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Transcriptomic and functional insights across different genetic mutations of ALS iPSC-derived Neural progenitors and Motor neurons

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Student declaration

I declare that the PhD thesis titled “Transcriptomic and Functional Insights Across Different Genetic Mutations of ALS iPSC-derived neural progenitors and motor neurons” represent my own work and that it has not been submitted, in whole or in part, in any previous application for a degree. I generated and analyzed the data herein reported. The following experiments have been conducted in collaboration:

- Blood samples used to generate iPSCs were collected by Prof. Letizia Mazzini and Prof. Fabiola De Marchi, ALS Centre, Neurology Unit, Maggiore della Carità Hospital, Department of Translational Medicine, University of Piemonte Orientale.

- RNAseq data analysis was performed in collaboration with the laboratory of Prof. Sandra D’Alfonso (University of Piemonte Orientale).

- The protocol for the differentiation of induced pluripotent stem cells (iPSCs) cells into motor neurons (MNs) was acquired during a visiting period at the Norwegian Core Facility for Human Pluripotent Stem Cells in the laboratory of Prof. Joel Clinton Glover (University of Oslo).

This PhD thesis is intended to remain confidential, as part 1 and part 2 of the work have not yet been published. Some of the results presented and discussed in this thesis has been accepted for publication in the journal “Cells”, as noted in the text. The work I carried out during my PhD program is not limited to what is included in this thesis, as I also contributed to the following publications:

- “Factor VIII promotes angiogenesis and vessel stability regulating extracellular matrix proteins”. *Haematologica*. 2024. C. Olgasi, A. Cucci, I. Molineris, S. Assanelli, F. Anselmi, C. Borsotti, C. Sgromo, A. Lauria, S. Merlin, G.E. Walker, S. Oliviero, A. Follenzi. DOI: 10.3324/haematol.2024.285089

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ACKNOWLEDGES

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Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal and heterogeneous neurodegenerative disorder affecting upper and lower motor neurons (MN). Its complex phenotypic variability and unknown molecular mechanisms continue to pose significant challenges for therapeutic development. Currently, there is no definitive cure, and patients are treated to slow down disease progression and alleviate symptoms. However, the drugs used have significant limitations in terms of efficacy and tolerability therefore other clinical approaches are needed. Stem cell therapy for ALS is emerging but despite the large number of basic, translational, and preclinical studies the outcomes so far have been relatively modest. Therefore, cellular models that can be used for drug screening or to investigate in depth the molecular mechanisms of the disease are needed. For this purpose, induced pluripotent stem cells (iPSCs) are largely used in preclinical studies. In this work, we generated iPSCs by reprogramming CD34⁺ cells isolated from the peripheral blood of healthy donors and ALS patients carrying different mutations (*C9orf72*, *TARDBP*, and *KIF5A*). Obtained iPSCs were characterized by the expression of the typical stem cell markers (OCT4, SOX2, KLF4, and c-MYC, SSEA3, and Tra1-60) and their ability to express the three germ layer markers after embryoid bodies generation. The analysis of the transcriptomic profile of healthy and ALS iPSCs and iPSCs-derived neurospheres showed a downregulation of genes related to extracellular matrix (ECM) in ALS iPSCs, indicating a potential common early signature in ALS pathogenesis. In contrast, iPSCs-derived neurospheres exhibited broader deficits in neuronal development pathways, such as axiogenesis, synaptic membrane formation, axon development, and neuron projection development. However, these transcriptomic signatures were not uniformly conserved across all patients, reflecting the underlying genetic and phenotypic heterogeneity of the disease. To investigate the impact of the observed transcriptomic changes on cell function, a neurite outgrowth assay was performed on iPSC-derived neurospheres. Interestingly, the ALS neurospheres showed a significant reduction in neurite branching, branch points, and overall network complexity compared to healthy neurospheres, suggesting a diminished capacity to form functional neural network. To further validate whether these impairments persist in MNs, healthy and ALS iPSCs were differentiated into MNs demonstrating a reduction in neuronal density, axonal length, and axonal diameter in ALS-derived MNs compared to healthy ones. Finally, the analysis of neural markers demonstrated that ALS-iPSCs derived MNs had generally lower levels of key maturation markers (as ChAT, TUB β 3, and MAP2, SYP, SYT, NeuroD1, NFH, NaV1.1 and Kv2.1) suggesting a lower capacity to acquire mature neuronal phenotype compared to healthy ones. Overall, this work lays the foundation to better understand ALS-associated molecular mechanisms and underscores the potential of patient-derived iPSC models for disease modeling and future therapeutic discoveries.

Abstract (Italiano)

La sclerosi laterale amiotrofica (SLA) è un disturbo neurodegenerativo progressivo che colpisce i motoneuroni superiori e inferiori (MN). La SLA presenta una variabilità fenotipica e i meccanismi molecolari coinvolti non sono del tutto conosciuti. Ad oggi, non esiste una cura definitiva e la terapia prevede il trattamento dei sintomi per rallentare la progressione della malattia. I farmaci utilizzati presentano però limitazioni in termini di efficacia e tollerabilità, rendendo necessario lo sviluppo di altri approcci clinici. Attualmente, le cellule staminali sono utilizzate in studi clinici con risultati non del tutto soddisfacenti. Pertanto, la generazione di modelli cellulari basati sull'utilizzo delle cellule staminali pluripotenti indotte (iPSC) può essere utile per lo screening farmacologico di nuovi farmaci o per approfondire i meccanismi molecolari della malattia. In questo lavoro, abbiamo generato le iPSC riprogrammando le cellule CD34+ isolate dal sangue periferico di donatori sani e pazienti affetti da SLA con diverse mutazioni (*C9orf72*, *TARDBP* e *KIF5A*). Le iPSC ottenute sono state caratterizzate per l'espressione dei marcatori tipici delle cellule staminali (OCT4, SOX2, KLF4, c-MYC, SSEA3 e Tra1-60) e per la loro capacità di esprimere i marcatori dei tre foglietti embrionali dopo la formazione dei corpi embrioidi. L'analisi del profilo trascrittomico delle iPSC sane e malate e delle neurosfere derivate da iPSC, ha mostrato un'espressione ridotta dei geni correlati alla matrice extracellulare (ECM) nelle iPSC da pazienti SLA, indicando una possibile comune patogenesi della malattia. Al contrario, le neurosfere derivate da iPSC hanno mostrato deficit più ampi nei pathways di sviluppo neuronale, come la genesi degli assoni, formazione della membrana sinaptica, sviluppo assonale e sviluppo delle proiezioni neuronali. Tuttavia, le differenze trascrittomiche non sono condivise fra tutti i pazienti, riflettendo l'eterogeneità genetica e fenotipica della malattia. Inoltre, mediante il saggio del neurite, abbiamo valutato l'impatto dei cambiamenti trascrittomici sulla funzione cellulare dimostrando una riduzione significativa nella ramificazione dei neuriti, nei punti di diramazione e nella complessità generale della rete nelle neurosfere derivate da pazienti SLA rispetto alle sane. Per confermare se queste alterazioni persistano nei MN, le iPSC sane e da pazienti SLA sono state differenziate in MN, dimostrando una riduzione della densità neuronale, della lunghezza assonale e del diametro assonale nei MN derivati da SLA. Infine, l'analisi dell'espressione dei marcatori neuronali ha dimostrato che i MN derivati da iPSC da pazienti SLA avevano generalmente livelli inferiori dei principali marcatori di maturazione (come ChAT, TUB β 3, MAP2, SYP, SYT, NeuroD1, NFH, NaV1.1 e Kv2.1), suggerendo una capacità ridotta di acquisire un fenotipo neuronale maturo rispetto a quelli sani. Nel complesso, questo lavoro pone le basi per comprendere meglio i meccanismi molecolari associati alla SLA e sottolinea l'importanza dell'applicazione dei modelli cellulari basati su iPSC come modello di malattia utile per lo sviluppo di nuovi approcci terapeutici.

Introduction

1. Amyotrophic lateral sclerosis: symptoms, incidence and genetics

Amyotrophic lateral sclerosis (ALS) is a progressive and fatal neurodegenerative disorder (NDD) that primarily affects upper and lower motor neurons (MNs), located in the motor cortex, brainstem nuclei, and the anterior horn of the spinal cord [1]. These MNs control voluntary muscle movements essential for walking, talking, chewing, and breathing. As ALS advances, MNs degeneration leads to muscle weakness, atrophy, and ultimately, the complete loss of voluntary motor function. The early symptoms of ALS, including muscle twitches (fasciculations), cramps, spasticity, and localized muscle weakness- often delay diagnosis.

As the disease advances, patients experience increasing difficulty with essential functions such as swallowing (dysphagia), speaking (dysarthria), and breathing (dyspnea) [2]. The relentless progression of the disease ultimately results in respiratory failure, the leading cause of mortality in ALS, with a median survival of three to five years post-diagnosis. However, around 10% of patients survive for a decade or longer. ALS is now recognized as a multisystem disease with significant phenotypic heterogeneity, including cognitive dysfunction and behavioral changes.

Based on symptom onset, two forms of ALS can be recognized:

- Spinal onset ALS, which occurs in approximately 75% of cases and initially affects one limb, causing asymmetrical muscle weakness. This form often impairs walking and manual dexterity, with gradual progression to other body regions. Generally, spinal onset ALS proceeds more slowly than bulbar onset (Figure 1).
- Bulbar onset ALS accounts for about 25% of cases, first impacting speech and swallowing muscles. Patients often present dysarthria and dysphagia, which may progress to a complete inability to speak. Within two years, most patients will also experience limb involvement. Due to its early impact on vital functions such as respiration and nutrition, bulbar onset is generally associated with worse prognosis. Many individuals with this form also develop pseudobulbar symptoms, including involuntary emotional expressions, and may suffer from cognitive impairments associated with frontotemporal dementia (FTD) (Figure 1) [3].

ALS has a global incidence of 1.75–3 cases per 100,000 individuals per year and a prevalence of 10–12 cases per 100,000 in Europe [4], with approximately 1,000 new cases diagnosed annually in Italy [5].

Worldwide, the prevalence of ALS is estimated to range from 200,000 to 300,000 cases, including about 5,000 cases in Italy [6]. The incidence of ALS increases significantly in individuals aged 45–75 years, the age group at the highest risk for developing the disease [7].

Symptoms typically occur between the ages of 58 and 63 years in sporadic ALS (sALS) cases and between 40 and 60 years in familial ALS (fALS) cases. ALS predominantly affects white males, who are more likely than females to develop sporadic limb-onset ALS. The male-to-female ratio for ALS is approximately 1.2–1.5, indicating a slight male predominance [8]. Several environmental factors, such as trauma, smoking, intense physical activity, and exposure to toxic agents, have been identified as potential risk factors [9]. However, the role of these factors is not yet fully understood, as ALS is believed to result from the interplay between genetic predispositions and environmental influences.

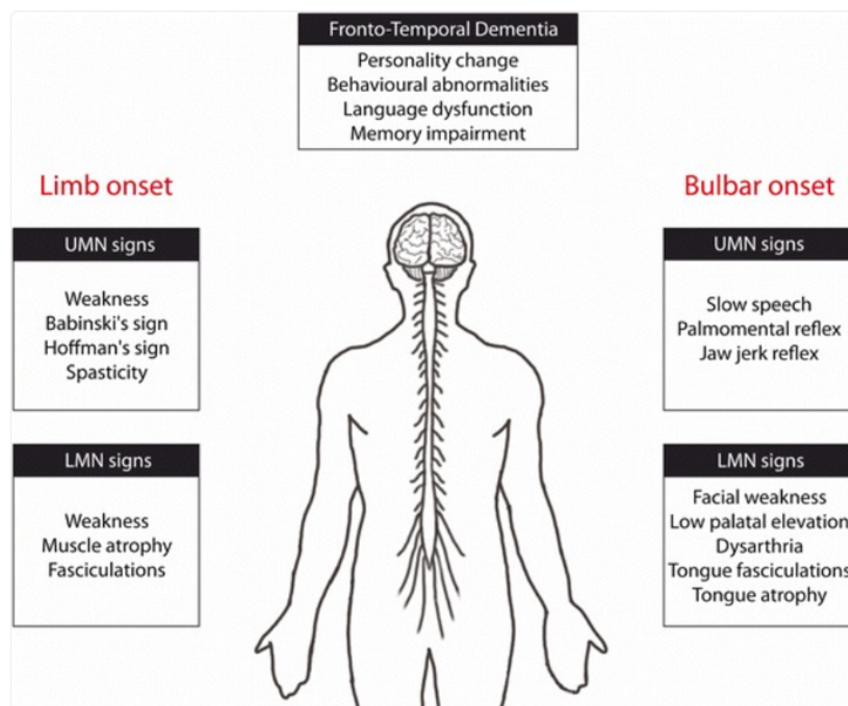


Figure 1: Clinical manifestation of ALS.

The main clinical characteristics of Amyotrophic Lateral Sclerosis (ALS) are represented in an illustrative scheme which divides into limb and bulbar categories based on the initial site of onset. The disease affects both upper motor neurons (UMNs) and lower motor neurons (LMNs) manifesting with different signs based on the affected area. The upper section of the image shows the typical cognitive symptoms linked to frontotemporal dementia (FTD) which include personality changes and behavioral abnormalities together with language impairment and memory deficits. Limb onset (left): UMN signs: weakness, spasticity, Babinski sign, Hoffmann sign; LMN signs: weakness, muscle atrophy, fasciculations. Bulbar onset (right): UMN signs: slow speech, abnormal reflexes; LMN signs: facial and tongue weakness and atrophy, dysarthria, tongue fasciculations [10].

1.1 Genetic of ALS

ALS can be classified into two main forms based on heritability: familial (fALS) and sporadic (sALS). FALS accounts for 5–10% of cases and is inherited in an autosomal dominant manner [11]

while sALS represents 85–90% of cases and occurs without a known family history [12]. Although multiple factors contribute to ALS, a strong genetic component has been identified. Genome-wide association studies (GWAS) and next-generation sequencing have been crucial for the investigations of ALS-associated genes and improved the understanding of the genetic basis of fALS [13]. In contrast to fALS, where genetic screening can often provide predictive insights, sALS remains largely unpredictable due to the absence of clear hereditary patterns. Beyond genetics, various environmental exposures have been proposed as potential contributors to sALS [14]. Actually, ALS is recognized as a polygenic disease meaning that more than one gene is implicated in the disease [15,16]. While over 50 potentially causative or disease-modifying genes have been identified, pathogenic variants in *SOD1*, *C9ORF72*, *FUS*, and *TARDBP* are the most frequently observed, whereas mutations in other genes are relatively rare [17,18].

1.1.1. SOD1

The *SOD1* gene, the first identified genetic cause of ALS, encodes a metalloenzyme of 153 highly conserved amino acids involved in neutralizing reactive oxygen species (ROS) within mitochondria [19]. Mutations in *SOD1* contribute to ALS pathogenesis through both loss- and gain-of-function mechanisms, leading to impaired ROS degradation, increased oxidative stress, and toxic protein interactions [20]. A key pathogenic feature is the formation of misfolded *SOD1* aggregates, which disrupt proteostasis by overloading the proteasome and impairing autophagic clearance, resulting in neuronal toxicity [21]. Additionally, *SOD1* mutations exacerbate oxidative stress and mitochondrial dysfunction, promoting lipid peroxidation, DNA damage, and dysregulation of calcium homeostasis. Axonal transport is also compromised due to impaired microtubule dynamics, affecting mitochondrial and synaptic vesicle trafficking [22]. Furthermore, neuroinflammation driven by activated microglia and astrocytes amplifies MNs degeneration, in particular microglia expressing mutant *SOD1* shifts from a neuroprotective phenotype at disease onset to a cytotoxic phenotype during disease progression [23]. A recent meta-analysis found that pathogenic variants in *SOD1* account for approximately 15–30% of fALS and fewer than 2% of sALS cases [13].

1.1.2. TARDBP

The *TARDBP* gene encodes the transactive response (TAR) DNA-binding protein 43 (TDP-43), a DNA/RNA-binding protein normally localized in the nucleus and shuttles in and out for protein transport [24]. The protein plays a crucial role in several aspects of RNA metabolism, including mRNA splicing, RNA transport, translation, and microRNA biogenesis [25]. While 40 missense mutations associated with the *TARDBP* gene have been identified but the effects of these mutations

on protein function and ALS protein aggregation are still under investigation [26]. When mutated, TDP-43 binds to ubiquitin and forms aggregates, leading to the formation of inclusions found in the MNs and brains of ALS patients. In particular, TDP-43 mutations result in loss and gain of function. The gain of function arises from aggregate formation, while the loss of function is associated with the depletion of nuclear TDP-43. In conditions of nuclear excess, TDP-43 binds to the 3'UTR of the pre-messenger RNA TARDBP, regulating polyadenylation and alternative splicing and maintaining TDP-43 levels in check. However, in ALS, nuclear depletion of TDP-43 disrupts this balance, causing excessive expression of TARDBP and contributing to TDP-43 proteinopathy. Today, TDP-43 is considered the most relevant biomarker for ALS [27].

1.1.3. C9orf72

C9orf72 is a (G4C2) hexanucleotide repeat expansion in the first intron of *C9orf72* gene which in healthy condition the number of repetitions is between 2 and 20, while in mutated condition over hundred repetitions have been counted. This repetition occurs in approximately 34% of fALS and 5% of sALS cases in the European population [28]. In neurons *C9orf72* also regulates autophagy and vesicular trafficking and affects dynamics and endosomal recycling at synapses. The age of onset for patients carrying this mutation is very varied. Non-coding G4C2 expansion leads to two major effects: a loss of function due to the hypermethylation of the CpG rich promoter region resulting in the transcriptional silencing, and a gain of function which is related to the abnormal production of RNAs that form toxic dipeptide repeat proteins (DPRs)[29,30]. The toxic accumulation of these DPRs in neurons impair nucleocytoplasmic transport, nucleotide metabolism, lysosomal processes, and cellular metabolic pathways [31].

1.1.4. FUS

Another protein belonging to the group of DNA/RNA binding proteins is FUS which is predominantly located in the nucleus, and it is encoded by Fused in Sarcoma (*FUS*) gene [31,32]. This protein, in normal condition, shares several physiological roles with TDP-43 protein such as: regulating transcription, RNA splicing, RNA transport, and DNA repair [27]. Mutations in the *FUS* gene, located on chromosome 16p11.2, are associated with various forms of NDDs, including juvenile ALS [31], early onset ALS, and fALS with FTD. FUS is a ubiquitously expressed protein involved in gene expression regulation, RNA metabolism, nucleocytoplasmic transport, DNA repair, and paraspeckle formation [24,33]. Under normal conditions, FUS localizes to the nucleus, but pathogenic mutations in the nuclear localization signal disrupt its transport, leading to cytoplasmic accumulation [34]. Over 50 autosomal dominant *FUS* mutations have been identified, including missense, insertion,

deletion, and splicing variants [35]. The pathogenic mechanism remains debated, with evidence supporting both loss- and gain-of-function models. Loss-of-function theories suggest that nuclear depletion impairs FUS-dependent processes, but knockout models in mice have failed to replicate ALS phenotypes [36,37]. In contrast, gain-of-function models suggest that cytoplasmic FUS accumulation induces neurotoxicity, either through direct aggregation toxicity or increased soluble FUS levels disrupting stress granule dynamics [31,38]. Some studies propose that aggregation may serve as a protective role against toxic soluble FUS [39]. Additionally, *FUS* mutations have been linked to RNA splicing defects, DNA damage, and impaired autoregulation [40,41].

1.1.5. KIF5A

The above mentioned are the oldest and most frequently mutated genes but a recent international GWAS has identified a unique form of ALS linked to mutations in the C-terminal region of the KIF5A protein [42]. *KIF5A* gene encodes a member of the kinesin family of proteins that function as a microtubule motor in intracellular organelle transport and cell division and is exclusively expressed in neurons. Kinesin structure consists of 2 identical heavy chains and 2 identical light chains. The heavy chain is composed of 3 domains: an N-terminal globular domain, a long alpha-helical coiled coil domain, and a small C-terminal globular domain. The N-terminal domain performs its motor action thanks to the ATPase activity that allows the protein to move and transport cargo in an anterograde manner along the microtubule. Mutations which affect this domain and abolish motor activity are the cause of hereditary spastic paraplegia and Charcot-Marie-Tooth disease [43]. On the other hand, ALS-KIF5A mutation affects the C-terminal tail which is crucial for binding and transport cargos. In the mutation based on the exon skipping Δ Exon27, the normal protein C-terminal 34 amino acids are replaced with 39 aberrant amino acids. It may involve dysfunctional kinesin and impaired cargo transport or a toxic gain-of-function mechanism causing detrimental effects for example: the formation of cytoplasmic aggregates at the plus end of microtubules, which are neurotoxic and lead to altered axonal transport. The altered C-terminal tail compromises the autoinhibition of KIF5A, relieving motor autoinhibition and increasing motor self-association [44]. This results in drastically enhanced processivity on microtubules, although the consequence of this increased processivity on neuronal health is not yet known. Patients with mutations in C-terminal KIF5A protein have a particular form of ALS exhibiting a median age of onset at 46.5 years and a slow progression [42]. Although there are several mutations associated with ALS and each of them act differently, affecting different pathways, all of them share the same disease phenotype, characterized by motor-motor degeneration. Mutations in these genes disrupt essential cellular processes, ultimately leading to motor neuron degeneration.

1.1.6. Other genes involved in ALS

Recent discoveries of novel genes associated with ALS have significantly expanded our understanding of its genetic landscape. Many of these genes are involved in critical cellular processes, including RNA metabolism (e.g. *ATXN2*, *SETX*) [45,46], protein homeostasis and trafficking (e.g., *OPTN*, *VCP*, *TBKI*) [47,48], cytoskeletal and axonal dynamics (e.g., *DCTN1*, *PFN1*, *NEK1*) [49], and mitochondrial function (e.g., *CHCHD10*, *C19ORF12*) [50]. However, establishing a causal relationship between many of these genetic variants and ALS remains challenging, particularly due to the limited cohort size. Additionally, several genetic modifiers influence disease susceptibility and progression. For example, *UNC13A* variants have been linked to both increased ALS risk and reduced survival, while intermediate-length expansions of *ATXN1* and *ATXN2* increase disease susceptibility, particularly in C9orf72 expansion carriers. Genetic heterogeneity of ALS highlights the urgent need for large-scale sequencing studies to drive the discovery of new therapeutic strategies [18].

2. Molecular mechanisms of ALS

The pathogenesis of ALS involves multiple molecular mechanisms, reflecting the heterogeneity and complexity of the disease. Understanding the molecular and cellular basis of MNs dysfunction is crucial for developing targeted therapies to mitigate the progression of ALS. Many ALS-related genes result in several primary molecular alterations, including protein conformational instability and aggregation, impaired RNA trafficking, and abnormalities in the regulation of cytoskeletal dynamics and axonal transport. These initial mechanisms lead to the activation of numerous secondary pathological processes, such as endoplasmic reticulum (ER) stress, activation of autophagy, proteasome dysfunction, glutamate-mediated excitatory toxicity, alterations in mitochondrial function, disruption of axonal transport, dendritic structural changes, and a significant neuroinflammatory response (Figure 2) [51].

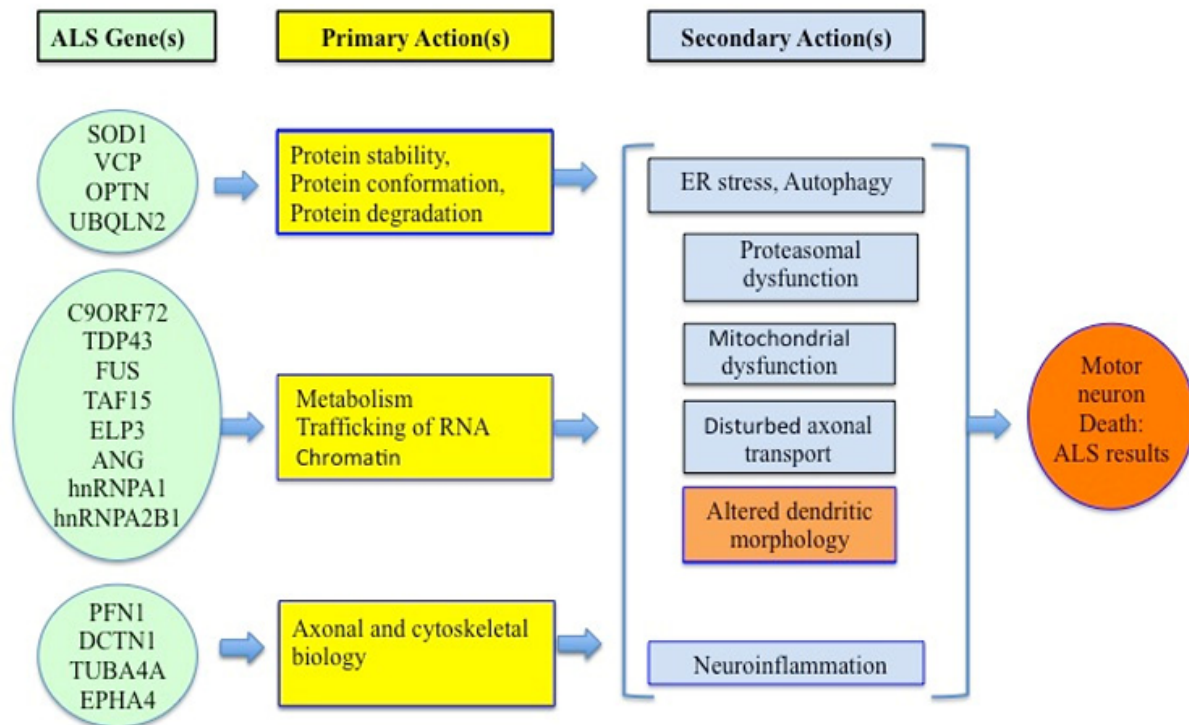


Figure 2. Summary of the main molecular mechanisms associated with ALS.

Mutations in ALS-associated genes result in primary actions in which several molecular processes are altered. Subsequently, these result in secondary actions ultimately leading to the death of MNs and ALS manifestations. Primary actions: Each gene group alters fundamental cellular processes (protein metabolism, RNA, cytoskeleton). Secondary actions: Primary defects cause endoplasmic reticulum stress, mitochondrial dysfunction, neuroinflammation, disturbed axonal transport, and alterations in dendritic morphology [51].

2.1 Proteins aggregate

Protein homeostasis is crucial for neuronal survival and function. Disruptions in this delicate balance result in the misfolding and accumulation of proteins, ultimately driving neuronal dysfunction and cell death [52]. Mutations in *SOD1*, *C9orf72*, *TDP-43*, *UBQLN2* and *VCP* genes directly compromise the ubiquitin proteasome system and the autophagic pathway, promoting the accumulation of toxic proteins [53].

Missense mutations in the *SOD1* gene lead to the formation of insoluble protein aggregates, which impair enzyme activity and initiate the unfolded protein response (UPR) in the endoplasmic reticulum. This activation of cellular stress pathways, if unresolved, results in progressive cellular damage and neuronal death [54]. A similar phenomenon is observed with mutations in the *C9orf72* gene, where the expansion of G4C2 nucleotide repeats causes an anomalous production of toxic RNAs and proteins that interfere with the correct folding and degradation of proteins [54]. This

accumulation overwhelms the protein degradation system, including the proteasome and autophagy, leading to ER stress. Similarly, the TDP-43 mutations cause cytoplasmic aggregation in MNs, impairing proteasome function and disrupting the cell ability to remove misfolded proteins.

