,* 2014).
The fusion process enables sharing mitochondrial metabolites, DNA, proteins, and three large GTPases are essential for mitochondrial fusion, the transmembrane mitofusins Mfn1 and Mfn2 (Santel and Fuller, 2000; Rojo *et al.*, 2002), and an inner membrane GTPase dynamin optic atrophy 1 (OPA1) (Alexander *et al.*, 2000; Delettre *et al.*, 2000).
The fission process allows mitochondrial motility and isolation of damage parts of the network prior to disposal by mitophagy (Chan, 2012). The molecule responsible for fission are a dynamin-related protein 1 (Drp1). These two opposite pathways were in imbalance in ALS.
About this imbalance, studies in various models have helped to clarify their actions on the disease. In SOD1G93A expressing SH-SY5Y and NSC-34 cell models, a decrease in OPA1 and increase in Drp1 levels result in a morphological fragmented mitochondria and results in a toxic SOD1 cells. When an overexpression of glutaredoxins 2 are added, the increase in solubility of mSOD1 in mitochondria reduces fragmentation and preserves it function and neuronal cells from apoptosis (Ferri *et al.,* 2010). When *in vivo*, the levels of Mfn1 and OPA1 decrease progressively while the levels of Drp1 phosphorylated at Ser616 and Fis1 remain stable in the spinal cord of SOD1G93A transgenic mice (Liu *et al.*, 2013). For its part, in SOD1G93A mouse muscle fibres there was no change in Mfn1, Mfn2 and Drp1 levels (Liu *et al.*, 2013).
Overexpression of WT TDP-43 resulted in reduced mitochondrial length and density in neurites of primary MN which was sensitive to Mfn2 levels, features further exacerbated by ALS-associated TDP-43 mutants Q331K and M337V (Wang *et al.*, 2013). Another study with the same model of transgenic mice expressing WT TDP-43 shows increase levels of Fis1 and activated phosho-Ser616 DRP1 and a reduction in Mfn1 (components of the machinery of mitochondrial fission), correlated with aberrant mitochondrial morphology and clustering, showed reactive gliosis, axonal and myelin degeneration, gait abnormalities, and early lethality (Y. F. Xu *et al.*, 2010). Contrasting with this study, the same group reported no changes in the levels of phospho-Ser616 Drp1, Fis1 or Mfn1 in mutant TDP-43 M337V transgenic mice, despite a similar mitochondrial phenotype (Xu *et al.*, 2011). It is suggested for such data

contrasts, different pathways lead to fragmentation on the mitochondrial network in WT and mTDP-43 transgenic mice.

Another possible way is external factors linked to disease result in mitochondrial fragmentation. A study of mitochondria functionality performed in TDP-43 A382T patient fibroblast showed Fis1 levels were significantly increased compared to control cells (Onesto *et al.,* 2016).

The relevance of this exacerbation in ALS is not yet fully understood, but several authors suggest pathways and mechanisms for this event. Hoitzing *et al.,* suggest that smaller mitochondria tend to be less energetically favourable and more prone to ROS accumulation induce damage if no fusion back to the network, may exacerbate mitochondrial damage (Hoitzing *et al.,* 2015).

Ashrafi *et al.,* suggest that fragmented mitochondrial network may correlate directly with reduced MMP, ATP levels and increased ROS reported in ALS. Regarding mitochondrial fragmentation it may also reflect mitophagy, a physiological quality control response to mitochondrial damage, since one of the first steps of mitophagy is to isolate damaged mitochondria from the mitochondrial network (Ashrafi and Schwarz, 2013). As disease progresses damage accumulates and as suggested by Song *et al.,* mitochondrial fragmentation may become self-propagating.

Actually, the inhibition of mitochondrial fission by overexpression of dominant negative Drp1 K38A rescued SOD1 G93A induced mitochondrial morphology fragmentation and trafficking defects and increased MN viability (Song *et al.,* 2013). Another study show that the promotion of mitochondrial fusion by co-expressing Mfn2 in WT or TDP-43 M333V expressing primary MN rescued mitochondrial fragmentation (Wang *et al.*, 2013).

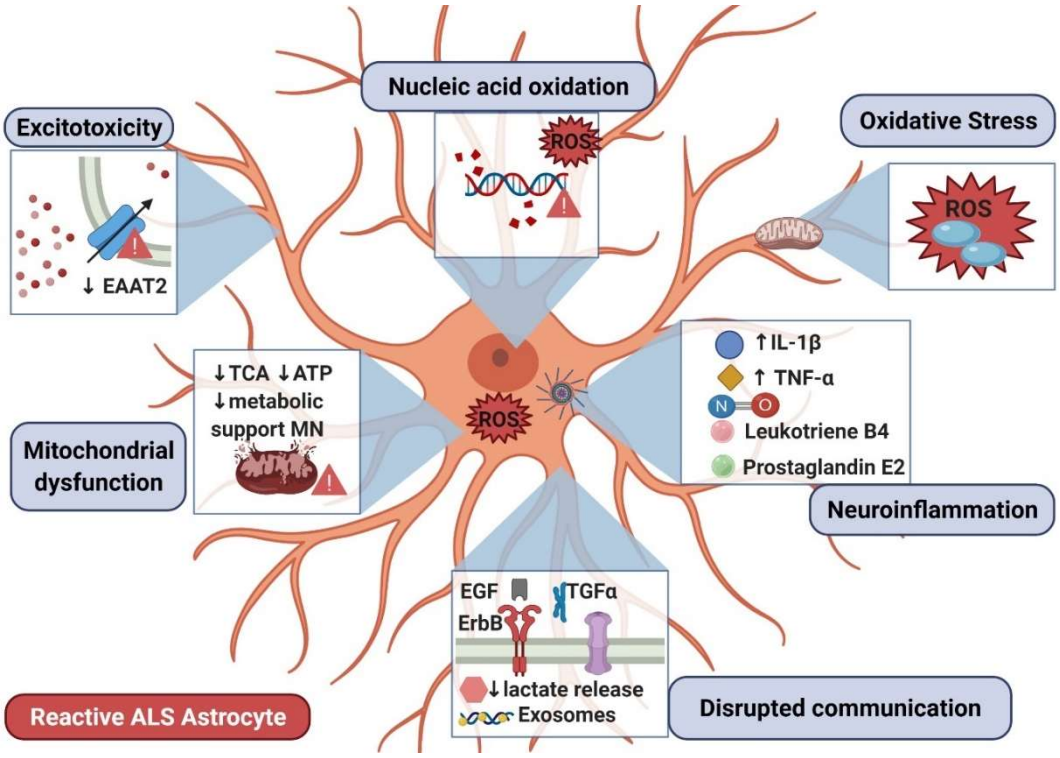

Figure I.5 The signature of astrocyte in ALS. Mechanisms that affect ALS astrocytes: decreased re-uptake of glutamate by the EAAT2 transporter glutamate in the synaptic cleft leads to disruption of intracellular calcium homeostasis causing excitotoxicity; Oxidative stress arises from an imbalance between the generation of ROS and the ability of the SOD1 to remove ou repair the damage caused by ROS; Neuroinflammation involves the overproduction of pro-inflammatory cytokines and other neurotoxic or neuroprotective molecules. ALS, amyotrophic lateral sclerosis; ATP, adenosine triphosphate; EAAT, excitatory amino acid transporter; EGF, epidermal growth factor; IL, interleukin; MN, motor neuron; NO, nitric oxide; ROS, reactive oxygen species; TCA, tricarboxylic acid; TGFα, transforming growth factor alpha; TNF-α, Tumor necrosis factor-α. (Artwork by Isabel Maurício)

1.5 The aberrant phenotype and astrocyte-mediated toxicity to motor neuron

The glial cells in ALS and in particularly astrocytes, revels a predisposition to became neurotoxic when subjected to cellular stress. Such vulnerability might be associated with permanent epigenetic changes, prompting an activated phenotype. After activation, the neurotoxic astrocyte phenotype seems to be maintained by mitochondria dysfunction (Cassina *et al.*, 2008), oxidative stress (Barber and Shaw, 2010), disrupted inflammatory signalling (Liu and Wang, 2017). Such assumptions led to the prediction thar MN degeneration in ALS could be initiated by the emergence of phenotypically aberrant astrocyte playing an active pathogenic role during disease progression (Cassina *et al.*, 2005; Pehar *et al.*, 2006).

In a SOD1G93A rat model, aberrant astrocyte is characterized by expression of astrocytic markers, such as glial fibrillary acid protein (GFAP), S100 calcium-binding protein (S100B), and connexin 43 (Cx43). These cell is typically localized in areas surrounding the dying MN in the ventral horn of the spinal cord (Trias *et al.*, 2013).

Cx43 is the predominant connexin in astrocytes, and some functions attributed to this connexin are homeostatic buffering, synchronization of astrocyte networks, metabolic support to neurons, modulation of synaptic activity and plasticity (Giaume and Liu, 2012; A. *et al.*, 2017). Previous studies have shown an increase in Cx43 expression in cortical and spinal cord (Trias, *et al*., 2018) in ALS SOD1G93A rodent models (Díaz-Amarilla *et al.*, 2011; Keller, *et al*., 2011; Cui *et al.*, 2014; Gomes *et al.*, 2019) and is functional contribution to toxicity to MN is reveal in a study with SOD1G93A mouse model and iPSC-derived astrocytes. This increased expression of Cx43 result in increased gap junctions and hemichannel activity, and the modulation of Cx43 can mitigate SOD1G93A mediated astrocyte toxicity to MN (A. *et al.*, 2017).

Similar to Cx43, S100B is also overexpressed and with increased proliferative capacity in ALS astrocytes (Díaz-Amarilla *et al*., 2011). Several studies appoint to an aberrant expression of S100B in ALS. In patients, S100B is increased in cortex astrocytes, astrocytes, and MNs in the spinal cord and its levels are also increased in the CSF, corresponding to a worsening of the disease (Kamo *et al.*, 1987; Migheli *et al.*, 1999; Süssmuth *et al.*, 2010). In rodent model, S100B astrocytes overexpression in the spinal cord (Shobha *et al.*, 2010; Trias *et al*., 2018), in SOD1G93A mice, the increase of S100B concerns a subpopulation of “aberrant” astrocytes and characterized by a overexpressed Cx43 and GLT1, and by an increased toxicity towards MNs (Díaz-Amarilla *et al.*, 2011; Serrano *et al.*, 2017). Another astrocytic marker that is also found to be increased in this models is Ki-67, a cellular marker for proliferation associated to cell hypertrophic shape and increased cell area (Sofroniew and Vinters, 2010). In a mice pup model SOD1 revealed a signature for this reactive properties of astrocytes, high S100B, Cx43, Ki-67, and decreased levels of GFAP and GLT1 (Gomes *et al.,* 2019).

1.6 Involvement of astrocytes and microRNA dysregulation in ALS neuroinflammation

Mature microRNAs (miRNAs) are a class of endogenous non-coding single-stranded, with approximately 22 nucleotide RNA molecules length, which regulate gene expression post-transcriptionally by base-pairing with their target mRNAs (Stenvang and Kauppinen, 2008). For the last few years, miRNAs has been described as important actors in several biological processes such regulation of development, behaviour, cell fate specification, apoptosis, metabolism and responses to physiological and environmental changes (Ambros and Ruvkun, 2018; Brites, 2020). Some of this miRNAs have been defined as inflamma-miRs since they are associated with inflammatory pathways (Tahamtan *et al.*, 2018), and more specific, miR-155, miR-146a, miR-124 and miR-21 (Brites, 2020). Their dysregulation in ALS are found in many studies, and suggested as biomarkers in therapeutic approaches (Ricci *et al*., 2018).

Upregulation of miR-155 in sALS (Butovsky *et. al.*, 2016) and in SC of mSOD1 mice in pre-symptomatic and symptomatic stages (Cunha *et al.*, 2018). Upregulation of miR-124 in mSOD1 astrocytes contributes to MN dysfunctions (Pinto *et al.*, 2017; Wang *et al.*, 2018). The overexpression of miR-21 in astrocytes as described by Bhalala *et al*., *(*Bhalala *et al.*, 2012) attenuate the hypertrophic profile while its inhibition led to increased hypertrophic profile, suggesting increased reactivity and inflammatory profile. A recent study conducted by Barbosa *et. al*., (Barbosa *et al.*, 2021), in order to revert the downregulation of mir-146a, and respectively the astrocyte inflammation, reveals that dipeptidyl vinyl sulfone as the ability in modulating miR-146a expression and increase expression (Falcão *et al.*, 2017).

1.7 Models for studying astrocytes in ALS

To reach today's point of knowledge about the astrocyte and its role in ALS, detailed in vitro studies have been conducted over the decades, which have allowed us to understand the physiological and pathological entanglement of the disease, as well as its interaction with the surrounding cells. One of the most used models was the rodent model, which despite its advantages as less complex systems, has limitations in the molecular mechanism and ethical issues regarding its use. This limitation is currently circumvented with the use of in vitro patient astrocyte models. A brief description about them follows in the following sections.

