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Disease-Modifying Therapies in Amyotrophic Lateral Sclerosis

Adriano Chiò,[a,b,c] Letizia Mazzini,[d,e] Gabriele Mora[f]

[a]ALS center, 'Rita Levi Montalcini' Department of Neuroscience, University of Torino, Turin, Italy

[b]Azienda Ospedaliero-Universitaria Città della Salute e della Scienza di Torino, Turin, Italy

[c]Institute of Science and Technology of Cognition, National Council of Research, Rome, Italy

[d] Department of Neurology, Università del Piemonte Orientale, Novara, Italy

[e]ALS Center, Azienda Ospedaliero-Universitaria 'Maggiore della Carità' Novara, Italy

[f]ALS Center, Istituti Clinici Scientifici Maugeri, IRCCS Milano, Milan, Italy

Corresponding author: Adriano Chiò, 'Rita Levi Montalcini' Department of Neuroscience, via Charasco 15, 10126 Torino, Italy. Email address: adriano.chio@unito.it

Abstract

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder of adult life, causing weakness and wasting of voluntary muscles, associated in about 50% of cases with a cognitive impairment. Pathologically, the disease is characterized by a degeneration of upper and lower motor neurons. A hallmark of the pathological process is the aggregation of the protein TDP43 in the cytoplasm of affected neurons detected in almost 97% of cases. About 15% of cases has a family history.

Currently, only two drugs have been demonstrated to be effective in ALS, riluzole and edaravone, which show only modest effects on disease progression. The quest for disease-modifying therapies in ALS has several obstacles, the most important being the sub-optimal quality of the design of clinical trials, and the clinical and pathological heterogeneity of the disease.

In this paper the pathological mechanisms relevant to ALS and current and future pharmacological and non-pharmacological trials, including gene and stem cells therapies, will be presented.

Keywords: amyotrophic lateral sclerosis; pathogenic mechanisms; clinical trials; drugs; stem cells

Highlights:

- Amyotrophic lateral sclerosis is a fatal neurodegenerative disease characterized the loss of upper and lower motor neurons and by the impairment of frontal cortices

- No effective disease-modifying therapy for ALS are available, with the only exception of riluzole, an antiexcitotoxic drug, and edaravone, an antioxidant and mitochondria acting agent.

- More than 40 drugs are currently under study as possible therapies for ALS, with several potential mechanisms of action

- Several phase 1 or 2 trials are ongoing on mesenchymal and neural stem cells as possible treatment for ALS

List of abbreviations

AD-MSC	Adipose-derived MSC
ALS	Amyotrophic lateral sclerosis
ALS/PDC	ALS/Parkinson-dementia complex
ALS2	Alsin
ALSFRS-R	ALS functional rating scale revised
AMPA	alpha-amino-3-hidroxy-5-methyl-4-isoxazole-propionic acid
ANG	Angiogenin
ANXA11	Annexin A11
ASO	Antisense oligonucleotide
ATXN2	Ataxin 2
BBB	Brain blood barrier
BMAA	β-N-methylamino-L-alanine
BM-MSC	Bone marrow-derived MSC
C5aR1	C5a receptor
C9orf72	Chromosome 9 open reading frame 72
CCS	Chaperone-for-SOD
CHCHD10	Coiled-coil-helix coil-helix-domain containing protein 10
CHMPB2	Charged multivesicular body protein B2

CNS	Central nervous system
CSF	Cerebrospinal fluid
DCTN1	Dynactin 1
DLK	Dual leucine zipper kinase
DPR	Dipeptide repeat proteins
DSB	Double-strand breaks
EAAT	Excitatory amino-acid transporters
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
ERBB4	Chorion protein gene ErB.4
ESC	Embryonic stem cell
FIG4	Phosphatidylinositol 3,5-bisphosphate 5-phosphatase
FOXP3	Forkhead box P3
FTD	Frontotemporal dementia
FUS	Fused in sarcoma
FVC	Forced vital capacity
G4C2	GGGGCC
GEF	GDP/ GTP exchange factor
GMP	Good manufacturing process

HMN	Hereditary motor neuropathy
HNE	4-hydroxy-2-nonenal
HNRNPA1	Heterogeneous nuclear ribonucloeprotein A1
hNSC	Human neural stem cell
HRE	Hexanucleotide repeat expansion
HRV	Human endogenous retroviruse
IL2	Interleukin 2
IL2RA	IL2 receptor subunit alpha
IL6	Interleukin 6
INSTI	Integrase strand transfer inhibitor
iPSC	Induced Pluripotent Stem Cell
JNK	c-Jun N-terminal kinase
KIF5A	Kinesin family member 5A
MAOB	Monoamine oxidase B
MATR3	Matrin 3
MIF	Macrophage migration inhibitory factor
MiToS	Milano-Torino staging
MN	Motor neuron
MNDA	N-methyl-D-aspartate

MNTF	Motoneuronotrophic factor
MPO	Myeloperoxidase
MSC	Mesenchymal stem cell
mTORC1	Mechanistic target of rapamycin complex 1
NAIP	Neuronal apoptosis inhibitory protein
NEK1	Never in mitosis gene A-related kinase 1
NRTI	Nucleoside reverse transcriptase inhibitors
NSC	Neural stem cell
3-NT	3-nitrotyrosine
NTF	Neurotrophic factor
OPTN	Optineurin
8-oxoG	8-Oxo-deoxyguanosine
PB	Phenylbutyrate
PBMC	Peripheral blood mononuclear cells
PEG	Percutaneous enteral gastrostomy
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
PET	Positron emission tomography
PFN1	Profilin 1
PINK1	PTEN-induced kinase 1

RAN	Repeat-associated non-AUG-dependent
RBP	RNA-binding protein
RCT	Randomized controlled trial
RIPK1	Receptor-interacting serine/threonine-protein kinase 1
RISC	RNA-induced silencing complex
RNP	Ribonucleoprotein
ROCK	Rho kinase
ROS	Reactive oxygen species
SCF	Stem cell factor
SETX	Senataxin
SG	Stress granule
SIGMAR1	Sigmar nonopioid intracellular receptor 1
siRNA	Small interfering RNA
SMA	Spinal muscular atrophy
SMAT	Spliceosome-mediate RNA trans-splicing
SOD1	Cu-Zn Superoxide dismutase 1
SPG11	Spastacin
SQSTM1	Sequestosome 1
SSB	Dingle-strand breaks

TARDBP	TAR DNA-binding protein 43
TBK1	TANK-binding kinase 1
TDP43	Transactive response DNA binding protein 43
Tregs	Regulatory T-cells
TUBA4A	Tubulin alfa 4A
TUDCA	Tauroursodeoxycholic acid
UBQLN2	Ubiquilin 2
UNC13A	Unc-13 homolog A
VAPB	Vesicle associated membrane protein associated protein B
VCP	Valosin Containing Protein
VPA	Valproic acid
WJ-MSC	Wharton´s Jelly-derived MSC
XPO1/CRM1	Exportin-1

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder of the adult life, characterized by a progressive loss of cortical and spinal motor neuron, at bulbar and spinal level, causing the weakness and wasting of muscle of limbs, chest, neck, and oropharyngeal area, associated with pyramidal signs. In about 50% of cases patients also present a cognitive impairment ranging from dysexecutive impairment to frontotemporal dementia (FTD), due to the involvement of prefrontal cortices; therefore ALS and FTD can be considered part of a pathological spectrum. The disease is invariably fatal, within 2 to 5 years from onset; the death is usually due to respiratory failure(van Es et al., 2017).

About 15% of cases have a family history for ALS or FTD, while the remaining present with an apparently sporadic disease. More than 30 genes have been related to ALS, the most common being *C9orf72, SOD1, TARDBP* and *FUS* (Chia et al., 2018).

Pathologically, the disease is characterized by a selective degeneration of motor pathways, with the death of upper and lower motor neurons. A hallmark of the pathological process is the aggregation of the protein TDP43 in the cytoplasm of affected neurons detected in almost 97% of cases; the only exceptions are the patients carrying mutations of *SOD1* and *FUS* genes, in whom a cytoplasmic accumulation of SOD1 and FUS protein, respectively, is seen.

Currently, only two drugs have been demonstrated to be effective in ALS, namely riluzole, marketed in 1996, and edaravone, marketed in 2016 in several Asian countries, in USA and Canada and in Switzerland, but not yet in the European Union. Riluzole acts principally, though not exclusively, as an anti-excitotoxic drug, while edaravone main activity is as antioxidant. It is generally recognized that there is an urgent need for effective therapies in this devastating disease, and although the pacing of clinical trials in ALS is accelerating, positive results are still lacking. After 1980 more than 80 RCTs on ALS have been published(Petrov et al., 2017), mostly with

negative results. However, these RCTs may be relevant for improving the design of future trials (see section Lessons for previous clinical trials).

The quest for a disease-modifying therapy in ALS has several obstacles, the most important being the sub-optimal quality of the design of clinical trials, the heterogeneity of the clinical picture of the disease, and the heterogeneity of the pathological processes and mechanisms leading to the degeneration of motor neurons. Murine models that are carriers of the ^{G93A}SOD1 mutation remain the most widely used at preclinical level since they closely resemble ALS disease as we know at clinical level and many clinical trials on humans are based on previous results in this animal model. However, they do not always adequately represent the disease or are sometimes methodologically flawed(Benatar, 2007). Guidelines have been published in order to improve the conduction and interpretation of preclinical animal research in ALS (Ludolph et al., 2010).

In this paper we will briefly discuss our knowledge concerning the pathological mechanisms relevant to ALS and that can be the rationale for the choice of therapeutic strategies, and we will go through the current and future trials. We will also consider non-pharmacological approaches, including gene and stem cells therapies.

2. Lessons from previous ALS clinical trials

After 1980 more than 80 RCTs on ALS have been performed and published(Beghi et al., 2011; Mitsumoto et al., 2014; Petrov et al., 2017) and only two drugs, riluzole and edaravone, emerged as moderately effective in slowing the progression of ALS. There are several reasons for this disappointing failure.

2.1. ALS pathogenic mechanisms

The incomplete knowledge of the basic mechanisms leading to motor neuron degeneration in ALS is one of the most important reasons for the failure of clinical trials in ALS. Most hypotheses of

ALS pathogenesis have been derived from studies on ex-vivo tissues of patients, genetics, and preclinical studies on animal or cellular models. With the exception of genetics, several mechanisms have been poorly replied and their bases remain incompletely demonstrated. Overall, no pathogenic mechanism clearly emerged as pivotal in driving motor neuron death, and therefore limiting the rationale for several experimental drugs utilized in the last years. Given the large number of pathogenetic events described in ALS, a multi-targeted approach that involves different therapeutic strategies should be probably considered.

It is now emerging the concept that ALS is not determined by a single mechanism but by a cascade of events that act serially, in which motor neuron death is merely a final common outcome. This idea is supported by the observation that, similarly to cancer(Armitage and Doll, 2004), there is a linear relationship between the log incidence and log age of onset of ALS, consistent with a multistage model of disease(Al-Chalabi et al., 2014). The slope estimate suggests that ALS is a 6-step process in sporadic ALS, which becomes shorter in presence of genetic mutations (*SOD1*, 2 steps; *C9orf72*, 3 steps; *TARDBP*, 4 steps)(Chiò et al., 2018).

2.2. ALS clinical heterogeneity

The clinical heterogeneity of ALS has been largely neglected in the design of pharmacological trials in ALS, increasing the risk of underpower the trials themselves, in particular small phase 2 trials. ALS clinical phenotypes show great differences in term of disease progression, with median survival ranging from 1.2 years in respiratory and 2.1 years in bulbar phenotypes to 6.0 years in predominant upper motor neuron phenotype(Chiò et al., 2011a). ALS phenotype may be considered a multi-dimensional space-time process and is determined by the different spatial extent of anatomical lesions as well as by the varied diffusion of these lesions over time in each patient. Moreover, ALS phenotypes are driven by several factors, such as patients' age at onset, sex, and genetics(Chiò et al., 2020). The apparent focal onset of ALS may point toward a specific trigger of the disease, while the spatial-temporal combination of motor and cognitive events leading to the

clinical onset and progression of ALS may represent the failure of the cortico-motorneuronal system to compensate a decline already started during the preclinical phase of the disease, characterized by a differential susceptibility of the motor and prefrontal cortices and bulbar and spinal motor neurons to the pathological process influenced by aging, sex, gene variants and other, still unexplored, factors(Chiò et al., 2020).

2.3. Shortcomings of study design

The assessment of the clinical efficacy of a drug is based on randomized controlled trials (RCTs). The design of RCTs performed in ALS has been criticized because of various shortcomings (Table 1). Also, some, if not all, of efficacy measures in ALS trials, used as primary or secondary endpoints, have been disputed (Table 2). It is generally recognized that the identification of a specific biomarker for ALS usable as a proxy of disease progression represents a major goal of future research.

The recent publication of a prognostic algorithm based on a large sample of ALS patients from 14 different European centers(Westeneng et al., 2018) is a step toward the identification of a measure that can facilitate trials design, since it allows to predict a composite survival outcome (time between onset of symptoms and non-invasive ventilation for more than 23 h per day, tracheostomy, or death). The algorithm is based on relatively few variables (age at onset, diagnostic delay, slope of the ALS functional rating scale revised (ALSFRS-R), forced vital capacity percent of predicted, bulbar onset, definite ALS according to El Escorial criteria revised, concurrent frontotemporal dementia, and *C9orf72* repeat expansion).

Similarly, traditional end-points such as survival or ALSFRS-S slope, whose clinical meaning has been disputed, could be replaced by measures able to indicate to the slowing in anatomical spreading, as the King's staging(Balendra et al., 2015; Roche et al., 2012), or the delay in reaching the loss of meaningful clinical milestones (swallowing, communicating, moving, breathing), as the

MiToS staging (Chiò et al., 2015; Tramacere et al., 2015). Interestingly, MiToS staging has been recently incorporated as primary endpoint in an RCT with guanabenz in ALS(Bella et al., 2017).

Acknowledging these issues, guidelines for the design and implementation of clinical trials in ALS have been recently published(van Eijk et al., 2019b). These guidelines are based on a consensus conference held in Airlie House in 2016 and cover 9 areas: (1) preclinical studies; (2) biological and phenotypic heterogeneity; (3) outcome measures; (4) therapeutic and symptomatic interventions; (5) recruitment and retention; (6) biomarkers; (7) clinical trial phases; (8) beyond traditional trial designs; and (9) statistical considerations.