2.2 Alterations in RNA metabolism

TDP-43 and FUS, recognized as RNA-binding proteins, are crucial for RNA processes like splicing, polyadenylation, mRNA stability, localization, and translation [24,55]. Under normal conditions, these proteins are in the nucleus and participate in RNA metabolism, but in ALS, they can mislocalize to the cytoplasm inducing a toxic gain of function that drives to neurodegeneration [56,57]. Bidirectional transcription of G4C2 repeats in the *C9orf72* gene generates sense and antisense RNAs forming toxic foci that sequester RNA-binding proteins like TDP-43. These RNA foci frequently localize in the nucleoli, with a higher density in antisense RNA foci than in sense RNA foci. Furthermore, antisense RNA foci are associated with TDP-43 aggregation in the cytosol of *C9orf72* patient motor neurons. Postmortem studies of ALS patients tissues reveal antisense RNA foci in 78.7% of neurons and 24.9% of glial cells within motor regions of the brain and spinal cord [58].

2.3 Oxidative stress and Mitochondrial dysfunction

Oxidative stress and mitochondrial dysfunction are key pathogenic mechanisms in ALS. Oxidative stress occurs when there is an imbalance between elevated reactive oxygen species (ROS) production and the cell ability to neutralize them. Excess ROS targets proteins, lipids, RNA and DNA, disrupting essential cellular processes and contributing to neuronal damage [59,60].

In fALS, mutations in genes such as *SOD1*, a decrease in antioxidant enzymatic activity occur, thus inducing ROS increase [61] with an accumulation of reactive species. This loss of function reduces cell ability to protect against oxidative damage, increasing MN weakness [62]. Specifically, oxidative changes in nerve cells result in structural changes, including protein glycoxidation and lipid peroxidation, observed in MNs of patients with fALS [63,64]. Mitochondrial dysfunction is also a key mechanism involved in ALS. Mitochondria, which are vital organelles responsible for ATP, calcium regulation, and apoptosis, appear to have altered morphology and function in ALS MNs and in *SOD1*G93A and TDP-43A315T mouse models [58]. The accumulation of mutant proteins caused by *SOD1* mutation leads to vacuole formation in the mitochondrial membranes and loss of function [65]. *FUS* mutations also lead to protein accumulation in the cytosol, resulting in mitochondrial fragmentation and increased ROS [66,67]. Further, mitochondrial damage can also interfere with calcium homeostasis and increase excitotoxic response to glutamate [68]. Additionally, elevated intracellular calcium related to mitochondrial dysfunction can originate apoptotic signals, resulting

in further cell death and neurodegeneration. Mitochondrial impairment is part of a cycle that accelerates the progression of ALS. Damaged mitochondria can be identified and targeted for degradation by the process of mitophagy, mediated by key proteins such as NIP3-like protein X (NIX) or PTEN-induced putative kinase protein 1 (PINK1)-E3 ubiquitin ligase parkin [46]. In ALS, however, mutations in *OPTN* and *TBK1*, two genes related to mitophagy, decrease the cellular ability to clear nonfunctioning mitochondria, allowing damaged mitochondria to remain and exacerbate injury to neurons [47,69]. Finally, disruptions in axonal mitochondrial transport influence neuronal survival [70]. Mitochondria need to be moved along the axon to meet local metabolic requirements, particularly in synapses, which are known to use large quantities of energy. Unusually, it has been found that mitochondria are mislocalized or distanced from sites of action where they are needed, which also contributes to denervation and degeneration of neurons. Abnormal interactions of *SOD1* mutations with dynein-actin, which mediates axonal transport, and the anti-apoptotic protein Bcl2, are at the prediction basis for these phenomena [71].

2.4 Axonal transport

The exact ways that axonal transport defects aggravate pathogenesis in ALS are not yet fully understood, but mutations in proteins related to axonal transport, as well as defects in microtubule stability, hyperphosphorylation of motor proteins, and ultimately defects in interactions between cargo and motor proteins, have been implicated [72]. The integrity of the neuronal cytoskeleton, which involves neurofilaments, governs axonal stability, electrical signal conduction, and axonal transport. Dysfunction in the neurofilament cytoskeletal network is often seen in ALS in part caused by mutations in the *NEFH* gene that affect the cross-linking characteristics of the heavy neurofilament chain, leading to accumulation of neurofilaments and defective axonal transport [73]. Neurofilaments also interact with actin and microtubules, creating a cytoskeletal network that stabilizes the axonal structure. Mutations in the *PFN1* gene, which codes for profilin I [74], interfere with actin polymerization and subsequently deteriorate transport, thus contributing to degeneration [75]. Instead, microtubules are made up of α - and β -tubulin and are necessary for axonal transport mediated through the molecular motors dynein-dynactin and kinesins. Mutations in the *TUBA4A* cause microtubule instability [76], while mutations in the *DCTN1* gene lead to dysfunctional interaction of dynactin with microtubules, thus impairing transport [76]. Kinesins KIF5A, KIF5B and KIF5C are necessary for anterograde transport, and mutations in their gene cause dysfunction in mitochondrial transport and accumulation of neurofilaments.

3. ALS Diagnosis

The diagnosis of ALS is a comprehensive process that begins with identifying clinical signs associated with both primary and secondary MNs. Given the absence of a single definitive test, a combination of diagnostic approaches is required to ensure accuracy and early detection. These include a complete medical history and neurological examination to assess reflexes, muscle strength, and motor responses [77]. The main used diagnostic techniques are genetic testing for specific mutations, electromyography (EMG) to evaluate nerve conduction velocities and neuroimaging methods to analyze brain activity and functional changes [78]. The availability of these methods not only enhances diagnostic precision but also helps distinguish ALS from other neurological disorders, allowing for timely intervention and optimized treatment strategies. Consequently, there is a growing emphasis on identifying reliable biomarkers that can enhance diagnostic accuracy and provide prognostic information. Biomarkers detectable in blood, such as neurofilament light chain and glial fibrillary acidic protein, have shown promise in reflecting neurodegenerative processes specific to ALS. Several studies highlight the identification of novel biomarkers that could aid diagnostic accuracy and provide prognostic information. Pathologically, ALS is associated with abnormal protein aggregation in MNs and glial cells, which contributes to disease progression. Given these challenges, biomarkers, particularly those detectable in cerebrospinal fluid (CSF), blood, and urine play a crucial role in elucidating disease mechanisms and improving diagnostic accuracy [79]. Among blood-based biomarkers, neurofilament light chain (NFL) has emerged as one of the most promising candidates due to its high specificity and sensitivity in reflecting neuronal axonal injury. NFL levels have shown to decrease in response to treatment with Riluzole, supporting their potential use in assessing treatment response [80]. Glial fibrillary acidic protein (GFAP) has also gained attention, particularly for its potential role in assessing non-motor aspects of ALS, such as cognitive impairment and systemic metabolic changes [81]. In addition to neuronal biomarkers, muscle-derived markers such as creatinine and creatine kinase (CK) have been explored for their prognostic potential. CSF has emerged as a source for biomarker discovery in ALS due to its ability to reflect neurodegenerative processes occurring within the blood–brain barrier. The upregulation of ubiquitin C-terminal hydrolase L1 (UCHL1), microtubule-associated protein 2 (MAP2), macrophage capping protein (CAPG), and glycoprotein non-metastatic melanoma protein B (GPNMB) in CSF suggests their potential role in neuronal injury and neuroinflammation in ALS [82].

4. Current treatment

Currently, there is no effective treatment to halt or reverse ALS progression and patients are treated to slow down disease progression and alleviate symptoms. The drugs recognized for the treatment of ALS symptoms are: Riluzole, Edaravone and Tofersen. Riluzole has an anti-glutaminergic action as it inhibits voltage-gated sodium channels decreasing the release of glutamate in the pre-synaptic space and, consequently, counteracts its neurotoxic effect, consequently it acts by reducing excitotoxicity caused by high levels of glutamate [83]. Clinical trials demonstrated how treatment with this drug slightly improved the life expectancy of patients [84]. Another drug is Edaravone which has not been approved in Italy. It works by eliminating hydroxyl radicals and lipid peroxides thus preventing neural damage consequently the oxidative stress which is recognized as an ALS hallmark [85]. Studies are still in course to better understand the side effects and its effectiveness in neutralizing the high levels of oxidative stress markers. Finally, in 2023, the Food and Drug Administration (FDA) approved Tofersen for the treatment of ALS patients carrying the SOD1 mutation. Tofersen is an antisense oligonucleotide consisting in short, synthetic single-stranded DNA molecules, designed to bind complementary sequences in target RNA transcripts and modulate gene expression. Tofersen acts through the degradation of *SOD1* mRNA through RNase H-mediated cleavage, thereby leading to the reduction of the toxic SOD1 protein [86]. Studies demonstrated that it cannot cross the blood-brain barrier, and the intracranial administration is able to reduce *SOD1* mRNA and protein levels in the central nervous system of patients with SOD1 mutations in whom there is a delay in the progression of the disease [87]. To act on other molecular mechanisms or to combat some manifestations, other drugs such as Baclofen, tizanidine and benzodiazepines have been tested and can be used to relieve spasticity in patients with ALS. However, there are significant limitations in terms of efficacy and tolerability therefore other clinical approaches are needed.

5. Stem cells therapy and clinical trials

Stem cell therapy for ALS is emerging as a new treatment plan to reduce the spread of neurodegeneration in the body, regenerate neurons that have already been affected, and protect those that are still healthy. Although numerous pre-clinical studies have shown positive results, on the other hand, translation into the clinic is progressing more slowly. A review of clinical studies registered on ClinicalTrials.gov since 1998 reveals 63 stem cell-based studies for ALS, of which only 11 are currently ongoing [88]. For all clinical studies, several aspects must be considered, including the source of the stem cells, the delivery mechanism, and the methods for determining clinical outcomes

of the therapy [12,89]. Many studies have used adult stem cells, especially human mesenchymal stem cells (hMSCs), which are administered primarily by intrathecal injections, or surgical implantation into the spinal cord or cerebral cortex [90]. Of note among these is the Phase III NurOwn® study using autologous bone marrow-derived MSCs, which achieved positive biological endpoints such as an improvement in neuroinflammation and neurodegeneration [91]. More recently, human neural progenitor cells (hNPCs) expressing glial cell-derived neurotrophic factor (GDNF) have been evaluated, demonstrating long-term graft survival and sustained production of overall neurotrophic factor [92]. Although early-stage clinical trials support the feasibility and safety of stem cell therapies in ALS, further large-scale, multicenter studies with standardized protocols are needed to definitively determine their clinical efficacy and optimize treatment strategies [93]. Despite the large number of basic, translational, and preclinical studies based on stem cell therapies, there has not been a corresponding surge in clinical trials, and the outcomes so far have been relatively modest. This gap is partly due to the challenges associated with the absence of clear guidelines for their clinical translation. Preclinical studies also exhibit considerable variability, influenced by differences in cell sources, cell numbers, and delivery methods. Additionally, to study ALS progression, the animal models used, mainly transgenic mice and rats, often fail to accurately replicate the disease progression or reflect human phenotypes. For this purpose, the use of induced pluripotent stem cells (iPSCs) can help in the understanding the pathogenic mechanism by differentiating them into the desired cell type and using the obtained cells as model of the disease or to test specific drugs.

6. Induced Pluripotent Stem Cells (iPSCs)

The full understanding of the pathogenic mechanisms leading to MNs degeneration in ALS remains elusive. Over the years, several disease models have been developed to replicate, at least partially, the complexity of ALS and the interactions among the principal cell types: MNs, astrocytes, muscle cells, and microglia. Among these models, transgenic mice and rats expressing the human mutant *SOD1* gene (G93A) have proven valuable in uncovering fundamental key mechanisms such as neuroinflammation, protein aggregation, and neuromuscular denervation [94–96].

Despite extensive research, no single animal model has successfully replicated the complex pathology observed in human ALS. In this context, the advent of human pluripotent stem cell technologies, specifically human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs), has opened new opportunities for in vitro ALS modeling and can be used to test new transplantation strategies and cellular therapies, and pharmaceutical treatments.

While hESCs offer promising potential for cell replacement therapies, their use is accompanied by ethical concerns. The solution was proposed in 2006 by Takahashi and Yamanaka with a study which demonstrating that somatic cells could be reprogrammed into “artificial” stem cells. These reprogrammed cells, called iPSCs, exhibited the same key characteristics as ESCs, such as the ability to self-renew, proliferate, and differentiate into various cell types [97,98]. The reprogramming strategy was based on the use of a combination of transcription factors and four key factors were ultimately identified as essential for reprogramming: *Oct4*, *Sox2*, *Klf4*, and c-Myc. Their ectopic expression was sufficient to revert somatic cells to a pluripotent state, showing an ESC-like morphology. In particular, *Oct4* belongs to Octamer binding protein family and is necessary for the formation of the internal cell mass of embryos and for the maintenance of the pluripotency of ESC [99–101]. The *Sox2* gene, called SRY- (Sex determining Region Y-) box2, encodes a transcription factor belonging to the family of Sox proteins that bind to DNA through their HMG domain [99]. The absence of *Sox2* in embryos causes a failure at implantation related to an impaired epiblast, demonstrating its role in embryonic development [100]. *Oct4/ Sox2* synergy regulates the expression of *Nanog* [101] a homeodomain transcription factor which also plays a crucial role in maintaining pluripotency by inhibiting differentiation pathways. The *Klf4* gene, also called Kruppel-like zinc-finger protein 4, encodes a transcription factor involved in left-right asymmetry during embryonic development. *Klf4* binds directly to the heterodimer *Oct4-Sox2* and tetrameric complexes containing DNA of the target gene are needed for somatic cell reprogramming [102]. The *c-Myc* gene encodes a transcription factor and binds to DNA through its helix-loop-helix domain. C-Myc is involved in the maintenance of the pluripotency of the ESC and its removal is associated with a decrease in reprogramming efficiency [103]. Due to the oncogenic nature of c-Myc, alternative reprogramming strategies that bypass it have been developed over time. These strategies often involve the use of other transcription factors, such as *Nanog* and *Lin28*, to enhance the reprogramming efficiency while minimizing the risk of tumor formation [104,105]. By omitting *c-Myc*, these protocols created a safer method for generating iPSCs, thereby reducing the oncogenic risks associated with the reprogramming process [106]. iPSCs exhibit similar characteristics to ESCs, including the ability to differentiate into derivatives of the three germ layers: ectoderm, mesoderm and endoderm, a similar expression of pluripotency markers such as *Oct4*, *Sox2*, *Klf4*, and surface proteins SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81. Furthermore, iPSCs show high telomerase activity, which is essential for genomic stability and cellular longevity [107]. Moreover, the ability of these transcription factors to promote the reprogramming is strictly modulated by the changes in chromatin structure and the recognition of pluripotency associated sequences on the DNA resulting in changes of the transcriptome that attend reprogramming. Nevertheless, studies demonstrated that somatic cells

maintain an epigenetic memory that interferes with the reprogramming process, inducing iPSCs through senescence and apoptosis. This incomplete reprogramming results in an inability to fully establish pluripotency, thus delaying the efficiency of the reprogramming process [108]. The persistence of these epigenetic signatures can be exploited to induce the differentiation of iPSCs towards the desired cell type [109,110].

6.1 Generation of iPSCs

Since their discovery, the generation of iPSCs has evolved through the selection of different somatic cell sources and the discovery of numerous reprogramming methods to improve their efficiency and safety. The first cell type used was represented by human and murine fibroblasts [98], then other types were added: keratinocytes [111], hepatocytes, gastric epithelial cells, neural stem cells, adipose stem cells, pancreatic cells, cord blood cells, blood progenitor cells, urinary cells, stromal cells derived from menstrual blood [112,113] and peripheral blood cells. Although fibroblasts are still the most used adult cell type to obtain iPSCs, there are several disadvantages that lead to defining this cell type as not optimal source for iPSC reprogramming, such as: the need for biopsy, the long time for cell expansion and finally the fact that fibroblasts are considered as a heterogeneous population [114]. Promising sources have been identified that resulted in the obtaining of bona fide iPSCs with higher efficiency. Peripheral blood mononuclear cells (PBMCs) can be considered a valid alternative, as they can be isolated from the peripheral blood, cord blood or bone marrow through density gradient. Moreover, their maintenance and expansion in culture is easier compared to other cells sources (eg. fibroblasts, keratinocytes, adipocytes). Several cells population can be distinguished from isolated PBMCs: monocytes, natural killer cells, lymphocytes dendritic cells, hematopoietic stem cells and hematopoietic progenitor cells. Recent studies have highlighted the efficiency of PBMCs reprogramming, evidencing the high rate of success from small blood samples (1-10 ml) [115] or even finger pricking [116]. Among the various cell types present in blood, PBMCs, including T cells, B cells, and CD34⁺ hematopoietic progenitors, have been successfully reprogrammed using both lentiviral vectors [117], Sendai virus [118], episomal vectors [104], and synthetic mRNA [119] (Figure 3). The importance of an initial activation and expansion phase for T cells before reprogramming has been emphasized. This employs IL-2, which promotes T cell growth and expansion, and anti-CD3/CD28 microbeads, which provide signals that mimic the natural activation of T cells [120]. T-cell development involves rearrangements in the T cell receptor (TCR) genes, while B cells undergo rearrangements in immunoglobulin (IgG) genes. This is useful for identifying the source of the resultant iPSCs [121]. Reprogramming B cells has proven to be more challenging, but was attained by Bueno et al., who utilized a non-integrating tetracistronic OSKM-expressing

Sendai virus, with transient co-expression of C/EBP α together with OSKM increased the yield of iPSCs [122]. The use of T and B cells, however, is limited by their immunological memory. This problem can potentially affect the differentiation of the resultant iPSCs into other cell types and eliminates the possibility of obtaining naïve T and B cells (without rearrangements in TCR and IgG genes) from the iPSCs. Finally, CD34⁺ cells, although rare in peripheral blood, exhibit high proliferation rates and are particularly amenable to reprogramming. These progenitors can be enriched through culture with cytokines such as SCF, Flt3-L, TPO, and IL-3, and subsequently reprogrammed using lentiviral vectors or non-integrative methods [116,123,124]. Moreover, to generate iPSCs a specific reprogramming strategy can be used. Several strategies were employed to obtain a good yield of iPSCs expressing pluripotent markers, in particular gene-delivery of transcription factors (OCT4, SOX2, KLF4, and c-MYC) has been granted thought viral, non-viral methods and, also with new reprogramming method based on the use of RNA, protein and microRNA [125]. Among the viral methods, retroviral vectors, lentiviral vectors, PiggyBac transposons were used, but random integrations and mutagenesis can occur [116,126]. On the other side non-viral methods, like episomal and adenoviral vectors avoid integration but show lower efficiency, but collectively, the non-DNA based approaches and optimized culture conditions can provide promising avenues for safely generating iPSCs with minimal risk of insertional mutagenesis and other genomic modifications [127] (Figure 3).

6.2 iPSCs applications in ALS

iPSCs offer a patient-specific, renewable source for generating diverse cell types, including neurons and cardiomyocytes, which are usually available only postmortem [128]. They provide cellular models that accurately recapitulate disease mechanisms, enabling detailed studies of pathogenesis and comparative analyses between healthy and diseased cells. Moreover, iPSCs serve as a helpful tool for drug screening and toxicity testing, significantly improving the drug discovery process [129]. Genome editing of iPSCs with mutations such as *SOD1*, *FUS*, and *C9orf72* has successfully corrected aberrant transcripts in derived MNs, allowing the identification of aberrant transcripts and the elimination of pathological phenotypes, underlining the therapeutic potential of this approach [130,131]. The understating of ALS mechanism has been accelerated through the advent of iPSCs technologies which offer the possibility to obtain all cell types, with the same genetic background of patients, involved in the pathogenesis and progression of ALS, such as: MNs, astrocytes, oligodendrocytes and microglia [132] (Figure 3). Replacement of damaged MNs emerges as an interesting approach in ALS. Although the transplant of differentiated MNs may seem to be the best solution, their non-dividing nature represents an obstacle. Furthermore, transplanted MNs must

establish precise axonal connections to target muscles and form functional synapses, so direct replacement of degenerated MNs with iPSC-derived MNs may not be a practical strategy for ALS treatment [132]. The iPSCs differentiation into MNs represent the first step necessary to translate the practical use of these cells from the laboratory into clinical practice. Several protocols aiming to differentiate MNs via small molecules exhibit common stages such as: ectodermal induction, neural progenitor generation, and a final MNs maturation [133]. It is notable that a crucial step commonly employed in studies on ALS model transplantation is the creation of neural progenitor cells (NPCs) from iPSCs. The transplantation of iPSC-derived NPCs (iNPCs) directly into the spinal cord of transgenic rats carrying a human mutated SOD1(G93A) gene showed an efficient engraftment in the spinal cord, higher rate of cell survival and differentiation into mature motor neurons [134] (Figure 3). In 2020 a group demonstrated that NPCs have also a neuroprotective effect and preserve perineuronal nets around MNs but in SOD-1 environment seem to be missing the necessary cues to promote precursor maturation into MNs [135]. The transplantation of iNPCs exercise a positive effect into the host tissue through the release of growth factors and the transformation into MNs which extend their axon along motor axonal tract in the ventral spinal cord of an immunodeficient rat [136]. In summary iNPCs represent the best choice to overcome the ethical problems related to the use of ESC-derived and fetal-derived progenitors [137] therefore they offer themselves as cell sources for autologous transplantation. Advances in microfluidic systems and three-dimensional cultures have developed ALS models by enhancing the formation and assessment of neuromuscular junctions, thereby supporting detailed electrophysiological, genetic, and pharmacological investigations [138–140]. Unfortunately, there are still some limitations related to the use of iPSCs-derived cells that slow down their use for clinical studies, such as tumorigenesis caused by incomplete differentiation, donor genetic variability, and immune rejection. Currently, the most practical approach for iPSC therapy is based on the use of iPSC generated from various HLA homozygous donors ensuring the maximum possible population coverage. The most HLA homozygous iPSC bank initiatives, have been stated in Japan, Europe, and the United States to support this strategy [141]. Despite the limitations of iPSCs, their use in ALS field can allow the observation of the early stages of degeneration, investigate the cellular and molecular mechanisms involved in the selective weakness of MNs, and test therapeutic strategies in a patient-specific system. To generate the desired cell type, specifically MN, from iPSCs, it is essential to closely mimic the *in vivo* mechanisms that drive neurogenesis during development.

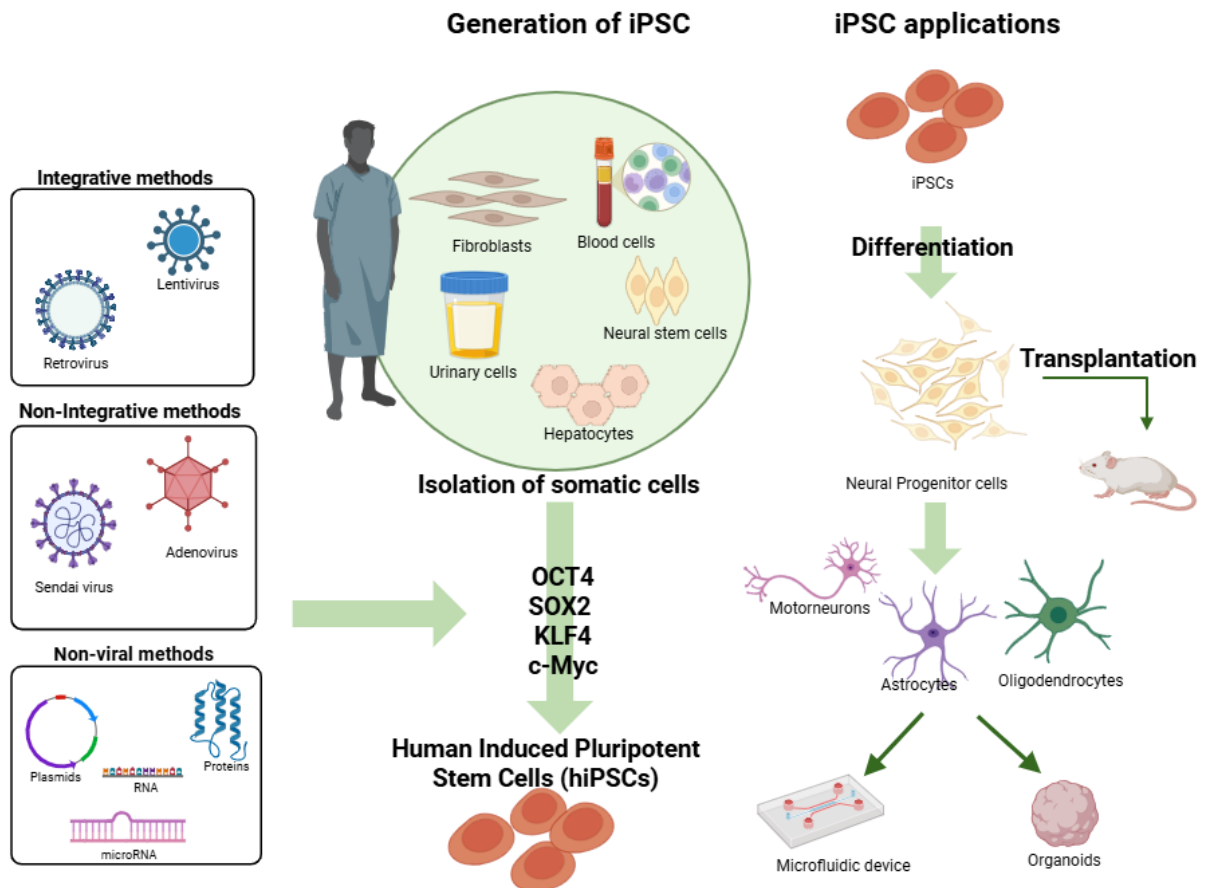


Figure 3. iPSCs generation and their application to study ALS.

The image shows the generation process followed to obtain iPSCs. Somatic cells (blood cells, fibroblasts, hepatocytes, urinary cells, and neural stem cells) can be reprogrammed with different strategies: viral and non-viral (i.e., plasmids, microRNA, and proteins). The first group can be divided into integrating (i.e., lentivirus and retrovirus) and non-integrating (i.e., Sendai virus and adenovirus). These techniques enable the introduction of Yamanaka factors (OCT4, SOX2, KLF4, and c-MYC) into somatic cells, reprogramming them into iPSCs. iPSCs have the potential to differentiate into several types of cells. For the study of ALS, iPSCs are commonly differentiated into neural progenitor cells, which are frequently used for transplantation into mouse models. Neural progenitor cells can further differentiate into motor neurons, astrocytes, and oligodendrocytes used to model disease with organoids and microfluidic devices. The image is generated with BioRender.

7. Motor neuron specification during neurogenesis

MNs are specialized neurons essential for controlling both voluntary and autonomic movement. Based on the location of their cell bodies, MNs are classified into two categories: Upper Motor Neurons (UMNs) and Lower Motor Neurons (LMNs). UMNs cell bodies are in the cerebral cortex and project exclusively into the CNS, forming glutamatergic synapses with the LMNs. Conversely, LMNs are in the motor nuclei of the brainstem and the ventral horn of the spinal cord projecting their axons outside the CNS to innervate skeletal muscles and peripheral structures. LMNs

can be further differentiated into three subtypes based on the nature of their peripheral target, including branchial motor neurons, visceral motor neurons, and somatic motor neurons [142]. Therefore, a critical division between these various classes of MNs will be important for studying ALS and developing relevant *in vitro* experimental models. Spinal MN development is a coordinated process that occurs during embryogenesis, starting with neural induction of the dorsal ectoderm and continuing through axial patterning and terminal differentiation. Starting from embryogenesis, a fertilized egg undergoes cell divisions to produce a blastula. In a subsequent phase, called gastrulation, some cells move into the blastula, creating the three basic germ layers in triploblastic organisms: endoderm, mesoderm, and ectoderm [143]. The ectoderm has a major role in a process called neurulation, in which it generates the superficial ectoderm, the neural crest cells, and the neural tube. Inhibition of bone morphogenic protein (BMP) and Activin signaling, mediated by molecules such as Chordin, Follistatin, and Noggin [144], is essential for the specification of the neuroectoderm from the dorsal region of the ectoderm. Furthermore, the contribution to this process is granted by the activity of fibroblast growth factor (FGF), epidermal growth factors (EGF), and Wnt [145].