1.7.1 Primary cultures of rodent astrocytes

Much of the knowledge about the astrocyte has been driven by primary culture assays of astrocytes. Questions about the role of this actor in both physiological and pathological states have been unraveled with primary culture assays. However, these cultures do not fully mimic complex events that occur *in vivo*, such as amino acid neurotransmission and calcium signaling (Lange *et al.,* 2012), but it is still a valuable tools (electrophysiological, molecular, genetic) in the study of ALS astrocytes. The studies of functional receptors expressed by astrocytes and respective responses can be study in real time cultures, showing functional roles that would otherwise be difficult to achieve. The question always remains to what extent one can transfer from primary cultures of astrocytes to functional rodent models and human astrocytes *in vivo* (Kimelberg *et al*., 2000; Foo *et al.*, 2011; Kimelberg, 2010).

1.7.2 *In vitro* studies

Much of today's knowledge about the pathological mechanisms of ALS is due to studies conducted in *in vitro* models. The *in vitro* model has the advantage of being a less complex system, thus allowing to reveal the specificity of cellular mechanisms (Gois *et al.*, 2020). Regarding genetic mutations in TARDPB, FUS and C9orf72 it has been possible to perceive their connection to the development of the disease in fALS and sALS (Myszczynska and

Ferraiuolo, 2016), and those involving the neurodegeneration of the MN in culture as pathogenic mechanisms and toxicity (Nagai *et al*., 2007; Thonhoff *et al*., 2009).
Among the *in vitro* studies, neurons culture expressing mSOD1 allowed the understanding of mechanisms associated with ALS such as excitotoxicity, mitochondrial dysfunction, formation of reactive species, proteins inclusions, axonal transport dysfunction, cell death and the role of astrocytes in toxicity and death of MN.

1.7.3 Cell reprograming technologies

In line with the evolution of reprogramming technologies, the conversion of adult human fibroblasts into iPSCs (Yamanaka *et al*., 2007) with the use of selected transcription factors has opened up new opportunities to study new fields of ALS, with particular emphasis on the sporadic disease (Myszczynska and Ferraiuolo, 2016). Since then, multiple direct conversion methods have been developed to iNPCs (Meyer *et al.,* 2014) and MNs (Son *et al*., 2011) from ALS patients.
Neural progenitor cells (NPCs) were isolated from post-mortem spinal cord samples of ALS patients, cultured, and differentiated into MNs, astrocytes and oligodendrocytes *in vitro* (Hester *et al.,* 2011). Such a landmark in technology provide model all forms of ALS *in vitro* without introducing major epigenetics alterations in cells. Later, primary astrocytes were isolated from spinal cord and brain biopsies of ALS patients (Re *et al*., 2014) providing new insights into the physiopathology of ALS.
Below is a brief description of each of the reprogramming techniques.

1.7.3.1 Astrocyte differentiation from iPSCs

The recent advances in stem cell biology have provided opportunities to develop disease-specific cell types, allowing to understand mechanisms that contribute to pathogenesis of disease. The ability to reprogram somatic cells into iPSCs provide advantages to generate patient-specific iPSCs that carry the same genetic information, in particular, the same mutations that may contribute to the disease process also allowing the study of sALS cases not possible with animal models (Ilieva *et al*., 2009; Roybon *et al.*, 2013; Richard and Maragakis, 2015).
The cells are easily obtained and derived from ALS patients with both familial and sporadic forms and allows to differentiate these cells into multiple subtypes.
iPSCs were first characterized by Yamanaka and Takahashi (Takahashi and Yamanaka, 2006) with their reprogramming from mouse somatic cells. iPSCs are pluripotent stem cells that are generated directly from adult cells. In this study, the authors used cultured skin fibroblasts from adult individuals introducing four transcription factors, POU5F1 and Oct4 (Oct3/4), sex

determining region Y-box 2 (Sox2), c-Myc oncoprotein (c-Myc), and Kruppel like factor 4 (Klf4), under embryonic stem cell (ESC) culture conditions. They designed these cells like iPSC, which exhibit the morphology and growth properties of ESC and express embryonic stem marker genes. The introduction of the obtained cells into nude mice via subcutaneous, result in tumours containing a variety of tissues from all three germ layers (endoderm, mesoderm, and ectoderm) (Takahashi and Yamanaka, 2006).

Advantages of this new technology are basically two and led to great changes: overpassed ethical concerns regarding the use of ESCs and more important, the use of stem cells for disease modelling.

In the subsequent year, Takahashi *et al.,* (Yamanaka *et al.,* 2007), developed the methodology to obtain human iPSC. The authors generated IPS cells from adult human dermal fibroblasts with the four factors (Oct3/4, Sox2, c-Myc, and Klf4). Once again, human iPS cells were similar hESCs in morphology, proliferation, surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes, and telomerase activity. As in the previous study, these cells could differentiate into cell types of the three germ layers, either *in vitro,* or in teratomas. The great finding of this study was the confirmation that iPS cells can be generated from human fibroblasts (Yamanaka *et al.,* 2007).

Despite these unique advantages, one important caveat with the iPSCs models is that a countless of epigenetic changes due to reprogramming and lengthy process of cell selection and *in vitro* cultures might contribute to the variations in the system (Richard and Maragakis, 2015). However, iPSCs were shown to recapitulate after differentiation *in vivo* biological markers of ALS nerve cells (neurons, motor neurons, astrocytes, oligodendrocytes) and that of other ALS-relevant cell subtypes.

1.7.3.2 Astrocyte differentiation from iNPCs

Despite patient-specific iPSCs have been used in ALS in last years, proved to be a lengthy process in a disease with a short life span. This limitation was circumvented by the direct conversion of human skin fibroblasts into neural progenitor cells and subsequent differentiation into induced astrocytes (iAstrocytes) (Meyer *et al.*, 2014). This conversion is carried out using retroviral vectors containing the same reprogramming factors used for iPSCs generation (Sox2, KLF4, Oct3/4, c-Myc) to convert human fibroblasts into iNPCs in approximately 6 to 10 days (Meyer *et al.,* 2014). The iNPCs grow as neurospheres when plated in un-coated dishes and start expressing markers like Pax6 and Nestin. These cells have the potential of tripontency, upon different protocols, to oligodendrocytes, neurons and astrocytes, generating stable expandable cells that can be cryopreserved for more than 12 passages and uniformly display morphological and molecular features. In this case, for astrocyte differentiation, the

iNPCs are seeded in a low density and in 10% FBS medium for at least 7 *days in vitro* (DIV). After, cells acquire characteristics of mature astrocytes (iAstrocytes). According to this protocol, it is possible to obtain mature astrocytes directly transdifferentiated from fibroblasts between 3 to 5 weeks (Meyer *et al.*, 2014).
In contrast to iPSCs, iNPCs do not exhibit a tumorigenic potential.

1.7.3.3 Highlights of iNPCs as providing advantages as ALS model

Regarding to donnor cells, fibroblasts are the cell type of choice for iNPS, since skin biopsy samples are easy to obtain from donors, both disease or health, and can be expanded in culture (Mertens *et al.*, 2016). In the generation of iNPCs only one pro-neurogenic factor is necessary. iNPCs can be generated within 2–3 weeks and can serve as expandable tri-potential intermediates, conferring on them remarkable properties with regard to long-term self-renewal, tripotential differentiation and responsiveness to regionalization cues, and they can be differentiated into cultures of functional neurons in 1–6 weeks (Falk *et al.,* 2012). The possibility of generating donor-specific glial cells from patients is one of the most important contributions of the stem cell field. The individual's identity can be recapitulated in culture, and the preservation of these unique features may differ between the different methods. This is particularly important for attempts to model and understand complex multigenic, sporadic and epigenetic diseases. iNPCs diferentiation has made it possible to study familial and sporadic forms such as ALS (Burkhardt *et al.*, 2013).

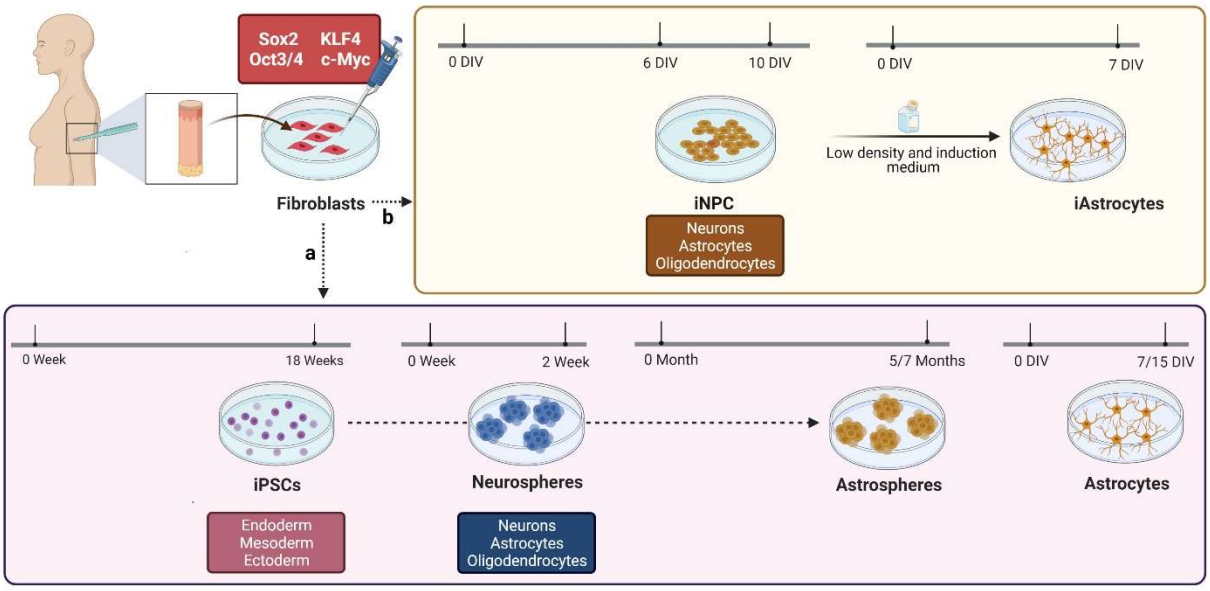

Figure I.6 *In vitro* culture of direct reprogramming human adult fibroblasts to astrocytes. In a process namely transdifferentiation, using retrovirus carrying the Yamanaka factors (Oct3/4, Sox2, c-Myc, and Klf4), human fibroblasts are converted to induced pluripotent stem cells (iPSCs)(**a**), in a process that could take place as long as 18 weeks. On the other hand, we can directly transdifferentiated into induced neuronal progenitor cells (iNPCs) in approximately 10 days (**b**). Given its characteristic pluripotency, iPSCs can generate three germ layers (endoderm, mesoderm, ectoderm). After 2 weeks induction for neuroectoderm lineage and growing as embryoid bodies, iPSCs progress to neurospheres which in turn can give rise to neurons, astrocytes and oligodendrocytes in specific conditions. For astrocyte differentiation, astrospheres are directly generated from iPSCs, or from neurospheres, in a process that could take place up to 7 months, followed by 15 DIV in culture for astrocyte maturation. Based on their multipotency, iNPCs can generate three neural cell types (neurons, astrocytes and oligodendrocytes). Regarding astrocyte maturation, iNPCs are plated in a low density and in induction medium for 7 DIV**,** in a condition where cells acquire a maturate state. iNPCs, induced neuronal progenitor cells; iPSCs, induced pluripotent stem cells; Oct3/4, POU5F1 and Oct4; Sox2, sex determining region Y-box 2; c-Myc, c-Myc oncoprotein; Klf4, Kruppel like factor 4. (Art work by Isabel Maurício)

1.8 ALS: specific inducers or general inflammatory pathway implicated in neurotoxic reactive astrocytes

Astrocytes represent the main glial cell in the CNS, being responsible for all aspects of metabolic support, nutrition, ion control and transmitter environment, BBB regulation and defense (Valori *et al.,* 2014). Faced with an inflammatory state or trauma, astrocytes undergo a morphological transformation called reactive astrocytosis (Sofroniew, 2015). Liddelow *et al* performed an elegant study in which determined that, in the face of an induced neuroinflammatory condition, astrocytes assume a reactive state phenotype, which he called A1. This reactivity was achieved when induced by activated microglia, and its strongest inducers of a partial A1 phenotype were IL-1α, TNF, and complement component 1, subcomponent q (C1q) both *in vitro* and *in vivo*. These three cytokines are highly expressed by microglia in inducing A1 astrocytes, although others cytokines are expressed (Liddelow *et al.*, 2017). The A1 astrocytes lost the ability to promote neuronal survival and outgrowth,

synapse formation, myelin debris, and are abundant in neurodegenerative diseases, such ALS and AD, which presence contribute to neurodegeneration and disease progression. The reason why the CNS produces a reactive astrocyte is still poorly understood, but it seems to be a mechanism to preserve neuronal function, in which dysfunctional neurons are removed or disabled, or given the ability of this astrocyte to secrete the complement cascade components, it seems to play a role in clearance of bacteria or viruses by the immune system. Other studies suggest the contribution of astrocytes in neurodegeneration. The generation of functional astrocytes from iPSCs derived from familial early-onset type AD patients with mutations in presenilin-1, demonstrated that astrocytes had increased β-amyloid production, altered cytokine release pattern and altered Ca^{2+} homeostasis (Oksanen *et al.,* 2017). This study relied on the analysis of the pattern of cytokine release after inflammatory-derived factors stimulation over astrocytes from patient and control, namely, interleukin-1β (IL-1β) and TNF-α, pro-inflammatory mediators known to be increased in AD brain (Sofroniew, 2015). The inflammatory stimulation led to astrocytes which manifest hallmarks of AD pathology, suggesting that the inflammatory response is related to Aβ pathology. These data allow us to confirm that iPSCs confer an excellent research tool not only to mimic cellular pathology but also to identify early changes in the pattern of cytokine release and the role of astrocytes in neurodegeneration. Another iPSC-based study have shown aberrant morphological changes in early-onset familial form of AD and late-onset sporadic form of AD astrocytes (Jones *et al.,* 2017).