In parallel, guidelines for the conduction of clinical trials in ALS addressed to industry have been recently issued by the European Medicine Agency (EMA) (EMA/531686/2015) and the Food and Drug Administration (FDA-2013-N-0035).

2.4. Pharmacogenetics interactions

Pharmacogenomics is the study of the influence of genetics in drug response, namely the influence of acquired and inherited genetic variation on drug response in patients. The gene-drug interactions can occur at pharmacokinetics (drug absorption, distribution, metabolism, and elimination) and pharmacodynamics (effects mediated through a drug's biological targets) levels. The aim of pharmacogenomics is therefore to optimize drug therapy, with respect to patients' genotype, and to increase the efficiency and minimize the adverse effects of a drug. Excellent examples of application of pharmacogenomics in neuropsychiatry are related to antiepileptic(Balestrini and Sisodiya, 2018) and antipsychotic drugs (Zhang and Malhotra, 2018).

In ALS recent studies have shown that genetic polymorphism can potentially modify the effect of drugs. Following the failure of several RCTs on lithium carbonate (Chiò et al., 2010; UKMND-LiCALS Study Group et al., 2013; Verstraete et al., 2012), a post-hoc meta-analysis of patients enrolled in 3 clinical trials with lithium carbonate confirmed that treatment was overall ineffective,

but that a genetic subgroup of patients (carrying *UNC13A* C/C genotype, rs12608932) may have benefited from this treatment(van Eijk et al., 2017). A subsequent analysis of two other RCTs with creatine monohydrate and valproic acid (VPA) in ALS assessed the genetic interaction with *C9orf72* expansion and *UNC13A, SCFD1, SARM1*, and *MOB* common polymorphisms(van Eijk et al., 2019a). A dose-response pharmacogenetic interaction between creatine and the A allele of the MOBP genotype (rs616147) was found, suggesting a qualitative interaction in a recessive model. Taken together these studies indicate the importance of incorporating genotypic information in ALS clinical trials, but also highlight the challenges for future pharmacogenetic trials.

3. ALS pathogenic mechanisms and therapeutic strategies

In this section the most important pathogenic mechanisms considered to be relevant in ALS will be briefly described and the corresponding therapeutic interventions will be reported. A list of current pharmacological trials in ALS is shown in Table 3.

3.1. Excitotoxicity

Glutamate is the main excitatory neurotransmitter in the central nervous system (CNS). Excessive activation of glutamate receptors and failure in the clearance of neurotransmitter from the synaptic cleft or increased post-synaptic sensitivity to glutamate results in accumulation of the excitatory mediators that cause injury to neurons; this mechanism, called excitotoxicity, is considered relevant in the pathogenesis of different neurological and psychiatric disorders (Olloquequi et al., 2018). The role of excitotoxicity in ALS is supported by many evidences, although the primary mechanism of glutamate toxicity in ALS is still unknown. Increased levels of glutamate have been detected in the CSF of ALS patients, in particular in spinal onset subjects(Spreux-Varoquaux et al., 2002). Glutamate toxic effects are mediated through calcium-dependent pathways; motor neurons are particularly susceptible to this mechanism because of their reduced Ca^{2+} buffering

capacity(Grosskreutz et al., 2010). Hyperexcitation of two glutamate receptors has been demonstrated in ALS, i.e. the alpha-amino-3-hidroxy-5-methyl-4-isoxazole-propionic acid (AMPA) and the N-methyl-D-aspartate (NMDA) receptors (Carriedo et al., 1996; Van Den Bosch et al., 2006). Studies also implied the reduced clearance of glutamate from the synaptic cleft into astrocytes (Foran and Trotti, 2009) due to a reduced expression of astrocytic excitatory amino-acid transporters (EAATs)(Bristol and Rothstein, 1996). Riluzole inhibits the release of glutamate and inhibits post-synaptic NMDA and AMPA receptors. Therefore, neuronal protection against excitotoxicity remains a major therapeutic target in ALS.

3.1.1. Therapeutic approaches to excitotoxicity

Riluzole is one of the drugs currently marketed for ALS. In the original phase 3 RTC, riluzole tablets at a daily dose of 100 mg showed an increase of survival of ALS patients of about 3 months compared to those treated with placebo (Lacomblez et al., 1996). The effect of riluzole is complex, and not completely understood. Riluzole directly inhibits the kainate and NMDA receptors, stimulates glutamate uptake and prevents glutamate release from presynaptic terminals (Dorst et al., 2018). Riluzole is also available as oral suspension specifically designed for patients with severe dysphagia or enteral nutrition via gastrostomy (Dyer and Smith, 2017). Two other oral formulations of riluzole are also being studied: dissolving tablets (BHV-0223) (NCT03520517) and oral soluble film (ROSF) (NCT03679975).

Perampanel is an antiepileptic drug which acts blocking the ability of the neurotransmitter glutamate to utilize AMPA receptors to excite neurons. This anti-excitotoxic effect is considered an interesting target for the treatment of ALS. Three placebo-controlled trial of perampanel with different design and doses in ALS are underway (NCT03020797, NCT03019419 and NCT03377309).

Memantine is a NMDA receptor antagonist. Positive results from a open label pilot trial of 20 slow progressor patients were followed by negative results in a phase 2 study, though such study was

underpowered (de Carvalho et al., 2010; Levine et al., 2010). A new multi-centered double blind, placebo-controlled study is ongoing (NCT02118727), evaluating Memantine at 20 mg BID in patients currently taking Riluzole. The purpose of this study is to determine if memantine can slow down disease progression and improve neuropsychiatric changes of patients with ALS.

3.2. Oxidative stress

CNS is particularly susceptible to oxidative stress because of several reasons: the neuronal membrane contains a high abundance of polyunsaturated fatty acids; it has a high rate of consumption of oxygen; it contains high concentrations of redox-active transition metals but a relatively low concentration of antioxidants(Contestabile, 2001). Oxidative stress damages critical cellular macromolecules, which can eventually lead to cell death by necrosis or apoptosis (Klaunig et al., 1998). Several oxidative stress biomarkers have been found to be elevated in ALS serum and CSF compared to healthy controls, including 4-hydroxy-2-nonenal (HNE), 3-nitrotyrosine (3-NT), 8-Oxo-deoxyguanosine (8-oxoG)(Chico et al., 2017). Besides *SOD1*, other ALS-related genes are involved in oxidative stress, such as *TARDBP, ALS2*, *VAPB,* and *ANG* (D'Amico et al., 2013; Hardiman et al., 2017). Other direct evidences of the involvement of oxidative stress in ALS are the findings of oxidative damage to proteins, lipids, and DNA in post-mortem CNS in ALS patients(Agar and Durham, 2003). It has also been shown that when cultured astrocytes undergo oxidative damage, there is an impairment of glutamate transport, linking oxidative stress to excitotoxicity (Tilleux and Hermans, 2007) .

3.2.1. Therapeutic approaches to oxidative stress

Several drugs have been tested in ALS acting on oxidative stress, namely vitamin E, coenzyme Q10, and acetylcysteine, but none of them proved to be effective in RCTs. Edaravone is the first new treatment licensed for ALS since riluzole in the 1990s. Edaravone is a free radical scavenger

thought to reduce oxidative stress, it has been marketed for over 20 years as a therapy for acute ischemic stroke. Since oxidative stress has long been implicated in ALS, edaravone has been proposed as a possible treatment for ALS. Pre-clinical studies gave encouraging results, but an initial placebo-controlled phase 3 study (MCI186-16, NCT00424463) did not meet the primary outcome(Abe et al., 2014). A post-hoc analysis of MCI186-16 trial revealed that a subpopulation of patients may have benefited of the drug, i.e. patients of 20−75 years with ALS of grade 1 or 2 in the Japan ALS Severity Classification, scores of at least 2 points on all 12 items of ALSFRS-R, forced vital capacity of 80% or more, definite or probable ALS according to the revised El Escorial criteria, and disease duration of 2 years or less(Takei et al., 2017). Accordingly, a placebo-controlled phase 3 enriched trial including only patients with these clinical characteristics was performed (Writing group edaravone, 2017). This study demonstrated a significantly smaller decline of ALSFRS-R score in patients treated with i.v. edaravone compared to those who received placebo. Edaravone has been approved in several countries in Asia, in USA, in Canada and in Switzerland.

Methylcobalamin is an active vitamin B_{12}. It functions as a coenzyme for homocysteine remethylation and inhibits neuronal degeneration by decreasing levels of homocysteine. In a recent study(Kaji et al., 2019) no significant differences were detected in primary endpoints. However, post-hoc analyses of methylcobalamin-treated patients with disease onset ≤12 months showed longer intervals to the primary event and less decreases in the ALSFRS-R score than the placebo group. On this basis, a Japanese phase 3 study is now ongoing to examine the clinical efficacy and safety of ultra-high dose (50mg, i.m., twice a week) methylcobalamin in retarding the progression of symptoms in ALS patients within 12 months after the clinical onset (NCT03548311).

Ranolazine, a FDA approved drug for angina which inhibits the late Na+ current and intracellular Ca2+ accumulation, may be neuroprotective in ALS by reducing neuronal hyperexcitability. A phase 2 trial dose-ascending with Ranolazine 500 mg and 1000 mg is underway (NCT03472950).

The impairment of iron metabolism is reported in preclinical models of ALS both in sporadic and genetic forms (*SOD1* and *C9orf72*). In the ^{G93A}SOD1 mouse model, the use of iron chelators has demonstrated neuroprotection and increased life expectancy (Golko-Perez et al., 2017, 2016). A good safety profile was observed using a chelator, deferiprone, in a pilot human study(Moreau et al., 2018). Currently the efficacy of this new treatment is being evaluated in a randomized, double-blind, placebo-controlled, multicenter phase 2-3 study (NCT03293069).

Verdiperstat is an irreversible inhibitor of myeloperoxidase (MPO), an enzyme that passes blood brain barrier and acts as a key driver of pathological oxidative stress and inflammation in the brain. Verdiperstat has been selected as an investigational therapy and a phase 3 in ALS is planned (https://alsnewstoday.com/2019/09/23/healey-amg-center-mass-general-launches-first-als-platform-trial-five-candidate-treatments/). A Phase 3 clinical trial is currently ongoing to evaluate the efficacy of Verdiperstat in multiple system atrophy (*NCT03952806)*.

Urate is an endogenous antioxidant system and a major defense against oxidative stress. Urate has demonstrated promising neuroprotective effect in neurodegenerative diseases, particularly in Parkinson's Disease. Higher urate levels are an independent predictor of slower progression and prolonged survival in ALS (Paganoni et al., 2012). A recent pilot phase 1 study of Inosine in ALS (NCT02288091) showed safety and feasibility of urate elevation(Nicholson et al., 2018). A multi-center phase 2 trial to confirm these findings with longer exposure time is ongoing (NCT03168711).

3.3. Mitochondrial dysfunction

Mutations in several ALS-associated genes address to the involvement of mitochondrial dysfunction in disease pathogenesis. TANK-binding kinase 1 (TBK1) phosphorylates both optineurin (OPTN) and sequestosome 1 (SQSTM1), and the TBK1/OPTN interaction and OPTN

phosphorylation are required to target these proteins to damaged mitochondria(Richter et al., 2016). The disruption of the TBK1/OPTN complex and subsequent accumulation of dysfunctional mitochondria may be an important hub in the disease process. Mutations in *CHCHD10* are associated with structural abnormalities in mitochondria and defects of respiratory chain defects(Bannwarth et al., 2014). The recently described mutations in *KIF5A* are all localized in the C-terminal cargo recognition domain (Nicolas et al., 2018), a region that is essential for binding and regulating axonal transport of mitochondria(Campbell et al., 2014). Another ALS-related gene, *VCP*, is essential for mitochondrial quality control through PINK1/Parkin and its mutations impair this function(N. C. Kim et al., 2013). Moreover, VCP mutations in ALS induce mitochondrial uncoupling and reduced ATP levels(Bartolome et al., 2013). Protein aggregation, a common process in ALS pathology, has deleterious effects on mitochondria function. For example, the aggregation of mutant SOD1 within mitochondria causes mitochondrial vacuolation through expansion of the intermembrane space(Higgins et al., 2003). *OPTN* codifies for a protein acting as an autophagy receptor inducing mitophagy of damaged mitochondria (Wong and Holzbaur, 2014). Considering the high energetic demand of motor neurons, also related to the length of their axons, it is evident that the perturbation of mitochondrial function has profound negative effect on these cells. This justifies therapeutic intervention directed to restore mitochondrial function.

3.3.1. Therapeutic approaches to mitochondrial dysfunction

Rasagiline is a monoamine oxidase B (MAOB) inhibitor and is marketed as a disease-modifying drug in Parkinson's Disease. Rasagiline reduces dopamine catabolism by inhibiting MAOB. Besides it found to be effective in prolonging survival the ^{G93A}SOD1 mouse suggesting its therapeutic potential in this disease(Waibel et al., 2004). Several trials on Rasagiline were performed in ALS. An open-label phase 2 trial demonstrated that the drug significantly increased the mitochondrial membrane potential (JC-1 red/green fluorescent ratio 1) and decreased apoptosis markers (Bcl-2/Bax ratio) in the blood(Macchi et al., 2015). Two other placebo-controlled phase 2

trials evaluated safety and efficacy of Rasagiline in larger cohorts, but both failed to identify any effect on the primary endpoints(Ludolph et al., 2018; Statland et al., 2019) (NCT01879241, NCT01232738). However, a post-hoc analysis stratifying the trial participants into two groups on the basis of median ALSFRS-R progression rate at baseline suggested that rasagiline might positively modify disease progression in a subset of patients, i.e. participants with an ALSFRS-R rate of decline greater than 0.5 points/months (Ludolph et al, 2018).