The neural plate extends and rises at the edges to create the neural folds that eventually come together at the dorsal midline, resulting in the neural tube, which is the primordium of the central nervous system. This process occurs in a progressive manner along the rostro-caudal axis, from the level of the future mesencephalon both anteriorly and posteriorly. Upon closure, the neural tube is organized along two major axes: rostro-caudal and dorso-ventral. In the first, retinoic acid (RA), synthesized by the enzyme aldehyde dehydrogenase 1 family member A2 (RALDH2) in the para-axial mesoderm, is essential for the division of the neural tube into anterior regions (rhombencephalon and spinal cord)[144]. This gradient of RA opposes FGF and Growth Differentiation Factor 11(Gdf11) signals, which favor more caudal identities [145]. Positional regulation along this axis is further refined by *Hox* genes, which control spinal cord regionalization and, together with co-factors such as FoxP1, contribute to the specification of neuronal subtypes. Patterning along the dorso-ventral axis is guided by opposing signals: Sonic Hedgehog (Shh), secreted by the notochord and the floor plate, directs ventral specification, while BMP and TGF β , produced by the roof plate, determine dorsal identities [146] (Figure 4). The ventral compartment is divided into five progenitor domains (p0–p3 and pMN), each responsible for the generation of specific types of interneurons (V0–V3) and MNs. The specification of each domain is the result of a delicate balance between Shh-induced (class II: Olig2, Nkx2.2, Nkx6.1) and Shh-repressed (class I: Pax6, Irx3, Dbx1, Dbx2) transcription factors [146–148]. These proteins interact with each other by mechanisms of reciprocal repression, which allow the precise definition of the boundaries between adjacent domains. For the generation of MNs, oligodendrocytes transcription factors 2 (Olig2) expression in MN progenitors induces Neurogenin

2 (Ngn2), which is not only responsible for cell cycle exit but also for the activation of terminal genes such as Homeobox gen 9 (Hb9), motor neuron homeobox 1 (Mnx1), ISL LIM homeobox 1 (ISL1), ISL LIM homeobox 1 (ISL2), and LIM homeobox 3 (Lhx3) [149]. Therefore, to differentiate MNs from iPSCs, it is crucial to replicate the molecular signaling pathways that guide MN specification during embryogenesis.

7.1 From iPSCs to MNs

iPSCs are a tool for the *in vitro* generation of human MNs, replicating key stages of embryonic development by using specific molecular signals. Considerable efforts have been made to obtain highly pure and high-quality MN cultures, since one of the main limitations of this approach is related to the functional immaturity of iPSC-derived cells. Such immaturity represents a significant complication for the study of neurodegenerative diseases that predominantly affect adult and fully mature neuronal cells. Although the protocols present in the literature may differ in duration, most of them rely on the use of small molecules in combination with chemically defined culture media, to drive neuronal differentiation in an efficient and reproducible way. Some protocols involve the use of iPSCs colonies, others use iPSC single cells, while alternative approaches are based on the formation of embryoid bodies (EB) [150–152]. Despite this methodological heterogeneity, neural specification *in vitro* is commonly induced by dual inhibition of the TGF- β and BMP signaling pathways, achieved through the combined use of the small molecules SB431542 and LDN193189 [153]. This treatment reproduces the morphogenetic signals that, *in vivo*, induce the formation of the neural plate from the ectoderm. Subsequent regionalization along the dorsal-ventral axis of the neural tube is driven by the activation of Sonic Hedgehog (SHH)-mediated signaling. This activation can be induced by the addition of recombinant protein SHH or by the combined use of RA and Purmorphamine (PMA), a Smoothed agonist capable of stimulating the SHH receptor and promoting ventral specification of neuronal progenies [154]. In parallel, caudalization of iPSC-derived neuroepithelial cells is induced by the activation of the canonical WNT signaling pathway via the glycogen synthase kinase 3 (GSK-3) inhibitor CHIR-99021, which effect is potentiated by the concomitant administration of RA, mimicking endogenous posteriorizing signals [155,156]. Finally, modulation of NOTCH signaling plays a crucial role in balancing neural progenitor proliferation and terminal differentiation. Inhibition of this pathway, for example by using the γ -secretase inhibitor Compound E, promotes MN maturation [150]. The terminal phase of differentiation is further supported by the addition of neurotrophic factors such as Insulin-like growth factor 1 (IGF-1), GDNF and Brain-derived Neurotrophic Factor (BDNF), which promote survival, neurite outgrowth and functional maturation of MNs in culture [156] (Figure 4).

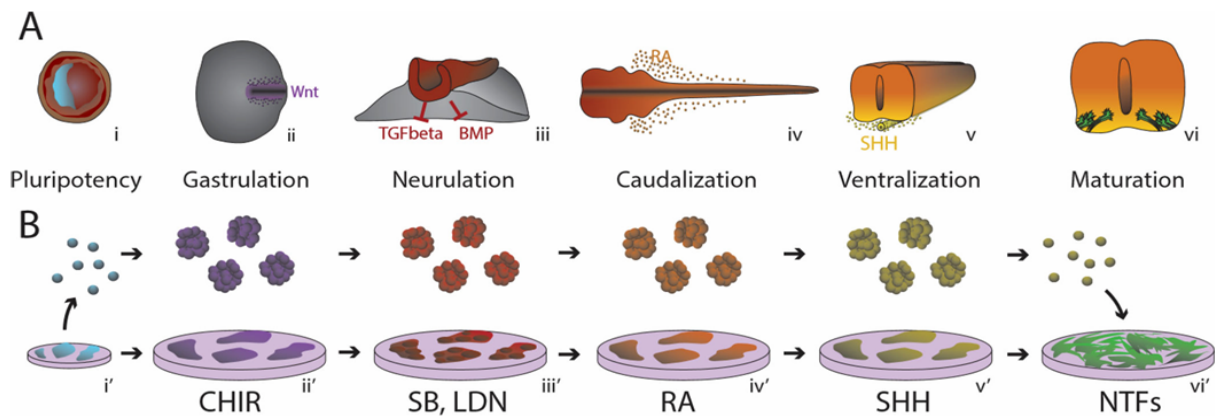


Figure 4. Embryonic and *in vitro* development of MNs.

In vitro neuronal differentiation follows the stages of embryonic development. **A)** Schematic representation of the developmental stages: (i) Pluripotency, (ii) Gastrulation (Wnt activation), (iii) Neurulation (TGFβ and BMP inhibition), (iv) Caudalization, (v) Ventralization (SHH-induced), (vi) Neuronal maturation. **B)** Protocol that mimics the developmental stages using small molecules: (i') pluripotent stem cells, (ii') treatment with CHIR (Wnt activator), (iii') treatment with SB and LDN (TGFβ/BMP inhibitors), (iv') treatment with RA, (v') treatment with SHH, (vi') treatment with neurotrophic factors (NTFs) to promote neuronal maturation. doi:10.1038/nn.4273.

In recent years, protocols for differentiation of iPSCs into MNs have undergone significant optimizations, allowing a substantial increase in yield. The first approaches, based on long treatments with RA and SHH, achieved very low yields, around 20%. By improving the timing and duration of exposure to these signals and integrating strategies such as dual-SMAD inhibition and activation of WNT signaling, protocols were able to produce up to 95% of MNs in less than a month. Such progress was made possible by a refined understanding of the temporal and dose-dependent role of small molecules mimicking endogenous morphogenetic signals, during the phases of neural induction, caudalization and ventralization. It is now clear that not only the choice of molecules, but especially the timing of their administration and the duration of exposure represent critical variables for the success of neuronal specification [133]. In parallel, the use of key transcription factors of MNs development, such as Ngn2, ISL1 and LHX3, has emerged as an effective strategy to directly target cell fate, further improving efficiency and reducing the heterogeneity of derived populations [157]. This approach offers a complementary and, in some cases, faster route to obtain functional MNs *in vitro*. In conclusion, iPSC-based models enable the study of early degenerative events, provide insight into the cellular and molecular mechanisms underlying the selective vulnerability of MNs, and offer a platform for testing patient-specific therapeutic approaches envisioning future clinical applications.

Aim of the thesis

ALS is a progressive and fatal neurodegenerative disorder (NDD) that primarily affects MNs, leading to the gradual loss of voluntary muscle control, muscle weakness and eventual paralysis. Respiratory muscles dysfunction is the main symptom associated with a lower survival rate. The urgent need for more effective treatments and a deeper understanding of the molecular mechanisms underlying ALS has driven extensive research utilizing cellular models including motoneurons (MNs) derived from induced pluripotent stem cells (iPSCs). These models offer valuable insights into disease pathology and potential treatment avenues.

This doctoral thesis is structured around two main aims:

1. The first aim involves the generation and characterization of iPSCs derived from healthy donors and ALS patients carrying familial mutations in *KIF5A*, *C9orf72*, and *TARDBP*. iPSCs were differentiated into neurospheres, three-dimensional aggregates of neural progenitor cells that recapitulate early neurodevelopmental stages. To investigate ALS-related molecular dysregulation across early neurogenic stages, transcriptomic analyses was performed on both iPSCs and iPSC-derived neurospheres. This approach aims to identify differentially expressed genes and dysregulated pathways, and to determine whether early transcriptional changes are already present in ALS patient-derived cells prior to and during neural commitment. The results related to this aim have been included in a scientific manuscript actually accepted.
2. The second objective of this thesis involves the differentiation of both control and ALS patient-derived iPSCs, harboring familial mutations, into neurospheres and, subsequently, into mature MNs. This process aims to not only to establish and refine a robust, reproducible differentiation protocol toward the MN lineage, but also to allow the identification of disease-relevant phenotypic differences emerging throughout neuronal development.

First Aim

Manuscript accepted

Article

Identification of Transcriptomic Differences in Induced Pluripotent Stem Cells and Neural Progenitors from Amyotrophic Lateral Sclerosis Patients Carrying Different Mutations: a Pilot Study

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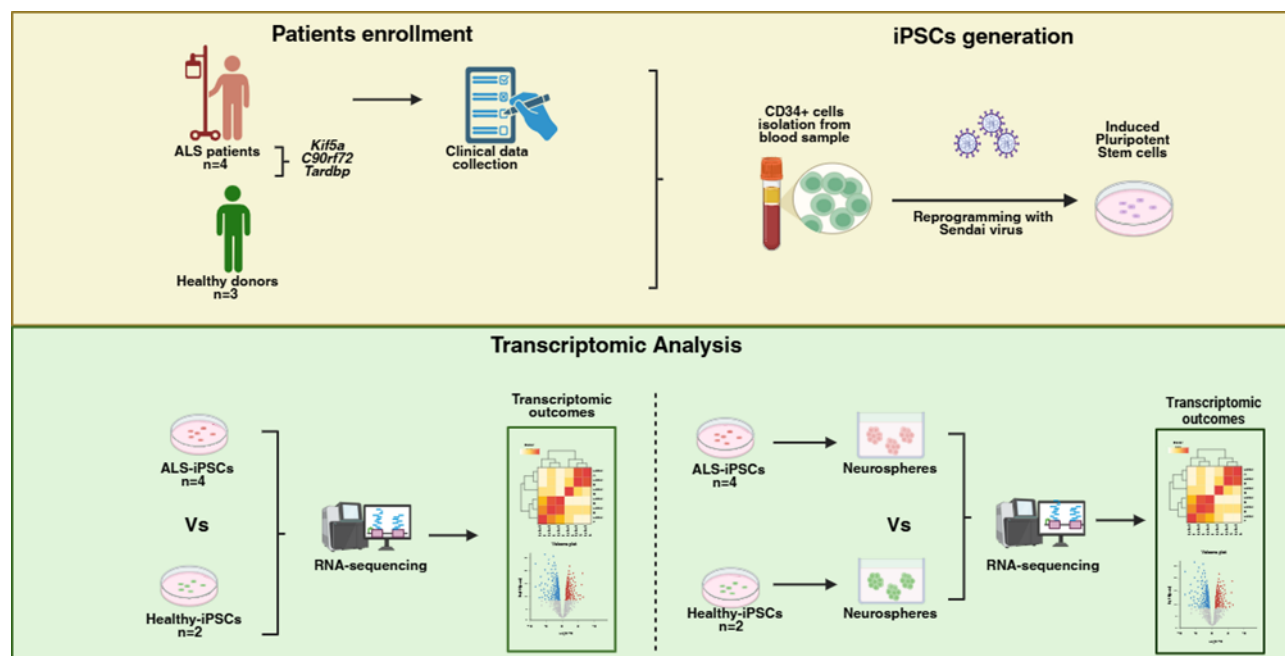
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Graphical Abstract



Abstract: Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease affecting motor neurons with a phenotypic and genetic heterogeneity and elusive molecular mechanisms. With the present pilot study, we investigated different genetic mutations (*C9orf72*,

TARDBP and *KIF5A*) associated with ALS by generating induced pluripotent stem cells (iPSCs) from peripheral blood of ALS patients and healthy donors. iPSCs showed the typical morphology, expressed stem cell markers both at RNA (*OCT4*, *SOX2*, *KLF4* and *c-Myc*) and protein level (Oct4, Sox2, SSEA3 and Tra1-60). Moreover, embryoid bodies expressing the three germ layers markers and neurospheres expressing neural progenitor markers were generated. Importantly, the transcriptomic profiles of iPSCs and neurospheres were analyzed to highlight the differences between ALS patients and healthy controls. Interestingly, the differentially expressed genes (DEG) shared across all ALS iPSCs are linked to extracellular matrix, highlighting its importance in ALS progression. In contrast, ALS neurospheres displayed widespread deficits in neuronal pathways, although these DEGs were varied among patients, reflecting the disease's heterogeneity. Overall, we generated iPSC lines from ALS patients with diverse genetic backgrounds offering a tool for unraveling the intricate molecular landscape of ALS, paving the way for identifying key pathways implicated in pathogenesis and the disease's phenotypic variability.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that affects upper and lower motor neurons (MNs), leading to the loss of voluntary muscle control, muscle weakness, and eventual paralysis. ALS is predominantly a multifactorial disorder characterized by great phenotypic heterogeneity, cognitive dysfunction and behavioral changes. Indeed, there are two main forms of ALS, according to the disease's family history: sporadic ALS (sALS), which represents 85-90% of cases, occurring without a known family history; and familial ALS (fALS), that accounts for 10-15% of cases, where a positive family history of ALS is reported [1]. Multiple factors contribute to the development of ALS, with a significant genetic component being identified. Pathogenic sequence variations in several genes have been strongly associated with the disease. In particular, mutations in *SOD1*, *C9orf72*, *FUS*, and *TARDBP* genes account for about 48% of fALS and about 5% of sALS cases within populations of European origin[2]. The GGGGCC hexanucleotide repeat expansion in the first intron of *C9orf72* is the most common genetic cause of ALS accounting for about 40% of fALS. This mutation causes an abnormal production of RNAs that produce toxic dipeptide repeat proteins (DPRs) accumulation, impairing nucleocytoplasmic transport, nucleotide metabolism, lysosomal processes, and cellular metabolic pathways [3]. *C9orf72* (chromosome 9 open reading frame 72) regulates autophagy and vesicular trafficking in neurons, and it is involved in actin dynamics and endosomal recycling at synapses [4]. The *TARDBP* gene encodes the transactive response (TAR) DNA-binding protein 43 (TDP-

43), which plays a crucial role in RNA metabolism. To date, approximately 40 different *TARDBP* missense variants have been identified. Mutated form of TDP-43 binds to ubiquitin and forms aggregates, leading to the formation of inclusions found in the MNs and brains of ALS patients [5]. More recently, thanks to next-generation sequencing approaches, over 40 new ALS genes have been identified accounting for a rarer proportion of ALS cases. Among these, a recent study identified *KIF5A* as a novel ALS gene through rare variant burden analysis [6]. *KIF5A* encodes a member of the kinesin family of proteins that function as a microtubule motor, necessary for organelle trafficking, cell division and axonal maintenance. Genetic analyses have identified multiple loss-of-function mutations in ALS: these include frameshift and splice site mutations that lead to the skipping of *KIF5A* exon 27 and the production of a truncated protein with impaired cargo-binding function and axonal transport disruption [6,7]. Although the different ALS-related mutations affect distinct molecular pathways, all of them lead to the common outcome: MNs degeneration. Currently, there is no effective treatment to halt or reverse ALS progression, and patients are treated to slow down disease progression and alleviate symptoms. Therefore, the urgent need for more effective treatments and a deeper understanding of the molecular mechanisms underlying ALS has driven the use of cellular models such as the induced Pluripotent Stem Cells (iPSCs) [8–10]. In this pilot study, we generated iPSCs by reprogramming CD34+ cells isolated from peripheral blood of four ALS patients carrying different mutations. iPSCs were further differentiated into neurospheres, a critical intermediate stage in MNs differentiation, characterized by self-renewal, multipotent differentiation, and able to closely replicate early neurodevelopmental processes. Transcriptomic analysis highlighted common transcriptomic signatures and disease-related enriched pathways in ALS iPSCs. On the other hand, transcriptomic analysis of neurospheres revealed a modulation of neural-specific pathways, thereby highlighting a dysregulation of the neural function already at the stage of progenitor cells. In conclusion, these findings could provide the molecular basis for ALS modeling and potentially reveal key transcriptomic insights into distinct mutations.

2. Materials and Methods

2.1. Patients

Three healthy donors and four patients diagnosed with ALS and carrying three different heterozygous mutations (*KIF5A*, *C9orf72* and *TARDBP*) were recruited at the Expert ALS Center of the Maggiore della Carità University Hospital, Novara, Italy. According to El Escorial Criteria–Revised [11,12] we included only patients classified as “definite ALS”. Demographic and clinical information of ALS patients are reported in Table 1. All enrolled subjects provided written

informed consent to participate in the study and blood samples were collected upon genetic test result notice (within six months of clinical diagnosis). The study has been approved by the ethical committee of the Maggiore della Carità Hospital (protocol 1102/CE, study number n. CE 233/20). In addition to genetic analysis, for demographic and clinical variables, of each included patient, we considered gender, age at onset, positive family history for ALS, cognitive status, disease phenotype (prevalent upper or lower MNs; spinal/bulbar), disease duration from symptoms onset to death, cognitive status [13].

2.2. CD34+ Isolation

Mononuclear cells were purified by gradient centrifugation on Ficoll (GE Healthcare) and CD34+ cells have been isolated by using MACS MicroBeadKit (Miltenyi Biotec) according to the manufacturer's protocol. Isolated cells were cultured for 3 days in StemPro34 medium (Gibco) supplemented by 100ng/ml of hSCF, 50 ng/ml of IL-3 and 25 ng/ml of GM-CSF (Immunotools)[14].

Table 1. Demographic, genetic, and clinical features of included fALS patients. All patients were of Caucasian ethnicity. Cognitive status is classified based on the Strong criteria. UMN: upper motor neuron; ALS-ci: ALS-cognitive impairment. *Disease course complicated by adrenal cancer and prolonged bed rest.

Subject	Mutation	Age at disease onset	Age at diagnosis	Gender	Family history	Phenotype	Cognition	Age at death	Disease duration (months from onset to death)
ALS_001	<i>KIF5A</i> (exon 27 3' splice junction variant, c.3020+1G>A)	61	62	Male	Mother, maternal grandfather and great-grandfather, three first degree cousins	Prevalent UMN (spinal)	ALS-ci	66	60*
ALS_002	<i>TARDBP</i> (c.1144G>A, p.A382T)	63	64	Male	brother	Prevalent UMN (bulbar)	ALS-ci	67	50
ALS_003	<i>C9orf72</i> (intron 1 GGGCC repeat pathogenetic expansion)	57	58	Male	father	Classic (spinal)	ALS-ci	60	30
ALS_004	<i>TARDBP</i> (c.1144 G>A, p.A382T)	61	65	Male	father	Prevalent UMN (spinal)	normal	-	84 (alive)
H_001	-	43	-	Male	-	-	-	-	-
H_002	-	58	-	Female	-	-	-	-	-

H_003	-	60	-	Male	-	-	-	-	-
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2.3. iPSCs Reprogramming

Four days after isolation, 9×10^4 cells were transduced with Sendai reprogramming vectors kit (ThermoFisher) at different multiplicity of infection following Manufacturer's instructions. After three days, transduced cells were plated on a layer of recombinant Vitronectin (ThermoFisher) with StemPRO34 medium without cytokines. Gradually medium was replaced with Essential8 medium (Gibco) to maintain iPSCs in culture.

2.4. iPSCs Culture

15/20 days after reprogramming iPSCs appeared and a clonal selection was performed. The single clones were manually picked up and transferred to a well of a 24 vitronectin-coated plate. For iPSCs culture and clones' stabilization several splitting ratios were used. Cells were kept in culture with Essential8 medium (Gibco), and medium was changed every day.

2.5. Alkaline Phosphatase Staining

Alkaline Phosphatase (AP) was performed as previously described [14]. Briefly, iPSCs were fixed with 4% of PAF and stained using the AP detection kit (Millipore) according to the manufacturer's protocol.

2.6. RNA Isolation and RT-PCR

RNA extraction was performed using Trizol reagent (Invitrogen). 1µg of total RNA was reverse transcribed with RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) and the cDNA was used to perform PCRs. All the PCRs were performed with Platinum™ Taq DNA Polymerase (Invitrogen). Primers, annealing temperatures and product sizes are listed below. PCR products were resolved in 2% agarose gels. Primers used are listed in Supplementary Table 2.

2.7. Immunofluorescence Staining

iPSCs were cultured on cover glass in 24 well plate. Cells were fixed in cold PFA 4% for 10 min and permeabilized with 0,5% PBS-TritonX100 and incubated with blocking buffer (5% goat serum, 1% BSA, 0.1% Triton X-100 in PBS) for 1h at room temperature (RT). Primary antibodies were used with the correct dilution in 5% goat serum, 1% BSA, 0.1% Triton X-100 in PBS and incubated for 2 hours at RT. Secondary antibodies were prepared in 0,5% PBS-TritonX100 and incubated for 45 minutes at RT. Nuclei were stained with DAPI (Sigma). Primary and secondary antibodies with the dilutions are detailed in Supplementary Table 3.

2.8. Embryoid Bodies Generation

When all iPSCs lines showed stable morphology without any type of differentiated cells and 85% of confluence was reached, they were detached and plated in a low adhesion well with Essential8 for 5 days.

2.9. Neurospheres Generation

Healthy and ALS iPSCs were detached when reached 80% of confluence, dissociated and 15×10^4 cells were plated with Essential 8 and 10 μ M of Rock Inhibitor (Y-27632) (Sigma-Aldrich). The induction of Neuropheres differentiation has been achieved using medium composed by: DMEM/F12 (Gibco), Neurobasal medium (Gibco) 0,5X N2 (Gibco), 0,5x B27 (Gibco), Glutamax (Gibco), Non-Essential Amino acids (Gibco) supplemented by small molecules as SB431542 (Stem Cell Technologies), LDN193189 (Stem Cell Technologies), CHIR99021 (Stem Cell Technologies), Ascorbic acid (Sigma), all-trans RA (Stem Cell Technologies) and Purmorphamine (Stem Cell Technologies). For Neuropheres generation we followed the main steps described in literature [15,16].

2.10. Western Blot

iPSCs were lysed in RIPA buffer supplemented with 100X protease inhibitors (Thermo Fisher Scientific). Protein concentration was determined using the BCA Protein Assay Kit (Thermo Fisher Scientific). Equal amounts of protein samples (20 μ g) were resolved by SDS-PAGE using different gel concentrations depending on the target protein. Membranes were incubated overnight at 4°C with the following primary antibodies: Anti-TDP-43 (1:1000), Anti-C9orf72 (1:1000), Anti-KIF5A (1:1000), Anti-vinculin (1:3000, SantaCruz). Proteins were visualized using enhanced chemiluminescence (ECL) (Bio-rad) and detected using a ChemiDoc imaging system (Bio-Rad).

2.11. Mutational Analysis of iPSCs

To confirm the occurrence of each of the mutations identified in the ALS patients from which iPSCs have been derived, we performed Sanger sequencing on ABI PRISM 3130XL for the mutations in *KIF5A* and *TARDBP* genes, and PCR-tandem repeat and analysis on ABI PRISM 3130XL for the *C9orf72* pathogenetic GGGGCC tandem repeat expansion. For each patient, we confirmed the heterozygous mutation originally identified in PBMC from peripheral blood in the corresponding iPSCs utilized in this study (Supplementary Figure 1).

2.12. RNA-Sequencing

The transcriptome of iPSCs and neurospheres derived from four male patients with pathogenetic sequence variations in three different ALS genes, namely *KIF5A* (exon 27 3' splice junction variant, c.3020+1G>A, NM_004984: chr12:57582630 G>A, hg38, ALS_001), *TARDBP* (p.Ala382Thr, c.1144G>A, NM_007375.4, chr1-11022553 G>A, hg38 ALS_002 and ALS_004) and *C9orf72* (intron 1 GGGGCC repeat pathogenetic expansion, ALS_003). Two gender-matched healthy controls (H001 and H003) were included in the study. For each subject, a technical duplicate was obtained from iPSCs at passages p17-p25 and from neurospheres resulting in a total of 24 samples.

RNA extraction was performed using the miRNeasy Micro Kit (QIAGEN) according to the manufacturer's instructions. For each sample, 500 ng of RNA were used for library preparation with the Illumina Stranded Total RNA Prep with Ribo-Zero Plus kit. The first 10 samples (iPSCs of the four patients and the first healthy control) were sequenced on Illumina NextSeq 550 sequencer (Illumina, San Diego, California, USA), while the remaining 14 samples (iPSCs of the second control and all the neurospheres) were sequenced on Novaseq sequencer (Illumina, San Diego, California, USA). Initial quality checks on reads were assured using FastQC [17]: the average phred score quality for each base was ≥ 30 , therefore reads have not been trimmed. RSEM computational pipeline [18] was used to quantify gene expression levels using STAR aligner [19]. GRCh38/hg38, annotated with the Ensembl v113 database, was used as reference genome. Only genes with a TPM > 1 in at least one sample were considered to be expressed and underwent further analysis, after batch effect correction. Statistical and graphical computations were performed in the R environment (www.r-project.org). PCA (Principal Component Analysis) plotting was performed using the "prcomp" package of R on the matrix of expression data. Differentially expressed genes (DEGs) were calculated using DESeq2 [20] with $|\log_2FC| > 2$ and $p_{adj} \leq 0.05$ as parameters to define the statistical significance of differential gene expression. Enrichment analysis has been conducted with Metascape [21] (min overlap=3; pval cut-off=0.01; min enrichment= 1.5).

2.13. Data Availability

Anonymized data and RNA-Seq supporting the findings of the study will be made available by the corresponding author, upon reasonable request, for any qualified investigator.