Knowing that the A1 phenotype is achieved through an activation that mimics microglia-derived factors and understanding that an inflammatory-derived factors induction also leads to astrocyte state of neurodegeneration in AD, we intent to understand the behaviour of both activations on sALS iAstrocytes. We acknowledge that the iPSCs as a disease modelling is important in diseases such as ALS, but we will be more ambitious and use iNPCs derived from sporadic ALS patient and a non-ALS patient.

1.9 Aims

As described by Meyer *et. al.,* (Meyer *et al.*, 2014) and lately explored in our recent study (Gomes *et al.,* 2022), direct conversion from patient fibroblasts into neuronal progenitor cells, and iAstrocytes is an efficient manner to investigate the neurotoxic mechanisms of the ALS pathological astrocytes and their molecular profile. More specifically, the application of this technique to investigate the sporadic form of the disease, which is the most prevalent form and the most challenging, is very promising to explore astrocyte signature and testing of target-driven medicines.

Several models have been used in ALS to trace the early development events of this disease, mainly with the SOD1G93A (mSOD1) mice. However, the translation of findings in animals to humans is problematic and can now be surpassed by the utilization of astrocytes directly converted from ALS patient fibroblasts with the additional advantage of retaining the individual age-associated signature (Gatto *et al.*, 2021) . With such a possibility we are also in conditions to investigate the effects of distinct inflammatory mediators as contributing and aggravating the ALS disease progression, in order that we can find more effective targets to develop innovative therapeutics.

Our group showed recently (Gomes et al. 2020) that astrocytes isolated from the cortical brain and the SC of the mSOD1 mice model exhibited different reactive phenotypes, which may derive from regional specific cues associated to pathological conditions in the surrounding tissue. Indeed, besides sensing differently the presence of inflammatory mediators, astrocytes may also acquire different reactive phenotypes determined by translational or local inflammation, mainly when derived from microglia.

In the present study we used the direct conversion of fibroblasts from a selected human sporadic patient fibroblast into iNPCs, which were obtained by Drª Cátia Gomes at Drs Kathrin Meyer and Brian Kaspar lab at the University of Ohio (Meyer *et al.*, 2014). iNPCs were later differentiated into iAstrocytes in our lab in Lisbon (Gomes *et al.*, 2022) using the protocol implemented by Drª Cátia Gomes for her doctoral thesis. The global aim of this thesis was to used those iAstrocytes from the sporadic patient to test their response toward the influence of neuroinflammation induced by: (i) TNFα+IL1β, generally derived from peripheral immune cells, as used in a previous study (Oksanen *et al.*, 2017); and TNFα+IL1α+C1q as lately proposed for the microglial cytokines responsible for the astrocyte neurotoxic phenotype (Liddelow *et al.,* 2017). Only few studies were developed in iAstrocytes from ALS sporadic patients and the impact of such immunomodulation never addressed, as far as we know.

Specific aims were to:

1. Generate iAstrocytes from the iNPCs generated from a patient with the sporadic form of ALS (sALS-iAstrocytes) and from a healthy control line;
2. Assess the differential sALS-iAstrocytes morphologies (arborised, polarised and fibroblast-like) upon TNF-α+IL-1β immunostimulation and interaction with TNF-α+IL-1α+C1q as cytokines released by microglia activation;
3. Investigate differences by the two activations on alterations of mitochondria dynamics in sALS-iAstrocytes using the MitoTracker Red;
4. Identify whether sALS-iAstrocytes pathological phenotype (dysregulation of GFAP, S100B, Cx43 and Ki-67) show a distinct profile upon each of the two tested stimulations;

5. Explore if the activation of sALS-iAstrocytes inflammasome components differed by the action of the translational and regional tested inflammatory mediators.

Chapter II

Material and Methods

2. Experimental outline

In diseases with a short life span and unclarified disease mechanisms, such as ALS, having a study model that is as similar as possible to the human disease is crucial, especially to investigate the sporadic cases no mimic by any animal model and where 90% of patients are included. This study brings a methodology that uses the reprograming technology that allowed us to investigate astrocyte pathological phenotype and cell response to two different immunostimulant conditions, one more closely recapitulating the peripheral inflammation (TNF-α+IL-1β) (Oksanen *et al.,* 2017) and the other more central derived from reactive microglia (TNF-α+IL-1α+C1q) (Liddelow *et al.*, 2017). We used iNPCs generated from the fibroblasts of a patient presenting a sporadic case (sALS), by direct conversion (Meyer *et al.,* 2014), which were produced by the PhD student Cátia Gomes during her training at the University of Ohio (Gomes *et al.*, 2022). These cells were sent to our laboratory and differentiated into iAstrocytes. We used a female ALS patient and a matched control (Table II.3) to obtain the respective iAstrocytes, which were characterized for morphology, mitochondria activation, reactive markers, and inflammasome activation, in naïve cells and after both stimulations.

2.1 Supplements and chemicals

Dulbecco's modified Eagle's medium-Ham's F12 medium (DMEM-Ham´s F-12), DMEM high glucose w/o pyruvate, FBS and Penicillin/Streptomycin were purchased from Biochrom AG (Berlin, Germany). Antibiotic-Antimycotic, Bovine serum albumin (BSA), Paraformaldehyde (PFA) and Hoechst 33258 dye were from Sigma-Aldrich (St. Louis, MO, USA). B27™ serum was purchased from Gibco (Thermo Fisher, MA, USA). IL-α was purchased from Cell Signaling (Danvers, Massachusetts, USA), TNF-α from PeproTech (London, UK) and C1q from MyBioSource (San Diego, CA, USA). TipleXtractor, Xpert cDNA Synthesis Mastermix Kit and Xpert Fast SYBR Mastermix (Uni) BLUE were purchased from GRISP (Porto, Portugal). miRCURY LNATM Universal RT microRNA PCR were obtained from Exiqon (Vedbaek, Denmark). PowerUp™ SYBR™ Green Master Mix were obtained from Applied Biosystems, Life Technologies. Mitotracker® Red CMXRos is from Invitrogen Molecular Probes™ (Eugene, Oregon, EUA). All the other common chemicals were of analytical grade and were purchased either from Sigma-Aldrich or Merck.

2.2 Antibodies

The set of primary and secondary antibodies used in immunocytochemistry assays is shown below in Table II.1 and Table II.2, respectively.

Table II.1 List of antibodies used in immunocytochemistry, with the respective brand and dilution.

Primary Antibody	Source	Specie	Dilution ICC
GFAP	Novocastra, GFAP-GA5, Leica	Mouse	1:100
S100B	Dako, AbCam, ab52642	Rabbit	1:200

GFAP, Glial fibrillary acid protein; S100B, S100 calcium-binding protein B

Table II.2: List of antibodies used in immunocytochemistry, with the respective brand and dilution

Secondary Antibody	Source	Dilution ICC
Anti-mouse Alexa 594	Invitrogen Corporation, A-11012	1:1000
Anti-rabbit Alexa 488	Invitrogen Corporation, A-11008	1:1000

2.3 Primers

The set of primers used in polymerase chain reaction (PCR) to amplify protein-coding genes was purchased from Invitrogen Corporation (Carlsbad, California, EUA), and presented below in Table II.3.

Table II.3: List of primer sequences used in polymerase chain reaction (PCR) to amplify protein-coding genes

Gene	Forward primer sequence	Reverse primer sequence
β-actin	5'-CAGAGCCTCGCCTTTGCCGA-3'	5'-ATCCATGGTGAGCTGGGGGC-3'
GFAP	5'-GAGGTTGAGAGGGACAATCT-3'	5'-GCTTCATCTGCCTCCTGTCTA-3'
S100B	5'-TGTAGACCCTAACCCGGAGG-3'	5'-TGCATGGATGAGGAACGCAT-3'
Cx43	5'-GTTCAATCACTTGGGTGAC-3'	5'-AGTTGAGTAGGCTTGAAC-3'
Ki-67	5'-TGATGGTTGAGGCTGTTCCTTGATG-3'	5'-TCCTTTGGTGGGACCAAGACCTG-3'
NLRP3	5'-ACAATGACAGCATCGGGTGT-3'	5'-AACCAGCTACAAAAAGCATGGA-3'
IL1-R1	5'-GAGCGGCAGGAATGTGAAA-3'	5'-GAGGGTGGGTCTACCTGGA-3'
IL-1B	5'- GGGCCTCAAGGAAAAGAATC-3'	5'- TTCTGCTTGAGAGGTGCTGA-3'

GFAP, glial fibrillary acid protein; S100B, S100 calcium-binding protein B; Cx43, conexin 43; Ki-67, proliferation marker Ki-67; NLRP3, nucleotide binding oligomerization domain (NOD)-like receptor protein; IL, Interleukin

2.4 Equipment

Fluorescence microscope (model AxioScope.A1) with integrated camera (AxioCamHRm) was purchased from Carl Zeiss, Inc. (Germany) and optical microscope

with phase-contrast equipment (Olympus, model CK2-TR) were used for evaluation of cell morphology. In order to ensure a stable environment to optimal cell growth (37ºC and 5% CO_2), cell cultures were maintained in HERAcell 150 incubators (Thermo Scientific, Waltham, MA, USA) and the work performed in sterile conditions in a HoltenLamin Air HVR 2460 (Allerod, Denmark). Eppendorf 580R (Eppendorf, Hamburg, Germany) centrifuge was used for different experimental procedures. QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, Life Technologies) was used for qRT-PCR. NanoDrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) was used to quantifying total RNA.

2.5 Skin fibroblast isolation and reprogramming

Human skin fibroblast samples were obtained from one non-ALS control and one ALS patient, diagnosed with sALS (Table II.4). Informed consent forms were obtained from the two subjects before sample collection. The isolation of patient skin fibroblasts and subsequent maintenance were performed in the University of Ohio, as published (Meyer *et al.,* 2014). Briefly, approximately 30 min after sampling, 2 cm^2 skin samples were washed with phosphate buffered saline (PBS) and placed in a 10 cm^2 dish with epidermal side down; 0.5 cm^2 strips were cut and incubated with 25 ml of 0.05% trypsin/EDTA (Invitrogen Corporation, Carlsbad, CA, USA) in a 50 ml conical tube at 37°C for 45 min. After that, 20 ml of DMEM containing 10% fetal bovine serum (FBS, Gibco/ThermoFisher, Waltham, MA, USA) were added and cells were centrifuged at 350 g for 4 min. The cell supernatant was aspirated and fresh DMEM containing 10% FBS was added, and cells were centrifuged at 350 g for 4 min. The cell supernatant was aspirated and fresh DMEM containing 10% FBS was added before plating cells in a six-well plate, as described (Gomes *et al.*, 2022). Preparation of retrovirus for reprogramming and conversion into iNPCs was performed as described (Gomes *et al.,* 2022). Briefly, DNA for klf-4, Oct3/4, Sox2 or c-Myc, obtained from Addgene (Cambridge, MA, USA), was transfected with the packaging plasmids CMV GagPol and CMV VSV-G in human embryonic kidney carcinoma 293 cells (Hek293T), using the CaCl2 transient transfection method. Viral particles were then obtained from the supernatants of Hek293T cells, collected for 3 consecutive days beginning 48 h after transfection. The concentration of the virus capable of promoting around 70–80% positive cells was selected for each factor, to be used thereafter in the transdifferentiation procedure.

Table II.4: Demographic information of the iAstrocytes-derived fibroblasts lines used in this study

Cell line	Clinical Features	Sex	Age at biopsy (years)	Onset Type	Symptoms onset to biopsy (months)
CTRL	Non-ALS	Female	64	Not applicable	
ALS	Sporadic disease	Female	69	Distal upper extremity	30

CTRL, control; ALS, amyotrophic lateral sclerosis

2.6 Conversion of ALS patient fibroblasts into iNPCs

The direct conversion of ALS patient fibroblasts to iNPCs was carried out according to the method developed by Meyer and colleagues (Meyer *et al.,* 2014). Briefly, one day after seeded into one well of a six-well plate at a density of ~10^5 cells/ml, skin fibroblasts from patient and non-ALS control were transduced with a mixture of retroviral vectors expressing Klf4, Oct-3/4, Sox2 and c-Myc (with a multiplicity of infection of 10 for each viral vector). The following day, regular fibroblast medium (DMEM plus 10% FBS) was added. After this recovery time, and to promote iNPC conversion, cells were washed with 1X PBS and incubated with fresh medium consisting of DMEM/F12, 1% N2, 1% B27 and containing fibroblast growth factor (FGF, 10 ng/ml), epidermal growth factor (EGF, 10 ng/ml) and heparin (5 µg/ml). This medium was changed every day thereafter. Once the cells change shape and form sphere-like structures (around 7 days post-transduction), meaning that iNPC culture is established, medium is switched to iNPC medium consisting of DMEM/F12, 1% N2, 1% B27, and FGF (20 ng/ml) (Figure II.1).