3.4. Autophagy and protein quality control

Macroautophagy, the most frequent form of autophagy, is an evolutionarily conserved and highly regulated catabolic process in which cellular contents such as misfolded proteins and damaged organelles are targeted to lysosomes for degradation. Numerous studies have demonstrated that defects in the autophagy pathway cause neurodegeneration(Menzies et al., 2017). Many proteins of the ALS/FTD spectrum participate in the autophagy pathway, suggesting that this is a major pathogenic mechanism in these disorders(Almeida and Gao, 2016). TPD-43 aggregates cause loss of nuclear functions caused by chronic sequestration in RNP granules resulting in reduced expression of autophagic factors that function at various events within the autophagy lysosomal pathway (Soo et al., 2015; Stoica et al., 2014). Receptors for selective autophagy, such as SQSTM1/p62 and OPTN, as well as TBK1 were shown to be involved in the degeneration of motor neurons (Freischmidt et al., 2015; Maruyama et al., 2010; Teyssou et al., 2013). Mutations in *C9orf72*, the most common cause of ALS, have been proposed to downregulate autophagy(Ji et al., 2017). *C9orf72* is a main component of GDP/ GTP exchange factor (GEF) for Rab8 and Rab39, which is crucial for autophagosome maturation. Other ALS-related genes whose products are involved in autophagy are *UBQLN2*, a molecular chaperone binding partner to direct misfolded proteins to the proteasome(Hjerpe et al., 2016), and *VCP*, an ATP-dependent enzyme involved in several aspects of protein quality control(Meyer et al., 2012). Lastly, recent findings have demonstrated the localization of mutant *SOD1* in stress granules(Gal et al., 2016) where the protein

can cause endoplasmic reticulum (ER) stress through blockade of ER-associated degradation (ERAD)(Nishitoh et al., 2008). Furthermore, mutant SOD1 protein has been linked to impaired proteasome function in pre-clinical models of ALS(Kabashi et al., 2004; Urushitani et al., 2002), likely contributing to an acceleration in the accumulation of misfolded mutant SOD1 protein in a pathogenic loop.

3.4.1. Therapeutic approaches to autophagy and protein quality control

Arimoclomol increases the production of heat-shock proteins, which amplify the function of lysosomes. Arimoclomol may reduce the levels of protein aggregates in the motor nerves, a possible cause of ALS, by boosting expression of chaperonins Hsp70 and Hsp90 which help newly synthesized proteins properly fold (Kalmar et al., 2014). The heat shock response promotes natural folding of nascent proteins and refolding of damaged or mutated proteins via enhanced heat shock protein expression, a mechanism of action that is thought to be highly relevant to an essential and early pathophysiological event that leads to neurodegeneration in ALS (Kalmar et al., 2014). A phase 2 placebo-controlled trial on arimoclomol in *SOD1* patients with rapidly progressive disease showed that the drug was safe and well tolerated and observed a consistent directional benefit of arimoclomol over placebo across predefined efficacy endpoints (Benatar, 2007). A larger phase 3 trial on sporadic and familial ALS patients with arimoclomol is underway (NCT03491462).

Guanabenz is an alpha-2-adrenergic receptor agonist, with the ability to modulate the synthesis of proteins by the activation of translational factors preventing misfolded protein accumulation and endoplasmic reticulum overload. On this basis, a placebo-controlled phase 2 trial with guanabenz in ALS has been performed with a futility design and using a novel endpoint, i.e. the progression to higher stages of MiToS staging(Bella et al., 2017) (Eudract no. 2014-005367-32).

A phase 1 trial on L-serine has been performed in ALS(Bradley et al., 2017), on the basis of the ability of L-serine to inhibit β-N-methylamino-L-alanine (BMAA), a neurotoxic substance linked to Guam ALS/PDC, misincorporation into motor neuron proteins via L-seryl tRNA synthetase, which

causes protein misfolding. Since L-serine proved to be safe, a 6-months phase 2a open-label trial is ongoing in a larger series of ALS patients (NCT03580616).

Also related to protein misfolding as a possible target for ALS treatment is the observation that heat shock protein B8 (HSPB8) recognizes and promotes the autophagy-mediated removal of misfolded mutant SOD1 and TDP-43 fragments from ALS motor neurons (Cristofani et al., 2019). Moreover, HSPB8-BAG3-HSP70 maintains the so-called granulostasis, a surveillance mechanism that avoids the conversion of dynamic SGs into aggregation-prone assemblies, which are a hallmark of ALS(Alberti et al., 2017). Colchicine enhances the expression of HSPB8 and of several autophagy players while blocking TDP-43 accumulation in neurons. In addition, colchicine anti-inflammatory activity may also improve the pathological process of ALS. These observations prompted a phase 2 placebo-controlled study of colchicine in ALS (NCT03693781).

A placebo-controlled phase 2 trial assessing rapamycin in ALS is underway (NCT03359538). The action of rapamycin is based on mTORC1 inhibition. Mechanistic target of rapamycin complex 1 (mTORC1) targets regulatory proteins in cell signaling and regulates autophagy by inhibiting the unc-51-like kinase 1 complex. Inhibition of mTORC1 by rapamycin stimulates autophagy, through the formation of autophagosome from the phagophore. Several non-routine laboratory studies will be performed, including quantification and characterization of Tregs, lymphocytes phenotype, mTOR, downstream pathway activation in peripheral blood mononuclear cells (PBMC), inflammasome components in PBMC and proinflammatory cytokine production in monocytes, peripheral biomarkers (Mandrioli et al., 2018).

3.5. Neuroinflammation

During the last years, research on innate immune system, including the complement system, has shown its novel roles during development, homeostasis, and ageing of the CNS. Several studies have indicated that the aberrant activation of the complement system in the central nervous system

may be involved in the pathophysiology of ALS. This observation makes the innate immune system a potential therapeutic target in ALS.

3.5.1. The role of microglia.

Previously, CNS has been considered an 'immunologically privileged' environment with a relative absence of immunologically reactive cells; however, it is now clear that CNS contains a highly compartmentalized and specialised immune system, protected by specific barriers(Engelhardt et al., 2017). Besides, until few years ago it was thought that microglia could have either neuroprotective or neurotoxic effects in the course of neuroinflammation. Under this concept, microglia was classified as M1 phenotype, responsible for releasing inflammatory cytokines and removal of injured cells, and M2 phenotype, capable of releasing neuroprotective anti-inflammatory cytokines (Zhao et al., 2013). It has been hypothesized that the M2 microglia increases in the early phase of the disease thus slowing disease progression, while in advanced phases M1 microglia may predominate, further exacerbating motoneuron injury (Henkel et al, 2009). More recently, microglia cells are considered to be more complex and dynamic, with the possibility to have different and opposing actions at the same time (Sasaki, 2017).

The imbalance between M2 and M1 is heavily influenced by regulatory T-cells (Tregs). These cells are marked by CD4 and IL2RA (IL2 receptor subunit alpha, CD25). Their development and maintenance depend on the transcription factor forkhead box P3 (FOXP3). Regulatory T-cells encourage M2 differentiation and impede the activity of cytotoxic T-lymphocytes (Tc, including natural killer T-cells). Lower levels of Tregs and of FOXP3 have also been correlated with the rate of clinical progression of ALS (Henkel et al., 2013).

Neuroinflammation, a fundamental component of ALS pathophysiology, is induced by activated microglia, astrocytes, and infiltrating CD4+ and CD8+T lymphocytes(Liu and Wang, 2017), but is has been generally considered as a secondary mechanism in motor neurons degeneration.

Nevertheless, some studies have found that microglial pathology correlates with disease progression(Brettschneider et al., 2012; Turner et al., 2004).

Recent studies have supposed that neuroinflammation may in fact be a primary event, resulting from a negative interaction between the degenerating motor neurons and surrounding cells(Jara et al., 2017).

3.5.2. The role of complement

In the ^{G93A}*SOD1* transgenic mouse it has been reported that motor neurons upregulate the production of C1q and C4 during disease progression(Ferraiuolo et al., 2007). This production of C1q and C4 has been confirmed in studies from human postmortem ALS spinal cords and brains(Sta et al., 2011) and the finding of increased levels of C3 and C4 in the cerebrospinal fluid (CSF) of ALS patients(Ganesalingam et al., 2011; Tsuboi and Yamada, 1994). In the motor neurons of transgenic ^{G93A}*SOD1* mice and in post mortem ALS patients it has been found an upregulation of the C5a receptor (C5aR1)(Lee et al., 2017; Woodruff et al., 2008). The detrimental role of C5aR1 is further confirmed by the significant increase of survival in the $_{G93A}$SOD1 C5aR1 knockout mouse model (^{G93A}SOD1 x C5aR1−/− mice)(Woodruff et al., 2008). Finally, the treatment of ^{G93A}*SOD1* mice with a C5a1 receptor antagonist improves symptoms and prolongs survival (Lee et al., 2017). Taken together, these data strongly implicate the C5a/C5aR1 axis in ALS and identify it as a target for therapy in ALS(Carpanini et al., 2019).

3.5.3. Therapeutic approaches to neuroinflammation

Two drugs acting on C5 have been planned for trials in ALS. Ravulizumab, a long-acting C5 inhibitor, is a humanized monoclonal antibody designed for the treatment of paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremic syndrome (McKeage, 2019; Stern and Connell, 2019). A phase 3 placebo-controlled trial in ALS is planned. Zilucoplan is a synthetic peptide that

binds complement component 5 (C5) and inhibits its cleavage into C5a and C5b, thus preventing the overactivation of the complement system. A placebo-controlled phase 2 trial in ALS is planned.

Ibudilast (MN-166) is an anti-inflammatory and neuroprotective oral agent, and a first-in-class, small molecule inhibitor of phosphodiesterase-4 and -10, as well as macrophage migration inhibitory factor (MIF). A phase 2 trial on ALS patients showed that the drug was safe and had potential benefit on survival (Brooks et al., 2018). A phase 2b/3 trial is planned (NCT02238626).

Tocilizumab is an IL6 receptor antibody currently approved for the treatment of rheumatoid arthritis. A proof-of concept study in 10 sporadic ALS patients demonstrated that tocilizumab infusions resulted in down regulation of inflammatory genes (in particular IL1β)(Fiala et al., 2013). A placebo-controlled phase 2 study has been performed in U.S. to determine the safety and tolerability of intravenous administration of 8 mg/kg of tocilizumab every 4 weeks and to describe the expression of pro-inflammatory genes in PBMCs and the ability of the drug to reduce the expression of pro-inflammatory genes in PBMCs and pro-inflammatory cytokines in the CSF of ALS patients (NCT02469896). The results of the trial have not yet published.

IC14 is a biologic monoclonal antibody drug that inactivates CD14 cells and in circulation. CD14 is a main regulator of the immune response and is also a key organizer of responses by the brain's most common immune cells to infection and injury. Hyper-activated CD14 results in damaging inflammation that kills healthy brain and other cells in diseases such as ALS (Reed-Geaghan et al., 2010; Zondler et al., 2017). A placebo-controlled phase 2 trial in a small series of ALS patients with a rapidly progressive disease (NCT03508453) is underway. Another open-label phase 2a clinical trial of intravenous IC14 in rapidly progressive ALS patients is ongoing (NCT03474263). Interestingly, one of the primary outcome measures of this trial will be microglial activation assessed in the motor region measured by [^{11}C]-PBR28 positron emission tomography (PET).

IPL344 activates the PI3K-Akt signaling pathway in a variety of cells, including neurons, inducing anti-inflammatory processes. An open-label phase 1-2 trial on IPL344 administered intravenously is underway in ALS (NCT03652805, NCT03755167).

A recombinant chimeric monoclonal antibody acting as modulator of immune system (IC-14) is under study in a placebo-controlled phase 2 trial in a small series of ALS patients with a rapidly progressive disease (NCT03508453).

Activation of the Rho kinase (ROCK) pathway appears to be instrumental for the modulation of the microglial phenotype: increased ROCK activity in microglia mediates mechanisms of the inflammatory response and is associated with improved motility, increased production of reactive oxygen species (ROS) and release of inflammatory cytokines(Roser et al., 2017). The ROCK inhibitor fasudil, an isochinoline derivative that was originally developed as a vasodilatory drug, has demonstrated beneficial effects in cell culture and animal models of ALS(Takata et al., 2013). A phase 2a trial to evaluate fasudil in early-stage ALS started patient recruitment in 2019 (NCT03792490) (Lingor et al., 2019).

Masitinib, an oral tyrosine kinase inhibitor, has demonstrated promising preclinical activity in ^{G93A}SOD1 rat models, exerting neuroprotection via its immunomodulatory properties and in particular through targeting microglia, macrophage and mast cell activity, in both central and peripheral nervous systems. In addition, masitinib has been found to regulate the stem cell factor (SCF) receptor c-Kit(Dubreuil et al., 2009); interestingly, in the ^{G93A}SOD1 transgenic mice, the pre-treatment of donor bone marrow cells with SCF resulted in a neuroprotective effect (Terashima et al., 2014). A placebo-controlled phase 2 study demonstrated a slowing of ALSFR-R decline in a subpopulation of ‘normal progressors’ ALS patients (i.e. patients with an ALSFRS-R decline <1.1 points/month) but not in fast progressors (Mora et al., 2019). A phase 3 study is planned.

Several trials are related to the regulation of Treg cells. In a placebo-controlled phase 2 trial the safety and efficacy of low-dose interleukin 2 (IL-2) in ALS patients is tested. IL-2 is known to

increase the production of Tregs (NCT03039673). With a different approach, in a phase 2a trial autologous Treg cells are expanded ex-vivo and returned intravenously in combination with low-dose IL-2 (Thonhoff et al., 2018) (NCT04055623). This trial is based on a previous phase 1 study showing that the interference with the immune system using Treg cells slowed ALS progression (Sheean et al., 2018) (NCT03241784).

RNS60 is an immune-modulatory agent with neuroprotective properties in preclinical models of ALS(Vallarola et al., 2018). In ^{G93A}SOD1 transgenic mice, RNS60 treatment resulted in upregulation of FOXP3-expressing Tregs and activation of protective astrocytes and microglia, which rescued the motor neurons and ameliorated disease progression (Vallarola et al., 2018). An open-label phase 1 trial of RNS60 aimed at identifying changes in plasma biomarkers of neuroinflammation (IL-17, FOXP3) and [11C]-PBR28 PET uptake modification, a marker of microglia, did not find any significant effect (NCT02525471). A placebo-controlled phase 2 trial with RNS60 is underway (NCT03456882).

Given the role of adaptive immunity in ALS, the pathogenicity of some clostridial strains on motorneurons, the putative role of cyanobacteria in ALS development, and the increasing interest for microbiota in neurodegenerative disorders, raised the hypothesis that the modification of intestinal microbiota might affect ALS at its core(Erber et al., 2019; Tremlett et al., 2017). A placebo-controlled phase 2 clinical trial on fecal microbiota transplant (NCT03766321) in patients affected by ALS to assess the biological (increasing Treg cells number) and clinical effect of the treatment is ongoing.

Microglial activation is a common phenomenon in neurodegenerative diseases. Ciprofloxacin and celecoxib were used in combination in primary murine microglial cells and effectively reduced inflammation by switching pro-inflammatory cytokines M1 to anti-inflammatory M2(Dey et al., 2018). An open label study in ALS patients has been designed to assess safety, tolerability, disease progression and change in serum pNFH (NCT04090684).