3. Results

3.1. Generation of iPSCs from CD34⁺ Cells Isolated from ALS Patients and Healthy Donors

After informed consent, blood samples from four fALS patients (consistent with autosomal dominance inheritance; defined ALS based on El Escorial revised criteria)[11,12] and three healthy donors were collected to generate ALS and healthy iPSCs (Table 1). In detail, we isolated CD34⁺ cells from non-mobilized peripheral blood of four fALS patients carrying different mutations and from three healthy donors and we reprogrammed them with Sendai virus. Main demographic and clinical features are summarized in Table 1. We obtained a comparable number of iPSC colonies among healthy and ALS reprogrammed CD34⁺ cells (Figure 1A). An exception was observed in ALS patients harboring the *KIF5A* mutation, where a noticeable reduction in the number of isolated CD34⁺ cells and colonies was detected (Figure 1A). The efficiency of the reprogramming process was consistent among the different samples, leading to the generation of iPSC lines from both healthy donors and ALS patients (Figure 1B). Indeed, iPSCs showed the typical ESC-like morphology, with compact colonies with well-defined borders and were positive for alkaline phosphatase (AP) (Figure 1B). Furthermore, we sequenced ALS iPSC lines to assess genetic stability. Notably, even the mutation involving the hexanucleotide repeat expansion (G4C2) in *C9orf72* was preserved in iPSC, confirming that the reprogramming process did not alter the genetic features (Supplementary Figure 1).

A

Source	Donor	Mutation	ml of total Pheriperal blood	Number of CD34+ cells	Number of colonies
Pheriperal blood	Healthy	None	52 ml	200.000 CD34+	60
Pheriperal blood	Healthy	None	48 ml	350.000 CD34+	70
Pheriperal blood	Healthy	None	46 ml	310.000 CD34+	50
Pheriperal blood	ALS patient	<i>KIF5A</i> c.3020+1G>A	22 ml	160.000 CD34+	21
Pheriperal blood	ALS patient	<i>TARDBP</i> c.1144G > A	22 ml	200.000 CD34+	80
Pheriperal blood	ALS patient	<i>C9orf72</i> GGGGCC expansion	22 ml	150.000 CD34+	70
Pheriperal blood	ALS patient	<i>TARDBP</i> c.1144G > A	27 ml	210.000 CD34+	71

B

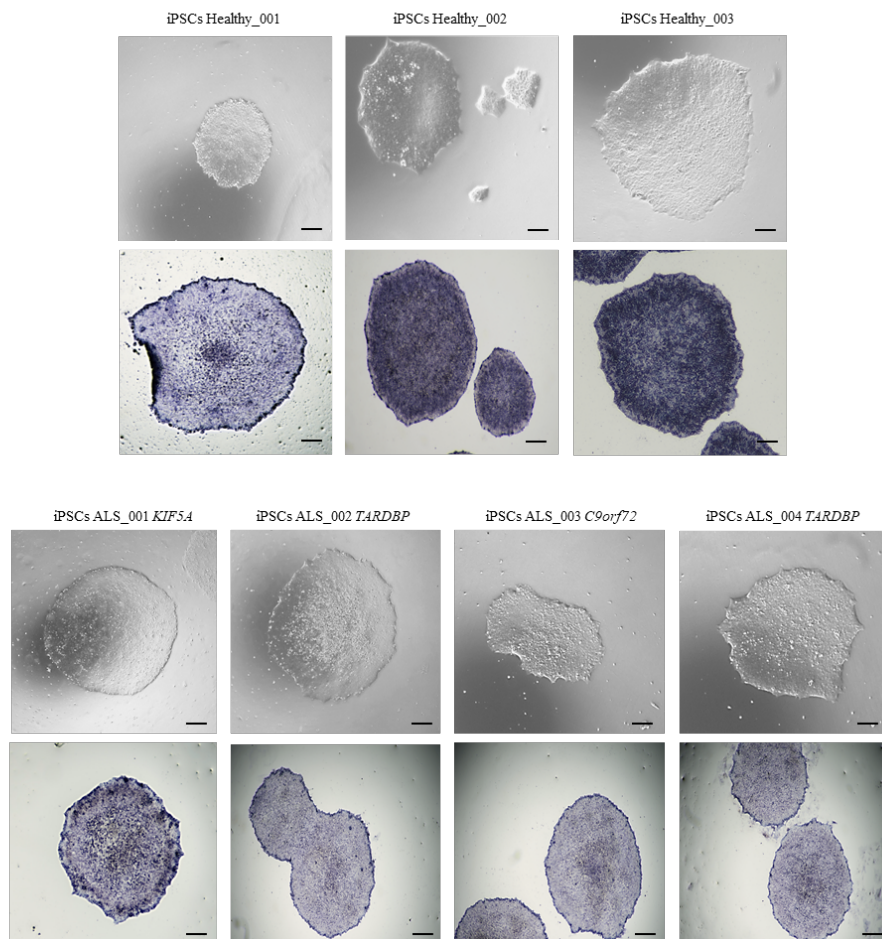
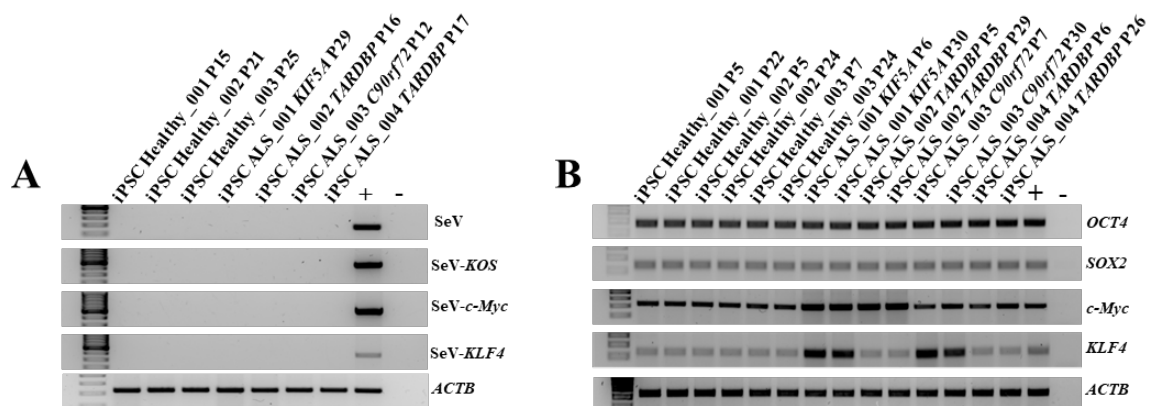


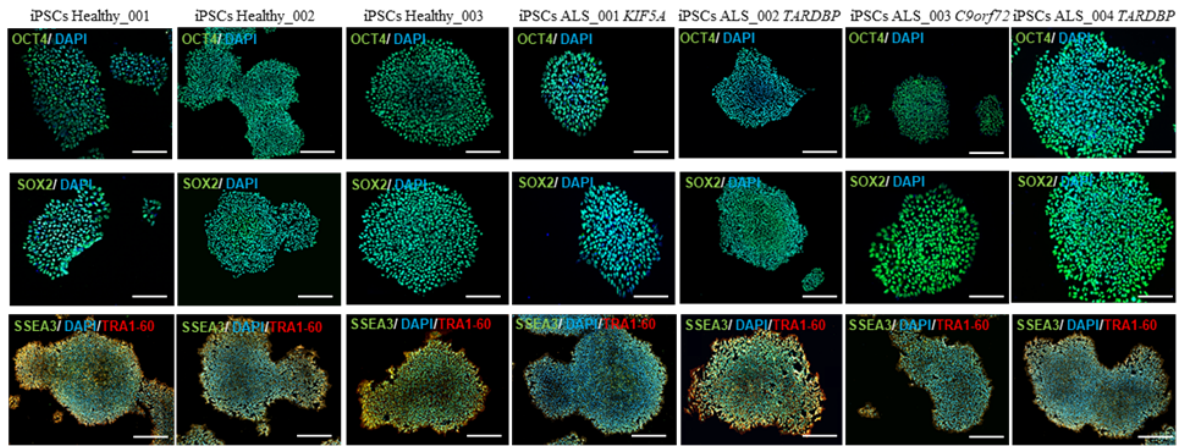
Figure 1. CD34+ cells reprogramming into iPSCs. A) List of ALS and healthy donors from whom iPSCs were generated. **B)** Upper panel: Representative phase-contrast microscopy showing ESC-like morphology of both healthy and ALS CD34+ cell-derived iPSCs. Lower panel: Positivity for alkaline phosphatase staining of healthy and ALS iPSCs. Scale bars, 100 μ m.

3.2. Characterization of iPSCs from CD34⁺ Cells Isolated from ALS Patients and Healthy Donors

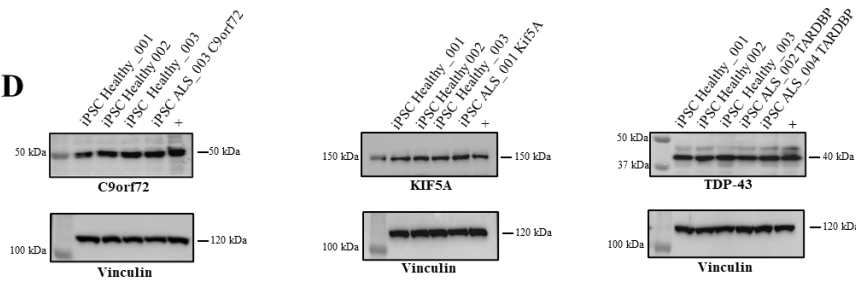
All iPSCs clones were characterized by the expression of exogenous and endogenous pluripotency markers. Considering the nature of the Sendai Virus the residual amount of virus in obtained healthy and ALS iPSC were evaluated. Specifically, we selected iPSC that had reached the 10th passage and by RT-PCR we demonstrated that there was no residual of the Sendai virus in transduced cells, confirming the silencing of exogenous markers compared to positive controls represented by iPSCs 15 days post-reprogramming (Figure 2A). On the contrary, endogenous markers were highly expressed at RNA level by iPSCs at the same passages (Figure 2B) demonstrating the stable reprogramming of iPSC and their sustained staminality. These data were demonstrated also at protein level showing a high expression of Oct4, Sox2, Ssea3 and Tra1-60 both in healthy and ALS iPSCs (Figure 2C). To further confirm the expression of ALS-associated mutated genes in the iPSC lines, we assessed protein levels for TARDBP, KIF5A, and C9orf72. As shown in Figure 2D, C9orf72, KIF5A, TDP-43, and were detected at protein levels in iPSCs. These results demonstrate that the ALS-related genes are expressed at both transcriptional and protein levels in the healthy and ALS iPSC lines. Finally, we exploited the ability of iPSCs to mimic embryogenesis at the level of the gastrula by generating embryoid bodies (EBs) from both healthy and ALS iPSCs (Figure 2E). We investigated the expression of key markers for the three germ layers by RT-PCR: *NES*, *NCAM*, and *OTX2* for the ectoderm; *ACTA2*, *TBXT*, and *TBX6* for the mesoderm; and *FOXA2*, *SOX17*, and *AFP* for the endoderm (Figure 2F). Our results demonstrated that all iPSC lines successfully generated EBs that expressed high levels of the typical markers of ectoderm, mesoderm, and endoderm confirming the differentiation potential of the obtained iPSCs.



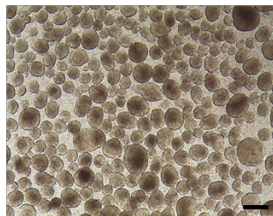
C



D



E



F

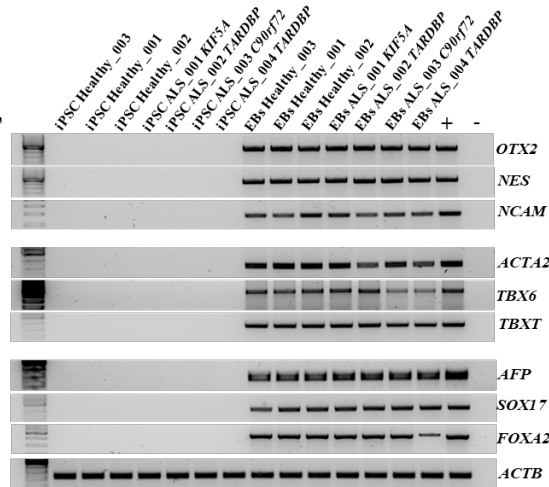
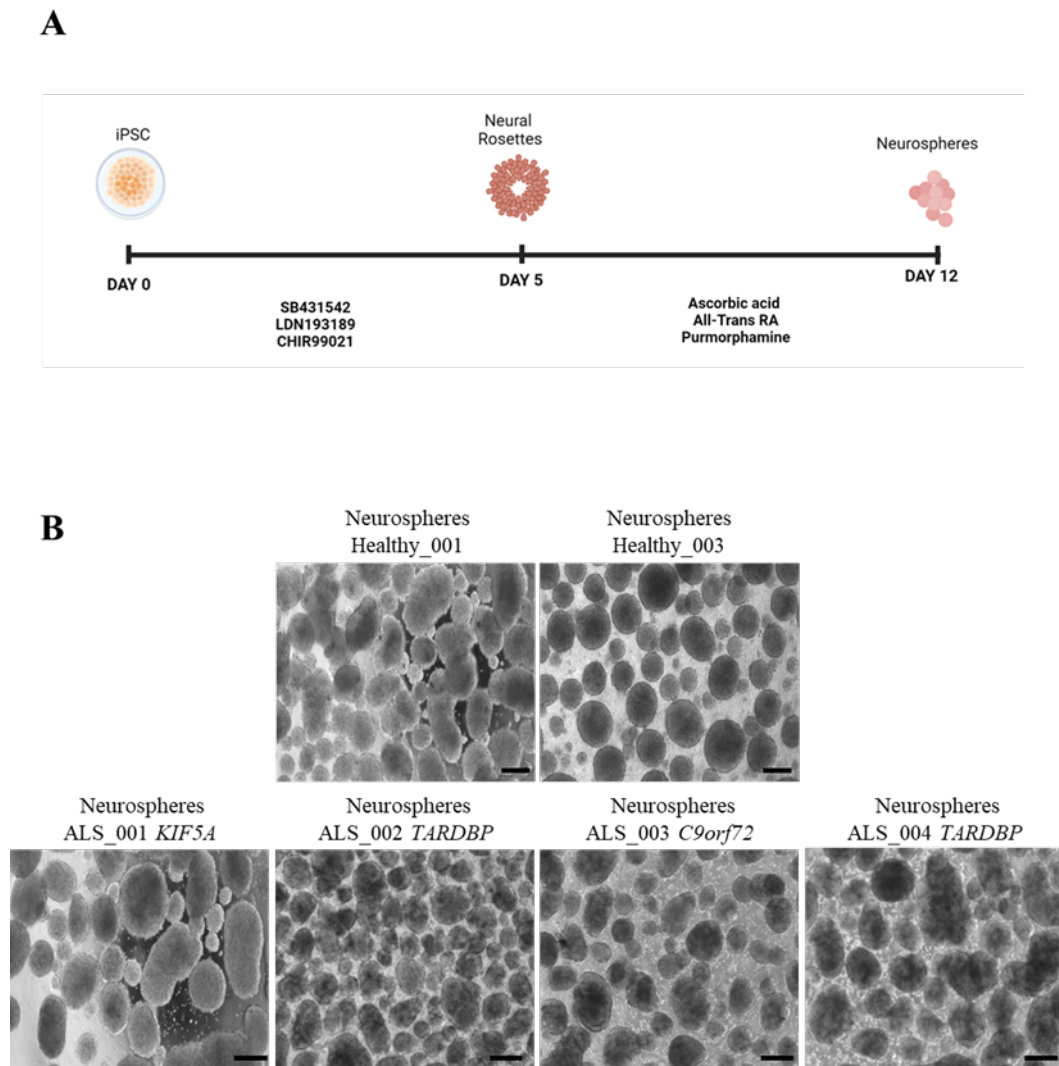


Figure 2. Characterization of healthy and ALS iPSCs. **A)** RT-PCR for exogenous factors on healthy and ALS iPSCs. iPSCs from a healthy donor 15 days after reprogramming with Sendai virus was used as positive control. **B)** RT-PCR for endogenous factors (*OCT4*, *SOX2*, *c-Myc*, *KLF4*) on healthy and ALS iPSCs. HEK293T cells transduced with the Sendai virus were used as positive control. **C)** Stem cell markers expression at the protein level was detected by immunofluorescence on healthy and ALS iPSCs. Markers used were: OCT4 (green), SOX2 (green), SSEA3 (green), TRA1-60 (red), and DAPI (blue). Scale bars, 200 μ m. **D)** Western blot analysis of C9orf72, KIF5A and TDP-43 expression. Positive controls: C9orf72: SH-SY5Y cell line; KIF5A: SH-SY5Y cell line; TDP-43: MDA-MB-231 cell line. **E)** Representative image of EB generated from the culture of iPSCs in low adhesion plate. Scale bar, 100 μ m. **F)** RT-PCR for the three germ layers markers on iPSCs-derived EBs: *OTX2*, *NCAM*, *NES* (ectoderm); *ACTA2*, *TBX6* and *TBXT* (mesoderm); *AFP*, *SOX17* and *FOXA2* (endoderm). All data are representative of three independent experiments.

3.3. Neurosphere Generation and Neuroectoderm Markers Expression

Envisioning future applications of obtained iPSCs in disease modeling we investigated their ability to differentiate into MNs and their commitment to the neuroectodermal lineage. Therefore, we induced neurosphere differentiation on two healthy iPSC and in all ALS iPSC (Figure 3A-B). The results showed that both healthy and ALS iPSCs were successfully differentiated into neurospheres, expressing high levels of *NES*, *PAX6*, and *SOX2* markers (Figure 3C) indicating the successful initiation of neural differentiation and the potential of these iPSC lines for further differentiation into MNs. Moreover, this differentiation step ensured that the cells had already undergone neuroectodermal commitment, making them a relevant model for subsequent transcriptomic analysis. The neurospheres obtained were therefore used for RNA sequencing to characterize their gene expression profile and gain deeper insights into their molecular identity.



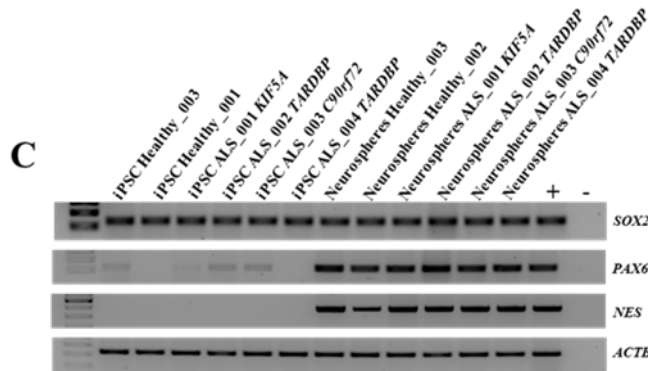


Figure 3. Neurosphere generation and characterization. Schematic representation of neurosphere differentiation. **B)** Representative images of neurospheres generated from healthy and ALS iPSCs. Scale bar, 100 μ m. **C)** RT-PCR for the expression of the neuroectoderm markers. Hela and iPSC were used as positive control. All data are representative of three independent experiments.

3.4. Transcriptomic Analysis of iPSCs and Neurospheres from ALS and Healthy Controls

To identify expression patterns and pathways implicated in ALS across different mutations, we performed whole-transcriptome sequencing (RNA-seq) analysis on technical duplicates from iPSCs and neurospheres, from the 4 patients carrying a pathogenetic mutation in one ALS gene, namely *KIF5A* (ALS_001), *TARDBP* (ALS_002 and ALS_004) and *C9orf72* (ALS_003), and two healthy gender-matched control (H00_1 and H00_3), for a total of 24 samples. The third healthy donor (H_002) was excluded from this analysis due to the gender mismatch with ALS cases. After PCA inspection (Supplementary Figure 2), no outlier was removed, with PC1 and PC2 explaining respectively 61,97% and 9,77% of variability. For both iPSCs and neurospheres, the transcriptomic profile of each patient has been compared to the healthy controls (Supplementary Figure 3, Supplementary Figure 4). By filtering for $p\text{-value adj} \leq 0.05$ and $|\log_2\text{FC}| > 2$, when comparing each ALS iPSCs with the healthy controls, we obtained 234 DEGs (41 upregulated and 194 downregulated genes) for ALS001_ *KIF5A* (Figure 4A); 1001 DEGs (443 upregulated, 558 downregulated genes) for ALS002_ *TARDBP* (Figure 4B); 171 DEGs (98 upregulated, 73 downregulated genes) for ALS003_ *C9orf72* (Figure 4C); and 444 DEGs (252 upregulated, 192 downregulated genes) for ALS004_ *TARDBP* (Figure 4D). For neurospheres, by applying the same cut-offs we obtained 85 DEGs (7 upregulated and 78 downregulated genes) for ALS001_ *KIF5A* (Figure 5A); 1334 DEGs (630 upregulated, 704 downregulated genes) for ALS002_ *TARDBP* (Figure 5B); 319 DEGs (113 upregulated, 206 downregulated genes) for ALS003_ *C9orf72* (Figure 5C), and 324 DEGs (171 upregulated, 153 downregulated genes) for ALS004_ *TARDBP* (Figure 5D). At iPSCs levels, only 10 differentially expressed genes were shared among all subjects (*HTR7*, *PCAT7*, *NAMPTP1*, *POM121B*, *MEG3*, *ABHD12B*, *MEG8*, *DLK1*, *CIDP1*,

CCDC144NL-ASI), while for neurospheres we identified 2 common DEGs (*PDZRN4*, *SLC35F4*) downregulated in all subjects. Subsequently, for each comparison an enrichment analysis by Metascape has been performed on the totality of DEGs. Most of the enriched pathways could be observed only in single comparisons and no common GO terms were found across all four patients for both iPSCs and neurospheres (Figure 6 A-D, Figure 7 A-D). However, an integrative and comparative analysis highlighted that some GO terms were shared at least by three subjects at iPSCs level (Figure 6E, Supplementary Figure 5, Supplementary table 1); for example, homophilic cell adhesion via plasma membrane adhesion molecules (GO:0007156), shared by ALS001_*KIF5A*, ALS003_*C9orf72* and ALS004_*TARDBP* (Figure 6 A-D); cell-cell adhesion (GO:0098609), shared by ALS001_*KIF5A*, ALS002_*TARDBP*, ALS003_*C9orf72* and ALS004_*TARDBP* (Figure 6E). Interestingly, we also found an enrichment in the GO terms related to extracellular matrix (ECM), including Naba Matrisome Associated (M5885), shared by ALS002_*TARDBP*, ALS003_*C9orf72* and ALS004_*TARDBP*, and extracellular matrix (GO:0031012) in ALS002_*TARDBP* and ALS003_*C9orf72*, suggesting a general role of ECM in ALS pathological process, independently of the mutation (Figure 6E). A deeper inspection of Matrisome GO term revealed that four genes (*FGF4*, *SCG2*, *GDF15*, *EDN1*) were shared among DEGs identified in at least three subjects, maintaining the same upregulated or downregulated trend in all the comparisons. Among the DEGs enriched in both the cell adhesion related pathways (GO:0007156 and GO:0098609), we also highlighted several genes belonging to the protocadherin family which are already known to be involved in disease process [22,23] and organized in a specific hub when studying the molecular interaction of ALS001_*KIF5A* DEGs by network analysis using String (Supplementary Figure 6). Regarding the transcriptomic profile of neurospheres, we could not identify pathways shared by at least three subjects. Indeed, most of the enriched pathways were specific of each subject carrying a different mutation and reflected the individual disease course (Figure 7A-D). Most similarities have been highlighted in the two TARDBP patients (ALS002_*TARDBP* and ALS004_*TARDBP*) and were associated with GO terms linked to extracellular matrix (Figure 7E). Generally, in all subjects the most enriched pathways at neurosphere level reflected neuronal processes, such as axon development (GO:0061564), regulation of dendrite development (GO:0050773), synaptic membrane (GO:0097060), neuron projection development (GO:0031175) (Figure 7A-D). To better understand the pathways specifically associated with the different mutations, we conducted enrichment analyses using Metascape, focusing separately on upregulated and downregulated genes for each of the four ALS patients (Supplementary Figure 8,9). In general, the enrichment analysis of upregulated genes revealed processes related to the known or potential functions of the mutated genes, both at the

iPSC and neurosphere levels. Conversely, in neurospheres, we observed that the majority of neuronal-related pathways were consistently downregulated across all subjects (Supplementary Figure 9).

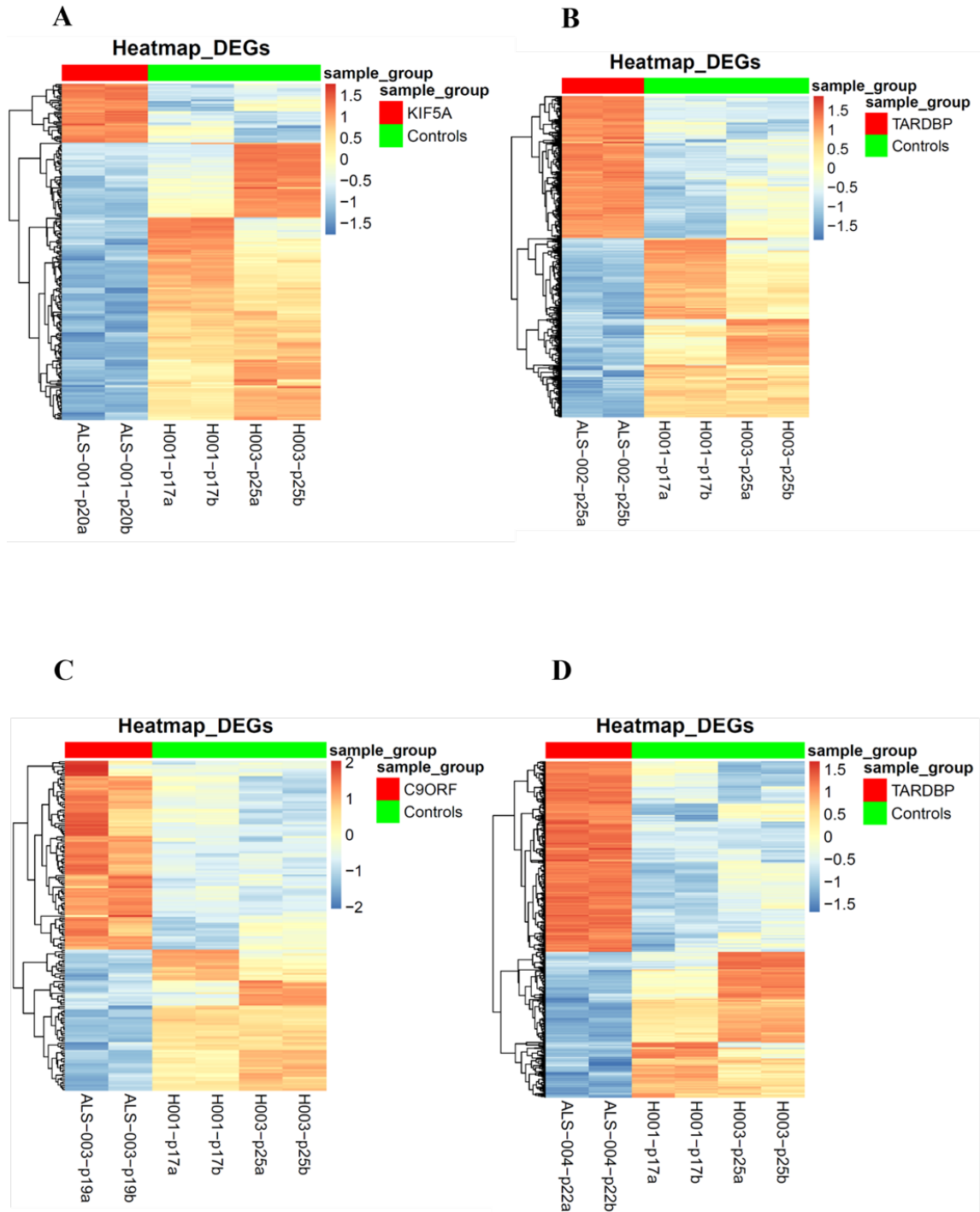


Figure 4. Heatmaps of the differentially expressed genes in iPSCs: Heatmaps of the differentially expressed genes of each of the four ALS patients in comparison to the matched healthy controls. Patients' differentially expressed genes are represented on the left (top red bar); healthy controls differentially expressed genes are represented on the right (top green bar). A: ALS001_KIF5A vs HC ; B: ALS002_TARDBP vs HC C: ALS003_c9orf72 vs HC; D: ALS004_TARDBP vs HC.