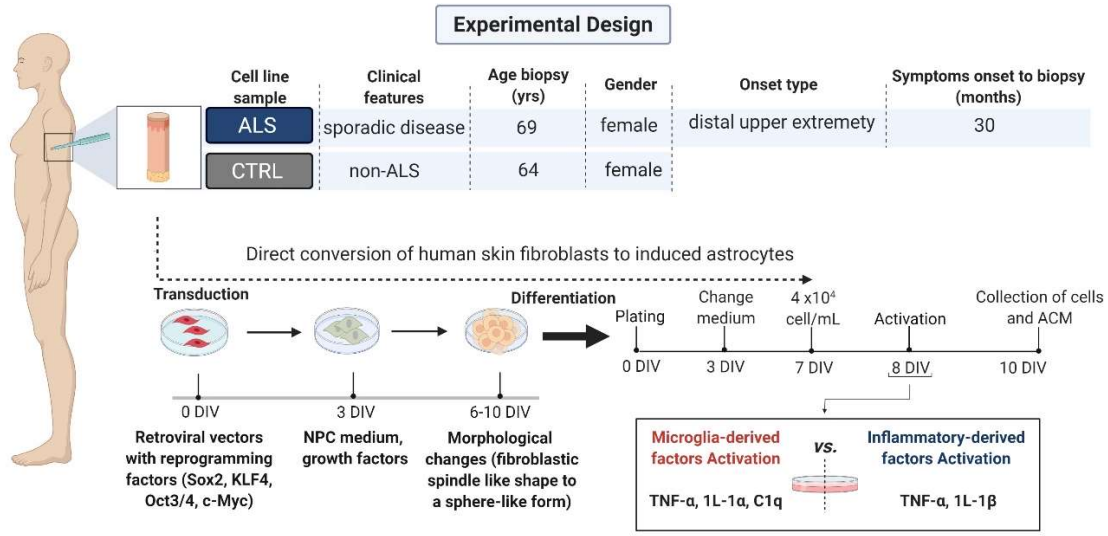

Figure II.1: Schematic representation of the experimental design for the direct conversion of human skin fibroblasts to induced astrocytes. Human fibroblasts from biopsy collection on a CTRL and a sALS female patient were differentiated into iAstrocytes in a culture of 10 DIV. Then, both non-ALS and sALS cells were full differentiated into a low-density medium for 7 DIV. In the next step, both non-ALS and sALS iAstrocytes were incubated with tumor necrosis factor (TNF)-α, interleukin(IL)-1α and C1q (usually

released by the activated microglia) or TNF-α and IL-1β (more representative of a generalized peripheral inflammation), for 48 h. At 10 DIV, cells were collected for different programmed assays, as well as their culture medium (ACM) containing the iAstrocyte secretome. ALS, amyotrophic lateral sclerosis; iAstrocytes, induced astrocytes; sALS, sporadic amyotrophic lateral sclerosis; CTRL, non-ALS female control; DIV, days *in vitro*. (Artwork by Isabel Maurício)

2.7 Differentiation of iNPCs into iAstrocytes

To differentiate iNPC into iAstrocytes, iNPCs were seeded at low density in a fibronectin-coated 10 cm^2 plate with medium containing DMEM, 10% FBS and 0.2% N2. At the end of 3 DIV, the medium was changed. iAstrocytes were characterized and maintained in differentiation/maturation conditions for 6 to 7 days, as we and others described (Meyer *et al.*, 2014; Gomes *et al.*, 2022). At day 7, cells were washed with EDTA (1:1000) in PBS and the incubated with accutase for 5 min at 37ºC, which action was stopped by addition if the iAstrocytes media. Cells were then centrifuged at 800 g for 4 min and the supernatant discarded. Finally, iAstrocytes were plated in 6- or 12-multiwell plates, previously coated with fibronectin, at a density of 4 x 10^4 cells/ml in iAstrocytes media. Bright field images corresponding to iAstrocytes differentiated from iNPCs at 7 DIV are represented in Figure III.1 in the Result Chapter III. iAstrocytes were maintained at 37ºC in a humidified atmosphere of 5% CO_2.

2.8 iAstrocyte immunostimulation

After 7 DIV, iAstrocytes were seeded into a six-well plate at a density of 4x10^4 cells/ml per well, from sALS patient and non-ALS control, and incubated with fresh medium consisting of DMEM, 0.2% N2, 1% FBS (free of exosomes). After 24 h, iAstrocytes were incubated for 48 h with medium containing microglia-derived factors, either TNF-α (30 ng/ml), IL-1α 3 (ng/ml) and C1q (400 ng/ml) (Liddelow *et al.*, 2017), or IL-1β (10 ng/ml) and TNF-α (50 ng/ml) (Oksanen *et al.,* 2017). All cells and conditioned medium containing the iAstrocyte secretome (ACM) were collected at 10 DIV.

2.9 Immunocytochemistry

After 10 DIV, astrocytes plated in coverslips were collected and fixed with 4% (w/v) of PFA. The immunocytochemistry protocol was based on prior studies from the group (Cunha *et al*., 2018). Briefly, coverslips were incubated 20 minutes with 0.2% Triton X-100 in PBS for cell permeabilization, 30 minutes with blocking solution 3% BSA in PBS, and overnight with primary antibodies at 4ºC. (Table II.2). In the following day, after three washes, coverslips were incubated for 2 hours at room temperature (RT) with species-specific fluorescent secondary antibodies (Table II.3). For nuclei staining, coverslips

were incubated with Hoechst 33258 dye (Sigma-Aldrich) and then mounted onto uncoated slides using PBS-Glycerol (1:1). Finally, fluorescence was visualized using AxioCamHRm camera adapted to an AxioScope A1®microscope (Zeiss, Germany) and Zen 2020 (blue edition) software. Merged images of UV and fluorescence from at least 10 random fields were acquired with 40X magnification per sample. Cellular morphology (area and Feret's diameter), as well as S100B mean fluorescence intensity levels were quantified for each cell by using ImageJ software.

2.10 RT-qPCR

Total RNA was isolated from iAST using TRIzol® Reagent (LifeTechonologies) and following manufacturer's instructions. Total RNA was quantified using Nanodrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and conversion to complementary DNA (cDNA) was performed with the Xpert cDNA Synthesis Mastermix Kit (Thermo Scientific). RT-qPCR was accomplished on a QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems) using Xpert Fast SYBR Green qPCR Master Mix (Thermo Scientific), under the following optimized conditions: 50ºC for 2 min followed by 95ºC for 2 min and finally 45 cycles at 95ºC for 5 sec and 62ºC for 30 sec. To verify the specificity of the amplification, a melt-curve analysis was performed immediately after the amplification protocol. The PCR was performed in 384-well plates, with the set of primers designed for protein-coding genes and cDNA (Table II.4). Each sample was measured in duplicated, and a non-template control (NTC) was included for each amplificated gene. Non-specific products of PCR were not found in any case. β-actin was used as an endogenous control to normalize gene expression levels.

2.11 Mitochondrial viability

To determine viable mitochondria, iAstrocytes were incubated for 30 min at 37°C with 500 nM of MitoTracker Red® solution as usual in our laboratory (Vaz *et al.,* 2015), and then fixed with 4% PFA. Cell nuclei were stained with Hoechst 33258 dye. Images were acquired as described above for immunocytochemistry. In this case, total mean fluorescence intensity was quantified by ImageJ software and normalized to the total number of cells in each field.

2.12 Statistical analysis

Results of at least three independent experiments were expressed as mean values ± SEM. Results of activated astrocytes were represented as fold *vs* non-activated CTRL samples. Differences between mean groups were determined by the two-tailed unpaired

Student's *t-test*. Welch's correction was applied when variances between groups were significantly different. Statistical analysis was performed using GraphPad PRISM 8.0 (GraphPad Software, San Diego, CA, USA). $p < 0.05$ was considered as statistically significant.

Chapter III

Results

3. Human iAstrocytes as an advanced and unique model to study sALS

ALS is a neurodegenerative disorder without treatment affecting MNs and glial cells. ALS astrocytes are highly toxic to MNs in either sALS or fALS cases (Gomes *et al.,* 2022). The sALS disease is the most complex to be investigated due to the absence of animal models, requiring the use of cells from these individuals. Here, we had the possibility to use here the direct conversion of human fibroblasts from a non-ALS control individual and a sALS case, both females (Table II.1), into NPCs followed by differentiation into iAstrocytes, as previously described (Meyer *et al.*, 2014).

The method requires one month of work in the laboratory and the generated iAstrocytes retain the ageing features of the donor individuals (Gatto *et al.,* 2021), making them a unique and valuable model to investigate astrocyte dysfunctionalities associated to a single patient, allowing the design of strategies to be applied in precision medicine. Thus, the direct conversion of fibroblasts into iAstrocytes is a better model than the generation of induced pluripotent stem cells, followed by differentiation into astrocytes, which is time-consuming, has low conversion efficiency and generates immature cells. Despite the limitation of only use one patient cell line, making difficult to obtain solid conclusions, the present work is innovative in assessing whether the response of the ALS pathological iAstrocytes differently respond to distinct immunostimulant conditions. With these data we expect to open new perspectives to be explored in future studies toward the development of strategies allowing the determination of patient astrocyte reactive profile and the best therapeutic approach with high promise for its translation to the clinic.

Although we followed established protocols (Meyer *et al.*, 2014; Gomes *et al.*, 2022), the work with these cell lines required the optimization of the protocol. iNPCs were differentiated into iAstrocytes in a 7-day process, with daily and brief observation of the development and differentiation of cells, as described in Material and Methods.

In Figure III.1 it can be seen bright field images of the differentiated iAstrocytes from iNPCs generated from non-ALS and sALS individuals as indicated in Table II.1. As a first look, iAstrocytes from the sALS case revealed the existence of morphometric alterations, clearly identified in the insets of the Figure III.1. Actually, the cells seemed to be more polarized with a narrowed soma, bipolar and few planar processes, with a morphology that was alike the rod shape described for the pathological microglia in some brain diseases (Giordano *et al.*, 2021). Therefore, we next decided to further explore the morphology of the control and patient cells lines upon the stimulation with TNF-α+Il-1β, the classical activation model (Oksanen *et al.*, 2017), or with TNF-α+Il-1β+C1q, recently proposed by Liddelow et al (Liddelow *et al.*, 2017). The later was described to correspond to factors released by the activated microglia and responsible

for the switch of the steady-state phenotype of astrocytes into the neurotoxic reactive cells.

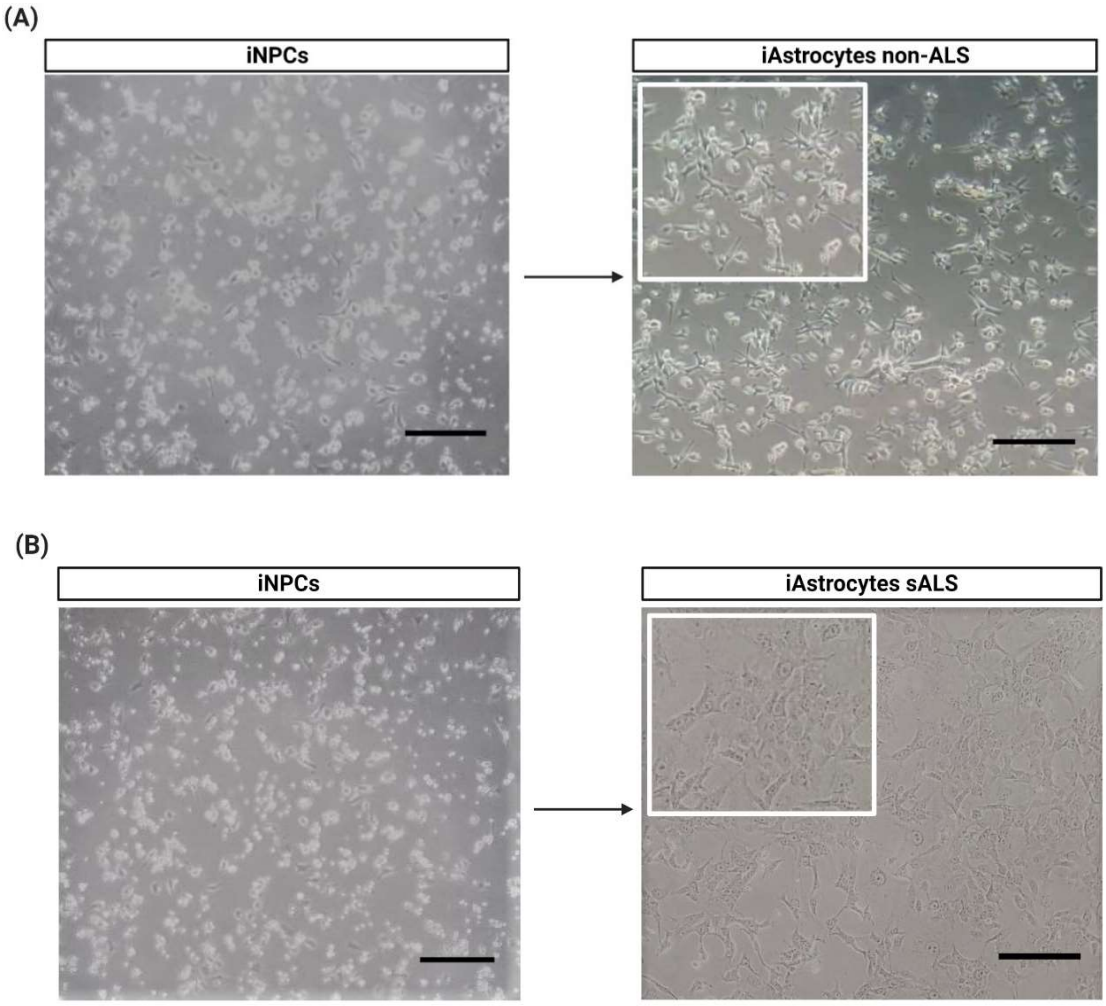

Figure III.1: Representative images of iAstrocytes differentiated from iNPCs of an ALS sporadic female patient and matched healthy non-ALS individual. (A) Bright field images of induced neural progenitor cells (iNPCs) and their differentiated iAstrocytes. iNPCs were seeded at low concentration and maintained for 7 days in astrocyte differentiation conditions. Top left image represents iNPCs as, sphere-like form in day 0, and on the right the iAstrocytes from the non-ALS day 7. (B) The same as (A) for day 0 (left, iNPCs) and day 7 (right, iAstrocytes) of cells from ALS sporadic patient. Scale bar represents 20 µm. ALS, amyotrophic lateral sclerosis; iNPCs, induced neural precursor cells; iAstrocytes, induced astrocytes; sALS, sporadic amyotrophic lateral sclerosis; iAstrocytes non-ALS, induced astrocytes from healthy non-ALS female individual.