Finally, low molecular weight dextran sulfate (ILB) will be studied in a phase 2a open-label trial (NCT03705390). ILB has several possible functions able to attenuate pathological process in ALS, including complement system protein inhibitor, stabilization of neurofilaments, attenuation of microglial activation, and normalization of mitochondrial function.

3.6. Apoptosis

Much interest has been raised by the possibility that a mechanism of programmed cell death, termed apoptosis, is responsible for the motor neuron degeneration in ALS. Apoptosis is regulated through a variety of different pathways which interact and eventually lead to controlled cell death. Apart from genetic regulation, factors involved in the control of apoptosis include death receptors, caspases, Bcl-2 family of oncoproteins, inhibitor of apoptosis proteins (IAPs), inhibitors of IAPs, the p53 tumor suppressor protein and apoptosis-related molecules. All these targets are highly druggable(Reyes et al., 2010). Activation of apoptotic signaling cascades has been observed in most ALS models, but this is mostly an indirect consequence of other toxic events (Smith et al, 2019). However, *SOD1* has been shown to directly influence apoptotic signaling by interaction with Bcl-2. Wild-type and ALS mutant *SOD1* (p.A4V, p.G37R, p.G41D and p.G85R) bind the anti-apoptotic factor Bcl-2, exposing the BH3 domain of Bcl-2 and thus inducing a pro-apoptotic gain of function of the Bcl-2 protein in the transgenic ^{G93A}SOD1 mouse and in the spinal cord of mutant *SOD1* A4V patients (Pedrini et al., 2010) . Another interesting mechanism is related to the dual leucine zipper kinase (DLK), a serine/threonine protein kinase that is a member of the mixed lineage kinase subfamily. Mixed lineage kinases are upstream MAP3Ks that activate the c-Jun N-terminal kinase (JNK) pathway. DLK is primarily responsible for activating JNK and mediating the apoptotic stress response in various cell types, specifically neurons(Ferraris et al., 2013). Inhibition and knockdown of DLK has been demonstrated to have neuroprotective effects in cellular and animal models of several neurodegenerative conditions including ALS(Le Pichon et al., 2017). Recently, in a

prospective study on ALS patients, a higher level of the endogenous antioxidative factor, neuronal apoptosis inhibitory protein (NAIP) in mononuclear cells has been associated with a smaller change in ALSFRS-R at 12 months, i.e. slower disease progression(Kano et al., 2018).

3.6.1. Therapeutic approaches to apoptosis in ALS

Tauroursodeoxycholic acid (TUDCA) is a hydrophilic bile acid that is normally produced endogenously in humans in the liver, by conjugation of taurine to ursodeoxycholic acid (UDCA). It is commonly used for treatment of chronic cholestatic liver diseases and for gallstone. Chronic administration of TUDCA in humans is safe and well tolerated. TUDCA reduces abnormally increased caspase activity, and negatively modulates the mitochondrial pathway by inhibiting Bax translocation, ROS formation, cytochrome c release, and caspase-3 activation (Amaral et al., 2009; Rodrigues and Steer, 2001; Vaz et al., 2015). A phase 2b placebo-controlled trial with orally administered TUDCA showed a reduction of ALSFRS-R decline in a small series of ALS patients(Elia et al., 2016). It is now underway a larger phase 3 RCT to establish the efficacy of the drug (NCT03800524). A RCT aiming at the reduction of apoptotic process in ALS evaluated the efficacy of sodium phenylbutyrate (PB), a small molecule targeting signals within a cells mitochondria and endoplasmic reticulum(Cudkowicz et al., 2009). It is now underway a phase 2 trial of AMX0035, a combination of TUDCA and PB (NCT03127514).

GDC-0134, an oral investigational drug designed to block pyrimidinyl-aminopyridine dual leucine zipper kinase (DLK) is also under study as potential treatment of ALS in a Phase 1, double-blind, placebo-controlled, single- and multiple-ascending-dose trial (NCT02655614).

DNL747 is an inhibitor of the receptor-interacting serine/threonine-protein kinase 1 (RIPK1) pathway. RIPK1 forms a signaling hub downstream of the TNF receptor pathway, which regulates inflammation. RIPK1 has been shown to initiate both necroptosis and apoptosis(Amin et al., 2018; Vandenabeele et al., 2010). RIPK1 has been shown to mediate axonal degeneration through

inflammatory and necroptotic processes in an ALS mouse model(Ito et al., 2016). On basis of this rationale, a placebo-controlled phase 1 study of DNL747 in ALS is ongoing (NCT03757351).

3.7. Nucleocytoplasmic transport

In the majority of patients with ALS and FTD, the pathologic hallmark of disease is the cytoplasmic deposition of the nuclear RNA binding protein TDP43(Neumann et al., 2006). The subcellular localization of TDP43 and related RNA binding proteins is critical for the function and the survival of neurons. TDP43 contain nuclear localization and nuclear export signals that facilitate rapid trafficking between the nucleus and cytoplasm. Disrupting the TDP43 nuclear localization signal enhances neurotoxicity (Barmada et al., 2010). The importance of nucleocytoplasmic transport in ALS has also been highlighted by the observation of mislocalization of importin a and b family proteins in a transgenic mouse model expressing mutant ^{G93A}*SOD1* (Zhang et al., 2006). Recent studies on *C9orf72* repeat expansions elucidated the molecular mechanisms underlying the involvement of nucleocytoplasmic transport in ALS/FTD pathogenesis. For example, in *C9orf72* iPSC-derived neurons, the nucleocytoplasmic Ran gradient has been shown to be decreased, and the nuclear import of proteins and nuclear export of RNAs compromised (Zhang et al., 2015). The alteration of nucleocytoplasmic transport in *C9orf72*-related ALS is probably caused by both toxic repeat RNAs and DPR proteins. RanGAP1 binds to GGGGCC repeat RNA in vitro, and colocalizes with G4C2 RNA foci in C9orf72 patient cells (Zhang et al., 2015). Therefore, GGGGCC repeat RNA might compromise nucleocytoplasmic transport through a physical interaction with RanGAP1.

3.7.1. Therapeutic approaches to nucleocytoplasmic transport

TDP43 contains a canonical leucine-rich nuclear export signal (NES) that is predicted to be a substrate of exportin-1 (XPO1/CRM1), a conserved nuclear export factor in mammalian cells. Recently novel selective inhibitors of nuclear export (SINE) compounds have been developed

(Archbold et al., 2018). SINE can prevent TDP43 nuclear export thus slowing or preventing neurodegeneration in models of ALS and FTD. BIIB100/KPT-350 is a small-molecule inhibitor of XPO1 that has been selected for a placebo-controlled phase 1 trial in ALS (NCT03945279).

3.8. DNA damage

While DNA is damaged in many ways over time, its integrity in mammalian postmitotic neurons has to be maintained for up to decades. The major cause of DNA damage in neurons are endogenous ROS, produced by mitochondrial respiration and other metabolic processes, that cause modifications of the structure of DNA, leading to single-strand breaks (SSBs) and, although less frequently, DNA double-strand breaks (DSBs)(Madabhushi et al., 2014). An example in ALS is represented by *FUS*, which participates in the formation of a D-loop during DNA DSB repair, suggesting a potential role in genomic stability (Baechtold et al., 1999). Several *FUS* mutations reduce the capacity of the protein to repair DNA DSB (Wang et al., 2013): for example, *FUS*-R521C interferes with the normal interaction between FUS and HDAC1 (Wang et al., 2013), and FUS-R521C transgenic mice show increased DNA damage in both cortex and spinal cord (Qiu et al., 2014).

DNA damage has been reported in spinal cord motor neurons of ALS patients with *C9orf72* repeat expansion (Walker et al., 2017). In induced pluripotent stem cells (iPSCs) derived from patients with *C9orf72* repeat expansion an age-dependent increase in DNA damage and oxidative stress has been reported (Lopez-Gonzalez et al., 2016). A recent study revealed a strong correlation in tissues of ALS patients of TDP-43 pathology with DSBs repair defects, and damage accumulation in the neuronal genomes of sporadic ALS patients (Mitra et al., 2019).

3.8.1. Therapeutic approaches to DNA damage in ALS

There are no clinical trials directly targeting DNA damage in ALS. However, several studies on preclinical models and patient-derived iPSC have been performed to restore DNA damage. ASOs targetingC9orf72 RNA and small molecules (KPT-276, orTMPyP4) treatments inhibited nuclear import impairment by the C9ORF72 repeat expansion in fly models, as well as in C9ORF72 iPSC-derived neurons, and reduced neurodegeneration (Zhang et al., 2015). A recent study reported that partial suppression of Ku80, a DNA repair protein, in *C9ORF72* iPSC-derived patient neurons through CRISPR/Cas9-mediated ablation or small RNAs-mediated knockdown reduced the apoptotic pathway, suggesting that the inhibition of the hyperactivated Ku80-dependent DNA repair pathway is a promising therapeutic approach in *C9ORF72* ALS (Lopez-Gonzalez et al., 2019).

3.9. RNA splicing/metabolism

The process of intron removal from pre-mRNAs is a highly regulated process and is determined by a complex interaction of more than 100 proteins and other regulatory elements. A misregulated splicing has been described in the cerebellum and frontal cortex of ALS patients with *C9orf72* repeat expansion and of sporadic patients (Prudencio et al., 2015).

Both TDP-43, the product of *TARDBP* gene, and FUS proteins have fundamental roles in the pre-mRNA splicing process (Buratti et al., 2001). The binding of TDP-43 to long introns affects the stability of these pre-mRNA targets, and the binding to exon-intron junctions regulates alternative splicing (Polymenidou et al., 2011).

Since the large majority of ALS and FTD cases display TDP-43 pathology, characterized by depletion of the protein in the nucleus and formation of deposits in the cytoplasm, it is generally recognized that the loss of normal TDP-43 function in splicing is a fundamental pathogenic factor in disease progression. Mutant TDP-43 can also have a gain-of-toxic-function manner (Arnold et al., 2013). Similarly, FUS interacts with U1, U11, and U12 snRNPs, which are mislocalized to the cytosol by mutant FUS (Sun et al., 2015). Other ALS-related genes implicated in RNA metabolism are *TIA1* (Mackenzie et al., 2017), *MATR3* (Johnson et al., 2014), *HNRNPA1* (H. J. Kim et al.,

2013) and *HNRNPA2/B1* (H. J. Kim et al., 2013). These findings highlight the important role of misregulation of splicing by different proteins in ALS/FTD.

3.9.1. Therapeutic approaches to RNA splicing/metabolism

There are currently no clinical trials targeting RNA splicing/metabolism in ALS. Proposed interventions are based on gene therapy, in particular antisense oligonucleodites (ASO), spliceosome-mediate RNA trans-splicing (SMAT) or small interfering RNAs (siRNAs)(Arechavala-Gomeza et al., 2014; Perrone et al., 2020; van Zundert and Brown, 2017).

3.10. Stress granules dynamics

Stress granules (SGs) represent a unique adaptive mechanism to conserve energy and favor survival in response to a stressful cellular environment by limiting protein translation(Anderson and Kedersha, 2008). SGs are non-membrane bound, cytoplasmic ribonucleoprotein (RNP) granules composed of mRNAs, translation initiation factors, 40S ribosomes, and many other RNPs (Protter and Parker, 2016). Besides the multifunctional roles in the cellular processes mentioned above, several ALS-related genes are also associated with SGs.

FUS is probably the clearest clue of the link between SGs and ALS. In fact, mutant *FUS* proteins show increased cytoplasmic localization and induce SGs formation (Bentmann et al., 2012; Bosco et al., 2010). Also, TDP-43 is associated with SGs through an interaction with the core SGs protein TIA1 (Bentmann et al., 2012; Freibaum et al., 2010). Other genes related to SGs formation with diverse mechanisms are *hnRNP A2* and *hnRNP A1* (H. J. Kim et al., 2013), *ANXA11* (Liao et al., 2019), *ATXN2* (Lee et al., 2018), *PFN1* (Figley et al., 2014), and *SOD1* (Gal et al., 2016). Finally, studies have implicated abnormal SG dynamics also in *C9ORF72*-related ALS through arginine-rich DPR proteins (Wen et al., 2014).

3.10.1 Therapeutic approaches to stress granules dynamics

There are currently no clinical trials directly targeting stress granules dynamics in ALS. Two recent genetic studies using animal models have demonstrated the potential therapeutic benefit of inhibiting RNP pathways. One study examined the effects of reducing ATXN2 levels in an animal model of ALS based on overexpressing wild-type TDP43(Becker et al., 2017). In this study, full knockout of ATXN2 increased the median lifespan of these animals by 80%, and ASO knockdown of ATXN2 increased their median lifespan by 35%(Becker et al., 2017). Cell stress disrupts the nuclear pore complex inducing the translocation of nuclear pore components to the cytoplasm where they colocalize with SGs (Zhang et al, 2018). In a Drosophila melanogaster model of ALS with overexpression of hexanucleotide repeat (G4C2)30 inhibitors of nucleocytoplasmic transport, such as the compound KPT-276, an exportin 1 inhibitor, suppress the pathological phenotype (Zhang et al, 2018). Many small-molecule inhibitors targeting known SG pathways are readily available, including those targeting the eIF4 pathway (involving mTOR), the eIF2α pathway (which includes the PERK and PKR pathways) and disaggregases (such as VCP, heat shock protein 104, transportin and importin (Wolozin and Ivanov, 2019). The inhibition of the PERK pathway with the PERK inhibitor GSK2606414 slowed disease progression in a TDP fly model (Kim et al., 2014).