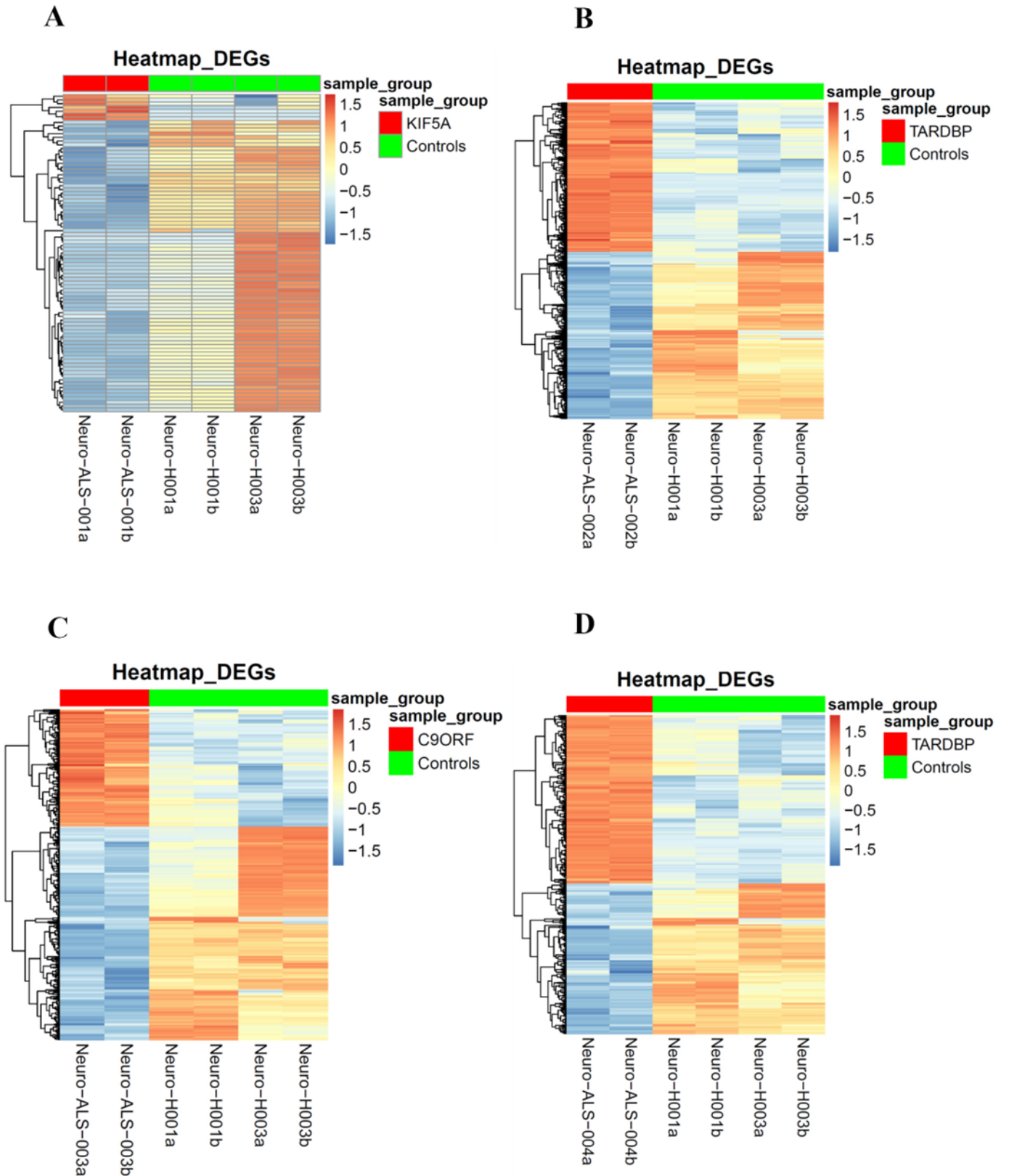


Figure 5. Heatmaps of the differentially expressed genes in Neurospheres: Heatmaps of the differentially expressed genes of each of the four ALS patients in comparison to the matched healthy controls. Patients' differentially expressed genes are represented on the left (top red bar); healthy controls differentially expressed genes are represented on the right (top green bar). A: ALS001_KIF5A vs HC ; B: ALS002_TARDBP vs HC C: ALS003_c9orf72 vs HC; D: ALS004_TARDBP vs HC.

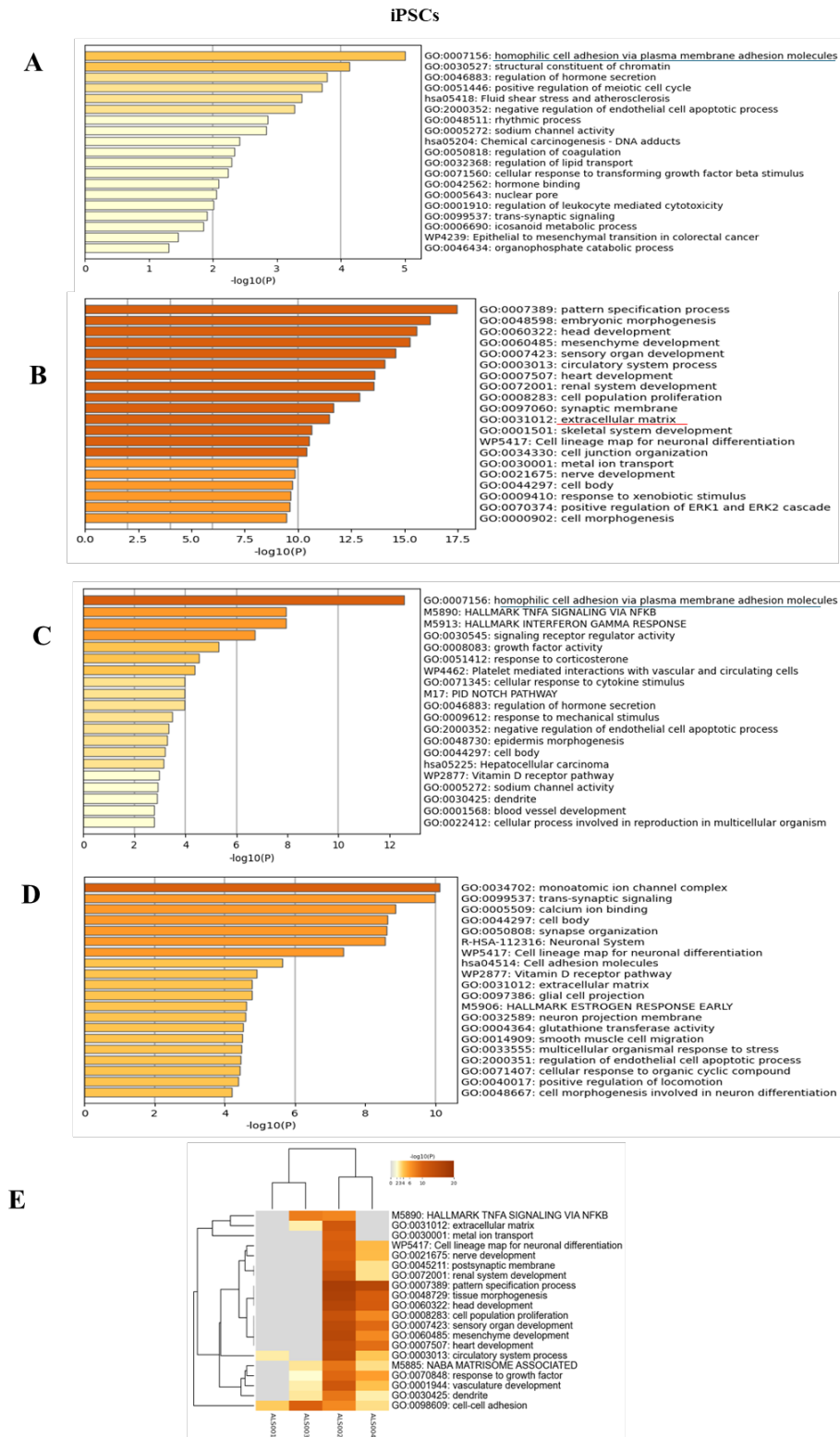


Figure 6. Enrichment analysis: Enrichment analysis results of the comparison between each of the four ALS patients and the matched healthy controls, at iPSC level. A: enrichment analysis for ALS001_KIF5A vs HC; B: enrichment analysis for ALS002_TARDBP vs HC; C: enrichment analysis for ALS003_c9orf72 vs HC; D: enrichment analysis results for ALS004_TARDBP vs HC; E: integrative and comparative enrichment analysis among the four subjects. The DEGs for all comparisons include either upregulated and downregulated genes. At iPSCs level, we underlined in different colours the shared pathways (blue for cell adhesion pathways and red for matrisome, extracellular matrix pathways).

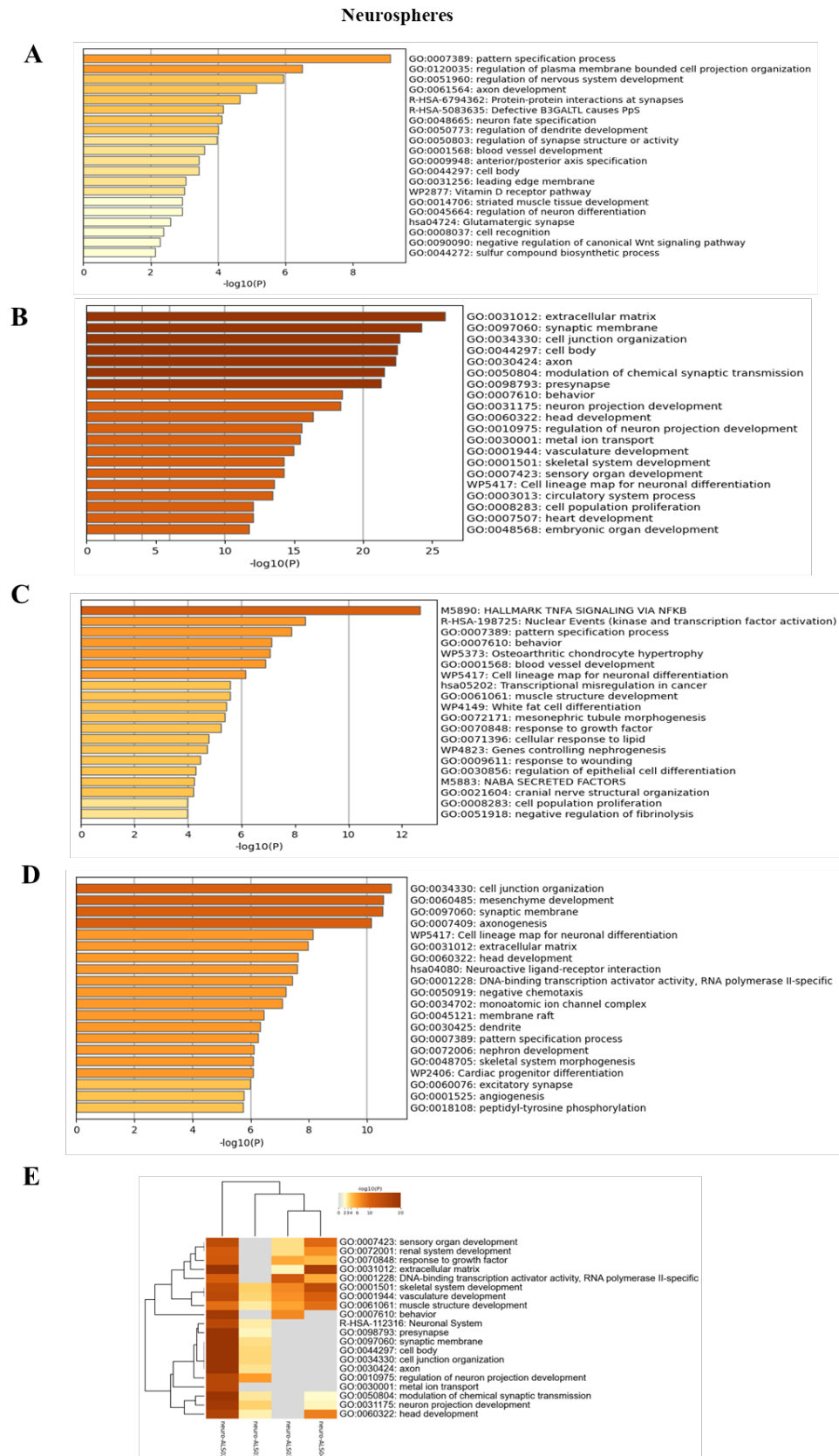


Figure 7: Enrichment analysis: Enrichment analysis results of the comparison between each of the four ALS patients and the matched healthy controls, at neurosphere level. A: enrichment analysis for ALS001_KIF5A vs HC; B: enrichment analysis for ALS002_TARDBP vs HC; C: enrichment analysis for ALS003_c9orf72 vs HC; D: enrichment analysis results for ALS004_TARDBP vs HC; E: integrative and comparative enrichment analysis among the four subjects. The DEGs for all comparisons include either upregulated and downregulated genes.

4. Discussion

ALS belongs to the class of neurodegenerative diseases characterized by the loss of neuronal function. ALS preferentially affects upper and lower MNs and can be classified into familial (fALS) and sporadic (sALS) ALS, with genetic mutations identified in over 40 genes including *SOD1*, *C9orf72*, *FUS*, *TARDBP*, and *KIF5A*. These mutations interfere with multiple cellular processes, such as nucleocytoplasmic transport and axonal and vesicular transport. In this study, we utilized iPSCs and neurospheres derived from four ALS patients carrying mutations in the *KIF5A*, *TARDBP*, and *C9orf72* genes, as well as from two healthy donors, to investigate transcriptomic differences associated with these ALS-related genetic mutations and to identify the corresponding molecular pathways. By differentiating iPSCs into neurospheres, we explored their potential to become MNs. Small molecules enhanced the efficient and specific conversion of both healthy and ALS iPSCs into neurospheres expressing the typical markers NES, PAX6 and SOX2. The transcriptomic profile of all ALS iPSCs was compared to two healthy gender-matched control (H_001 and H003) showing that only 10 differentially expressed genes (DEGs) were shared across ALS iPSCs, highlighting the wide heterogeneity of ALS pathogenesis. Interestingly, among these genes, *POM121B* was downregulated in all ALS iPSCs. This gene encodes for a transmembrane protein essential for nuclear pore complex assembly and nucleocytoplasmic transport of RNAs and proteins [24]. A nuclear depletion of *POM121B* has been observed in iPSC-derived neurons and postmortem tissue from *C9orf72* patients [25] and *TARDBP* iPSCs derived-MNs [26], suggesting its key role as an early pathogenic event and in the nuclear pore complex disruption. Intriguingly, *POM121B* was downregulated also in *KIF5A* iPSCs indicating a potential link between axonal transport defects and impairment in nucleocytoplasmic transport [27]. Considering that nucleocytoplasmic transport defects are a common hallmark of neurodegenerative diseases, future studies aiming in examining the nuclear protein complex expression in ALS may aid in the identification of a potential therapeutic target. By analyzing the common GO terms in ALS iPSCs, we found an enrichment in GO terms related to Matrisome-associated terms as extracellular matrix (GO:0031012) [28,29] cell-cell adhesion (“homophilic cell adhesion via plasma membrane adhesion molecules” (GO:0007156) and “cell adhesion via plasma membrane adhesion molecules” (GO:0098609) and a modulation of the Notch signaling pathway, highlighting its potential role in ALS pathophysiology. Indeed, Notch pathway regulates critical processes such as cell differentiation, survival, and neuroinflammation, all of which are disrupted in ALS [30,31]. Moreover, altered cell adhesion molecule (CAM) expression suggests a dysfunction that impairs neuronal differentiation, neuronal maturation, synaptic organization [32] and promote immune infiltration into CNS, contributing to neuroinflammation [33]. Finally, CAM closely collaborates

with the ECM, regulating processes that are fundamental for the homeostasis of nervous tissue. Among ECM genes and NABA pathways, the *GDF15* overexpression is of great interest given its emerging role as an ALS biomarker [34]. Considering that unprocessed *GDF15* remains bound to the ECM, this could suggest that its upregulation may act as a compensatory mechanism [35]. On the other hand, as a stress-responsive cytokine, *GDF15* may exert a protective effect by counteracting neuroinflammation, metabolic alterations, and ECM remodeling, thus mitigating neuronal damage.

Interestingly, an enrichment in GO terms in ALS iPSCs was directly correlated with the function of the mutated protein. Indeed, in ALS_001 *KIF5A* iPSCs, upregulated genes were enriched for the “transport vesicle membrane” (GO:00306580133), directly connected to *KIF5A* function [36]. In both *TARDBP* iPSCs, the “DNA-binding transcription factor activity” (GO:0001228) was enriched [34], reflecting the role of TDP-43 protein in transcription and splicing [37]. Finally, ALS_003 *C9orf72* iPSCs showed an enrichment in several pathways linked to immunity and inflammation (M5913 “hallmark interferon gamma response”, M5890 “TNF α signalling via NF- κ B”, WP619 “Type II interferon signalling”), underlining the specific role of *C9orf72* in eliciting immune activity and regulation highlighting the role of inflammation in neurodegeneration [38,39].

We further investigated whether transcriptomic differences also occur at neurosphere level. Interestingly, in iPSCs-derived neurospheres no common pathways were shared across all samples, suggesting that the molecular signature could reflect the distinct molecular mechanisms driven by the different ALS-related mutations. In the two *TARDBP* neurospheres, a significant upregulation of the ECM pathway was observed, suggesting that these alterations may influence cellular behavior, tissue remodeling, and neuroinflammation. Indeed, in ALS, excessive ECM remodeling could promote fibrosis, impair neuron-glia communication, and hinder neuro regeneration, ultimately exacerbating neurodegeneration [40]. A detailed analysis of the specific ECM components affected, such as collagen, laminins, or proteoglycans, could provide insights into disease progression and help identify novel therapeutic strategies.

Moreover, an enrichment of several GO terms related to key neuronal processes, such as axon development, dendrite regulation, synaptic membrane formation, and neuron projection development were found, suggesting that the disruption in these fundamental mechanisms might occur both from pathological changes in mature MNs and from early defects in differentiation and neurons network formation [41,42]. Indeed, the disruption of these processes may lead to impaired neuronal circuit formation, reduced synaptic plasticity, and deficits in neural communication, all of which are hallmarks of ALS pathology.

In conclusion, this study highlights the advantages of iPSC technology in ALS research by providing a renewable source of patient-specific cells to investigate the complex molecular landscape of ALS. The next step should focus on differentiating these iPSCs into MNs and other cell types to develop organoids containing ALS-derived cells, performing comprehensive transcriptomic and proteomic analyses, and investigating the functional consequences of ALS-associated mutations. Moreover, our study is perfectly in line with other international initiatives, such as Answer ALS, a large-scale resource for sporadic and familial ALS in United States combining clinical and multi-omics data from iPSCs and iPSC-derived MNs [43]. Although our work includes iPSCs derived from patients carrying different mutations, thus contributing to increase the diversity and representativeness of the available cellular models, it shows some limitations. The major concerns are the small sample size and limited number of control cell lines. For these reasons, this work should be considered as a pilot study. To strengthen the obtained results, future studies including a larger cohort and a higher number of independent lines for each genotype should be used. Given the urgent need for effective treatments for ALS, this study represents a step forward in leveraging stem cell technology and ALS study. The integration of iPSC with advanced transcriptomic analyses holds promise for uncovering the intricate network of pathways involved in ALS, ultimately leading to novel therapeutic strategies.

Authors contributions: CS, MT, CO, GV, BP, AC, performed research and analyzed data; CS, CO, GV, AC generated and characterized iPSC from healthy donors and ALS patients. CS generated neurospheres from iPSC. MT, FF, BP and LC performed and analysed RNA-Seq. FDM, LM selected ALS patients; LM, SD, AF and CO conceived the study, generated funding, designed the research, and analyzed data. CS, MT, CO, FDM, LM, SD and AF wrote the manuscript that was revised by all authors.

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Second Aim

Material and methods

Neurite outgrowth assay

To assess neurite outgrowth, neurospheres were generated from iPSCs. On day 12 of the differentiation protocol, cells were dissociated with StemPro Accutase (Thermo Fisher Scientific) and 5×10^3 cells were seeded in low-adherence 96-well plates with rounded bottoms. The culture medium was composed by a 1:1 mixture of DMEM/F12 and Neurobasal Medium (Gibco), supplemented with $0.5 \times \text{N2}$, $0.5 \times \text{B27}$, GlutaMAX and non-essential amino acids (Gibco), enriched with the following small molecules: SB431542 (Stem Cell Technologies), LDN193189 (Stem Cell Technologies), CHIR99021 (Stem Cell Technologies), Ascorbic acid (Sigma), all-trans RA (Stem Cell Technologies) and Purmorphamine (Stem Cell Technologies). The medium was changed every other day until day 20 when neurospheres were transferred to 96-well plates coated, with 0.1% polyethylenimine (PEI) and 20 $\mu\text{g/mL}$ laminin, to promote neurite attachment and sprouting. Cells were cultured for 1 week in the same medium changed every two days. Neurite growth was monitored by live-imaging using the Incucyte® SX5 system (Sartorius), with images acquired every 4 h for a period of 7 days. At the end of the experiment, cells were fixed with 4% paraformaldehyde and processed for immunofluorescent analysis, to evaluate β -III tubulin (TUB β III) expression.

Immunofluorescence analysis for neurite outgrowth

Fluorescence quantification was evaluated from seven squares per image obtained after overlaying a standardized grid on each image. Image J software was used to quantify TUB β 3 expressions [158]. The analysis of total branches, total branching point and circuitry was quantified by Wimasis Neurite Outgrowth Software.

Statistical analysis

Data obtained from ImageJ were analyzed by GraphPad Prism; values are expressed as mean and a One-way ANOVA was used to assess statistical significance.

MNs differentiations

The differentiation protocol of MNs shared the initial phase with that of neurospheres. On day 20, neurospheres were dissociated and neural progenitor cells were seeded in 24-well plates, pre-coated with 0,1% of PEI and laminin (20 µg/mL). NPCs were maintained in a medium containing: Ascorbic acid, Putrescine and All trans-RA until day 30. From that date, cells were fed every other day with a medium, enriched with the Glial-derived Neurotrophic Factor (GDNF) and BDNF Brain-derived Neurotrophic Factor (BDNF), to support neuronal maturation and survival. At the end of differentiation MNs were characterized by immunofluorescence and Real-Time qPCR. Primary and secondary antibodies used are listed in Table 1. During maturation step (from day 30 to 45) MNs growth was followed by Incucyte in order to identify morphological changes.

Analysis of Chat-positive MNs

Previously stained cells with antibodies against ChAT were analyzed using the Incucyte® live-cell system. For each field of view, images were acquired in both brightfield and the red fluorescence channel (corresponding to ChAT immunoreactivity). Image analysis was performed using ImageJ software. Brightfield images were used to define the total cell population. A threshold was manually adjusted to generate a binary mask, which was then used to create a region of interest (ROI) corresponding to the overall cellular area. This ROI was then applied to the corresponding red fluorescence image (same field), to restrict quantification of the ChAT signal to the total cell-containing area. For quantification, the mean fluorescence intensity was measured, providing an estimation of ChAT signal. Fluorescence intensity was then normalized to the corresponding brightfield reference (set to 100%) to calculate the percentage of ChAT-positive cells relative to the total population.

Real-time qPCR analysis

Real-Time qPCR was carried out by using the SyberGreen qPCR Mix (ThermoFisher) and by performing the reaction in a StepOnePlus™ instrument (Applied Biosystems). Gene expression was quantified using the $2^{-\Delta Ct}$ method, where ΔCt was calculated by normalizing to the ΔCt of the housekeeping gene, in this case B-actin. Primers used are listed in Table 2 below.