3.1 iAstrocytes from the sALS patient reveal smaller mean cell area, without manifesting marked morphometric differences by the two types of activation

Astrocytes are known to present different morphologies, whose representation may vary with astrocyte reactivity in ALS disease (Oberheim *et al.,* 2012). Here we compared sALS-iAstrocytes with healthy-iAstrocytes, using the staining with S100B, one of the markers more commonly used to identify astrocytes (Escartin *et al.*, 2021).

Cell shape analysis was carried out by discriminating three categories of astrocyte morphologies: arborized (with processes), polarized (thin, and process-devoid cells) and fibroblast-like (large volume, lacking processes, low surface area) (Fig. III.2). We used the evaluation on the methodology previously described (Jones *et al.,* 2017) for Alzheimer's Disease cases, either familial (FAD) or sporadic (SAD) and by Gomes *et al*., (Gomes *et al.*, 2020) for astrocytes derived, from the spinal cord and brain cortex of SOD1G93A (mSOD1) mice at postnatal day 7, cultured for 13 DIV.

We assessed morphological alterations of iAstrocytes after 48h incubation and S100B staining. As previously indicated, iAstrocyte morphological alterations were compared to the cell line from the non-ALS control and extended to the analysis after the two distinct forms of induction, TNF-α/IL-1α/C1q (like the microglia-derived factors) and TNF-α/IL-1β (reproducing the generalized inflammatory condition), as depicted in Figure III.3A-C. The arborised morphology was similarly exhibited in both sALS patient and non-ALS individual (Fig. III.3D). Slight differences, non-significative, were noticed for the non-ALS iAstrocytes by the two activation modes for the number of polarised cells that decreased (Fig. III.3E) and for the fibroblast-like cells that increased (Fig. III.3F). No modifications were present in sALS iAstrocytes. To note, however, that astrocytes from the sALS patient showed a smaller mean cell area ($p<0.05$ *vs.* matched non-ALS cells) that was sustained after activation, though not so markedly (Fig. III.3G). Similar behavior was noticed for the Feret's diameter (Fig. III.3H). Such data are in line with our first observation by the bright-field images pointing to smaller soma. In sum, the pathological iAstrocytes revealed morphometric alterations as compared to the non-ALS cell line, suggesting their stressed condition. For the immunostimulating conditions they did not significantly alter the morphology of iAstrocytes in either the patient or in the control samples, attesting that cell morphometry may not reproduce phenotypic, metabolic, or functional differences.

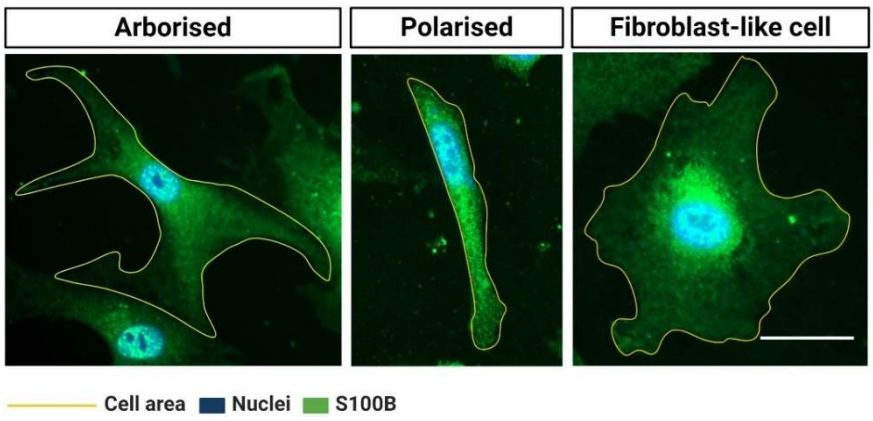

Figure III.2: Representative fluorescence images of arborised, polarized and fibroblast-like cell morphologies identified after S100B staining, a characteristic marker of astrocytes. IAstrocytes derived

from the non-ALS patient fibroblasts were used in this classification. Scale bar represents 40 µm. Yellow line was used to define cell area limitation.

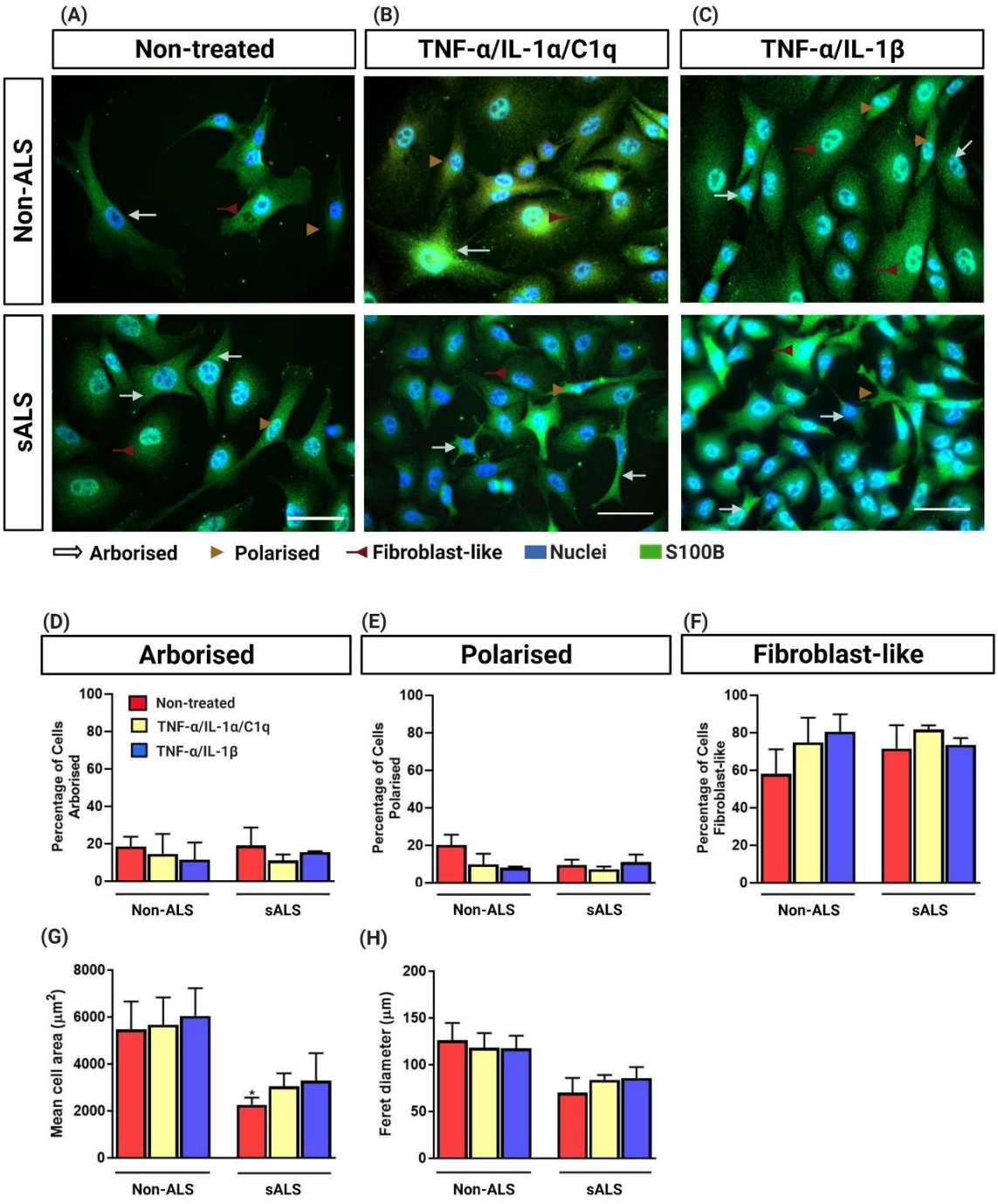

Figure III.3 Morphological features of non-ALS and sALS iAstrocytes non-treated or treated for 48h with either TNF-α+IL-1α+C1q, or TNF-α+IL-1β. (A) Non-treated non-ALS and sALS astrocytes (left images). (B) Astrocytes after microglia-derived factors activation (middle images). (C) Astrocytes after inflammatory-derived factors activation (right images). (D) Arborised astrocytic morphology percentage cells non-treated and activated, Polarised (E) and Fibroblast-like (F). (G) Mean cell area in non-ALS and sALS astrocytes before and after activations. (H) Feret diameter. Data are mean values ± SEM from at least three independent experiments. * p< 0.05 by two-tailed unpaired Student´s *t*-test vs. matched non-ALS cells. Scale bar represents 40 µm. Non-ALS, healthy non-ALS female individual; sALS, sporadic female patient Mean cell area and Feret's diameter were quantified for each cell using the ImageJ program, as described in the methods section.

3.2 Mitochondria dynamics is decreased in iAstrocytes from the sALS patient and not modified by the immunostimulating conditions

Mitochondrial dysfunction and oxidative stress are well described in ALS, either for MNs (Vaz *et al.,* 2015; Carrì, *et al.,* 2017) or astrocytes (Cassina *et al.,* 2008). As depicted in Figure III.4A,D sALS iAstrocytes show a decrease in mitochondrial viability ($p<0.05$). When activated with TNF-α/IL-1α/C1q (Fig. III.4B,E) the decrease was even noticed, relatively to non-treated, non-ALS iAstrocytes, represented as a broken line, though not significantly ($p=0.081$). When we used TNF-α/IL-1β (Fig. III.4C,F), a slight reduction was noticed in the control, but still a higher one was present in the sALS iAstrocytes, without statistical significance. These data confirms that the iAstrocytes from the sALS patient are dysfunctional and show a compromised mitochondria viability. Activation was not able to modify such deficit, and no difference from the control was observed because the control iAstrocytes also revealed a disturbed mitochondria upon the two activation conditions, as compared to the non-activated cells. To also note that the activations seem to cause the mitochondria delocalization to the peri-nuclear space suggesting a high-energy demand of nuclear processes (Fig. III.4 A-C).