3.11. Cytoskeleton integrity and axonal trafficking

Motor neurons are characterized by long axons and are therefore particularly susceptible to the disruption of axons metabolism and trafficking (Burk and Pasterkamp, 2019). It is therefore not surprising that several ALS-related genes (the most relevant being *PFN1, TUBA4A,* and *KIF5A*) are directly implicated in cytoskeleton or axonal trafficking and that their mutations impair axonal transport. In addition, it has been shown that mutations in TDP43 impair anterograde trafficking of TDP-43-positive RNA granules in both mouse neurons and human iPSC-derived motor neurons(Alami et al., 2014). Finally, several ALS genes (such as *FUS, HNRNPA1, HNRNPA2B1, VCP, TUBA4A, SOD1*, and *PFN1*) have been shown to undergo translation in the axon (Cagnetta et al., 2018), and therefore their mutations are likely to affect the axonal localization or translation of

their respective transcripts(Cook and Petrucelli, 2019). In addition, an heterogenous group of proteins related to axonal transport has been implicated in ALS. These include the superfamily of kinesins, a group of proteins mediating anterograde axonal transport, which is essential for synapse generation and for maintaining synaptic transmission (Hirokawa et al., 2009). Interestingly, missense mutations in the gene *KIF5A*, encoding a heavy chain kinesin, has been found to be related to hereditary spastic paraplegia (Reid et al., 2002), Charcot-Marie-Tooth type 2A neuropathy (Zhao et al., 2001) and amyotrophic lateral sclerosis (Nicholas et al, 2018). A second family of proteins includes dynein and dynactin. A dynein subunit was identified as a component of mutant SOD1-containing high molecular weight complexes prior to the onset of symptoms in ALS animal models (Zhang et al., 2007). Finally, mutations in the dynactin 1 gene (DCTN1), encoding the p150Glued subunit of dynactin, cause human distal hereditary motor neuropathy (HMN7B) (Puls et al, 2003) and transgenic mice expressing these mutations develop motor neuron degeneration (Laird et al., 2008).

3.11.1 Therapeutic approaches to cytoskeleton integrity and axonal trafficking

There are currently no clinical trials targeting cytoskeleton integrity and axonal trafficking in ALS. Known stabilizers of microtubules, such as paclitaxel, epothilones, and cabazitaxel, have been proposed for neurodegenerative diseases (Varidaki et al., 2018). A modulator of microtubules dynamics, noscapine, decreased hyperdynamicity, retrograde axonal transport and delayed onset improving motor performance in a *SOD1* transgenic mouse(Fanara et al., 2007). Conversely, epothilone D has been shown to accelerate disease progression in the ^{G93A}SOD1 mouse model of ALS(Clark et al., 2018), indicating that this approach should be considered with caution.

3.12. Other clinical trials employing drugs acting on different mechanisms

Reldesemtiv (CK-2127107) is a fast-skeletal muscle troponin activator (FSTA) that aims to slow the decline of muscle function in patients with ALS. Reldesemtiv is designed to bind to and slow the rate of calcium release from the troponin complex, a regulatory protein that plays an essential

role in the contraction of fast skeletal muscle fibers(Andrews et al., 2018). A placebo-controlled phase 2 trial of reldesemtiv performed in ALS (NCT03160898) failed to reach statistically significant differences in SVC decline compared with a placebo after 12 weeks. However, a post-hoc analysis combining the 3 groups of treatment demonstrated a 27% reduction in SVC decline and a 25% reduction of ALSFRS-R decline compared with the placebo.

Levosimendan binds selectively to troponin C sensitizing cardiac and skeletal muscles to calcium (Haikala et al., 1995), and an intravenous formulation of levosimendan is indicated for the treatment of acute worsening of severe heart failure. A placebo-controlled phase 2 trial of levosimendan aimed at evaluating the effect of the drug on sitting SVC did not reach the primary endpoint(Al-Chalabi et al., 2019). However, since a post-hoc analysis of the data showed that supine SVC favored levosimendan over placebo, a phase 3 trial is ongoing (NCT03505021).

Neuroinflammation and human endogenous retroviruses (HERV) are thought to have a role in the pathophysiology of amyotrophic lateral sclerosis (ALS) and treatments directed against endogenous retroviruses has demonstrated positive effects during in vitro and biomarker studies. Triumeq contains the booster-free integrase strand transfer inhibitor dolutegravir (50 mg) and the nucleoside reverse transcriptase inhibitors abacavir (600 mg) and lamivudine (300 mg). An open-label phase 2 trial of Triumeq (NCT02868580) demonstrated that the treatment was well tolerated. A favorable response on HERV-K expression levels was observed, accompanied by a decline in ALSFRS-R progression rate (Gold et al., 2019). A larger international phase 3 trial will be deployed to assess the effect of Triumeq on overall survival and disease progression.

Pimozide is an antipsychotic drug acting as a receptor antagonist of the D_2, D_3, and D_4 receptors and the 5-HT_7 receptor, used for the treatment of psychosis. It has also enhancing effects on the neuromuscular transmission in ALS(Patten et al., 2017). Although not effective in two mouse models of ALS (Pozzi et al., 2018), pimozide is investigated in ALS in a placebo-controlled phase 2 trial (NCT03272503).

The bioactive compounds of Cannabis sativa have shown antioxidant, anti-inflammatory and neuroprotective effects in preclinical models of central nervous system disease (Giacoppo and Mazzon, 2016). On this basis a placebo-controlled study on a cannabis-based medicine extract (CannTrust CBD Oil) is ongoing (NCT03690791).

CNM-Au8 is an orally administered gold nanocrystal suspension designed to overcome cells' bioenergetic imbalance, allowing cells to have the energy needed to function efficiently. The therapy is the first of a new class of neurotherapeutic medicines. It is designed to support bioenergetic cellular reactions and help remove the destructive byproducts of cellular metabolism that add to the breakdown of motor neurons in ALS. An open-label phase 2 trial of CNM-Au8 is underway in ALS patients, using an innovative primary endpoint, i.e. the ratio of the oxidized to reduced form of nicotinamide adenine dinucleotide (NAD+:NADH) measured non-invasively by 31phosphorous magnetic resonance spectroscopy (31P-MRS) (NCT04098406).

Copper delivery is another novel therapeutic approach in ALS. A pivotal pre-clinical study utilized the PET-imaging agent CuATSM, which is known to deliver copper into the CNS, and found that the drug prevented the early mortality of a ^{G93A}SOD1 mice with coexpression of Copper-Chaperone-for-SOD (CCS) protein. These mice showed also a marked increase of Cu, Zn SOD protein in their ventral spinal cord (Williams et al., 2016). On basis of this and other pre-clinical data, a placebo-controlled phase 2 trial has been started in ALS patients (NCT04082832).

GM604 is a peptide with a sequence identical to one of the active sites of human motoneuronotrophic factor (MNTF), an endogenous human embryonic stage neural regulatory and signaling peptide that controls the development, monitoring and correction of the nervous system. A small placebo-controlled phase 2a trial in ALS showed that GM604 was safe. GM604 decreased the plasma levels of Tau, SOD1 and TDP-43. No clear clinical benefit was detected (NCT01854294).

4. The genetics of ALS

Some 15% of ALS patients present a Mendelian ALS, i.e. have a family history for ALS or FTD. However, heritability of ALS has been estimated to be 61% (twin studies)(Al-Chalabi et al., 2010), 40-50% (patent-sib couples in a population based register) (Ryan et al., 2019), or 21% (genome-wide genotyping data) (Keller et al., 2014). These figures indicate that genetic therapy is highly relevant for the treatment of ALS. Currently some 30 genes with Mendelian pattern of inheritance have been reported to be related to ALS, although about 70% of pedigrees in Caucasian populations are determined by only four genes, i.e. *C9orf72, SOD1, TARDBP* and *FUS* (Table 4). In addition, some gene variants are likely to act as modifier of the phenotype. We will briefly discuss the major genes which are likely to be the first to be targeted by gene therapy.

4.1. C9orf72

C9orf72 is the most common gene in ALS, at least in the Caucasian populations, accounting for 30-40% of familial and 5% of apparently sporadic patients. In ALS patients the *C9orf72* gene is characterized by longer GGGGCC (G4C2) hexanucleotide repeat expansion (HRE) in the first intron (>30 HREs) than in healthy subjects (less than 23 HREs)(DeJesus-Hernandez et al., 2011; Renton et al., 2011). This expansion is also found in people affected by frontotemporal dementia (FTD), a disease characterized by loss of neurons in frontal and temporal lobes, and clinically by progressive behavioral and personality changes. Up to 50% of ALS patients with *C9ORF72* HREs develop various degrees of cognitive and behavioral impairment. The pathogenic mechanism of *C9orf72* has been related to three non-exclusive processes. First, the presence of the repeat expansion downregulates *C9orf72* gene expression leading to a loss of function. Second, HREs are bi-directionally transcribed into RNAs containing G4C2 and C4G2 repeats that aggregate in the nuclei of cells, sequestering RNA-binding proteins (RBPs) into intra-nuclear RNA foci. Third, repeat-containing RNAs can move to the cytoplasm, where they are translated into dipeptide repeat proteins (DPRs) through a non-canonical translation mechanism known as repeat-associated non-AUG-dependent (RAN) translation (Balendra and Isaacs, 2018).

4.2. SOD1

Mutations of the *superoxide dismutase 1* (*SOD1*) gene are found in 20% of FALS cases and in 2-4% of SALS cases. More than 180 missense and nonsense mutations have been reported (http://alsod.iop.kcl.ac.uk). The enzyme encoded by *SOD1* inactivates superoxide radicals converting them to O_2 and hydrogen peroxide (H_2O_2), which is, in turn converted to H_2O and H_2 by catalase(Radunovíc and Leigh, 1996). Our knowledge on *SOD1* mutations effects is largely based on transgenic mice, which bears the human transgene in several copies. The *SOD1* transgenic mice has shown that *SOD1* mutations cause a toxic gain-of-function. Patients carrying *SOD1* mutations have SOD1/ubiquitin-positive aggregates in the cytoplasm of motor neuron instead of TDP43 aggregates, which can be found in sporadic patients and patients carrying other ALS-related mutations, such as *C9orf72* and *TARDBP*(Shibata et al., 1996). The exact mechanism of SOD1 toxicity is unknown, but it has been hypothesized that mutations induce structural destabilization, prompting the misfolding of SOD1 protein followed by its aggregation (McAlary et al., 2016). Mutants *SOD1* trigger ER stress through interactions with Derlin 1 and have a direct effect on proteasome activity (Nishitoh et al., 2008). It is not clear why *SOD1* mutations affect only motor neuron, since SOD1 is a ubiquitous protein; it has been hypothesized that motor neurons are less able to correctly degrade unfolded/misfolded proteins.

4.3. TARDBP

The *TARDBP* (trans-activation element DNA-binding protein) gene is mutated in 5% of FALS cases and 1% in SALS cases. More than 50 different mainly missense mutations have been identified (Lattante et al., 2013). Most of these mutations are located in the 3′ region encoding a glycin-rich domain the gene product, TDP43. TDP-43, a protein for the most part expressed in the nucleus, participates in RNA metabolism through different mechanisms, including transcriptional regulation, splicing, mRNA stabilization, and miRNA processing. In ALS patients with or without

TARDBP mutations, TDP43 is mislocalized in the cytoplasm in the form of ubiquitin-positive aggregates(Scotter et al., 2015). Interestingly, in TDP43 aggregates also two adaptor proteins sequestosome 1 and ubiquilin 2 are found, both encoded by ALS-related genes (Lamark and Johansen, 2010). Several observations support an important role for autophagy in TDP43-linked pathogenesis. TDP43 interferes with autophagy also through SGs(Afroz et al., 2017). Abnormal post-translational modifications including hyperphosphorylation and aberrant cleavage are known to be the characteristics for TDP-43 inclusions (Sreedharan et al., 2008). It has been suggested that hyperphosphorylation of TDP43 residues may have a role in regulating its oligomerization or aggregation(Hasegawa et al., 2008). On these bases, targeting TDP43 hyperphosphorylation with specific kinases has been proposed as a therapeutic strategy in ALS(Alquezar et al., 2016; Li et al., 2017).

4.4. FUS

FUS (fused in sarcoma) missense and nonsense mutations are observed in 4% of FALS patients and 1% of SALS patients (Lattante et al, 2013). Most described mutations are localized in the 3′ region encoding an arginine/ glycine-rich region and a NLS domain (nuclear localization signal). FUS protein, which is essentially localized in the nucleus, regulates RNA processing, splicing, and mRNA trafficking (Deng et al., 2014). Patients with *FUS* mutations usually show cytoplasmatic aggregates immuno-reactive for FUS protein, which are considered essential for the degenerative process of neurons (Vance et al., 2013). *FUS* mutations have been implicated in several pathogenic mechanisms in ALS, such as ER stress, SGs dynamic, autophagy and RNA metabolism.

4.5. Possible approaches of gene therapy in ALS

Several gene therapy approaches have been proposed in ALS(Picher-Martel et al., 2016).

1. ASOs are short synthetic oligonucleotides (15-25 nucleotides) which bind targeted mRNA and can be administered by intrathecal or intracerebroventricular injections. ASOs reduce the expression of a specific protein though two main mechanisms: (a) the degradation of mRNA by endogenous RNase H or (b) the block of mRNA translation. ASOs are potentially useful in ALS by reducing the protein level of TDP-43, SOD1 of FUS or by targeting C9orf72 RNA foci.

2. RNA interference pathway through siRNAs, which are double-stranded RNAS. After strand unwinding, one siRNA strand binds argonaute proteins as part of the RNA-induced silencing complex (RISC) and is recruited to a target mRNA which is then cleaved (Bhandare and Ramaswamy, 2016). Specific pathways or genes can be targeted by siRNAs to rescue motor neurons (Imamura et al., 2017).

3. Another approach is antibodies directed toward misfolded proteins in order to reduce the toxic aggregates or to prevent the disease propagation between cells. Another possible mechanism of antibodies could be the pathological interactions between proteins by binding the interactions sites.

4. A fourth therapeutic approach is gene delivery to treat in particular loss-of-function mutations. Viruses can provide a functional replacement of a missing gene by mRNA or cDNA delivery. Using this approach, a phase 1, open-label, single-infusion, ascending dose, single-center clinical trial assessing gene-replacement therapy with onasemnogene abeparvovec (AVXS-101, Zolgensma) in spinal muscular atrophy (SMA type 1) infants has been recently completed (AVXS-101-CL-101; NCT02122952). AVXS-101 used a non-replicating adeno-associated virus (AAV) capsid to deliver a functional copy of the SMN1 gene to motor neuron cells in SMA patients as a one-time intravenous injection (Mendell et al., 2017). Zolgensma has been approved by the FDA to treat all SMA types in newborns through toddlers up to age 2 (https://www.fda.gov/vaccines-blood-biologics/zolgensma). The advantage of gene delivery by viruses is that they can cross the brain-blood barrier and consequently can be administered intravenously.