Table 1. List of antibodies used

Primary antibodies	Host	Reactivity	Manufacturer	Dilution	Catalog #
ChAT	Rabbit	Human	Invitrogen	1:500	PA5-29653
TUB β 3	Mouse	Human	Invitrogen	1:100	MA1-118
MAP2	Mouse	Human	Invitrogen	1:400	13-1500
Secondary antibodies	Fluorophores	Manufacturer		Dilution	Catalog #
Goat anti-Rabbit	AlexaFluor 546	Invitrogen		1:500	A-11012
Goat anti-Mouse	Alexa Fluor 488	Invitrogen		1:500	A-11001
Goat anti-Mouse	AlexaFluor 546	Invitrogen		1:500	A-11005

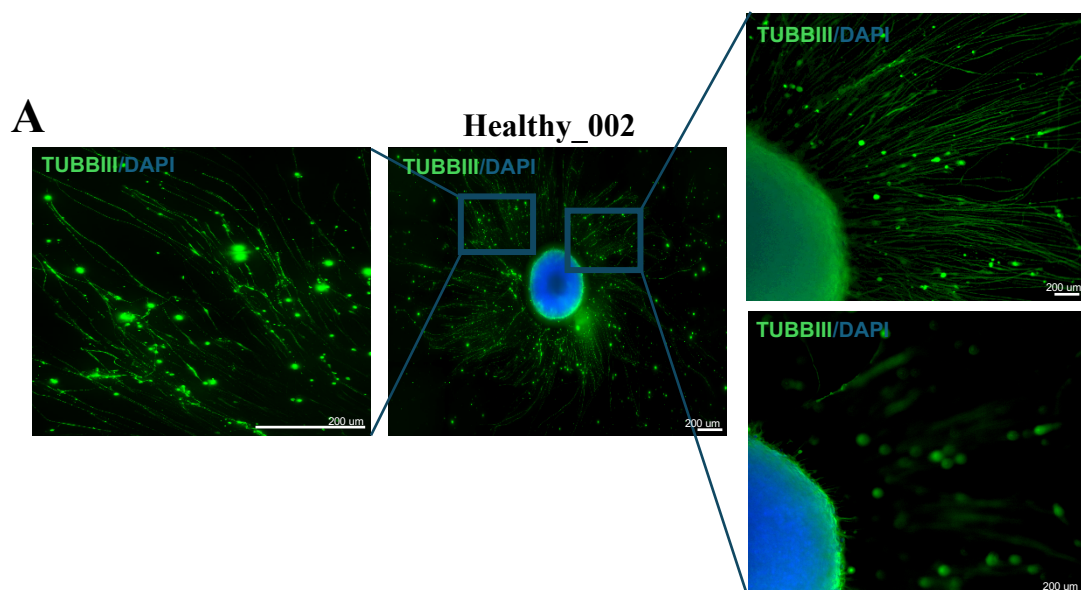
Table 2. List of primers used

Gene	Synthetic oligonucleotide	Attempted band
<i>SYP</i>	S: 5'-TTAGTTGGGGACTACTCCTCG -3'	131bp
	A: 5'-GGCCCTTTGTTATTCTCTCGGTA -3'	
<i>SYT1</i>	S: 5'-GTGAGCGAGAGTCACCATGAG -3'	175bp
	A: 5'-CCCACGGTGGCAATGGAAT -3'	
<i>NeuroD1</i>	S: 5'-GGATGACGATCAAAAGCCCAA -3'	179bp
	A: 5'-GCGTCTTAGAATAGCAAGGCA -3'	
<i>NFH</i>	S: 5'-CCGTCATCAGGCCGACATT -3'	161 bp
	A: 5'-GTTTTCTGTAAGCGGCTATCTCT -3'	
<i>Nav1.1</i>	S: 5'-AGCTCTGATGCAGGCCAATG -3'	360bp
	A: 5'-AGCTGCCTCCTGTTGCTTT -3'	
<i>Kv2.1</i>	S: 5'-CCATTCTGCCATACTATGTCACC -3'	196bp
	A: 5'-AGCAAGCCCAACTCATTGTAG -3'	

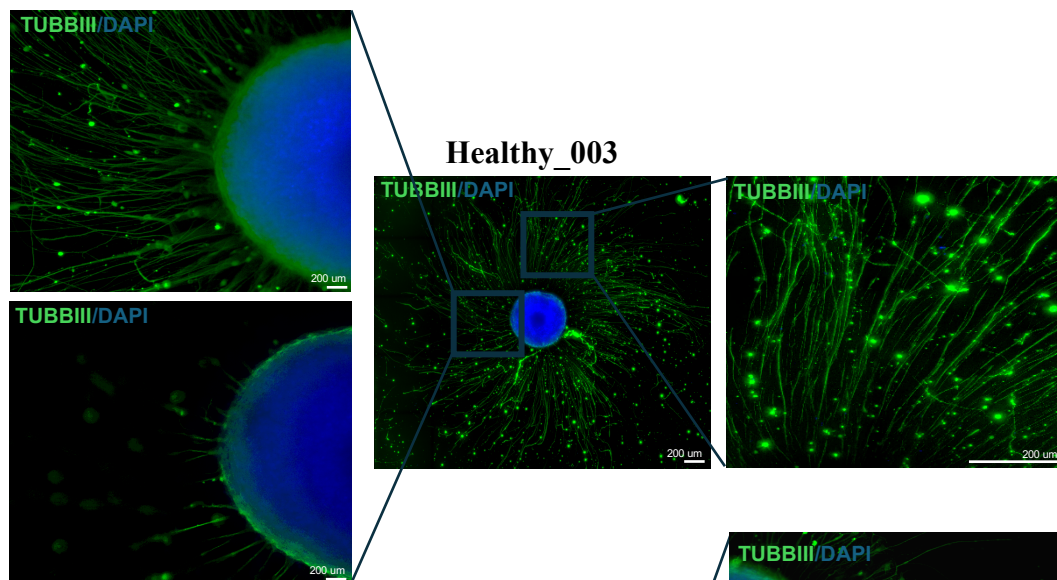
Results

Neurite outgrowth assay

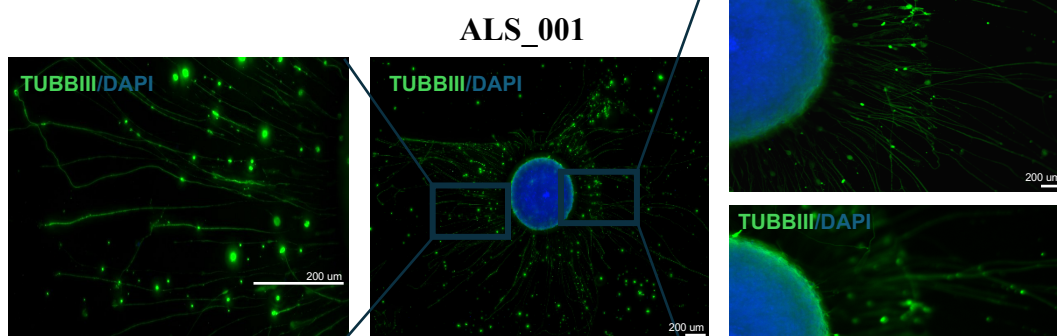
The ability of NPCs, that are in the form of 3D aggregates (neurospheres), to generate neurites was tested by a neurite outgrowth assay. Neurospheres growth was monitored by live-imaging for a period of 7 days and a video was recorded to capture the entire growth and extension process. After one week of culture, by immunofluorescence was evaluated the expression of (TUB β III), a neuronal marker commonly used to visualize and quantify neurite morphology. Qualitative analysis of the images showed a higher density and complexity of the neuritic network in neurospheres derived from healthy NPCs compared to ALS NPCs (Figure 1 A-F). Healthy neurospheres showed a homogeneous and plentiful distribution of neurites extensions compared to ALS neurospheres. This was particularly evident in TARDBP-derived neurospheres, where a reduction in the total number of neurites was observed. Moreover, in neurospheres obtained from KIF5A and C9orf72 iPSCs, the neural network appears to be less dense, even though their length was comparable to healthy NPCs. TUB β III fluorescence, was quantified by applying a standardized grid to the acquired images, and seven representative squares were selected for each image (Figure 2 A). The fluorescence intensity of the TUB β III signal was measured for each square, obtaining a quantitative estimation of neurites density, revealing a significant reduction in fluorescence intensity in all ALS neurospheres compared to the healthy neurospheres. Moreover, within ALS group it is possible to appreciate that in TARDBP (ALS_002) fluorescence intensity is lower (Figure 2 B). The results showed that ALS-derived samples followed a consistent trend of reduced neurite outgrowth compared to healthy controls.



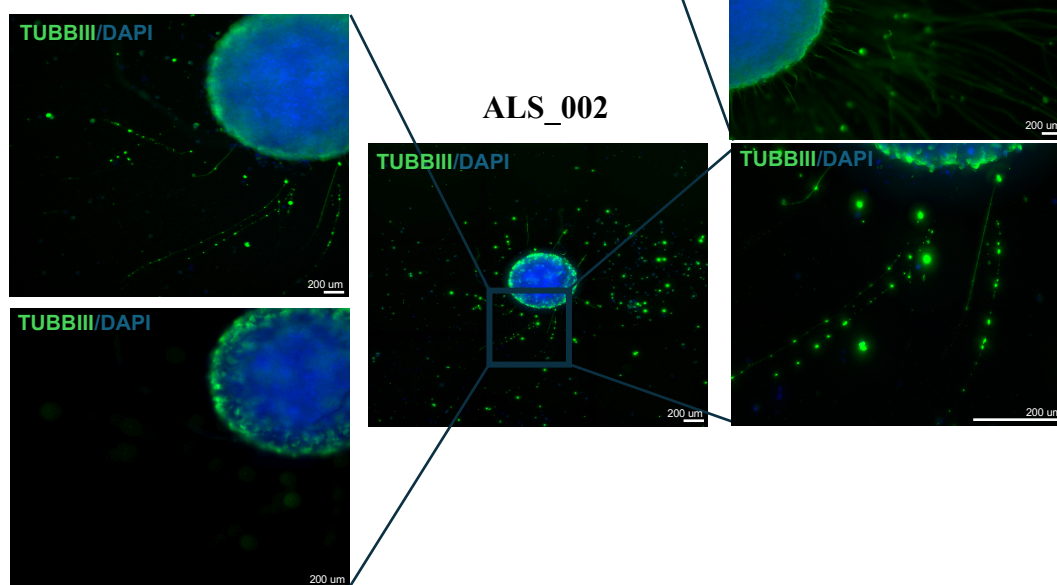
B



C



D



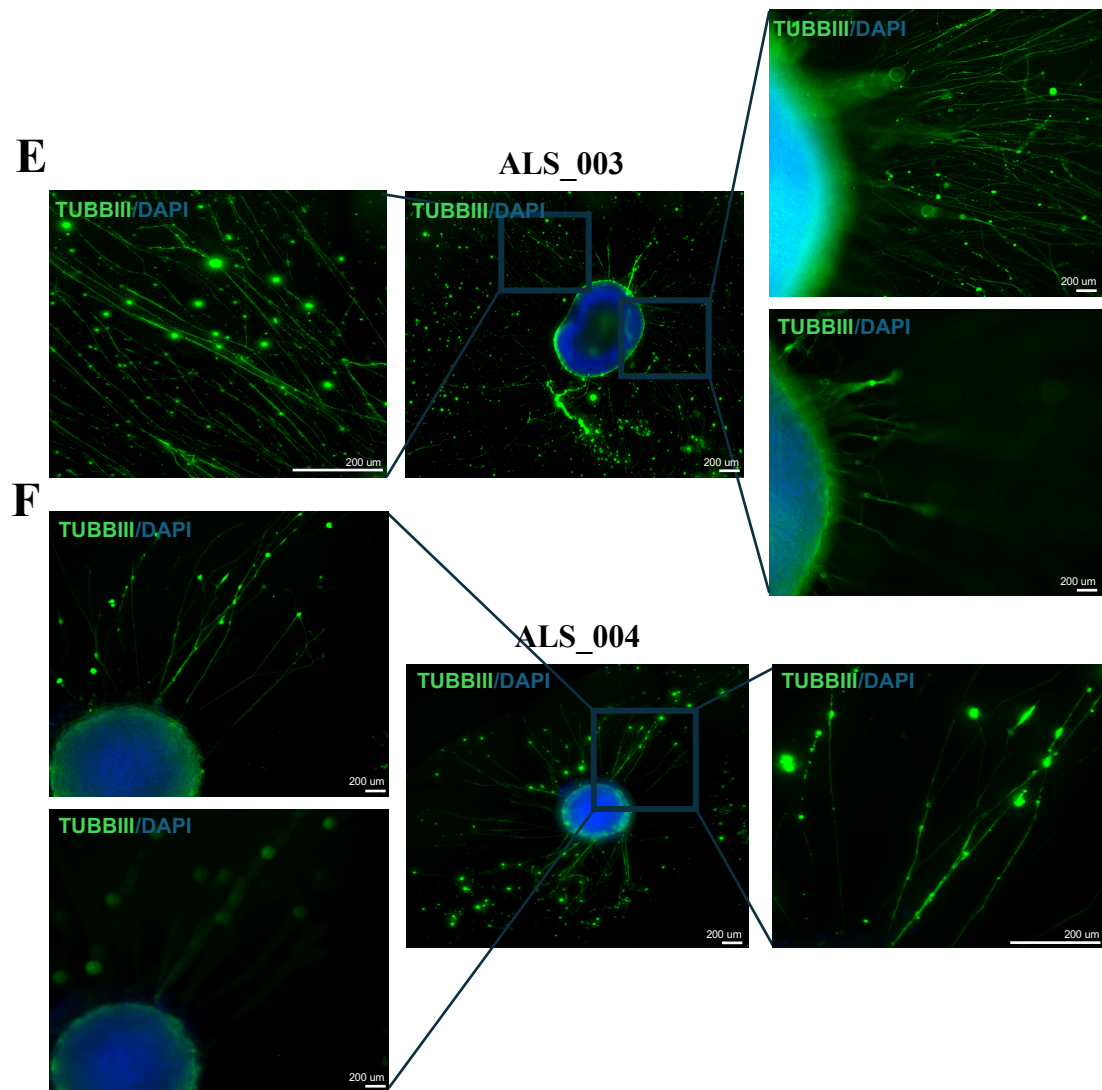


Figure 1. Evaluation of TUB β III fluorescence after Neurite outgrowth assay.

TUB β III expression was detected by immunofluorescence on healthy (H_002 and H_003) and ALS neurospheres (ALS_001, ALS_002, ALS_003 and ALS_004). Markers used were TUB β III (green) and DAPI (blue). Scale bars, 200 μ m. A) Images of H_002 neurospheres. B) Images of H_003 neurospheres. C) Images of ALS_001 neurospheres. D) Images of ALS_002 neurospheres. E) Images of ALS_003 neurospheres. F) Images of ALS_004 neurospheres.

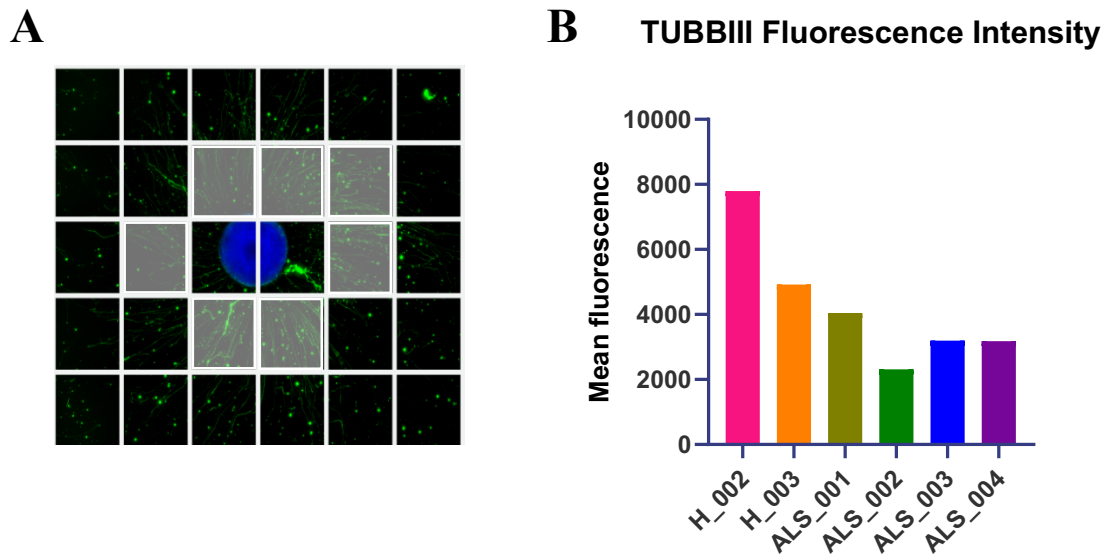
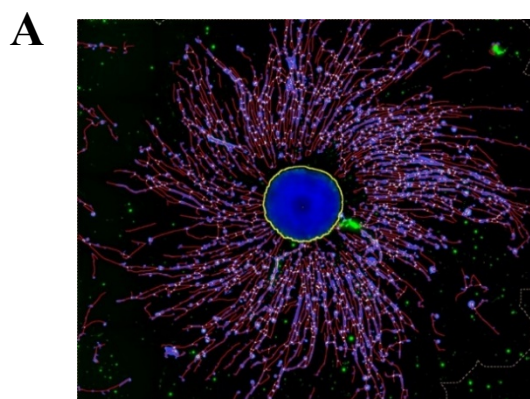


Figure 2. Quantification of TUB β III expression after Neurite outgrowth assay.

A) Representative method used to quantify the fluorescence of TUB β III. A reference grid was overlaid on each image, allowing the same number of squares (7) to be selected for each image and selected squares are evidenced in the representative figure. Fluorescence quantification of each square was evaluated using ImageJ. **B)** Quantification of TUB β III fluorescence. The experiment was done once and the values represented are relative to the average derived from the fluorescence values of the 7 squares.

Several key morphological parameters were analyzed, such as the total number of branches, neurites branching points and overall length of the neurite network (circuitry). The analysis highlighted a common trend in healthy NPCs with higher values in all the three parameters (Figure 2 A-D). This confirms a greater branching capacity, and a more complex neural network of healthy NPCs compared to the ALS NPCs. Moreover, the higher values in extension and complexity of the neural network, in total number of branches and in branching points observed in healthy NPCs were morphologically confirmed also by the immunofluorescence staining for β III-tubulin, showing a higher distribution and density of healthy neurites compared to ALS once.



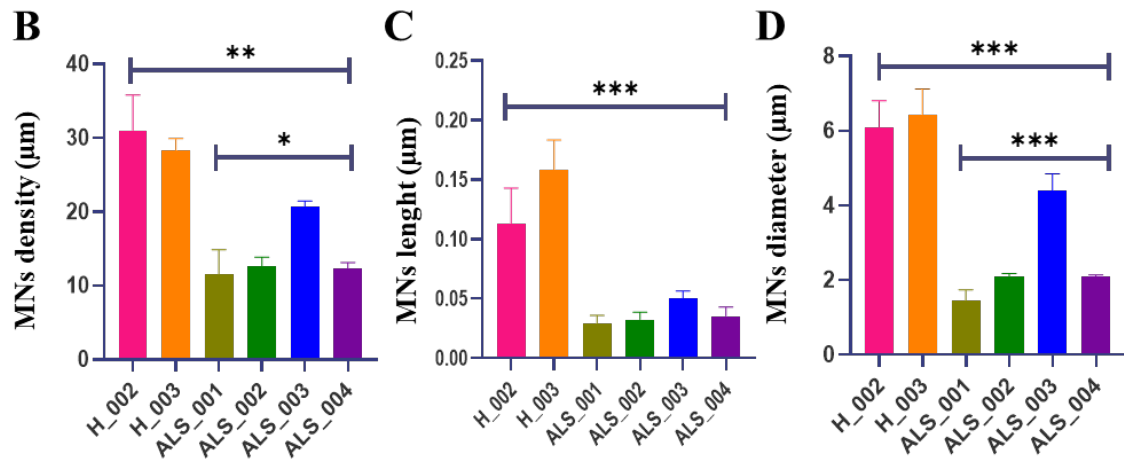


Figure 3. Evaluation of morphological parameters after Neurite outgrowth assay.

A) Representative image obtained with the Neurite outgrowth Software. Yellow circle: central area of neurospheres. Red lines: total number of neurites. Blue lines: secondary branching. White dots: branching points. **B)** Quantification of the total number of branches. **C)** Quantification of the total number of branching points. **D)** Quantification of circuitry length. All measurements are obtained in one biological replicate and performed in technical triplicates (one neurosphere per well) and expressed in the graphics as mean $\pm$ SD. Statistical analysis was performed by One-way ANOVA test (*** $p < 0.001$; ** $p < 0.01$).

MNs characterization

iPSCs from healthy donors and ALS patients were differentiated into MNs passing through neural Rosettes formation. At the end of the differentiation period (45 days) (Figure 4) the obtained MNs were characterized for their morphological features and for the expression of neuronal markers both at RNA (SYP, SYT, NeuroD1, NFH, NaV1.1 and Kv2.1) and protein (ChAT, TUB β III, MAP2) levels.

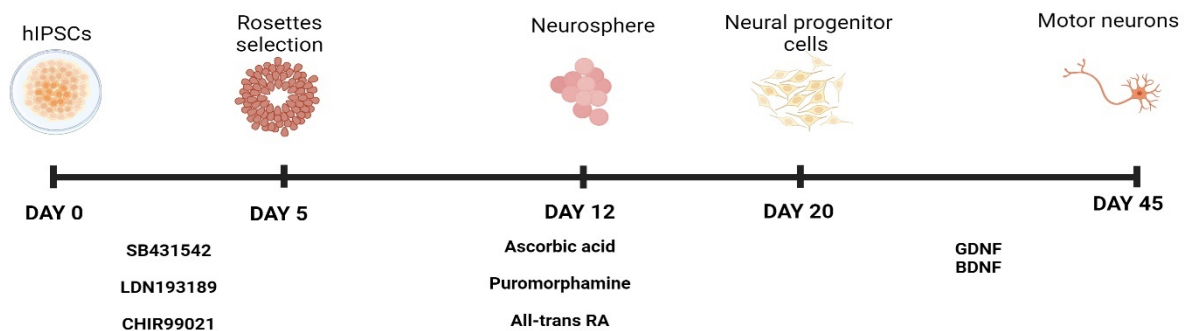


Figure 4. Schematic representation of MNs differentiation protocol.

iPSCs were induced through the formation of neural rosettes. Following their selection, the generation of neurospheres occurs. The dissociation of neurospheres enables the maturation of NPCs into mature MNs. The small molecules utilized include CHIR99021, LDN193189, SB431542, RA, ascorbic acid, purmorphamine, GDNF, and BDNF.

The images obtained by phase contrast microscopy highlighted morphological differences between the different cell groups (Figure 6 A-B, first column). In fact, healthy MNs showed a more developed and complex neural network than those obtained from ALS MNs. Nevertheless, among patients, the MNs line derived from the patient carrying the mutation in C9orf72 stands out for a greater extension and density of the neural network compared to the other mutations. Moreover, differences in terms of cell density, length, and thickness of the axon segment were highlighted.

The length of the MN was calculated by tracing the neurite from one soma to another demonstrating the highest length of the two healthy iPSCs-derived MNs, compared to the four ALS iPSCs-derived MNs. Finally, a clear difference between the healthy and the diseased group is reported by the data regarding the thickness of the neuron axon (Figure 5 A-C). By comparing the two groups it was visible that values regarding the ALS patients were about three times lower than the healthy ones.

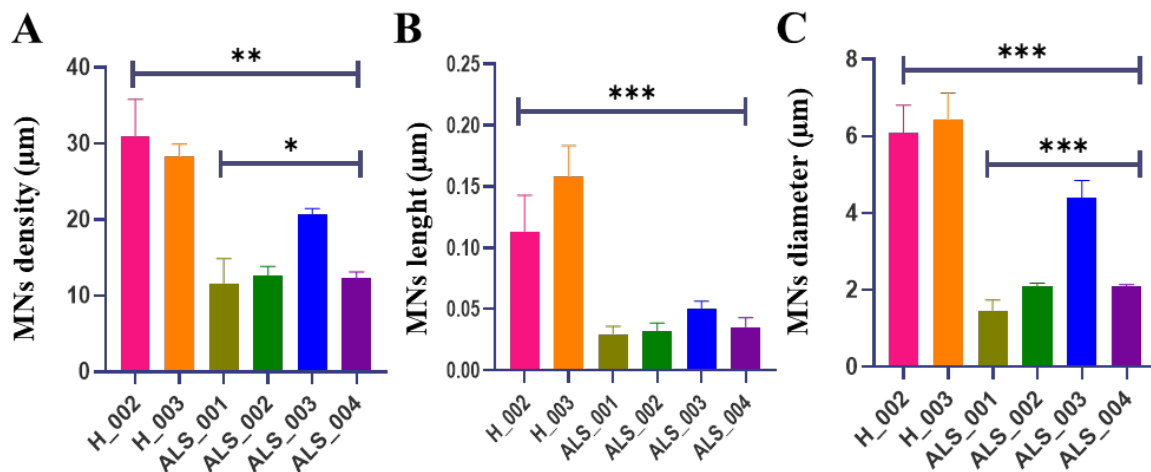


Figure 5. Evaluation of morphological parameters after MNs differentiation.

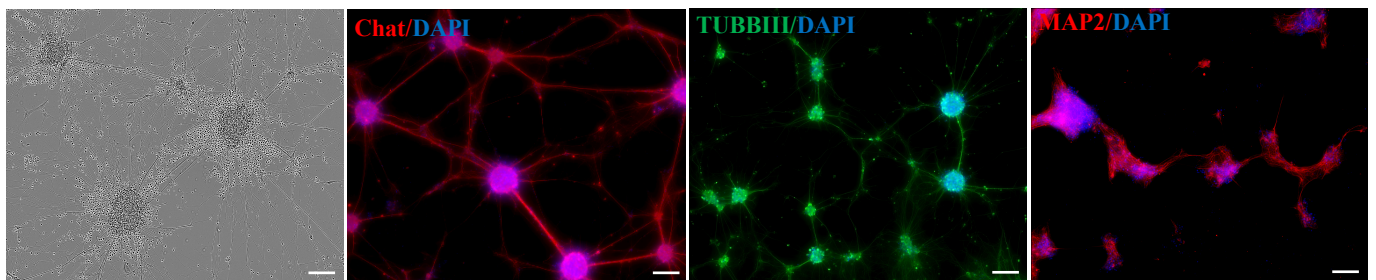
A) Quantification of MNs density. **B)** Quantification of MNs length. **C)** Quantification of MNs diameter (axon diameter). All differentiations were performed in two independent experiments. All measurements are obtained in two biological replicates: each of them represented by technical triplicate and expressed in the graphics as mean $\pm$ SD. Statistical analysis was performed by One-way ANOVA test (** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$).

To confirm neuronal maturation, the expression of choline acetyltransferase (ChAT), a specific marker expressed typically by mature MNs was analyzed. Immunofluorescence staining showed a positive expression of ChAT in all obtained MNs (Figure 6 A-B). In addition, the expression levels of TUBB3 and microtubule-associated protein 2 (MAP2), proteins associated with the structure and stability of the neuronal cytoskeleton, were evaluated. The analysis showed adequate expression levels of both markers in all cell lines, further confirming the occurred neuronal differentiation (Figure 6 A-B).