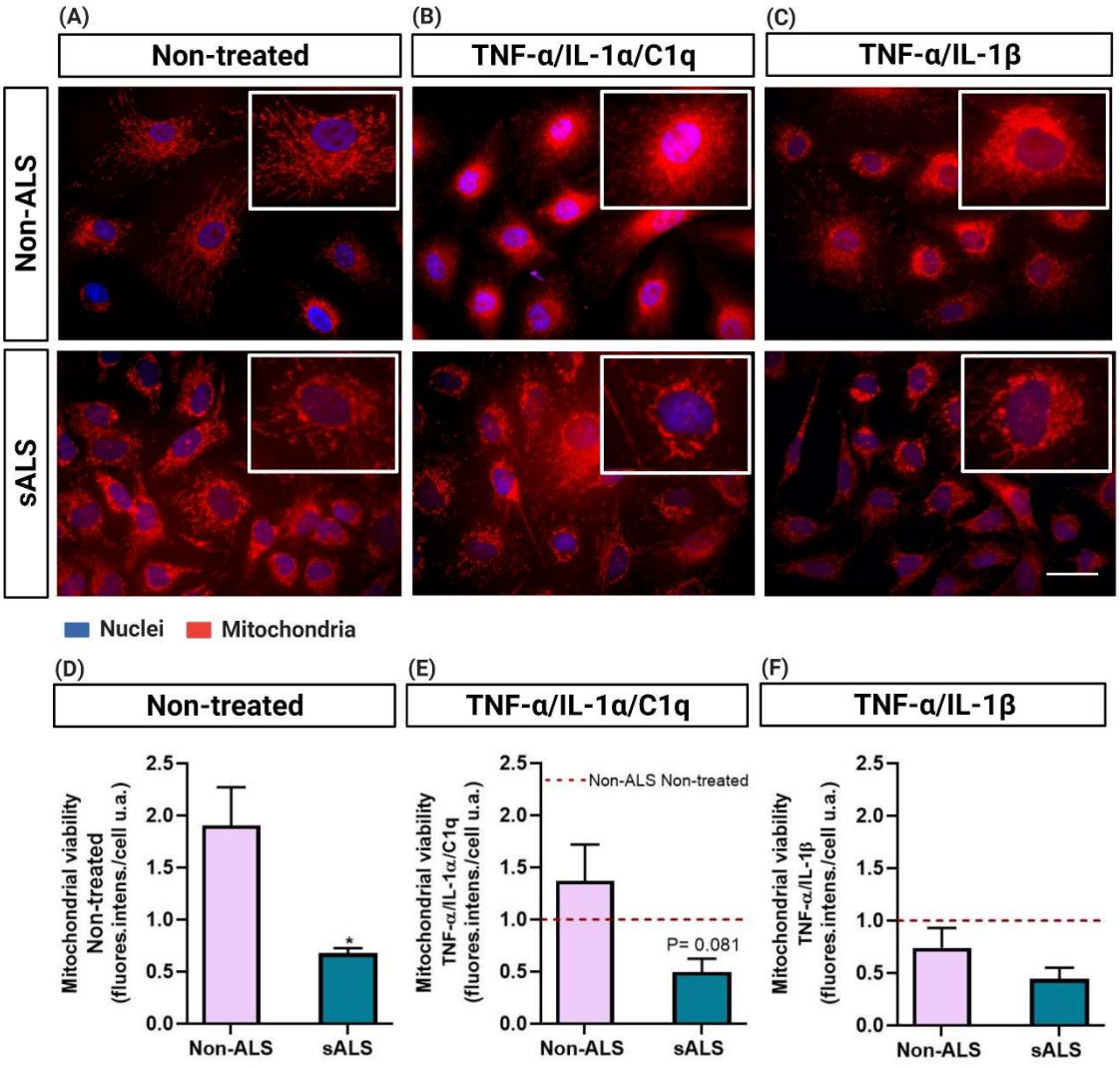

Figure III.4 Control and sALS iAstrocytes treated with MitoTracker Red before and after activation with TNF-α+IL-1α+C1q or TNF-α+IL-1β. iAstrocytes were either non-treated or treated for 48h with TNF-α, IL-1α, C1q and TNF-α, IL-1β, and incubated with MitoTracker™Red CMXRos at day 10 *in vitro*. Total mean fluorescence was quantified in each image and normalized to the total number of cells, as described in methods. A representative image from each condition is shown (A-C). (D) Fluorescence intensity results are depicted. Activations with TNF-α+IL-1α+C1q (microglia-derived factors) (E) and activation with TNF-α+IL-1β (inflammatory-derived factors) (F). Data are mean values ± SEM from at least four independent experiments. * p< 0.05 by two-tailed unpaired Student´s *t*-test with Welch´s correction when required. Scale bar represents 40 µm. Non-ALS, healthy non-ALS female individual; sALS sporadic female patient.

3.3 Reduction of Cx43/S1000B and increased Ki-67 gene expression levels characterize sALS iAstrocytes and only Cx43 is normalized upon immunostimulation

To better characterize the phenotype of sALS iAstrocytes, we analyzed the gene expression levels of the classical astrocytic reactive markers, which we also observed to be dysregulated in the mSOD1 mice (Cunha *et al.*, 2018; Gomes *et al.*, 2019; Gomes *et al.*, 2020; Gomes *et al.*, 2022).

The gene expression of Cx43 and S100B were found decreased, while that of Ki-67 was increased, and no changes were noticed for GFAP in the sALS iAstrocytes (at least $p<0.05$, Fig. III.5A-D). Upon the stimulating conditions, Cx43 in both the control and the sALS iAstrocytes was upregulated and the classical activation further enhanced the proliferative marker in the sALS iAstrocytes. On the other hand, the microglial-associated inflammatory condition led to a significant decrease of the GFAP gene expression level ($p<0.05$). No other modifications in the sALS iAstrocytes could be noticed by the added cytokines.

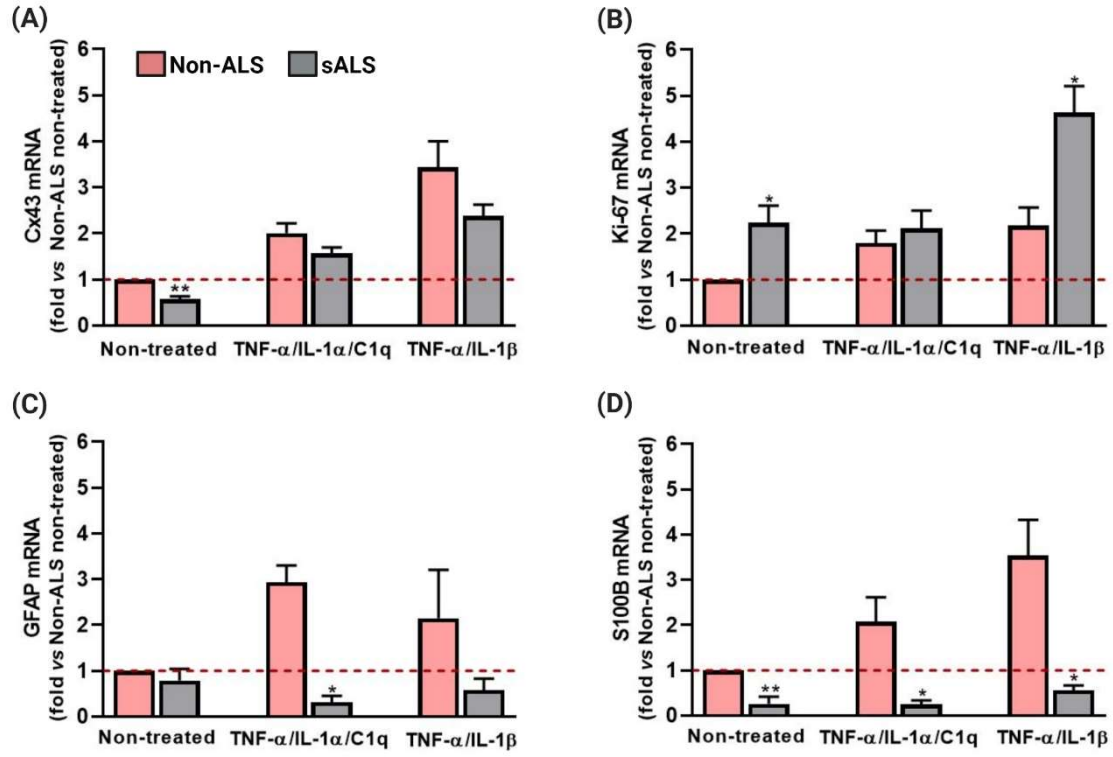

Figure III.5 Cx43, Ki-67, GFAP and S100B gene phenotypic markers in iAstrocytes from the non-ALS and sALS cell lines before and after immunostimulatory conditions. iAstrocytes were obtained as described in Materials and Methods and were analysed in the absence or in the presence of TNF-α/IL-1α/C1q or TNF-α/IL-1β factors for 48 h. mRNA levels of connexin 43 (Cx43) (A), Ki-67 (B), GFAP (C) and S100B (D) evaluated by RT-qPCR. Data are mean values ± SEM from at least three independent experiments and expressed as fold change *vs.* control non-activated (CTRL non-activated). *$p<0.05$ by two-tailed unpaired Student´s *t*-test with Welch´s correction when required. Scale bar represents 40 µm. Non-ALS, healthy non-ALS female individual; sALS sporadic female patient.

3.4 Immunocytochemical analysis of S100B reveals decreased levels in the sALS iAstrocytes upon immunostimulatory conditions

To confirm whether the depressed levels of S100B gene expression in the sALS iAstrocyte line we next assessed its protein levels by immunocytochemistry using specific antibodies (Fig. III.6A-C). While in Non-ALS cells we could observe a slight although not significant increased expression in the S100B levels upon stimulation of both types of activation (Figure III.6A-C), no marked differences were noticed between

in each of the tested immunostimulatory conditions in sALS iAstrocytes (Figure III.6A-C). In this case, the levels of S100 were significantly lower than the ones found in the non-ALS controls subjected to the same treatment ($p<0.05$ vs. respective non- treated cells).

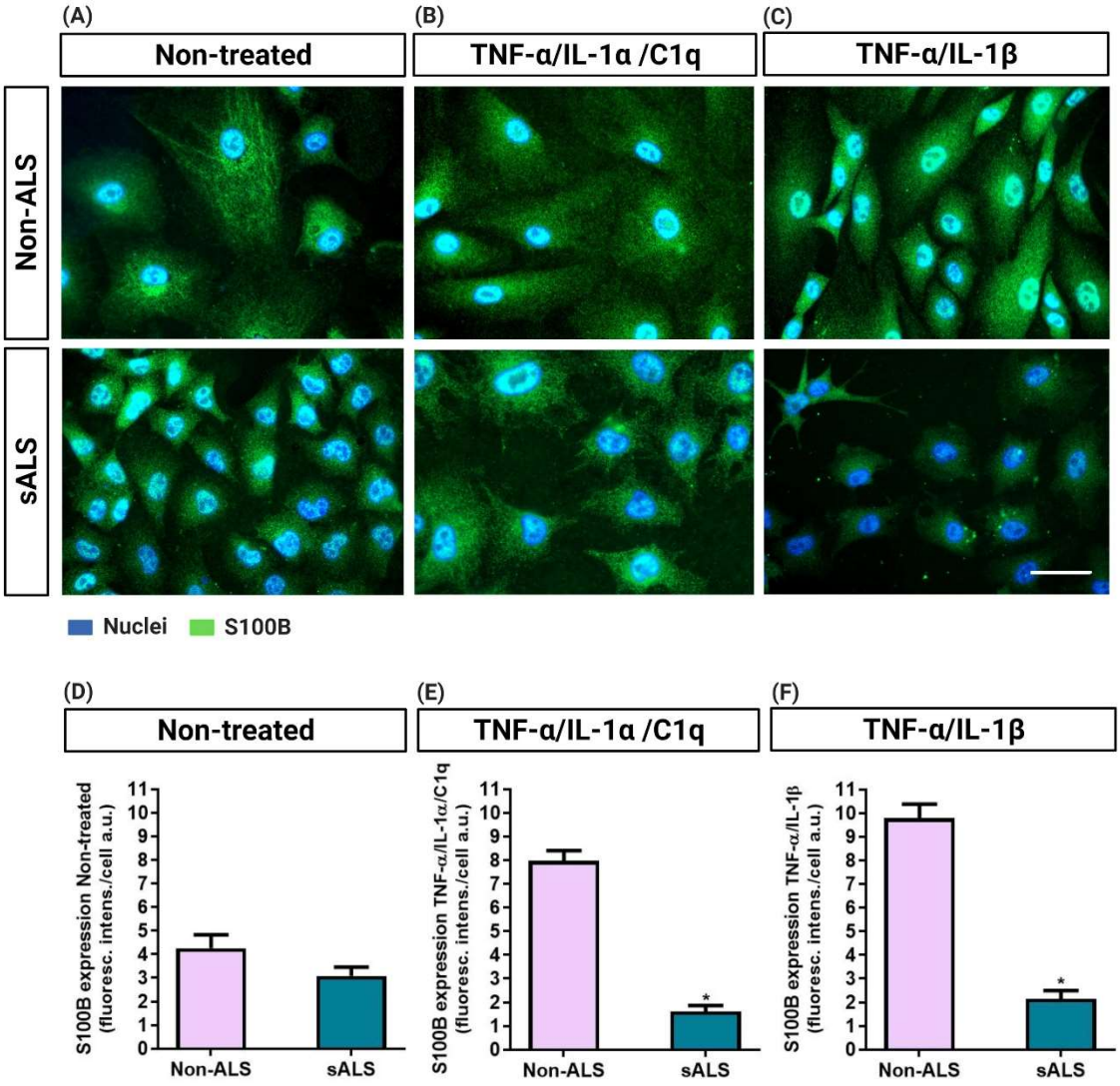

Figure III.6. S100B immunocytochemistry in iAstrocytes from the non-ALS and sALS cell lines before and after immunostimulatory conditions. iAstrocytes were obtained as described in Materials and Methods and were

analysed in the absence or in the presence of TNF-α/IL-1α/C1q or TNF-α/IL-1β factors for 48 h. Representative images of S100B immunocytochemistry for iAstrocytes in the absence (A) or presence of cytokines associated to microglia activation (B) or classical and generalized inflammation (C). Quantification in each of the conditions (D, E, F), where each cell was quantified in terms of mean fluorescence intensity, as indicated in Methods. Data are mean values ± SEM from at least three independent experiments and expressed as fold change *vs.* control non-activated (CTRL non-activated). * p< 0.05 by two-tailed unpaired Student´s *t*-test with Welch´s correction when required. Scale bar represents 40 µm. Non-ALS, healthy non-ALS female individual; sALS sporadic female patient.