4.5.1. Gene therapy in ALS: clinical trials

The first in human clinical trial of gene therapy in ALS was based on an ASO (ISIS 333611) targeted toward SOD1 (Miller et al., 2013). In this study, ISI 333611 was administered by intrathecal infusion using an external pump over 11·5 h at increasing doses (0·15 mg, 0·50 mg, 1·50 mg, 3·00 mg) to four cohorts of eight patients with *SOD1*-positive ALS (six patients assigned to ISIS 333611, two to placebo in each cohort). The drug was well tolerated and SOD1 protein level measured by ELISA assay in CSF decreased in most patients. This study prompted the starting of a second phase 1-2 trial with an ASO (BIIB067, Tofersen) targeted toward SOD1 in a larger number of ALS patients. The study showed a statistically significant reduction of CSF SOD1 in the 100-mg cohort (n=10) versus placebo (n=12) (p=0.002) and suggested substantial reduction of CNS tissue SOD1. Lowering of CSF phosphorylated heavy neurofilament and slowing of functional decline as measured by ALSFRS-R scores, slow vital capacity, and muscle strength were observed in the 100-mg cohort versus placebo (Miller et al., 2019). It is currently ongoing a multicenter phase 3 placebo-controlled trial of BIIB067 (VALOR 233AS101, NCT02623699).

A phase 1 trial targeting *C9orf72* with ASOs is ongoing (NCT03626012). The drug, BIIB078, targets specifically the *C9orf72* mRNA and reduces the production of the abnormal protein. Differently from *SOD1*, whose mutations cause a gain of function of the mutated protein, in the case of *C9orf72* several pathogenetic mechanisms are considered, including loss of function caused by a downregulation of the transcription of the gene, making more uncertain this approach. However, preclinical studies on induced pluripotent stem cells (iPSC) and fibroblast models from patients with *C9orf72*-related ALS and transgenic mouse models expressing human C9orf72 HREs showed encouraging results, reducing RNA foci, abrogating aberrant gene expression patterns and improving behavioral and cognitive deficits (Donnelly et al., 2013; Jiang et al., 2016), justifying the moving toward humans.

Therapy based on virus vectors is also arriving to human trials in ALS. AAV9 viral capsid, creating a new AAV9-SOD1-shRNA (AVXS-301) has been translatable to the clinic.

Intracerebroventricular delivery (ICV) of AVXS-301 directly into the CSF was performed in the ^{G93A}SOD1 ALS mouse model and in non-human primates (NHPs) to reduce SOD1 throughout the CNS. A one-time ICV administration of AVXS-301 lead to significantly improved motor function and prolonged survival in mice overexpressing human mutated *SOD1*. AVXS-301 has been also tested on NHPs to facilitate dose extrapolation to human patients; in these animals, a single lumbar intrathecal administration obtained efficient transduction and SOD1 downregulation throughout the entire CNS. Importantly, the administration of AVXS-301 appeared safe and well tolerated in both mice and NHPs. A phase 1 trial on AVXS-301 in *SOD1* mutated patients is going to be started.

5. Stem cells therapeutic approaches

Cell-based therapies have generated widespread interest as a potential therapeutic approach for ALS because they potentially target multiple pathogenic mechanisms and may replace lost or diseased cells. Cell therapy focused on motor neurons (MNs) replacement is a daunting challenge, given the system complexity and anatomically distributed sites of degeneration however transplanted stem cells can favorably influence non-cell-autonomous disease mechanisms through the secretion of neurotrophic factors and their differentiation into astrocytes and microglia (Lepore et al., 2008; Teng et al., 2012; Xu et al., 2006) The cellular microenvironment plays a critical role in disease onset and progression and seems the most important target of stem cell therapeutic strategy (Boido et al., 2014; Knippenberg et al., 2017; Vercelli et al., 2008). It must be kept in mind that regardless of whether strategies can be developed to replace lost motor neurons and increase cell survival, the primary neurodegenerative process is still ongoing in both residual and newly generated cells. Several preclinical in vitro and in vivo studies show great efficacy of Neural Stem Cells (NSCs) and Mesenchymal Stem Cells (MSCs) in addressing ALS pathogenic mechanisms and slowing the rate of progression of the disease (Boido et al., 2014; Knippenberg et al., 2017; Kondo et al., 2014; Thomsen et al., 2018; Uccelli et al., 2012; Vercelli et al., 2008). However, no conclusive results

have yet been reported on the most effective cell type and way of delivery to be used in transplantation. Moreover, different stem cell types that have been tested both in pre-clinical and clinical experiments do not possess the bona fide properties of stem cell, that is self-renewing and pluri/multipotency and are more properly classified as precursors or even differentiated cells. Identification of appropriate good manufacturing process (GMP)-compliant human SC lines that exhibit similar efficacy and safety profiles is needed. Validation of findings using such GMP-compatible lines is also a prerequisite to clinical testing.

5.1 Mesenchymal Stem Cells

MSCs have received special interest in the treatment of ALS given their excellent accessibility, relative ease of handling, extensively studied characteristics. Overall, the use of MSCs for the treatment of ALS holds great promise due to the capability of this cell population to provide therapeutic support through immune modulation, anti-inflammatory, and anti-apoptotic activity. Transplantation of MSCs also creates a neuroprotective environment to slow the loss of endogenous neurons as well as promoting the survival of transplanted NSCs and/or stimulating compensatory neurogenesis. However, there is little evidence for the differentiation potentials of MSC in neuron in vivo. Recently even the author that first identified MSCs (Caplan, 2018) recommended the term must be changed to Medicinal Signaling Cells (Caplan, 2010) to more accurately reflect the fact that these cells home in on sites of injury or disease and secrete bioactive factors (Meirelles et al., 2009) that are immunomodulatory and trophic (regenerative)(Caplan and Dennis, 2006), meaning that these cells make therapeutic drugs that are medicinal (Caplan and Correa, 2011). The critical role of neuroinflammation in favoring and accelerating the pathogenic process in ALS raised up the needs to target the cerebral innate immune cells as potential therapeutic strategy to slow down the disease progression. In this scenario MSCs have aroused great interest thank to their immunomodulatory properties, that have been largely ascribed to the release of extracellular vesicles (EVs), namely exosomes and microvesicles. There are a wide variety of sources from

which MSCs are acquired. Bone marrow-derived MSCs (BM-MSCs) have been most widely investigated since a long time ago however MSCs obtained from adipose (AD-MSCs) or umbilical cord tissues (Wharton´s Jelly: WJ-MSCs) have been also used in ALS clinical trial. MSCs derived from different sources possess characteristic features possibly related to their developmental origin, the age of the donor, and the isolation method. Since procedures for isolation, maintenance and differentiation of MSCs may vary and influence clinical outcome, there is an urgent need for standardization of general protocols to achieve a more unified therapeutic effect (Nicaise et al., 2011). The most efficient route of administration seems to be intraspinal injection with subsequent cell migration towards the damaged tissue. Intraspinal transplantation of MSCs at an early stage of progression in *SOD1* animal ALS model improved motor function, attenuated MNs loss and astrogliosis and slowed the progression of the disease (Vercelli et al., 2008). Similar results had been obtained with intrathecal injections probably mediated by the secretion of neurotrophic factors (Boido et al., 2014) or release of extracellular vesicles (Lopez-Verrilli et al., 2016). However, the real ability of these cells to cross the BBB or the meninges, has not been fully demonstrated yet(Bianco, 2014).

5.2. Neural Stem Cells

Another accessible source of SCs for human transplantation are NSCs. Capable of generating all the differentiated neural cells in the central nervous system, NSCs can be sourced from primary tissues, such as the fetal, neonatal, and adult brain, or from ESCs and iPSCs. Human neural stem cells (hNSCs) or neural progenitor lines, suitable for allogeneic transplantation, are usually obtained from the fetal Central Nervous System (Gelati et al., 2013; Guo et al., 2010; Vescovi et al., 1999). In ALS models, a growing number of studies show both *in vitro* and *in vivo* improvements in ALS pathologies and survival (Knippenberg et al., 2017; Teng et al., 2012; Xu et al., 2006). The most efficient route of administration seems to be intraspinal injection and strategies that include multiple injections along the spinal cord including cervical and lumbar levels have been more effective in

prolonging animal survival (Teng et al., 2012). However, transplantation of NSCs does not always yield predictable outcomes, and migration and differentiation may be significantly affected by the recipient's microenvironment. hNSCs have been shown to be resilient to transformation and genetically and functionally stable (Foroni et al., 2007; Mazzini et al., 2015), although others have reported a moderate degree of chromosomal instability (Diaferia et al., 2011) hNSCs can be expanded (for over 30 passages) without transformation and do not generate tumors in long-term transplantation studies (Mazzini et al., 2015). Whether NSCs can survive in the long-term within tissue or are subject to immune-mediated rejection is still a matter of debate. Several preclinical studies using ^{G93A}SOD1 rodent models (Knippenberg et al., 2017; Teng et al., 2012; Xu et al., 2006) have shown that implanted hNSCs can integrate into the tissue, differentiate into astrocytes, oligodendrocytes and neurons and form synapses with host neurons (Knippenberg et al., 2017; Xu et al., 2006). These experiments have also demonstrated that transplantation of NSCs can slow MN degeneration, ameliorate motor symptoms and prolong animal life span in a dose-dependent manner.

Pluripotent SCs such as Embryonic stem cells (ESCs) and, more recently, iPSCs represent a novel source of hNSCs (Kondo et al., 2014) that have also been used in animal models of ALS. These cells have the potential to replace diseased MNs and incorporate themselves into existing neural circuits if they can establish the necessary and appropriate synapses However, standardization of protocols and reproducibility of iPSC-derived hNSC lines should be improved before clinical application.

5.3. Stem cells: clinical studies

Although a number of pre-clinical studies have been launched and there has been much progress in the understanding of the role of stem cells for the treatment of ALS there is still much to be learned. Pre-clinical studies have shown variable effects depending on the types and sources of stem cells.

However, no comparative studies have been performed involving different sources, types i.e. undifferentiated versus differentiated, stages, doses and routes of stem cell transplantation to validate their optimum therapeutic outcome. The genomic, anatomical and physiological difference between rodents and humans may preclude the translation of results obtained in rodents to the treatment of ALS patients. Moreover, the different stages of ALS progression in humans may play a critical role in the outcome of the cell transplantation. Large animal models may overcome some of these issues, in particular the short life span of rodents. Ideally, pre-clinical and clinical evidence should be integrated into a coherent treatment protocol for guiding further advances in stem cell research leading up to the evidence-based implementation of cellular therapies.

Currently a few pre-clinical studies have paved the way for clinical translation and very few scientific clinical trials have been carried out so far Nearly two decades of phase 1/2 clinical trials with stem cells (SCs) have not provided definitive evidence for safety and efficacy in ALS patients(Abdul Wahid et al., 2016). Only the results of 23 clinical trials with stem cells have been published in international peer-reviewed scientific journals (Table 5) and their long-term outcomes are largely unavailable. All completed trials are phase 1/2 and recruited very few patients (median=18). As of November 2019, a search of ClinicalTrials.gov (using the search terms “stem cell” and “ALS”) identified 9 active clinical trials (Table 6). Among them only one is randomized double-blind placebo-controlled phase 3 trial. A phase 2 trial on a single intramuscular and intrathecal administration of mesenchymal stem cell (MSC)-neurotrophic factor (NTF) cells (autologous bone marrow-derived MSCs, induced to secrete NTFs) has been published indicating the safety early promising signs of efficacy of the treatment (Berry et al., 2019).

5.4. Potential Adverse Effects of Stem Cell Therapy

There are several challenges concerning the clinical translation of stem cell-based therapy such as tumorigenicity, immune rejection, contamination, genetic modification, uncontrolled migration and

growth, and unintended trans-differentiation. The major safety concern regarding stem cell-based treatments is the potential for tumor formation, which may occur due to graft overgrowth, residual pluripotent cells in the graft, or acquisition of tumorigenic mutations during cell culture. While tumors have not been seen post-grafting in animals, it must be acknowledged that the grafts are only present in the animals for few months. In contrast, neural grafts would be expected to be present for some years in patients; therefore, rigorous safety data will be necessary to ensure that tumor risk is negligible, even over long periods of times. Different approaches for testing the safety of cell therapy have been evaluated in clinical trials. The quality and well-defined characteristics of the stem cells are basic criteria that need to be ascertained prior to any clinical application. These procedures should be extensively reported. Since procedures for isolation, maintenance and differentiation particularly of MSCs may vary and influence clinical outcome, there is an urgent need for standardization of general protocols to achieve a more unified therapeutic effect. Equally important is the rigorous clinical trial design in order to ensure unbiased, reproducible, and valid safety outcomes. The primary safety outcomes for the trials should be carefully considered, together with the dose, route, and timing of cell administration. Differences in clinical trial designs have made difficult comparing the outcomes between studies. Minimally invasive procedures, such as intravenous route, may not present much safety issues related to the injection site as opposed to more invasive administrations (e.g. intrathecal, and intracerebral) which should be performed with much greater care by a skilled clinician. The risks of chronic immunosuppression are not insignificant, and must be considered in the risk-benefit analysis of any cell transplant strategy. All previous clinical studies have reported that both the cells and the procedures used were safe, but these results are still inconclusive because most studies lacked long-term follow-up and well-defined outcomes, and the cohorts of patients were small, heterogeneous and tended to have more advanced disease. Intrathecal delivery was mostly used for MSCs delivery and very few adverse effects of one-time dosing have been reported (headache, fever and back pain the most common). (Table 7) Multiple injections and highest dose of adipose tissue-derived MSCs were associated to

higher risk of lumbosacral radiculitis associated with CSF protein elevation and pleocytosis or with nodularities and enhancement in MRI study. However, CSF and MRI were reported as an outcome only in one paper (Staff et al., 2016). Transient pain and paresthesia were the most common side effects of surgical transplantation in the spinal cord (Table 8) no evidence of cord damage, syrinx, tumor formation or neurological deterioration had been reported.

5.5. Potential Efficacy of Stem Cell Treatments

Clinically, previous and current trials are primarily phase 1/2 trials with safety as the main objective. Most of them provided some indications of a possible transitory clinical benefit induced by the treatment, generally reflected by changes in the progression rate of the ALSFRS-R score and FVC. Both safety and efficacy results seem to be independent of the type and number of cells and the mode of delivery. These studies had many limitations for producing evidence to demonstrate the efficacy of cellular therapies. Most published clinical studies were small and single-center, with different recruited patient populations, therapeutic protocols, and outcome measures. The protocols for cellular expansion in most clinical studies are not reported or are suboptimal, hence some negative clinical results can be explained by the poor quality of the transplanted products. A compelling lesson learned from previous research is that confirmatory trials of the efficacy of new stem cell lines for the treatment of ALS should be initiated only after an evidence-based, treatment protocol is constructed. Questionable trials represent a waste of resources and time and generate an avoidable risk for patients. Moreover, it is important that definite negative results both from preclinical and clinical experiments are also published in a timely fashion. The preliminary efficacy measure detected by phase 2 studies should clearly indicate significant effect sizes at the endpoint of open-label studies before confirmatory studies are initiated. Ideally, pre-clinical and clinical evidence should be integrated into a coherent treatment protocol for guiding further advances in stem cell research leading up to the evidence-based implementation of cellular therapies.