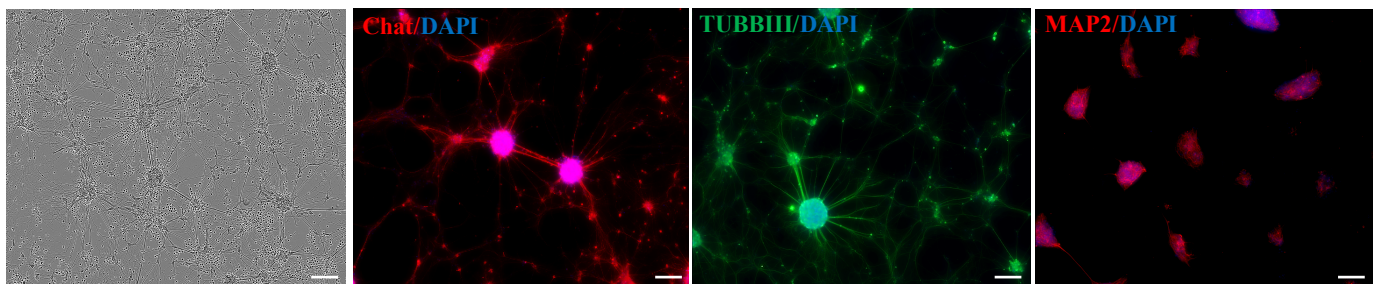
Healthy- MNs

A

H_002



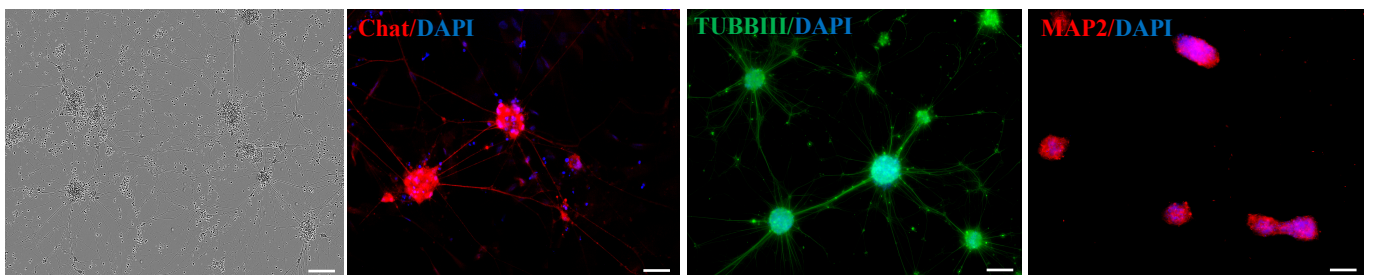
H_003



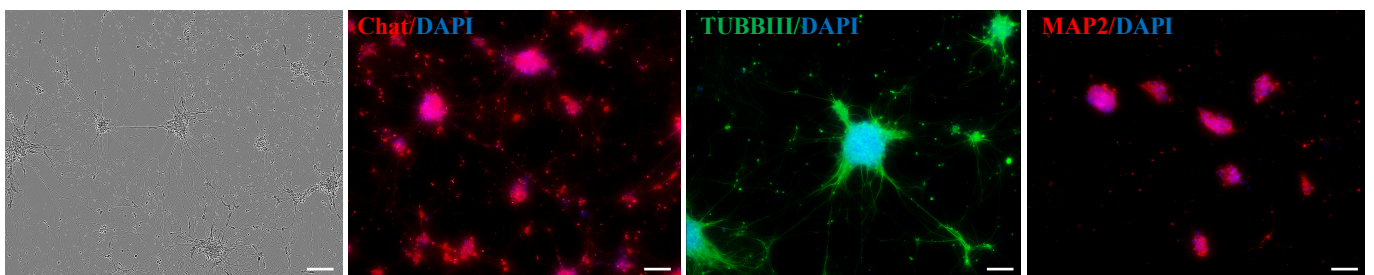
ALS MNs

B

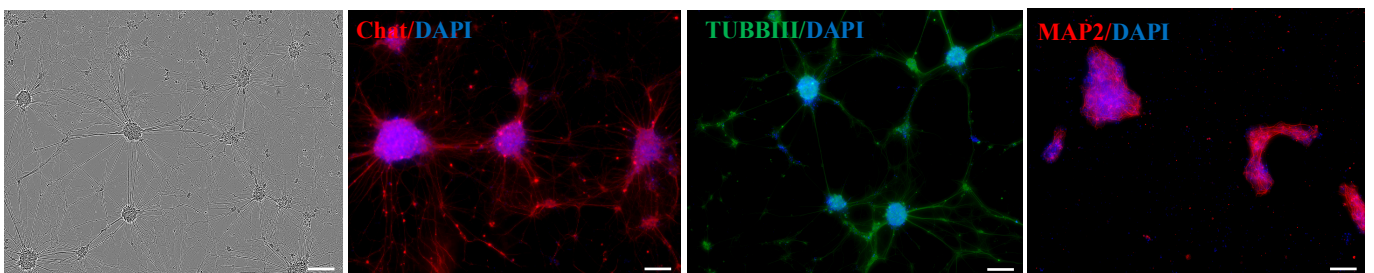
ALS_001



ALS_002



ALS_003



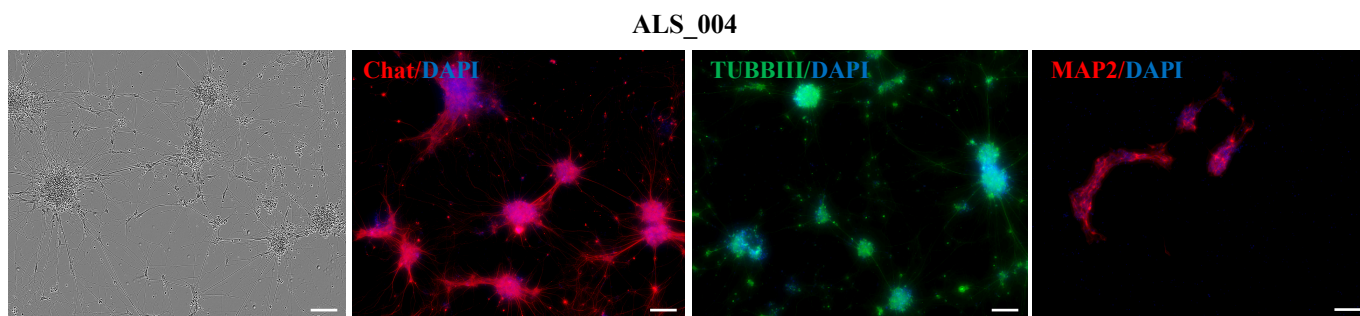
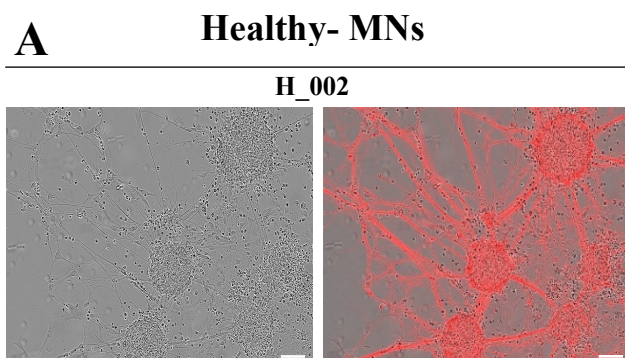


Figure 6. MNs characterization.

Specific neural markers at the protein level were detected by immunofluorescence on healthy and ALS MNs. Markers used were: ChAt (red), TUB β III (green), MAP2 (red) and DAPI (blue). Scale bars, 100 μ m. **A)** Representative images related to healthy MN lines: H_002 and H_003. **B)** Representative images related to ALS MN lines: ALS_001, ALS_002, ALS_003 and ALS_004. In sequence from left to right image of phase-contrast microscopy, immunofluorescence for Chat, immunofluorescence for TUBBIII and immunofluorescence for MAP2.

Due to the qualitative nature of the immunofluorescence technique, a quantitative analysis of Chat-positive cells was performed to evaluate their proportion within the total population of iPSC-derived MNs. This analysis was based on images acquired in both brightfield and red fluorescence channels (Figure 7 A-B).

The fluorescence signal from ChAT-positive cells was quantified and normalized to the total signal obtained from the corresponding brightfield images. This approach allowed for an accurate estimation of the proportion of ChAT-positive MNs in each sample. Healthy control lines showed a consistently high percentage of Chat⁺ cells (H_002: $\sim 94\% \pm \text{SD}$; H_003: $\sim 94\% \pm \text{SD}$), indicating efficient differentiation (Figure 7 C). Similarly, ALS patient-derived lines also showed a high percentage of Chat expression, although with greater variability across individuals (ALS_001: $\sim 90\% \pm \text{SD}$; ALS_002: $\sim 80\% \pm \text{SD}$; ALS_003: $\sim 96\% \pm \text{SD}$; ALS_004: $\sim 82\% \pm \text{SD}$). Despite the high percentage of ChAT-positive cells observed across all samples, a statistically significant difference was detected between healthy controls and most ALS patient-derived lines.



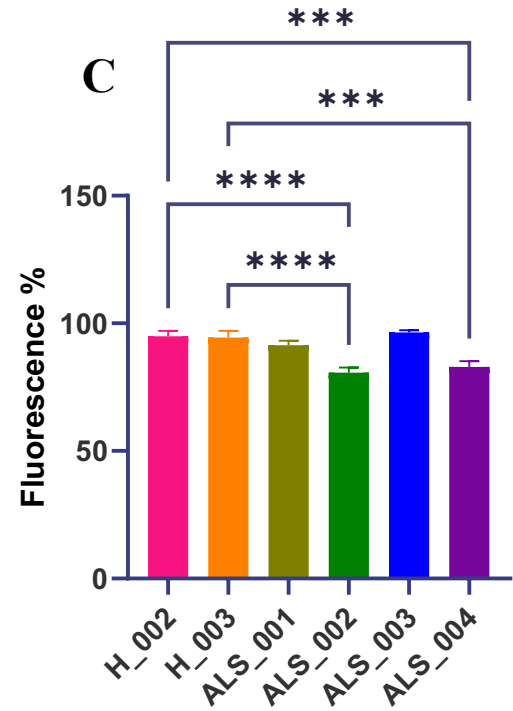
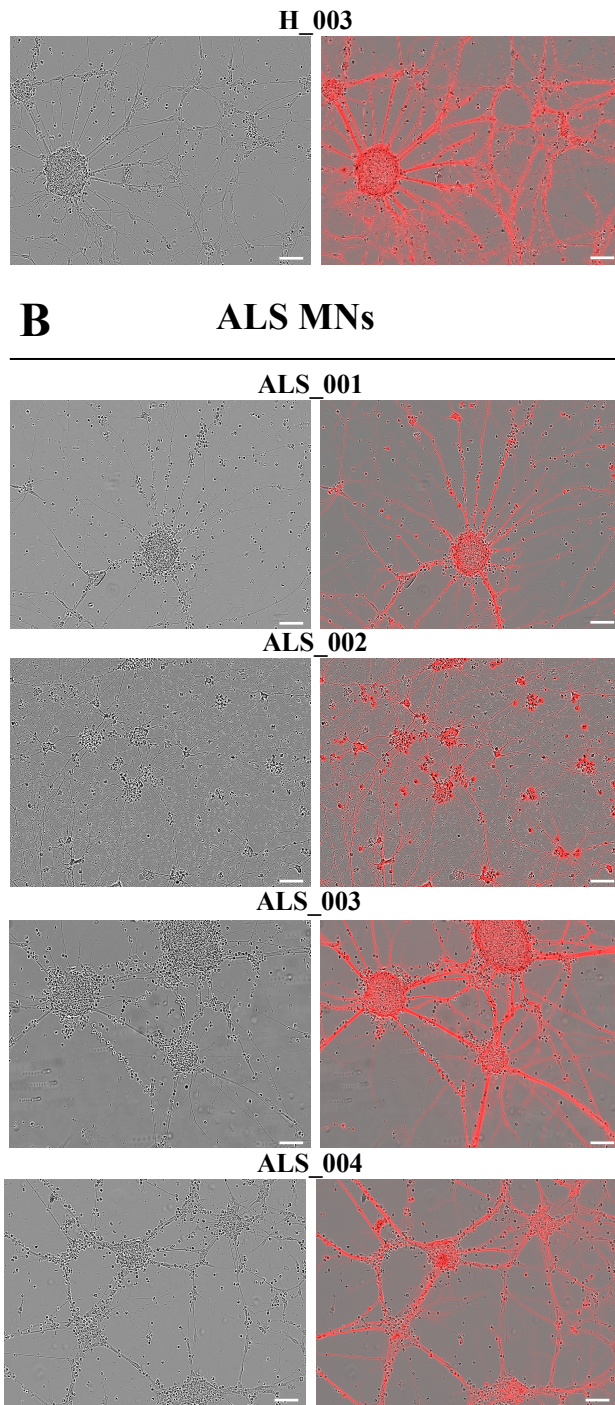


Figure 7. Quantification of Chat+ MNs.

A) Representative images of iPSC-derived MNs from two healthy control lines (H_002 and H_003). **B)** Representative images of iPSC-derived MNs from four ALS patient-derived lines (ALS_001, ALS_002, ALS_003 and ALS_004). In both panels (A and B), for each line, brightfield images are shown on the left and corresponding red fluorescence images for ChAT staining are shown on the right. **C)** Quantification of the fluorescence percentage of ChAT-positive cells relative to the total MN population. The analysis was performed using the mean percentage calculated from three biological replicates, each with three technical replicates (for each subject, three independent image groups were analyzed in triplicate). The values are expressed as the mean percentage of ChAT fluorescence normalized to the corresponding brightfield image $\pm$ SD. Statistical analysis was performed using One-way ANOVA test (** $p < 0.001$; **** $p < 0.00001$).

Expression levels of key genes involved in neuronal function were assessed as:

-Synaptophysin (SYP) and Synaptotagmin 1 (SYT1): synaptic markers essential for the formation and functioning of synapses.

-Neurofilament Heavy Chain (NFH), an essential structural component of the neuronal cytoskeleton.

-Neuronal Differentiation 1 (NEUROD1), a transcription factor implicated in neurogenesis and neuronal maturation.

-Sodium Voltage-Gated Channel Alpha Subunit 1 (Nav1.1) regulates the conduction of electrical signals through sodium channels

-Potassium Voltage-Gated Channel Subfamily B Member 1 (Kv2.1) regulates the conduction of electrical signals through potassium channels.

As shown in Figure 8 MNs derived from healthy iPSCs expressed significantly higher levels of SYP, SYT1, NeuroD1 and NFH compared to ALS-MNs, Finally, the expression of the genes related to ion channels, Nav1.1 and Kv2.1, was higher in healthy MNs than patients MNs, highlighting a possible impairment of the ability to generate and propagate the action potential.

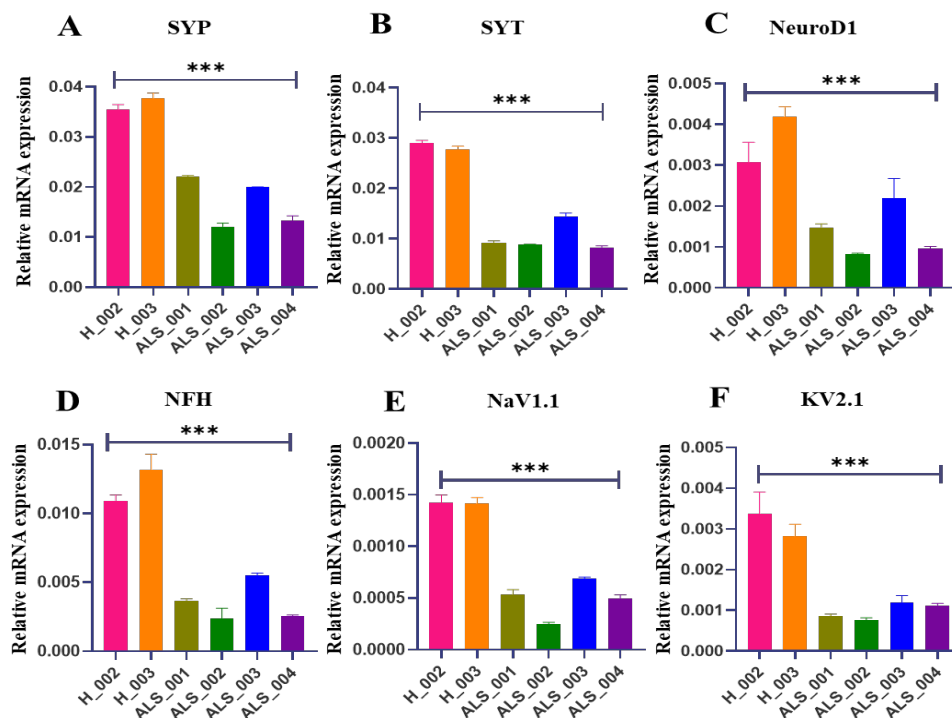


Figure 8. MNs characterization.

Quantification of the expression of neural markers to assess the maturation degree at the end of differentiation. Gene expression was evaluated by qPCR. All differentiations were performed in two independent experiments. All measurements are obtained in two biological replicates: each of them represented by technical triplicate. The values reported by graphs are the result of the average of $2^{-\Delta C_t}$ derived from both the technical triplicates. Data are presented as mean $\pm$ SD. Statistical analysis was performed using one-way ANOVA. (***) $p < 0.001$. **A)** Relative mRNA expression of SYP gene. **B)** Relative mRNA expression of SYT gene. **C)** Relative mRNA expression of NeuroD1 gene. **D)** Relative mRNA expression of NFH gene. **E)** Relative mRNA expression of NaV1.1 gene. **F)** Relative mRNA expression of KV2.1 gene

Discussion

ALS is a complex and multifactorial neurodegenerative disease characterized by the progressive loss of UMNs and LMNs; however, many advances have been made to better understand the molecular and cellular mechanisms underlying the disease. Unfortunately, many of these mechanisms remain unknown, preventing us from gaining a more specific picture of the disease and developing new therapeutic strategies.

In this context, iPSCs represent a useful cellular model that allows us to model *in vitro* the patient-specific disease. For this aim we generated iPSC from healthy donors and four ALS patients carrying different mutations and by transcriptomic analysis we identified common traits among the different mutations, such GO terms directly related to the role of the specific mutated genes. Subsequently, a comparative and integrative analysis allowed us to identify pathways shared by at least three patients. Among these common pathways, we have mostly focused on those related to cell-cell adhesion (homophilic cell adhesion via plasma membrane adhesion molecules (GO:0007156) and cell adhesion via plasma membrane adhesion molecules (GO:0098609), suggesting a dysfunction of adhesion molecules that can consequently lead to an alteration of the process of neural differentiation, maturation, and synaptic organization. Regardless of the individual mutations, we also highlighted a broad involvement in the pathogenesis of ALS of the ECM, in particular of the GO terms related to the ECM organization and the NABA matrisome. The consistent enrichment of genes in the ECM pathway in different models supports the notion that dysregulation of this pathway is a critical factor in disease pathology [159]. Disruptions in ECM integrity may lead to harmful remodeling in the pathological state, reinforcing the idea that the ECM pathway plays a significant role in the progression of neurodegenerative conditions in ALS.

Subsequently, by transcriptomic analysis of iPSC-derived neurospheres, we focused on the molecular deficits that could be present at early stages of neurogenesis, evaluating the GO terms that are specifically involved in neural processes. Indeed, we highlighted a down regulation of the terms related to axiogenesis, synaptic membrane formation, regulation of neuron differentiation, axon development, and neuron projection development. To further investigate the impact of the observed transcriptional variations on iPSCs-derived NPCs morpho-functionality, we performed an *in vitro* neurite outgrowth assay, and we analyzed the number of branches, the number of branch points, and the circuitry. All these parameters provide important information on the extension and spatial complexity of the neural network, reflecting the capacity of NPCs to form functional connections between neurons. ALS iPSCs-derived NPCs showed lower values for all the parameters taken into analysis compared to healthy iPSCs-derived NPCs, confirming their lower ability to generate a

functional neural network and therefore suggesting that the defects of the NPCs can have a long-term impact on neural maturation and functionality.

Interestingly, in TARDBP iPSCs-derived NPCs some of these altered parameters correlated with our transcriptomic data showing a downregulation of specific genes and pathways. In detail, in TARDBP iPSCs-derived NPCs, a compromised neurite outgrowth was observed, correlating with the downregulation of PHOX2B, a transcription factor necessary for axonal growth and associated with a reduced length of neurites [160] and the downregulation of pathways involved in neuronal development, such as "axon development" and "neuron projection development".

To further investigate whether the functional alterations observed in iPSCs-derived NPCs were also present in MNs, we differentiated healthy and ALS iPSCs into MNs. This was made possible through the collaboration with the Norwegian Core Facility for Stem Cells at the University of Oslo, ensuring access to advanced expertise and resources for robust and reproducible differentiation.

Morpho-functional characterization of obtained MNs showed significant differences in MNs density, length and diameter between healthy and ALS MNs with a lower ability of ALS MNs to form a complete axonal network. Interestingly, by comparing diseased MNs with each other, we observed that in C9orf MNs the density of the neural network and the extension of the axons from one soma to another, is higher than the other patients. These data are in line with the studies of Mehta et al. which demonstrated that C9orf72 iPSC-derived MNs showed defects in axonal length attributed to mitochondrial dysfunction, which determined a reduced energy availability, hindering normal growth [161]. Low levels of cell density, length and diameter were also found in KIF5A iPSCs-derived MNs. KIF5A mutation specifically causes a loss of autoinhibition, thus generating a constitutively active protein, impairing correct axonal transport [162]. The observed morphological alterations, therefore, could reflect a compromised growth capacity due to a defect in axonal transport mechanisms.

After verification of morphological differences between healthy and ALS iPSCs-derived MNs, we performed a more in-depth analysis at the level of gene and protein expression to determine if obtained cells had an adequate level of maturation. Through immunofluorescence we evaluated the expression of ChAT, a mature MN-specific marker, TUB β 3 and MAP2, essential for the stability of the neuronal cytoskeleton, demonstrating that were expressed in obtained MNs, confirming the achievement of neuronal maturation. In addition, the quantification of ChAT fluorescence provided an accurate estimation of Chat-positive MNs within the total iPSC-derived neuronal population. This approach, based on normalization of fluorescence signals to brightfield-acquired images, consistently reflects the degree of cholinergic differentiation. Nevertheless, the identification of other neuronal subtypes that may also be present within the cultures cannot be excluded. A more precise

characterization would require the use of double immunostaining with subtype-specific markers to distinguish between MNs and other neuronal populations.

Gene expression analysis allowed us to evaluate the profile of key genes associated with MN maturation and functionality. The obtained results showed a higher expression of most of the markers in healthy MNs, suggesting that probably, ALS mutations are also involved in MNs maturation.

In particular, the SYP and SYT1 genes, involved in synapse formation and function, were expressed at significantly higher levels in healthy MNs compared to ALS ones. This finding suggests that MNs derived from healthy controls reached a more advanced state of synaptic maturation. The reduction of NeuroD1 expression, a transcription factor essential for neurogenesis and neuronal maturation, in ALS MNs probably is related to an arrest or delay in neuronal maturation potentially linked to pathology. Similarly, the marked expression in healthy MNs of NFH, a component of axon cytoskeleton, suggests a good level of MNs maturation. Low NFH levels in the ALS group may be indicative of abnormal accumulation or fragmentation of neuro filaments, which may be related to the disease.

Finally, the ion channels Nav1.1 and Kv2.1, essential for the generation and propagation of the action potential, are more expressed in healthy MNs, confirming a presumably more efficient electrical functionality. The reduction observed in ALS MNs suggests a potential deficit in electrophysiological maturation or in the capacity for synaptic transmission that can be validated with future functional studies. Although the values obtained from the analysis of the main markers are not sufficiently high in all lines, it is important to underline the fact that the final outcome may depend on intrinsic factors of the line, genetic variability and intrinsic epigenetic factors.

In conclusion, this study supports the use of iPSCs as a valuable model to investigate ALS mechanisms by capturing patient-specific disease features. Due to the limited size of sample, this work can be considered a pilot study that provides important insights into how different mutations can lead to diverse molecular and phenotypic outcomes.

A relevant example is represented by patients ALS_002 and ALS_004, who share the same TARDBP mutation but exhibit distinct form of ALS, bulbar and spinal, respectively. While some transcriptomic patterns were shared, others diverged, suggesting that the same mutation can lead to variable molecular and phenotypic outcomes. This highlights the concept of an intra-mutational heterogeneity which may be influenced not only by the mutation and the individual genetic background but may be also the result of a patient-specific epigenetic mechanisms. Future studies investigating DNA methylation, histone modifications, and miRNA-mediated regulation, already known as implicated in the formation of pathological protein aggregates in ALS, could help to clarify how these factors contribute to distinct transcriptional and phenotypic profiles, even in the presence of a shared genetic

mutation. Overall, the transcriptomic analysis has enabled the identification of early transcriptional alterations particularly in cell adhesion genes while highlighting ECM-related pathways as a shared feature across different mutations. The observed downregulation of neuronal pathways in neurospheres, which is partially reflected at the functional level, suggests an early impairment of NPCs. Furthermore, the establishment of a robust differentiation protocol for NPCs into mature MNs provides a more refined and disease-relevant cellular model, advancing our understanding of ALS molecular mechanisms and potentially informing therapeutic strategies.

Endothelial Dysfunction Project

During the PhD I worked also on the role of coagulation FVIII beside coagulation using primary and iPSCs-derived ECs showing that FVIII is involved in the maintenance of hemophilic endothelial cells.

1 Endothelial Dysfunction

Endothelial dysfunction (ED) is a multifactorial alteration of vascular homeostasis, caused by a disruption of the endothelium ability to control vascular tone, permeability, cellular adhesion and inflammatory reactions [163,164]. Vascular homeostasis is maintained by a balance between endothelium-derived vasodilators and vasoconstrictors factors [165,166].

Disruption of this balance underlies overproduction of ROS and consequently oxidative stress [167], impaired nitric oxide (NO) bioavailability, and dysfunctions in vasoregulation, coagulation, and inflammation mechanisms [168].

This condition results in pro-inflammatory endothelial activation, characterized by upregulation of cytokines interleukin 1 beta (IL-1 β), interleukin 6 (IL-6), tumor necrosis factor alpha (TNF- α), and adhesion molecules vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1), worsening endothelial damage [169,170]. Alterations in blood flow, specifically reduction in physiological shear stress, also significantly affect endothelial function. Under normal conditions, laminar flow allows expression of anti-inflammatory and antithrombotic gene properties [171]. However, the presence of oscillatory flows induces mechanosensitive signals that alter the structure of the cytoskeleton, damage the endothelial glycocalyx, and compromise the integrity of intercellular junctions. This series of events influences vascular permeability and favors the migration of immune cells [172]. ECM is another key element that ensures the integrity of the vasculature, provides structural support and modulates regulatory signals affecting cytoskeletal organization and endothelial cell (EC) behavior, including adhesion, migration, and proliferation [173]. Alterations in ECM composition or structure, or modifications of cytoskeletal proteins such as vinculin, can impair endothelial function. Therefore, considering the crucial role of ED in the pathogenesis of most chronic diseases like cardiovascular, metabolic and neurodegenerative disease, it appears ever more relevant to investigate its implications in less studied contexts, like Hemophilia A, where vascular alterations may be important in the clinical picture of the disease [174,175].

2 Hemophilia A and Endothelial Dysfunction

Hemophilia is a rare genetic disorder characterized by a congenital deficiency of specific clotting factors, leading to an impairment of body hemostasis and an increased tendency to bleed [176,177]. FVIII gene (F8) is located on the X chromosome and when mutated, causes the X-linked disease of hemophilia A. The reduction or lack of plasma FVIII causes prolonged bleedings, either spontaneously or secondary to trauma, with the clinical severity proportional to the degree of FVIII reduction. To date, there is no definitive cure for HA, with the current therapeutic practice being a replacement therapy of recombinant human FVIII (rhFVIII) delivered by intravenous injections on an as needs basis to treat acute hemorrhages or to prevent bleeding events [178]. Alongside clotting factor advancements, another class of drugs called non-replacement therapy is emerging to overcome the difficulties of intravenous delivery and to improve the effectiveness of therapies in all patients, regardless of the inhibitors formation [179].

There are other important pathological conditions associated with FVIII deficiency, such as cartilage degeneration, bone remodeling, renal and cardiovascular diseases, and spontaneous intracranial hemorrhages. Approximately 3 to 5% of severe HA patients develop intracranial hemorrhages (ICH) in the perinatal period, with trauma the major causative factor [180].

Hemophilia A is also associated with chronic pain, joint impairment, and a reduced quality of life starting from an early age. Due to recurrent joint bleeding, one of the main and common long-term complications is the development of hemophilic arthropathy, although the pathological mechanism underlying its development is not yet fully understood [181–183]. The mechanism is multifactorial and vascular development and angiogenesis are an essential component of blood-induced joint disease. Recently, it was demonstrated an attenuated microvascular endothelial functionality in hemophilic patients and altered collagen levels in the plasma of HA patients, suggesting a dysfunction in ECs [184,185]. Parallel to ED, ECM degradation has been documented in HA patients, as demonstrated by elevated plasma levels of collagen XVIII, PRO-C4, C4M, and PRO-C8 [186,187]. These alterations suggest a compromised structural integrity of the vascular wall, which may further exacerbate vascular fragility and contribute to the increased susceptibility to joint bleeding and the progression of hemophilic arthropathy[186,187]. All this evidence suggests that the bleeding manifestations that characterize HA are not only linked to a coagulation deficit but also to an alteration of endothelial homeostasis which consequently leads to an alteration of vascular integrity.

3 Primary endothelial cells to study ED in Haemophilia A

ECs are the main FVIII producers, however, an association between endothelial fragility and the absence of or low activity of FVIII has never been explored [188–190]. Further, very little is known regarding the differences in the genetic profile between healthy and HA ECs. Healthy and HA ECs can be isolated from the peripheral blood, as blood outgrowth endothelial cells (BOECs), or can be differentiated from induced pluripotent stem cells (iPSCs), reprogrammed from circulating CD34⁺ cells [123,191]. Both EC models, once transduced with a lentiviral vector (LV) carrying the B domain deleted (BDD) form of coagulation FVIII (LV-FVIII) and implanted into a pre-vascularized medical device, or in association with microcarrier beads, were able to correct the bleeding phenotype of HA mice. Therefore, based on the clinical observation of a general endothelial fragility in HA patients, in our last published work we investigated the role of FVIII in the maintenance of EC stability by evaluating the differences in the functionality and in the transcriptomic profile of healthy, HA, and LV-FVIII-transduced HA ECs. BOECs have been used as cellular model to study the role of FVIII in the maintenance of ECs permeability, migration, and tubulogenesis. Specifically, our results demonstrate that FVIII promotes the activation of the Focal Adhesion Kinase (FAK/Src) signaling cascade, resulting in the modulation of ECM-associated gene expression, including that of Nidogen-2.