3.5 NLRP3 and IL1β gene expression levels are depressed in sALS iAstrocytes and NLRP3 though being upregulated by inflammatory stimulations still present lower values than the non-ALS iAstrocytes

The gene expression levels of inflammatory markers associated with inflammasome activation, NLRP3, IL1R1 and IL-1β, were analysed by RT-qPCR. Considering the receptor IL1R1, no gross alterations were noticed for sALS iAstrocytes either non-stimulated or after treatment with cytokines (Fig. III.7A). The NLRP3 inflammasome was found depressed in the sALS iAstrocytes ($p<0.05$, Fig.III7B). As expected, the stimulatory conditions led to its increase, but NLRP3 inflammasome was found still downregulated in sALS iAstrocytes as compared to the values we obtained in the non-ALS iAstrocytes ($p<0.05$). The activation of the inflammasome is required for the maturation of IL-1β (Kang *et al.,* 2017). In conformity, IL-1β was found downregulated in the sALS iAstrocytes in the absence of inflammatory conditions ($p<0.05$, Fig.III7C). However, such finding disappeared by the presence of cytokines, in contrast with the NLRP3 mRNA profiling.

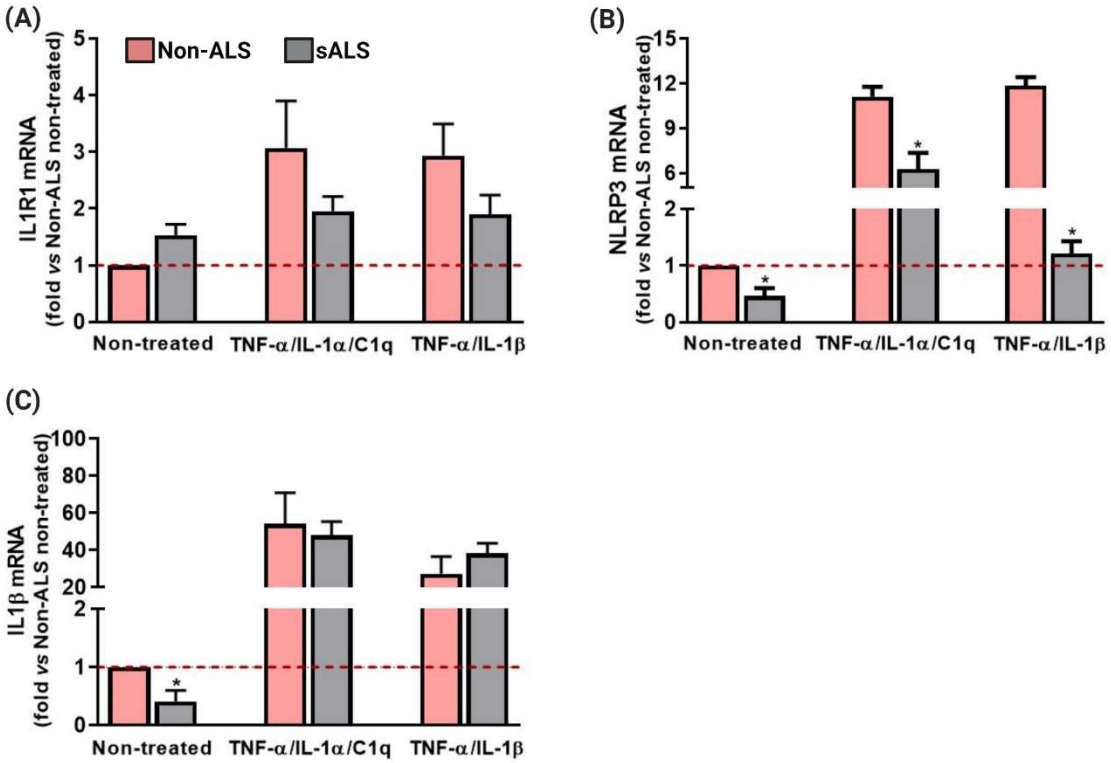

Figure III.7 Inflammasome signature in the sALS iAstrocytes relatively to the no-ALS cell line. mRNA levels of IL1R1 (A), NLRP3 (B) IL-1β (C). iAstrocytes were obtained as described in Materials and Methods and were analysed in the absence or in the presence of TNF-α/IL-1α/C1q or TNF-α/IL-1β factors for 48 h. Data are mean values ± SEM from at least four independent experiments. *p< 0.05 by two-tailed unpaired Student´s *t*-test. Non-ALS, healthy non-ALS female individual; sALS sporadic female patient.

3.6 sALS iAstrocyte phenotype signature differs from non-ALS cells in reactive markers and inflammasome components, and is mostly sustained under inflammatory conditions

When we get together the phenotype and inflammasome signature, it became clear the distinct signatures of non-ALS and sALS patient iAstrocytes, as summarized in the heat map depicted in Figure.III.8. Most dysregulated markers were observed in the absence of cytokines. Our data do not provide elements to conclude that the pathological phenotype of sALS iAstrocytes is aggravated by the presence of cytokines, suggesting that the pathological astrocytes are already dysfunctional and with a lower responsive phenotype when compared with the non-ALS cell line. Finally, only minor differences were observed between the effects produced by each of the inflammatory conditions that were tested, the one associated to the activation of microglia and the other to the classical and generalized inflammation in the sALS iAstrocytes. Differences were more evident for the non-ALS cells when comparing the effects on mitochondria viability and S100B immunocytochemistry, where the effects were more notorious for the classical activation.

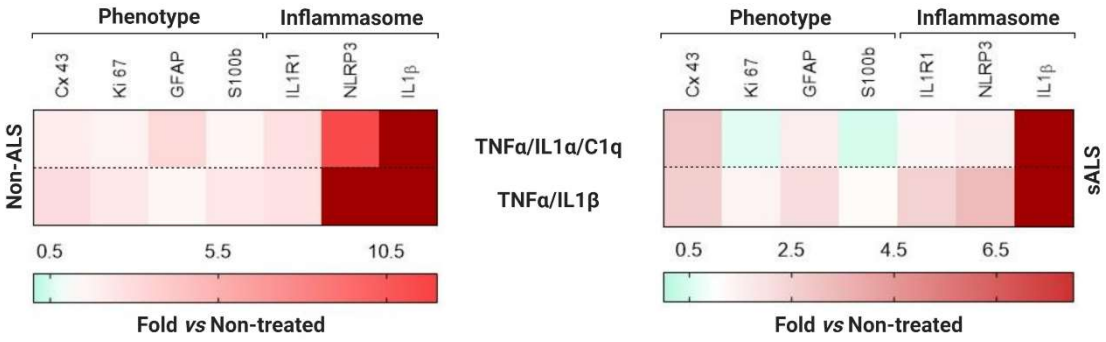

Figure III.8. Heat map summarizing the phenotypic reactive markers and the inflammasome signature of non-ALS iAstrocytes and sALS patient cells. iAstrocytes were obtained as described in Materials and Methods and were analysed in the absence or in the presence of TNF-α/IL-1α/C1q or TNF-α/IL-1β factors for 48 h. Data are fold *vs.* naïve iAstrocytes. Non-ALS, healthy non-ALS female individual; sALS sporadic female patient.

Chapter IV

Discussion

4. Discussion

ALS is a neurodegenerative disease characterized by progressive and irreversible degeneration of UMN and LMN, with a life expectancy of 3 years after diagnosis. The majority of ALS cases are sporadic (90-95%) and given the unknown genetic etiology, it is extremely difficult to design a suitable disease model (Fujimori *et al.*, 2018). Several experimental models (since invertebrates to vertebrates) have been used to better understand the complex pathological mechanisms associated to ALS (Clerc *et al.,* 2016). Systematic reviews on the various ALS experimental models with their advantages and disadvantages have been published (Van Damme *et al.,* 2017; Morrice *et al.,* 2018; Gois *et al.*, 2020; Xu *et al.*, 2021). The ability to reproduce the symptomatology, with the same progression, neurological and inflammatory mechanisms would be characteristics to consider in the ideal model. Moreover, considering the heterogenous phenotype of ALS patients, the possibility to investigate mechanisms and targets in patient neural cells would represent an advance toward the possibility of personalised therapeutic intervention.

Here, we used the direct conversion of human fibroblasts (Meyer *et al.*, 2014) from a non-ALS female as a control and a sALS female patient into tripotent iNPCs, followed by differentiation into iAstrocytes. The method is performed within one month of work in the laboratory. These iAstrocytes were shown to be comparable to native brain astrocytes (Caiazzo *et al.*, 2015). Importantly, the directly converted astrocytes retain the ageing features of the donor individuals (Gatto *et al.*, 2021), making them a unique and valuable model to investigate astrocyte dysfunctionalities associated to a single patient, allowing the design of strategies to be applied in precision medicine. Thus, the direct conversion of fibroblasts into iAstrocytes is a better model than the generation of iPSCs, followed by differentiation into astrocytes, which is time-consuming, has low conversion efficiency and generates immature cells.

Reactive astrocytes play an important role in neurotoxicity of ALS MNs via loss of normal functions or gain of abnormal effects (Sofroniew and Vinters, 2010; Liddelow *et al.*, 2017). The observation of such reactive neurotoxic phenotypic characteristics were found in patients and mSOD1 rodent models and considered to be therapeutic targets (Vargas and Johnson, 2010; Díaz-Amarilla *et al.*, 2011; Trias *et al.*, 2013; Pehar *et al.,* 2017; Cunha *et al.*, 2018; Izrael *et al.,* 2020; Vaz *et al.,* 2021; Ziff *et al.*, 2022). Though neurotoxic astrocytes were identified in iAstrocytes from sALS patients before (Meyer *et al.*, 2014), cells were not at that time explored for their reactive markers. Lately, our study with iAstrocytes from fALS and sALS cases identified the presence of different phenotypes based on reactive markers and inflammatory associated miRNAs (Gomes

et al., 2022). In the meantime, it was published that astrocytes become neurotoxic by cytokines released by activated microglia, *i.e.,* TNF-α, IL-1α and C1q (Liddelow *et al.*, 2017). Nevertheless, other studies have been considering the use of TNF-α plus IL-1β to enhance the reactive phenotypic profile of astrocytes (Oksanen *et al.*, 2017; Hyvärinen *et al.*, 2019). That said, there are no studies on the effects produced by the first or later immunostimulatory condition on the reactive profile of astrocytes and even less with iAstrocytes from a sALS patient.

We acknowledge that using only two cell lines is a limitation of the study, considering the diversity of reactive astrogliosis in neurodegenerative diseases (Moulson *et al.*, 2021) and the cellular diversity of astrocytes in ALS (Gomes *et al.*, 2020; Stifani, 2021). However, this is the first time that this type of study is performed in iAstrocytes under different conditions of activation. Most of the studies have been performed with transgenic cells, either from mice models or iPSCs, while ours represent an important step ahead to better understand astrocyte dysfunction in sALS patients using the direct conversion technique that we anticipated to open new avenues to be explored in future studies and with high promise for its translation to the clinic. Thus, recognising such limitation but also aware of the innovative approach here used, we proceeded to study the behaviour of astrocytes when subjected to the two types of activation.

In the present study we explored the aberrant phenotype of iAstrocyte reactive markers, mitochondria dynamics, cell morphology and inflammasome components. We assessed different iAstrocyte morphologies, namely, arborised, polarised and fibroblast-like cells, in line with a previous study by Jones *et al.,* (2017). Astrocytes from the ALS patient exhibited fewer polarised cells, an increased number of fibroblast-like cells although without statistical evidence. Nevertheless, it was clear the lower soma of the sALS iAstrocytes manifested by the lower soma, when compared to the non-ALS control sample. Changes in morphology have been pointed in patients in ALS models (Tripathi *et al.*, 2017; Yamanaka and Komine, 2018; Izrael, *et al.,* 2020), but data from patient astrocytes generated by reprogramming technologies do not include morphometric studies (Madill *et al.*, 2017; Birger *et al.*, 2019). Such kind of morphological alterations in astrocytes were previously described for the astrocytes isolated from the mSOD1 models, though reporting hypertrophy (Pehar *et al.,* 2017; Gomes *et al.*, 2020; Vaz *et al.,* 2021). However, other studies in iPSC-derived astrocytes from sporadic and familiar cases of Alzheimer's disease also reported a reduced soma and process volumes (Jones *et al.*, 2017).

Overexpression of TNF-α was shown to induce senescence, autophagy, and mitochondrial dysfunctions, that included more condensed and shorter mitochondria with perinuclear localization (Tyciakova *et al.*, 2021). Il-1β was also shown to cause

mitochondrial impairment and alterations in fusion/fission proteins when observed by Mitotracker Red (Batista *et al.*, 2021). Our data indicates that the iAstrocytes from the sALS female patient have a marked impairment of mitochondria, and so intense that no further changes could be observed when submitted to each of the immunostimulant conditions. These results contrast with those observed in the control iAstrocyte cell line, where the reduction of fluorescence was notorious, mainly upon TNF-α/IL-1β treatment. Mitochondrial dysfunction was associated to inflammatory activation of astrocytes (Rahman and Suk, 2020), but was also noticed in APOE4-expressing astrocytes (Schmukler *et al.,* 2020) and in mSOD1 astrocytes as reviewed *(Cassina et al.*, 2021). Our findings are interesting in validating that the direct conversion of patient fibroblasts into iAstrocytes sustain the reactive phenotype and the characteristic mitochondria pathology of ALS even in the absence of any proinflammatory stimulus. Interestingly, the mitochondria seemed to be more localized in the peri-nuclear space, suggesting a higher requirement in preserving metabolic functions and nuclear pathways, as an attempt to reduce oxidative stress. As in other studies, we may hypothesize that this change in mitochondrial dynamics may associate to morphological alterations and imbalance between the expression of fusion and fission proteins (Vaz *et al.,* 2015).