Basic information about dosing, time of administration, type of cells, route of administration, and other variables needs to be clarified. Patient selection is a very important variable, but to date, there is little agreement about this inclusion criterium. The more heterogeneous study population may mask efficacy of the study intervention on more specific subpopulations of patients such as genetic forms of the disease or restricted phenotypes (e.g. flail-arm or flail-leg variants).

Larger studies are needed, but these will be difficult to perform due to factors such as cost, and the balance between relative advantages and disadvantages of wide versus narrow criteria for enrolment. Recruitment for large controlled studies may also be a challenge due to the reluctance of patients to participate in placebo-controlled studies where they may not get treatment and potentially undergo harmful procedures (Atassi et al., 2016).

When examining the current literature and considering the design of future studies, it is important to select reliable outcome measures. Owing to the progressive damage of neural systems and the fact that the presentation and severity of the biological impairments and functional disability vary markedly across people with ALS a coordinated effort between basic and clinical researchers is needed to select and organize outcome measures assessment. An adequate interface between basic researchers and clinical experts in ALS is essential in establishing appropriate pathogenic targets and expected clinical outcomes. Different cell types (autologous vs allogenic) or ways of delivery (surgery vs intrathecally) may require different selection of primary outcome measures for assessing ALS patients. Another difficulty with primary outcome measures is the choice of the magnitude of improvement indicating a clinically significant effect size. In stem cells clinical trials it will be important to identify also clinically meaningful changes, not just those that are statistically significant. The current stage of the clinical research in this field requires to identify a primary outcome measure which can show an improvement that is clinically meaningful to the patient so that the risk of the procedure is reasonably balanced by the potential clinical benefits and the value of the resulting scientific knowledge.

ALS is a progressive chronic disease that takes several years before clinical manifestation of symptoms hence it is essential to determine the appropriate time window for transplantation during the disease progression. We can hypothesize that SC transplantation, at the onset of the disease, can be more effective when the motor neurons suffers the fewest alterations in microenvironment. However, patients at initial stage of the disease have higher risk of iatrogenic functional damage hence a balance between risk and potential benefit must be evaluated.

5.6. Ethical Issues in stem cell therapy

Specific guidelines for SC research, including the most recent revision of international guidelines in 2016 (Daley et al., 2016), have been published also in a version intended for patients. These guidelines and a recent Lancet Commission of Experts report (Cossu et al., 2018) stress the ethics of procurement, derivation, banking, distribution, and use of cells and tissues and helps assure patient safety and the integrity of the research process. Despite progress in this field has been slow many "stem-cell clinics" have appeared in the past few years, offering various types of treatments with a clear overestimation of effectiveness and an underestimation of risks. Such unregulated clinics are usually private organizations promoting the therapeutic benefits of MSCs. They generally offer little if any assurance of expertise, quality of care or ethical standards (Cote et al., 2017). The proliferation of these "clinics" has led to 'medical tourism' by ALS patients who are particularly vulnerable. Patients planning to undergo unproven SC interventions must be informed about the many risks and undefined benefits of these procedures. Researchers and media but also legislators have the responsibility to protect vulnerable patients from charlatans and "stem cell clinics"(Daley et al., 2016).

6. Conclusions and future perspectives

The last 3 decades have seen much hope and as much disappointment for promising therapies in ALS. Only two drugs, riluzole and edaravone, have shown to slightly positively affect the course of the disease. ALS community is still searching for an effective disease-modifying treatment of ALS. The advances in the knowledge of pathogenic mechanisms of ALS, mainly but not only through the development of pre-clinical models and, more recently, of iPSCs derived from patients, will allow more focused therapeutic approaches. However, the clinical and pathogenic heterogeneity of the disease still hamper the achievement of a therapy. It is getting clear for ALS researchers that a single therapeutic approach will reveal to be ineffective for all ALS patients. Instead, a personalized approach, based on the specific clinical and biological characteristics of subgroups of patients, will be more likely the avenue for effective targeted treatments. This notion is supported by current trials on specific ALS-related mutations, in particular *SOD1* and *C9orf72*, and by the demonstration that specific drugs may be particularly effective in defined subgroups of patients, as it has been shown in the case of edaravone trials.

The heterogeneity of the pathological mechanisms in ALS is reflected by the myriad of therapeutic approaches that are being followed, including oxidative stress, autophagy, apoptosis, excitotoxicity, nucleocytoplasmic transport, and neuroinflammation. Other important mechanisms have not yet been targeted for therapy, namely DNA damage, RNA splicing/metabolism, and axonal trafficking, likely because they are less easily druggable.

An improvement of the design of clinical trials in ALS is also urgent. Novel design should include more efficient and relevant outcome measures, including biomarkers sensitive to disease change, group-sequential and adaptive designs to shorten phase 2 trials, better patients' stratification. Also, the organization of multi-arm multi-stage platform trials could represent a cost-effective tool for testing several drugs in parallel with a single placebo arm, a strategy already utilized in cancer therapy (Park et al., 2019).

Due to the possible multiple pathogenic mechanisms acting in the same patient, it is also likely that effective therapy in ALS will be based on poly-pharmacological and maybe pharmacological-

cellular interventions. Therefore, we need to develop novel designs for assessing these multiple combined interventions in clinical trials.

The area of cellular therapies is growing, but it is still in its infancy. Centers of excellence for ALS and regenerative medicine are likely to facilitate the regulated conduct of a stem cells clinical trial, allowing rigorous evaluation of the safety and efficacy of cell therapy. A number of promising clinical trials for ALS patients are now on the horizon, and we hope that stem cells will enter the clinic in the medium-term future.

Finally, a strict and open collaboration between ALS research community, patients' advocates, regulatory authorities, and drug companies will be the ultimate key for the success of future clinical trials for this fatal disease.

Conflict of interest: Adriano Chiò serves on scientific advisory boards for Mitsubishi Tanabe, Roche, Cytokinetics, and Biogen. Letizia Mazzini and Gabriele Mora report no conflicts of interest.

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Table 1. Possible reasons for negative results of ALS RCTs

1. Rationale	Insufficient overall rationale
	Insufficient, negative or misinterpreted data from pre-clinical models
	Misinterpretation of phase 1 or 2 findings
	Excessive reliance on post-hoc analysis of phase 1 or 2 findings
2. Pharmacological issues	Inadequate drug passage of the brain-blood barrier
	Pharmacological interaction (riluzole, other drugs)
	Inadequate dose (too low)
	Drug not tolerated at the active dose (drug toxicity)
	Inadequate pharmacokinetics
	Pharmacogenetics issues
3.Trial design issues	Insufficient statistical power (sample size calculation)
	Inadequate number of patients enrolled
	Inadequate length of trial
	Inadequate inclusion/exclusion criteria
	Imbalance of concurrent treatments (riluzole, other drugs)
	Failure of randomization (imbalance of treatment arms)
	Heterogeneity of sample population (Phenotypic heterogeneity; Inclusion of long survivors; Inclusion of too advanced patients)
	Lack of pharmacological biomarkers (demonstration of the biological effect of the drug)
	Lack of biomarkers sensitive to disease progression
	Non-generalizability of enrolled population

	Inadequacy of measures of efficacy (see Table 2)
	Different population from phase 2 to phase 3
	Heterogeneity of patients' care by recruiting centers (lack of multidisciplinary treatment group)
	Excessive numbers of dropouts/premature discontinuations

Table 2. Measures used as endpoint in ALS clinical trials

Clinical biomarkers	Survival (time to death, tracheostomy o full time non-invasive respiratory support)
	ALSFRS-R
	Muscle Strength Testing
	Respiratory measures (FCV, SVC, Snip)
	Bulbar-specific measures
	Composite markers (Combined Assessment of Function and Survival, CAFS)
Neurophysiological biomarkers	Neurophysiological Index
	Motor unit estimation (MUNIX)
	Electrical impedance myography (EIM)
Muscle imaging	Magnetic Resonance Imaging
	Muscle ultrasound
Staging	King's staging
	MiToS staging
'Wet' biomarkers	Creatine
	Albumin
	Neurofilaments
Neuropsychological markers	Neuropsychological battery, ECAS

Neuroimaging biomarkers	Brain and spinal cord MRI
	Brain and spinal cord PET

Table 3. List of current pharmacological trials in ALS

Trial number	Location of treatment	Type of trial	Molecules	Mechanism of action	Type of delivery	Estimated patients' number	Status	Outcome
EXCITOXICITY								
NCT03520517	U.S.A.	Phase 1	Riluzole (BHV-0223)	Anti-glutammate	Dissolving tablet	22	Pending FDA approval	Safety and tolerability
NCT03679975	U.S.A.	Phase 1	Riluzole (ROSF)	Anti-glutammate	Soluble film	30	Pending FDA approval	Safety and tolerability
NCT03020797	U.S.A.	Phase 1	Perampanel	AMPA receptors antagonist	Oral	60	Ongoing	Safety

NCT03019419	Japan	Phase 2	Perampanel	AMPA receptors antagonist	Oral	60	Ongoing	ALSFRS-R
NCT03377309	Lebanon	Phase 1	Perampanel	AMPA receptors antagonist	Oral	20	Ongoing	Safety and tolerability
NCT02118727	U.S.A.	Phase 2	Memantine	NMDA receptors antagonist	Oral	90	Ongoing	ALSFRS-R
OXIDATIVE STRESS								
			Edaravone	Free radical scavenger	Intravenous		Marketed in U.S.A., Canada, Switzerland, some Asian countries	

NCT03548311	Japan	Phase 3	Methylcobalamin	Reduce oxidative stress by decreasing level of homocysteine	Oral	128	Ongoing	ALSFRS-R
NCT03472950	U.S.A.	Phase 2	Ranolazine	reduce neuronal hyperexcitability	Oral	20	Ongoing	Safety
NCT03293069	France	Phase 2/3	Deferiprone	Iron chelator, reduce oxidative stress	Oral	240	Ongoing	CAFS
			Verdiperstat	Inhibitor of myeloperoxidase			Pending regulatory approval	
NCT03168711	U.S.A.	Phase 2	Inosine	Anti-oxidant	Oral	30	Ongoing	Safety and tolerability
MITOCHONDRIAL DYSFUNCTION								

NCT01879241	Germany	Phase 2	Rasagiline 1 mg	MAOB inhibitor	Oral	252	Completed	Survival
NCT01232738	U.S.A., Canada	Phase 2	Rasagiline 2 mg	MAOB inhibitor	Oral	36	Completed	ALSFRS-R
AUTOPHAGY AND PROTEIN QUALITY CONTROL								
NCT03491462	U.S.A., Canada and Europe	Phase 3	Arimoclomol	Increase Hsp70 and Hsp90, reduce protein aggregates	Oral	231	Ongoing	CAFS
EU 2014-005367-32	Italy	Phase 2	Guanabenz	Alpha-2-adrenergic receptor agonist	Oral	208	Completed	Progress of disease (ALS-MITOS staging system), safety and tolerability
NCT03580616	U.S.A.	Phase	L-Serine		Oral	50	Ongoing	Safety and

		2		BMAA inhibitor				tolerability
NCT03693781	Italy	Phase 2	Colchicine	Enhance HSPB8	Oral	54	Ongoing	ALSFRS-R
NCT03359538	Italy	Phase 2	Rapamycin	mTorC1 inhibitor and enhance protein degradation	Oral	63	Ongoing	T-reg number
NEUROINFLAMMATION								
			Ravulizumab	Inhibitor of complement C5			Pending FDA approval	
			Zilucoplan	Inhibitor of complement C5			Pending FDA approval	
NCT02238626	U.S.A.	Phase	Ibudilast		Oral	71	Completed,	Safety and

		2	(former MN-166)	inhibitor of PDE-4 and -10 and MIF			pending FDA approval for phase 3	tolerability
NCT02469896	U.S.A.	Phase 2	Tocilizumab	IL6 receptor antibody	Intravenous	22	Completed	Safety and tolerability
NCT03508453	Australia	Phase 2	IC-14	Monoclonal anti CD-14 antibody	Intravenous	50	Not yet recruiting	Changing concentration of neurofilament
NCT03474263	Australia	Phase 2a	IC-14	Monoclonal anti CD-14 antibody	Intravenous		Ongoing	Glial activation measured by PET
NCT03792490	Deutschland	Phase 2	Fasudil	Rho kinase	Intravenous	120	Ongoing	Safety and tolerability

				inhibitor				
NCT03127267	Canada	Phase 3	Masitinib	Tyrosine kinase inhibitor	Oral	495	Not yet recruiting	ALSFRS-R
NCT03039673	France, UK	Phase 2	Interleukin-2	Increased T-regs	Subcutaneous	304	Ongoing	Survival
NCT04055623	U.S.A.	Phase 2	Autologous T-regs + Interleukin-2	Modulate immune system	Expansion and infusion Intravenous	12	Ongoing	Change in T-regs
NCT03456882	U.S.A., Italy	Phase 2	RNS60	Upregulation of T-regs and activation of protective astrocytes and	Intravenous and Inhaled	142	Ongoing	Farmacodynamic biomarkers

				microglia				
NCT03652805	Israel	Phase 1	IPL344	PI3K-Akt signaling activator	Intravenous	15	Ongoing	Safety and tolerability
NCT03755167	Israel	Phase 2	IPL344	PI3K-Akt signaling activator	Intravenous	15	Ongoing	Safety and tolerability
NCT03766321	Italy	Phase 2	Fecal microbiota transplant	Modulate immune system	Infusion through nasojejunal tube	42	Ongoing	Increase of T-reg number
NCT04090684	U.S.A.	Phase 1	Ciprofloxacin/Celecoxib	Regulate	Oral	30	Net yet recruiting	Safety and tolerability

				inflammation				
NCT03705390	UK	Phase 2	Low molecular weight Dextran sulfate	Complement system inhibitor, reduce microglial activation	Subcutaneous	15	Ongoing	Safety and tolerability
APOPTOSIS								
NCT03800524	Europe	Phase 3	Tauroursodeoxycholic acid (TUDCA)	Anti-apoptotic, inhibition of caspase-3	Oral	440	Ongoing	ALSFRS-R
NCT03127514	U.S.A.	Phase 2	Phenylbutyrate + tauroursodeoxycholic	Anti-apoptotic	Oral	132	Completed	ALSFRS-R, safety