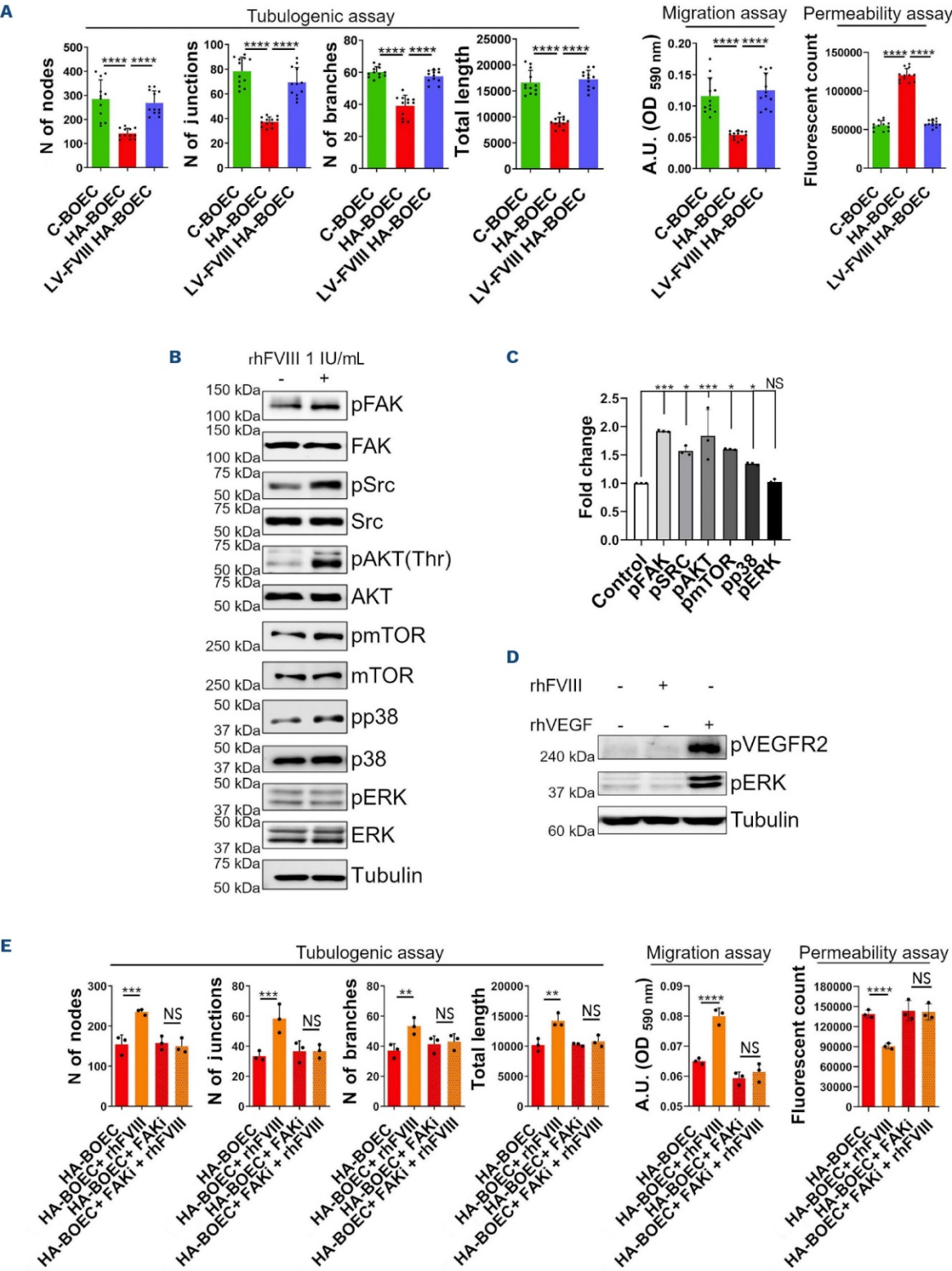
Factor VIII promotes angiogenesis and vessel stability regulating extracellular matrix proteins

Factor VIII (FVIII) is a crucial coagulation co-factor and mutations in the *F8* gene lead to hemophilia A (HA). Reduced activity of FVIII or its absence results in bleeding episodes, which can occur either spontaneously or secondary to trauma.¹ To date, there is no definitive cure for HA. The standard therapy involves prophylaxis with either replacement products, such as recombinant human FVIII (rhFVIII), or non-substitutive treatment as a gold standard for the severe cases to prevent bleeding events.² Besides general bleeding complications, HA patients have been recently reported to have attenuated microvascular endothelial function and altered plasma collagen level, suggesting a dysfunction in endothelial cells (EC).^{3,4} Endothelial dysfunction in these patients is evidenced by decreased flow-mediated dilation and reduced hyperemic velocity time integral (VTI) compared to those in healthy controls.^{5,6} Furthermore, studies involving FVIII-deficient mice have revealed substantial alterations in joint vascular remodeling and increased synovial vascular permeability following induction of hemarthrosis.⁷⁻⁹ This suggests a non-physiological angiogenic response that may exacerbate the severity of the bleeding episodes and the vulnerability of the vascular system in the absence of FVIII. Overall, these findings suggest that hemorrhagic events in HA patients might result not only from impaired clotting but also from vascular abnormalities, indicating the development of an endothelial dysfunction in these patients. Extensive research has established that EC are a primary source of FVIII, particularly liver sinusoidal EC¹⁰ while the specific role of FVIII in EC physiology remains poorly described. Therefore, we investigated the role of FVIII in EC function using blood outgrowth endothelial cells (BOEC), an optimal model for studying EC biology and for the development of therapeutic cell and gene therapy strategies.¹¹ In particular, BOEC obtained from patients with severe HA (HA-BOEC) displayed defective endothelial function, which was reversed by FVIII treatment. Consistent with this, FVIII was also required *in vivo* for proper angiogenesis and preservation of vessel integrity in a HA mouse model. Mechanistically, we found that FVIII induces EC signaling activating the focal adhesion kinase (FAK)/SRC proto-oncogene (Src) pathway regulating the expression of genes related to the basement membrane and the extracellular matrix (ECM). Overall, these data identify FVIII as a player in the control of vessel stability.

First, we demonstrated that, when cultured on Matrigel®, HA-BOEC formed a sparse and incomplete vascular network compared to BOEC derived from healthy donors (C-BOEC), which developed a complete and stable vascular network (Figure 1A). The functional impairment of HA-BOEC was also

evident in migration (Figure 1A) and permeability (Figure 1A) assays. Notably, HA-BOEC transduced with a lentiviral vector (LV) carrying the *F8* transgene (LV-FVIII) (LV-FVIII HA-BOEC) showed significantly improved tubule network formation, migration, and permeability, akin to the results seen in C-BOEC (Figure 1A). To determine whether acute treatment of BOEC with rhFVIII would also improve some EC functions, we challenged both C-BOEC and HA-BOEC with several doses of rhFVIII (5 IU/mL, 1 IU/mL, 0.5 IU/mL, 0.2 IU/mL and 0.1 IU/mL). Remarkably, while HA-BOEC evidenced marked improvements in tubule formation, migration, and permeability starting from a rhFVIII concentration of 0.2 IU/mL, C-BOEC showed no further increase in functionality following rhFVIII treatment (*Online Supplementary Figure S1A-D*), suggesting that the endogenous FVIII secretion by BOEC themselves is sufficient to guarantee their activity. We therefore hypothesized that FVIII could trigger a signaling pathway in EC which modulates angiogenesis as well as migration and permeability. Indeed, we found that rhFVIII induced the phosphorylation of FAK and Src along with the downstream targets AKT, mTOR, and p38, but not ERK (Figure 1B, C). This signaling cascade is widely recognized to control EC function upon several stimuli. However, the lack of vascular endothelial growth factor receptor 2 (VEGFR2) and ERK phosphorylation upon rhFVIII stimulation (Figure 1D) suggests that FVIII induces a different pathway compared to the classical one triggered by VEGF. Moreover, by comparing HA-BOEC *versus* C-BOEC, it was evident that the above-mentioned signaling pathway is activated in untreated C-BOEC (*Online Supplementary Figure S1E*), suggesting that FVIII can act as an autocrine factor.

To confirm that FVIII controls EC functions through FAK activation, we performed tubulogenesis, migration, and permeability assays on HA-BOEC treated first with defactinib, an inhibitor of FAK, and then exposed to rhFVIII. We observed that FAK inhibitor treatment significantly disrupted the FVIII-dependent response of HA-BOEC (Figure 1E). When transferred *in vivo* into an immunodeficient mouse model of HA, NOD-*scid* IL2Rg^{null} (NSG) (NSG-HA), C-BOEC expressing green fluorescent protein and embedded in Matrigel® plugs formed well-organized vascular structures while HA-BOEC exhibited a significant deficiency in vessel formation (Figure 2A). This impairment was effectively rescued by either transducing HA-BOEC with LV-FVIII or supplementing rhFVIII into the Matrigel® plugs (Figure 2A). Quantification of vessel density and diameter confirmed that both LV-FVIII transduction and rhFVIII treatment markedly improved the ability of HA-BOEC to form well-organized vessels in Matrigel® plugs implanted in HA mice (Figure 2B).



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Figure 1. Factor VIII restores the defective function of hemophilia A blood outgrowth endothelial cells through the focal adhesion kinase/SRC proto-oncogene pathway involvement. (A) *Tubulogenic assay.* Quantitative analysis of the number of nodes, junctions, branches, and total length of tubule networks using control (C)-blood outgrowth endothelial cells (BOEC) (N=4), hemophilia A (HA)-BOEC (N=4), or HA-BOEC transduced with a lentiviral vector (LV) carrying the *F8* transgene (LV-FVIII HA-BOEC) (N=4). The BOEC (5x10⁴) were placed on top of the Matrigel® and incubated overnight. ImageJ Angiogenesis software was used to quantify the number of nodes, junctions, branches, and total length of acquired images. *Migration assay.* Indirect measurement of cell migration through crystal violet staining elution of BOEC. BOEC were plated into the upper compartment of an 8-µm pore size Transwell at a density of 10⁵ cells per well in serum-free medium, while the lower compartment was filled with complete medium and incubated overnight. Migrated cells were stained with crystal violet that was eluted with acetic acid and quantified using a Victor spectrophotometer at 590 nm. *Permeability assay.* Permeability was measured by the extravasation of FITC-dextran across a monolayer of BOEC (8x10⁴ cells/well) cultured on a 0.1% gelatin-coated Transwell (3 µm pore) until confluence was reached. At the end of the culture, 5 µg/mL of FITC-conjugated 40 kDa dextran was added to the upper chamber and the fluorescence of the lower chamber was measured in the medium using the Victor spectrophotometer (490 nm [excitation]/520 nm [emission]). Fluorescence readings were normalized to dextran permeability in Transwell inserts without cells. (B) Western blot analysis of whole cell lysate from HA-BOEC treated for 15 min with or without 1 IU/mL of B-domain-deleted recombinant human factor VIII (rhFVIII) and stained with antibodies against pFAK, total FAK, pSrc, total Src, pAKT, total AKT, pmTOR, total mTOR, pp38, total p38, pERK, and total ERK. Tubulin was used as a loading control. (C) Quantification of increased phosphorylation of FAK, Src, AKT, mTOR, and p38 expressed as fold-change relative to untreated control. (D) Western blot analysis of whole cell lysate from HA-BOEC incubated for 15 min in the presence or absence of rhFVIII (1 IU/mL) or rhVEGF (50 ng/mL) and stained with antibody against phosphor vascular endothelial growth factor receptor 2 (VEGFR2) and pERK. Tubulin was used as a loading control. (E) Quantitative analysis of the number of nodes, junctions, branches, and total length of tubule networks performed on untreated HA-BOEC or HA-BOEC treated with 1 µM FAK inhibitor. All cells were incubated in the presence or absence of 1 IU/mL of B-domain-deleted rhFVIII overnight. Indirect measurement of cell migration by elution of crystal violet staining in HA-BOEC. Permeability assay results calculated on the extravasation of FITC-dextran through an intact monolayer of BOEC. Data in (A) and (E) are expressed as mean ± standard deviation. Statistical analysis was performed by a one-way analysis of variance test (****P<0.0001; ***P<0.001; **P<0.01; *P<0.05, NS: not significant). A.U.: arbitrary units; OD: optical density; FAKi: focal adhesion kinase inhibitor.

To further investigate *in vivo* the role of FVIII in angiogenesis, we assessed the vessel formation potential of murine EC in adult NSG versus NSG-HA mice into which Matrigel® plugs were implanted intradermally. In NSG mice, EC, identified by murine CD31 expression, built a well-organized vessel network, stabilized by α-smooth muscle actin pericytes (Figure 2C). Conversely, NSG-HA EC formed smaller and more disorganized vessels. The injection of NSG-HA mice with either 5x10⁸ transduction units/mouse of LV-FVIII or 2 IU of rhFVIII (every 2 days) promoted the formation of larger and more stable vessels (Figure 2C). The quantification of their density and diameter further indicated that FVIII contributes to *in vivo* angiogenesis in FVIII-deficient mice (Figure 2D).

Vessel permeability was evaluated *in vivo* by intravenous injection of Evans blue, an albumin-binding dye, into NSG versus NSG-HA mice. Under physiological conditions the endothelium is impermeable to albumin, so the Evans blue is retained within the blood vessels, while increased EC permeability of NSG-HA mice was demonstrated by Evans blue extravasation into the interstitial tissue (Figure 2E). Intriguingly, both LV-FVIII and rhFVIII (4 IU every 2 days for 20 days) were associated with a marked reduction in dye extravasation (Figure 2E). Taken together, these results indicate that FVIII plays a crucial role in angiogenesis and in the maintenance of vessel integrity.

To investigate the impact of FVIII on the EC transcriptome, we performed RNA sequencing, comparing HA-BOEC to C-BOEC, and LV-FVIII HA-BOEC to HA-BOEC (Figure 3A). A volcano plot revealed that 215 genes were upregulated and 155 genes downmodulated in HA-BOEC versus C-BOEC (Figure 3B), with the latter including predominantly genes

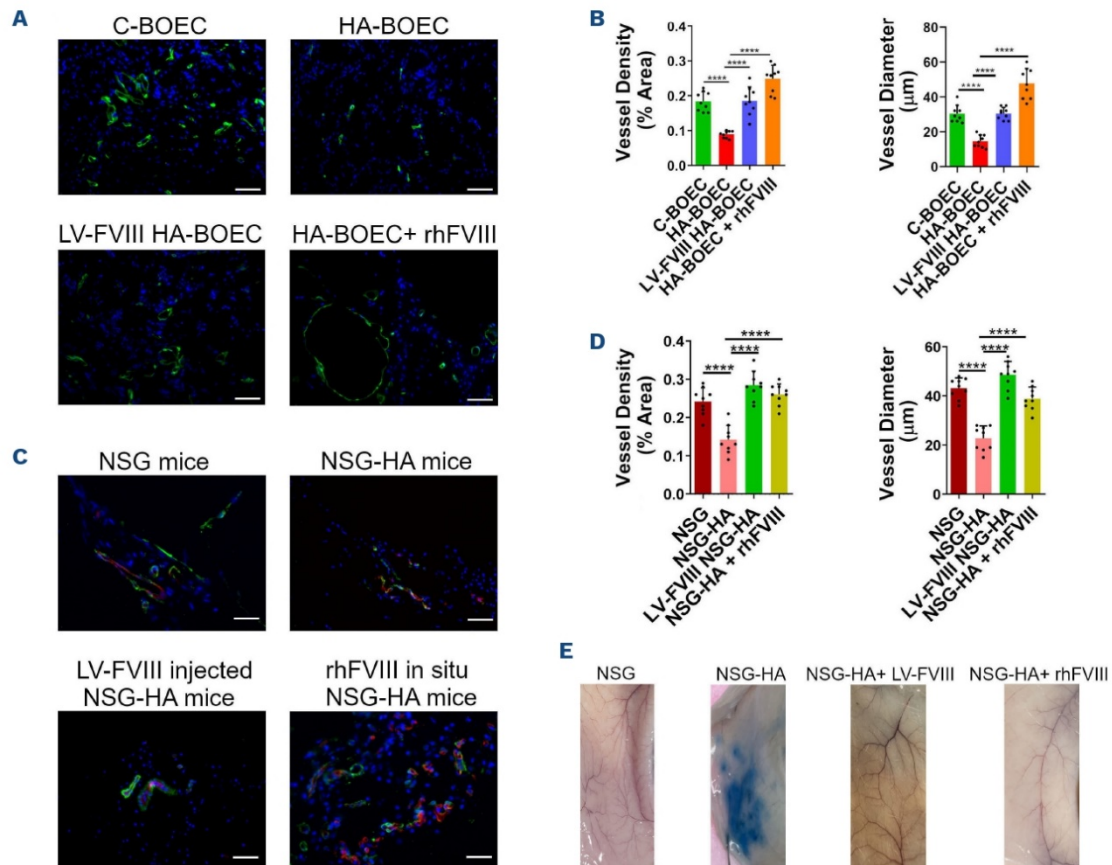
involved in ECM composition, such as the genes encoding collagen4a1 (*COL4A1*), nidogen2 (*NID2*), fibulin1 (*FBN1*), and peroxidasin (*PXDN*). Importantly, the expression level of most of these genes was significantly restored when FVIII was reintroduced into HA-BOEC (Figure 3A, C). Supporting our *in vitro* and *in vivo* findings, gene set enrichment analysis and gene ontology process analysis of differentially expressed genes identified pathways corresponding to vascular development, cell migration, regulation of cell adhesion, ECM organization, and integrin cell surface interactions. These pathways were downregulated in HA-BOEC versus C-BOEC and rescued by LV-FVIII transduction (*Online Supplementary Figure S2A, B*).

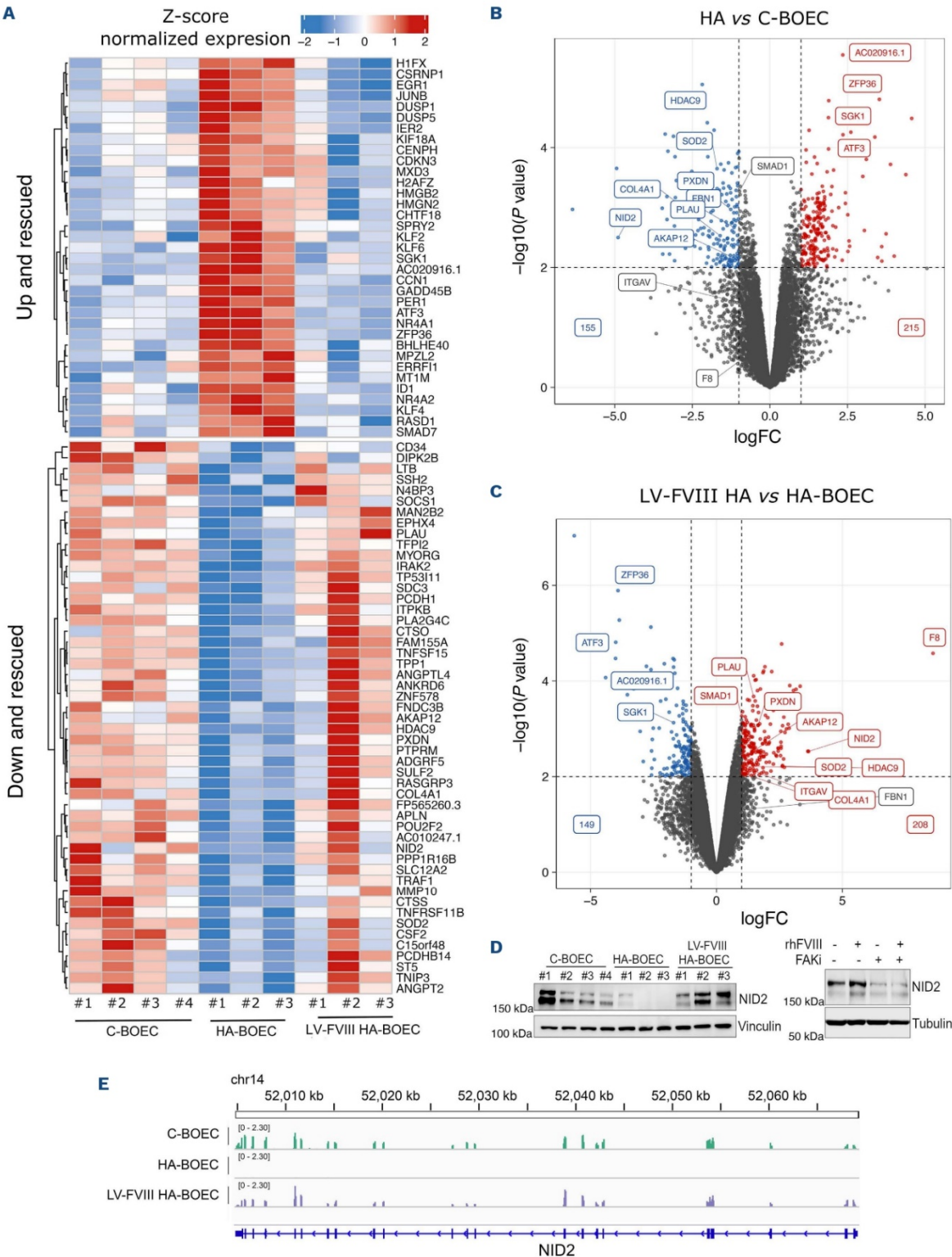
Finally, to gain deeper mechanistic insights into the role of FVIII in EC we focused on NID2, a glycoprotein critical for endothelial basement membrane stability, since it was one of the most significantly downregulated genes in HA-BOEC and was rescued by LV-mediated transduction of FVIII (Figure 3D, E). Importantly, FAK inhibition in EC treated with rhFVIII prevented a FVIII-induced increase of NID2 (Figure 3D, right panel). We performed a complementation assay by ectopically expressing NID2 in HA-BOEC (*Online Supplementary Figure S3A*), which led to the formation of stable tubule networks in Matrigel®, enhanced their migration capabilities and, remarkably, restored barrier integrity to levels comparable to those seen in C-BOEC (*Online Supplementary Figure S3B*). NID2 knockdown by shRNA in C-BOEC (*Online Supplementary Figure S3C*) significantly impaired EC tubulogenesis, migration and increased EC permeability (*Online Supplementary Figure S3D*). Treatment with rhFVIII failed to restore the impaired functions in these NID2-deficient EC (*Online Supplementary Figure*

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S3D), confirming that NID2 is required for FVIII-mediated regulation of fundamental EC activity. Taken together, our findings indicate that BOEC from HA

patients have impaired endothelial function, which is compensated by FVIII treatment. One of the main FVIII partners in coagulation is von Willebrand





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Figure 3. Factor VIII regulates the expression of genes related to the extracellular matrix such as nidogen 2 (*NID2*). (A) Heatmap showing the expression pattern of genes modulated by factor VIII (FVIII). The upper panel shows upregulated genes in hemophilia A (HA) blood outgrowth endothelial cells (BOEC) versus control BOEC (C-BOEC) and rescued in HA-BOEC transduced with a lentiviral vector (LV) carrying the *F8* transgene (LV-FVIII HA-BOEC) and vice versa in the lower panel. (B, C) Volcano plots showing differentially expressed genes in HA-BOEC versus C-BOEC (B) and LV-FVIII HA-BOEC versus HA-BOEC (C). Genes are classified as upregulated if they have a log-fold change >1 and *P* value <0.01, and as downregulated if they have a log-fold change <-1 and *P* value <0.01. (D) Left panel. Western blot analysis of *NID2* expression levels in C-BOEC (N=4), HA-BOEC (n=3), and LV-FVIII HA-BOEC (N=3). An anti-vinculin antibody was used to confirm equal loading. Right panel. Western blot analysis of *NID2* expression in HA-BOEC treated or not with B-domain-deleted recombinant human FVIII (rhFVIII) in the presence or absence of 1 μ M defactinib (a FAK inhibitor) for 48 h. An anti-tubulin antibody was used to confirm equal loading. (E) Genome browser view showing the average RNA-sequencing signal profile of the *NID2* gene in C-BOEC (N=4), HA-BOEC (N=3), and LV-FVIII HA-BOEC (N=3). RNA sequencing was performed in the Illumina sequencer NextSeq 500. Sequencing reads were aligned to the human reference genome (version GRCh38.p13) using STAR v2.7.7a0 5 (with parameters -outFilterMismatchNmax 999 -outFilterMismatchNoverLmax 0.04) and providing a list of known splice sites extracted from GENCODE comprehensive annotation (version 32). Expression counts were then analyzed using the edgeR package.

factor (VWF), reported to exert both pro- and anti-angiogenic effects. Indeed, reduced VWF expression in EC increased the cells' migration, proliferation, and angiogenesis *in vitro*. In a similar way, mice lacking VWF display elevated vessel formation and a large vascular network in the ear.¹² Although its mechanism of action in regulating vessel formation is yet not fully understood, VWF has been suggested to bind and recruit several angiogenic growth factors to the cell membrane thanks to its heparin-binding domain.¹³ Interestingly, EC lacking FVIII display the opposite behavior, showing impaired function. However, the mechanism of FVIII/VWF regulation in EC biology requires further investigation.

Through the activation of the FAK/Src pathway, FVIII regulates the expression of genes related to the structure of the ECM, which is known to play a critical role in maintaining the integrity of blood vessel structure, regulating EC migration and angiogenesis, and the formation of new blood vessels.^{14,15} The link found here between FVIII and regulation of ECM genes is concordant with the recent clinical findings of a reduction of plasma collagen level during hemarthrosis⁸ and the elevated plasma levels of collagen XVIII in HA patients correlating with higher annual bleeding rates.⁴ This evidence from the literature and our findings support the hypothesis that HA EC have significant deficits in ECM components, which can be mitigated by FVIII treatment. Moreover, it has been previously shown that *NID2* is crucial for endothelial basement membrane integrity, largely due to its interaction with collagen IV, laminins, and perlecan. Recent studies have further identified the role of *NID2* in regulating the EC phenotype, revealing its significant impact on ECM organization.^{16,17} Thus, the observed reduction in *NID2* expression in EC isolated from patients with severe HA may contribute to ECM impairment. Our results suggest that FVIII replacement therapy is not only fundamental to treat coagulation problems, but it might be helpful in maintaining the vascular integrity of HA patients by targeting several genes, such as *NID2*, involved in ECM stability.

From a clinical standpoint, these findings suggest that FVIII replacement therapy for HA patients could offer benefits that go beyond bleeding prevention, such as preservation of vascular health and resilience. This new perspective on

FVIII functions paves the way for future research aimed at developing more multidisciplinary approaches for treating HA, targeting both coagulation deficiencies and vascular stability.

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AF has made an international patent application (Number PCT/IB2017/054574. Title: Method for inducing and differentiating pluripotent stem cells and uses thereof). The other authors have no conflicts of interest to disclose.

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Contributions

CO, AC, and SA designed and performed the experiments and analyzed the data. CS, GW, and SM performed the experiments and analyzed the data. CB conducted the *in vivo* studies. IM, FA, and AL designed and performed the RNA-sequencing transcriptomic analysis. AF conceived the study. AF and SO generated funding, analyzed data and supervised the project. CO, AC, SA, AF, and SO wrote the manuscript, who was revised by all authors and approved the final version.

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Data-sharing statement

For original data, please contact the authors for correspondence.

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