Cx43 is a marker of astrocyte reactivity, astrogliosis and interconnectivity between astrocytes (A. *et al.,* 2017 ; Vicario *et al.*, 2017). Cx43 increased expression has been observed in human ALS neural tissues and in iPSC-derived astrocytes from sALS and fALS patients, as well as in rodent ALS models (Díaz-Amarilla *et al.*, 2011; Cui *et al.*, 2014; A. *et al.*, 2017; Gomes *et al.*, 2022). Unlike what it was suggested in the literature, the Cx43 mRNA expression was downregulated in the iAstrocytes from the sALS patient and increased upon the cytokines, as proposed in other studies *(Sáez et al.*, 2020), though slightly reduced as compared with the control cells. The reduction we observed in the iAstrocytes from the sALS case was indicated to be associated to a defence mechanism towards the increase of the cell survival in ALS (Almad *et al.,* 2022). However, it was also associated to a less resistance to oxidative stress-iAstrocytes death (Le e*t al.,* 2014). Our S100B data also revealed a different outcome from the literature review, specifically, in spinal cord of mSOD1 mice astrocytes (Díaz-Amarilla *et al.*, 2011) and in the cerebrospinal fluid from sALS patients (Shobha *et al.,* 2010). Intriguingly, reduction of S100B in ALS was shown to decrease the expression of proinflammatory genes (Serrano e*t al.*, 2017) and shown to promote neuronal survival (Villarreal *et al.,* 2011). The disparate results we noticed for Cx43 and S100B, deserve further investigation in other ALS cell lines and should be accompanied by the evaluation of intracellular Ca^{2+}.

Ki-67 was been widely used as a proliferative is a proliferation marker (Sofroniew and Vinters, 2010) and our previous studies supported its overexpression in several mouse and human models (Cunha *et al.*, 2018; Gomes *et al.*, 2019; Gomes *et al.*, 2022), together with a decrease in the gene expression of GFAP in the brain cortex of mature astrocytes in the mSOD1 mouse model (Gomes *et al.,* 2020). Data here obtained confirmed these aberrant characteristics of the ALS astrocyte phenotype, which were sustained even in the presence of the immunostimulatory events.

Such decrease of GFAP was already found in ALS rodent models (Yoshii *et al.*, 2011).

NLRP3 inflammasome activation in microglia has been associated to Alzheimer's disease (Hanslik and Ulland, 2020) and to the release of the downstream effectors IL-1β and IL-18 (Saresella *et al.*, 2016), being considered as a novel therapeutic intervention (Heneka, *et al.*, 2013). NLRP3 was lately indicated as a potential biomarker in ALS skeletal muscle (Moreno-García *et al.,* 2021) and found to be expressed by astrocytes in the mSOD1 mice and in the spinal cord of human sporadic patients (Johann *et al.*, 2015). Apart from this last study, no other data are described specifically for astrocytes when derived from human ALS patients. In addition, the dysregulation of NLRP3 inflammasome activation was shown to be involved in neuroinflammation, but inconsistent findings have also been described (S. Lin and Mei, 2021).

The expression of NLRP3 mRNA was decreased in the iAstrocytes from the sALS patients as compared with the control and in conformity a similar reduction was observed for IL-1β gene expression. As expected NLRP3 gene expression was elevated in the presence of both immunostimulatory conditions, but as shown previously for other assessed biomarkers, the NLRP3 in the pathological iAstrocytes was the less responsive to the presence of cytokines, and the classical activation without effect.

Taken together, our results provide new evidence indicating that directly converted human astrocytes from a sporadic patient, besides being a powerful tool to better understand disease mechanisms and test new therapeutic strategies, also show a defective phenotype that do not respond as the matched controls to the immunostimulatory stimulations. Similar observations were indicated to be observed in senescent astrocytes that showed decreased normal physiological function (Han *et al.*, 2020) attesting the pathological phenotype of iAstrocytes generated from the sALS female patient. After obtaining the data presented in this thesis we speculate that neuroinflammation can only be considered a valuable driver of pathology in ALS, and probably other neurodegenerative diseases, when cells are still responsive and can favour the release of more cytokines. In the case of this sample of diseased iAstrocytes we consider that they are not in conditions of fighting off foreign invaders, heal injuries or mop up debris (Sochocka *et al.,* 2017), i.e. with ability to perform the good attributes

of inflammation, thus contributing to inherent disease progression. Regarding the effects of the two tested stimuli, it seems that the classical one exerted more drastic effect that the one derived from microglia. Though more studies are required to validate or deny that our data can be extended to other iAstrocyte cell lines, it is unquestionable that the cells directly derived from the patients can be assessed for their intrinsic phenotype, thus supporting, and contributing to the development of personalized therapies and probably helping in patient stage classification.

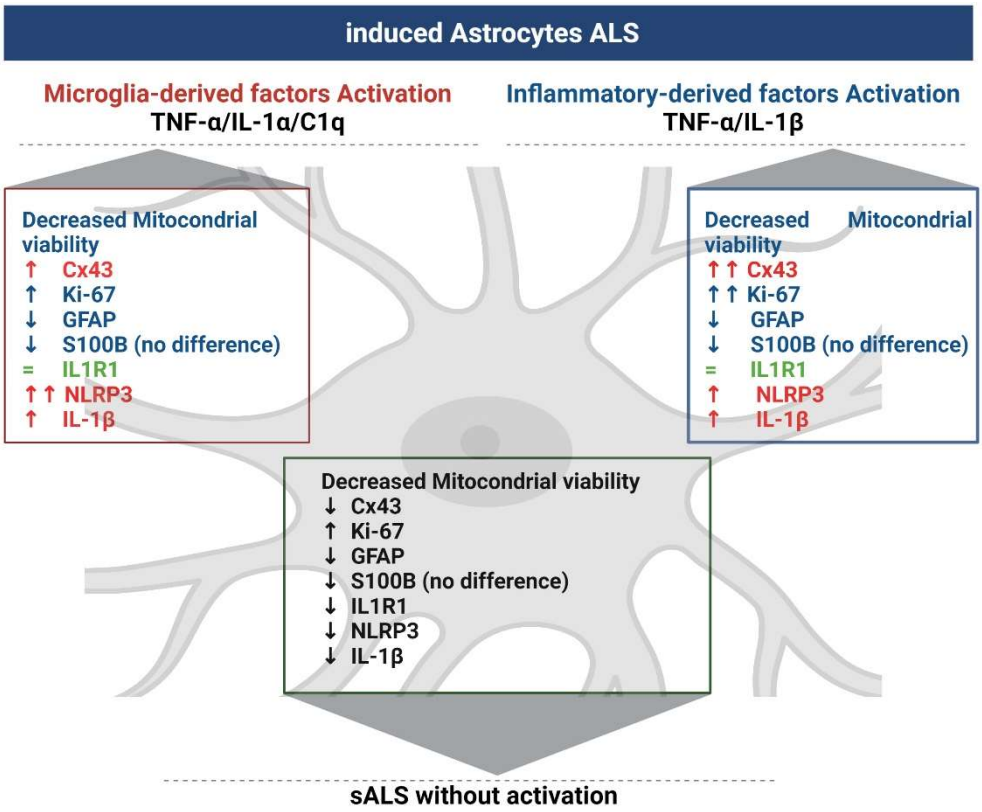

Figure IV.1 Schematic representation of the main findings of the present work. The use of iAstrocytes differentiated from neural precursor cells directly converted from fibroblasts directly converted from patient fibroblasts is a novel and unique tool to explore the disease mechanisms associate to the sporadic cases, for which there is no study model. Our cell line of iAstrocytes from a female patient with a sporadic form of ALS exhibited a specific phenotype characterized by: 1. Small soma not strongly modified by inflammation; 2. Decreased mitochondria viability and S100B that were sustained during induced immunostimmulation; 3. High cell proliferation rate; decreased Cx43 counteracted by the two inflammatory stimuli; 4. Decreased GFAP upon cytokines attributed to activated microglia; 5. Reduced NLRP3 and IL-1β counteracted by inflammatory conditions. Whether this profile is also exhibited by other ALS patient iAstrocytes deserves to be explored in the future. More information on the pathological pathways that were discovered can be found in the main test. (Artwork by Isabel Maurício)

Chapter V

Concluding Remarks

5. Concluding remarks

Astrocytes are one of the main players in the CNS homeostasis (Vasile *et al.,* 2017), but also one of the most reactive glial cells to environmental changes (Yamanaka *et al.,* 2008). In ALS, besides the degeneration of the MNs most of the disease progression is due to alterations in glial phenotypes, which include the dysregulation of several markers that characterize the astrocyte associated aberrancies and the release of toxic compounds (Brites and Vaz 2014; Vaz *et al.,* 2021). Models to mimic the disease pathogenesis in ALS, only reproduce some of the pathways of the familial cases, as the mSOD1 mouse model. However, many cases are not directly associated to the presence of familiar mutations and can only be investigated by using cells from the patients. Human cells derived from patients by reprogramming technologies (Meyer *et al.*, 2014) represent a novel tool that can help in establishing disease associated pathways and contribute to the identification of targets and development of therapeutic strategies. Moreover, with this technology it is possible to identify facets of patient-specific signatures. Lately inflammation has been claimed to represent a major driver force, but no definitive information was established on its role. In the experimental work inflammation can be reproduced either by using TNF-α plus IL-1β, the classical route (Oksanen *et al.,* 2017) or IL-1α, TNF-α and C1q indicated as being released by the activated microglia (Liddelow *et al.,* 2017).

In the present study, we used one iAstrocyte cell line generated from a sporadic ALS patient and its stimulation by the two types of immunostimulation. On one hand it allowed the definition of the phenotype associated to the pathology of ALS in this case and on the other hand the possibility to study the responsive characteristics to the treatment of the two types of cytokines. We provide evidence supporting the dysregulation of biomarkers associated to the astrocyte reactive characteristics in the sALS case, e.g., small soma, decreased mitochondria viability, low expression of S100B, Cx43, IL-1β and NLRP3 pointing to a malfunctioning and unresponsive cell. Cytokines did not aggravate the phenotype but only slightly ameliorated the expression of Cx43 and to some extent activated NLRP3, while the other markers were maintained dysregulated as in the naïve cells when compared to the matched control cell line.

Altogether, the results obtained in this work unveiled the pathological pattern of iAstrocytes derived from the sporadic ALS patient, with a phenotype that do not entirely reproduce neither the astrocytes isolated from the spinal cord or cortical brain of mSOD1 mice and highlighted the existence of cell strong deficiencies that make them incapable to respond and fight immunological stimuli.

Future perspectives

The present work unveils facets of one sporadic iAstrocyte cell line and validates the existence of individual signatures despite the common pathological features. The relevance that this finding can have to define the best therapeutic strategy is still to be clarified. We anticipate that the establishment of gene network plots using different sporadic and familial iAstrocyte lines from individuals with ALS may contribute to find central targets in the disease mechanisms to develop common treatments that can be used together with those more personalised, aiming at a translational strategy in ALS therapeutics. Further understanding on the response of such iAstrocyte lines to the presence of different inflammatory stressful conditions will also improve the knowledge about disease stages and approaches to be undertaken. Here we observed that the iAstrocytes from the sporadic patient already present an intrinsic pathology that turn them irresponsive to different cytokine activations. The presence of dysfunctional mitochondria may contribute to cell hypometabolism that may determine hypoxia-inducible factors determining increased cell proliferation, as observed in the present study. This can be beneficial or harmful to the hosting brain and may represent a new avenue in the future research to be developed. Low S100B mRNA gene levels, even in the presence of immunostimulants, can derive from a reduction in the differentiation potential of the pathological astrocyte lineage (Brozzi *et al.*, 2009), and not from the reprogramming technology we used once not observed in the control iAstrocytes. This should be explored toward the use of human astrocytes or human astrocyte like cell population as recently tested by Neuralstem Inc (Feldman *et al.*, 2014; Glass *et al.*, 2016). From our study the response to immunostimulation by the sALS iAstrocytes was clearly far away of that shown by control iAstrocytes, indicating that these cells cannot be neurosupportive to MNs in the tested stressful conditions. Preclinical studies are clearly warranted to define the loss of such important neuroprotective functions. A recent study identified that different mutations in ALS have distinct underlying molecular patterns and different impacts (Taha *et al.*, 2022), even in the absence of microglia, reinforcing astrocyte malfunction as a putative therapeutic target. How early astrocytes are dysregulated in ALS patients should be a matter of research in future studies using the iPSC-derived astrocytes and astrocyte direct conversion from the same ALS patient. In the presence of MNs and microglia using the same reprogramming technologies and biological systems based on organoids, spheroids or tricultures in organ-on-a chip, it would then be possible to follow ALS disease steps and test different regenerative strategies.

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