			acid (TUDCA)					
NCT02655614	U.S.A., Canada, Netherland	Phase 1	GDC-0134	dual leucine zipper kinase inhibitor	Oral	82	Ongoing	Safety and tolerability
NCT3757351	Netherland	Phase 1	DNL747	RIPK1 inhibitor	Oral	16	Ongoing	Safety and tolerability, PK, PD
NUCLEOCYTOPLASMIC TRANSPORT								
NCT03945279	U.S.A.	Phase 1	BIIB100	inhibitor of XPO1	Oral	40	Ongoing	Safety
OTHER MECHANISMS								
NCT03160898	U.S.A., Europe	Phase 2	CK-2127107 (Reldesemtiv)	Troponin	Oral	458	Ongoing	SVC

				complex activation				
NCT03505021	North America, Europe, Australia	Phase 3	Levosimendan	Action on Troponin C	Oral	450	Ongoing	SVC
NCT02868580	Australia	Phase 2a	Triumeq (dolutegravir 50 mg, abacavir 600 mg, lamivudine 600 mg)	Anti-retroviral	Oral	43	Ongoing	Safety
NCT03272503	Canada	Phase 2	Pimozide	D_2, D_3, D_4 receptor antagonist	Oral	100	Ongoing	ALSFRS-R
NCT03690791	Australia	Phase 3	Cannabis	Anti-oxidant,	Oral	30	Ongoing	ALSFRS-R, FVC

				anti-inflammatory				
NCT03843710	U.S.A.	Phase 2	CNM-Au8	Support energetic cellular reactions	Oral	24	Not yet recruiting	MR spectroscopy
NCT04082832	Australia	Phase 2	Cu-ATSM	Deliver Copper in CNS	Oral	80	Ongoing	ALSFRS, ECAS
NCT01854294	U.S.A.	Phase 2	GM604	Decrease endogenous human embryonic stage neural regulatory	Intravenous	12	Recruitment completed	Safety and tolerability, biomarker in CSF

				signaling peptide				
		GENETICS						
NCT02623699	North America, Europe	Phase 3	BIIB067 (Tofersen)	Antisense oligonucleotides against mutated SOD1 mRNA	Intrathecally	183	Ongoing	Safety, laboratory test and ALSFRS-R
NCT03626012	North America, Europe	Phase 1	BIIB078	Antisense oligonucleotides against mutated C9ORF72 mRNA	Intrathecally	80	Ongoing	Safety

Table 4. Mendelian genes related to ALS or ALS-FTD

Name	Frequency (family cases)	Type of trasmission	Name of the disease	Gene	Locus	Protein
Amyotrophic lateral sclerosis						
ALS1	20%	AD/AR	SOD1-FALS	SOD1	21p22.1	Cu-Zn Superoxide dismutase 1
ALS2	Rare	AR	Juvenile ALS type 3	ALS2	2q33	Alsin
ALS3	Single family	AD	FALS	-	18q21	Unknown
ALS4	Rare	AD	Distal henreditary neuropathy with pyramidal signs	SETX	9q34	Senataxin
ALS5	Rare	AR	Juvenile ALS/PLS	SPG11	15q15.1-	Spastacin

			with atrophy of corpus callosum		q21.1	
ALS6	2%	AD	FALS	FUS-TLS	16q12	Fused in sarcoma/translocated in liposarcoma
ALS7	Single family	AD	FALS	-	20tel	Unknown
ALS8	Rare	AD	SMAIV, SMA Finkel type (proximal)	VAPB	20q13	Vesicle associated membrane protein associated protein B
ALS9	Rare	AD	FALS	ANG	14q11.2	Angiogenin
ALS10	6%	AD	FALS	TARDBP	1p36	TDP-43
ALS11	Rare	AD	FALS	FIG4	6q21	Phosphatidylinositol 3,5-bisphosphate 5-phosphatase
ALS12	Rare	AR	FALS	OPTN	10p15-p14	Optineurin

ALS13	2%	AD	FALS	ATXN2	12q24.12	Ataxin 2, espansioni intermedie
ALS14	Rare	AD	FALS IBM-FTD	VCP	9p13.3	Valosin Containing Protein
ALS15	Rare	X-linked D	FALS	UBQLN2	Xp11.21	Ubiquilin 2
ALS16	Rare	AR	FALS	SIGMAR1	9p13.3	Sigmar nonopioid intracellular receptor 1
ALS17	Rare	AD	FALS	CHMPB2	3p11.2	Charged multivesicular body protein B2
ALS18	Rare	AD	FALS	PFN1	17p13.2	Profilin 1
ALS19	Rare	AD	FALS	ERBB4	2q34	Chorion protein gene ErB.4
ALS20	Rare	AD	FALS	HNRNPA1	12q13	Heterogeneous nuclear ribonucloeprotein A1
ALS21	Rare	AD	FALS	MATR3	5q31.2	Matrin 3

ALS22	Rare	AD	FALS	TUBA4A	2q35	Tubulin alpha 4A
ALS23	Rare	AD	FALS	ANXA11	10q22.3	Annexin A11
ALS24	Rare	AD	FALS	NEK1	4q33	Never in mitosis gene A-related kinase 1
ALS25	Rare	AD	FALS	KIF5A	12q13	Kinesin family member 5A
Amyotrophic lateral sclerosis and frontotemporal dementia						
ALS-FTD1	30%	AD	ALS with FTD	C9ORF72	9p21.2	Chromosome 9 open reading frame 72
ALS-FTD2	Rare	AD	ALS with FTD	CHCHD10	22q11.23	Coiled-coil-helix coil-helix-domain containing protein 10
ALS-FTD3	Rare	AD	ALS with FTD	SQSTM1/p62	5q35.3	Sequestosome 1
ALS-FTD4	Rare	AD	ALS with FTD	TBK1	12q14.2	TANK-binding kinase 1

Table 5. Summary of past stem cells clinical trials in ALS

Trial number	Location of treatment	Type of trial	Type of cells	Type of delivery	Patients number	Planned outcome	Results
MSCs							
(Mazzini et al., 2012, 2010, 2003)	Italy	phase 1	autologous bone marrow MSCs	surgical transplantation	7 + 10 + 2 (compassionate use)	safety + preliminary efficacy	safe in long term follow-up
(Blanquer et al., 2012, 2010)	Spain	phase 1	autologous bone marrow mononuclear cells	surgical transplantation (multiple)	11	safety + preliminary efficacy	safe; no changes in disease progression
(Staff et al., 2016)	US	phase 1,	autologous adipose-derived	intrathecal	27 (5/5/7/5/5)	safety + preliminary	safe

		dose escalation	mesenchymal stromal cells			efficacy + biomarkers	
(Deda et al., 2009)	Turkey	phase 1/2	BM-derived hematopoietic progenitor stem cells	surgical transplantation	13	safety	safe
(Karussis et al., 2010)	Israel	phase 1/2	autologous bone marrow MSCs	Intrathecal and intravenous	19	safety	safe
(Prabhakar et al., 2012)	India	phase 1/2	autologous BM-derived stem cells	intrathecal	10	safety + preliminary efficacy	trend to stabilization in ALSFRS-R
(Syková et al., 2017)	Czech Republic	Phase 1/2	ex vivo-expanded autologous BM-MSCs	intrathecal	26	safety + preliminary efficacy	safe; mild reduction in ALSFRS-R progression

(Oh et al., 2018, 2015)	Korea	phase 1 and phase 2	autologous bone marrow MSCs	Intrathecal (multiple)	8 (first trial); 33 (+ 31 controls second trial)	safety + preliminary efficacy + biomarkers	safe; reduced pro-inflammatory cytokines
(Petrou et al., 2016)	Israel	phase 2a, dose-escalation	autologous MSC-NTF (NurOwn®)	Intrathecal and intramuscular	12 (6 IM + 6 IT) + 14 (IM + IT)	safety + preliminary efficacy	safe; progression decline in intrathecal group
NSCs							
(Feldman et al., 2014; Glass et al., 2016)	US	phase 1 and 2, dose escalation	fetal NSCs	surgical transplantation	18 + 15	safety + preliminary efficacy	safe; no changes in disease progression
(Mazzini et al., 2019, 2015)	Italy	phase 1, dose esclalation	fetal NSCs	surgical transplantation	18	safety + preliminary efficacy	safe; 2 patients showed a transitory improvement of

							subscore ambulation in ALSFRS-R.
G-CSF							
(Zhang et al., 2009)	China	phase 1/2	G-CSF	Peripheral injection	13	safety + preliminary efficacy	safe; reduction in slope of decline of ALSFRS-R
(Tarella et al., 2010)	Italy	phase 1/2	G-CSF	Peripheral injection	24	safety + preliminary efficacy + biomarkers	safe; no clinical benefit
(Nefussy et al., 2010)	Israel	Phase 2	G-CSF	Peripheral injection	17 (+18 placebo)	safety + preliminary efficacy	safe; no differences in progression
(Chiò et al.,	Italy	Phase 1/2	G-CSF	Peripheral	24	safety +	safe; no clinical

2011b)				injection		preliminary efficacy + biomarkers	benefit
(Cashman et al., 2008)	Canada	phase 1/2	G-CSF	Peripheral injection	8	safety + preliminary efficacy	safe; no clinical benefit
(Appel et al., 2008)	US	phase 1/2	G-CSF + total body irradiation	Peripheral injection + total body irradiation	6	safety + preliminary efficacy	no benefit
(Martinez et al., 2009; Martínez et al., 2012)	Mexico	phase 1/2	G-CSF + CD133+ cells	Peripheral injection + intracortical	10 (first trial); 67 (second trial)	safety + preliminary efficacy	safe; mild improved survival
OECs							
(Huang et al.,	China	phase 2	fetal OECs	intracortical	15 (+ 20 not	safety +	slower rate of

2008)					randomized controls)	preliminary efficacy	decline in ALSFRS-R

ALSFRS-R: Amyotrophic Lateral Sclerosis Functional Rating Scale – revised; BM: bone marrow; G-CSF: Granulocyte-colony stimulating factor; MSCs: mesenchymal stem cells; NTF: neurotrophic factors; NSCs: neural stem cells; OECs: olfactory unsheathing cells

Table 6. Ongoing stem cells clinical trials in ALS

Trial number	Location of treatment	Type of trial	Type of cells	Type of delivery	Estimated patients' number	Status	Outcome
NCT03268603	US	phase 2, single group assignment	autologous adipose-derived MSCs	intrathecal (multiple)	60	recruiting	safety + preliminary efficacy
NCT03280056	US	phase 3, randomized, double-blind, placebo-controlled	autologous NurOwn® (MSC-NTF)	intrathecal (multiple)	200	active	safety + biomarkers
NCT03296501	Poland	phase 1, single group assignment	autologous adipose derived mesenchymal regenerative cells (ADRC)*	intrathecal (3 injections every months)	30	unknown	safety + preliminary efficacy

NCT03214146	Korea	phase 1, open label	HLA-haplo Matched Allogenic Bone Marrow Derived SCs	intrathecal (multiple)	6	active, not recruiting	safety
NCT02290886	Spain	phase 1/2, randomized, controlled with placebo	autologous adipose-derived MSCs	intravenous	40 (currently 52)	active, not recruiting	safety + preliminary efficacy
NCT02917681	Brazil	phase 1/2, unmask	autologous adipose-derived MSCs	intrathecal	28	unknown	safety + preliminary efficacy
NCT03482050	Israel	phase 1/2a, dose escalation	Astrocytes (AstroRx) derived from human ESCs	intrathecal (multiple)	21	recruiting	safety + preliminary efficacy
NCT02943850	US	phase 1, single group assignment	CNS10-NPC-GDNF**	surgical transplantation	18	active, not recruiting	safety + biomarkers
NCT02478450	unknown	phase 1/2a, non-	Q-Cells®***	surgical	30	not yet	safety +

		randomized, dose escalation		transplantation		recruiting	preliminary efficacy
EudraCT 2014-002228-28	Italy	phase 2	G-CSF	IV administration of G-CSG, stimulation of CD34+ cells	76	Recruiting	safety + preliminary efficacy

* ADRC: heterogeneous population of cells including multipotent adipose derived SCs, other progenitor cells, fibroblasts, T-regulatory cells and macrophages

** transplanted genetically modified neural progenitor cells (CNS10-NPC-GDNF)

*** adult stem cells, restricted in their ability to differentiate in glia (hGRPs)

Table 7. Adverse Events after Intrathecal SCs Infusion

Author **Cells** **Adverse Event**	**(Petrou et al., 2016)** **BM-MSCs**	**(Oh et al., 2018)** **BM-MSCs**	**(Staff et al., 2016)** **Adipose-MSCs**	**(Syková et al., 2017)** **BM-MSCs**
Headache	13/26 (50%)	9/33 (25%)	1/27 (4%)	7/26 (30%)
Fever	11/26 (42%)	18/33 (63%)	1/27 (4%)	_
Back/leg pains	8/26	16	7/27	_

	(30%)	(50%)	(26%)	
Neck stiffness	2/26 (7%)	_	_	_
Vomiting	3/26 (11%)	_	_	
Spasticity	1/26 (4%)	_	_	_
Bruising	1/26 (4%)	_	_	_
Thickened lumbosacral nerve roots	_	_	7/27 (26%)	_

BMSC Bone Marrow Mesenchymal Stem Cells

Table 8. Adverse Events after Intraspinal SCs Transplantation

Author **Cells** **Adverse Event**	**(Mazzini et al., 2008, 2004)** **BMSCs**	(Ruiz-López et al., 2016) **HBMSCs**	**(Mazzini et al., 2019, 2016)** **Fetal NSCs**	**(Glass et al., 2016; Riley et al., 2014)** **Progenitor NSCs**
Pain	11/19 (58%)	10/11 (90%)	8/18 (44%)	22/30 (73%)
Hypoesthesia	4/19 (21%)	7/11 (63%)	_	6/30 (20%)

Paresthesia	12/19 (63%)	6/11 (54%)	7/18 (38%)	4/30 (13%)
Urinary incontinence	_	_	_	2/30 (6%)
Tremor	_	_	1 /18 (5%)	2/30 (6%)
Respiratory disorders	_	1/11 (9%)	1/18 (5%)	1/30 (3%)
Acute Diabetes Mellitus	_	_	1/18 (5%)	2/30 (6%)
Pneumonia	_	2/11 (18%)	4/18 (22%)	3/30 (10%)

BMSCS Bone Marrow Mesenchymal Stem Cells

HBSCs Haematopoietic Bone Marrow Stem Cells

NSCs Neural Stem Cells