Universidade de Lisboa

Faculdade de Farmácia

Biological and Technical Advances in Therapies for Amyotrophic Lateral Sclerosis Disease

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Resumo

A Esclerose Lateral Amiotrófica (ELA) é uma doença neurodegenerativa fatal, caracterizada pela perda de neurónios motores e atrofia muscular progressiva. A ELA pode ser classificada em familiar (10% dos casos) se derivar de um padrão de hereditariedade autossómica dominante, ou esporádica (10%) se não estiver associada a um historial familiar. A ELA pode também ser classificada como bulbar ou medular, dependendo do local de origem. O seu prognóstico é a morte do paciente por falência respiratória, 2 a 5 anos após o início dos sintomas.

A ELA pode ter diversas manifestações motoras tais como fraqueza muscular progressiva, degeneração, fasciculações e contrações, mas também manifestações extra-motoras podendo ir ao encontro do diagnóstico de demência fronto-temporal (DFT).

As causas da ELA não são claras, porém existem diversos mecanismos patológicos associados. Uma das principais causas genéticas é a mutação no gene *SOD1*, causando patogenicidade através da perda de funções na proteína codificante assim como a sua agregação em neurónios motores e células da glia, despoletando outros mecanismos tais como neuro-inflamação, que contribuem para a progressão da doença. A causa genética mais comum é a expansão repetida hexanucleotídica no gene *C9orf72*, responsável pela maioria dos casos de ELA/DFT e cerca de 40% dos casos familiares de ELA.

Todas essas complicações tornam a vida dos pacientes bastante complicada. Infelizmente, não existem muitos medicamentos modificadores da doença aprovados para o tratamento da ELA, tornando o tratamento muito focado na gestão dos sintomas e na ajuda da melhoria da qualidade de vida dos pacientes. Os únicos medicamentos aprovados no mercado são o riluzol e o edaravone.

O objetivo principal desta monografia é fornecer uma visão geral e atualizada da doença em 2020 e a revisão das terapêuticas mais promissores a ser desenvolvidas de momento e respetivos ensaios clínicos de fase I, II e II.

Palavras-Chave: Esclerose Lateral Amiotrófica; ELA como uma doença multifatorial; Tratamento da ELA; Novas abordagens terapêuticas

Abstract

Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease characterized by loss of motor neurons and progressive muscular atrophy. ALS can be characterized as familial ALS (10% of the cases) if it has autosomal dominant inheritance patter or sporadic ALS (90% of the cases) if there is no family history. It can also be characterized in bulbar ALS or spinal ALS depending on the onset point. Its prognostic often is death by respiratory failure, 2 to 5 years after disease onset.

ALS can have very different motor manifestations such as progressive muscle weakness, wasting, fasciculations and cramps, but also non motor manifestations, a lot of the times meeting the diagnosis of fronto-temporal dementia.

What causes ALS is not clear, but there are a lot of genetic and pathological mechanisms studied that are associated with it. One of the main genetic cause is a mutation in SOD1 gene, causing pathogenesis by loss-of-functions of the codifying protein and cytoplasmic aggregations in neurons and glial cells, triggering other mechanisms such as neuroinflammation that contribute to disease progression. The most common genetic cause is a hexanucleotide repeat expansion in the *C9orf72* gene, responsible for most cases of ALS/FTD and about 40% of familial cases of ALS.

All those complications make a very difficult life for patients to take. Unfortunately, there are not so many disease-modifying drugs approved for the treatment of ALS, making treatment very focused in symptom management and in helping patients to have a better life quality. The only two drugs approved are riluzole and edaravone.

The main objective of this dissertation is to give an updated overview of the disease in 2020, and review some of the most promising studies being made concerning new therapy ideas and phase I, II and III clinical trials.

Keywords: Amyotrophic Lateral Sclerosis; ALS as multifactorial disease; ALS treatment; New therapeutic approaches.

Abbreviations

AAV – Adeno-associates virus

AGO – Argonaute

ALS – Amyotrophic Lateral Sclerosis

ALSFRS-R – ALS Functional Rating Scale

ANG - Angiogenin

ASO – Antisense oligonucleotide

AV – Adenovirus

CNS – Central Nervous System

crRNA - CRISP targeting RNA

CSF – Cerebrospinal fluid

C9orf72 - Chromosome 9 open reading frame 72

DNA - Deoxyribonucleic acid

DPRs – Dipeptide Repeats

DSBs - Double-strand breaks

EMG – Electromyography

fALS – Familial ALS

FTD – Fronto-Temporal Dementia

FUS – Fused in Sarcoma

GFP – Green Fluorescent Protein

IFN - Interferon

IL – Interleukin

iPSCs – Induced Pluripotent Stem Cell

LMN – Lower motor neuron

LV – Lentivirus

MN – Motor Neurons

miRNA – Micro RNA

mRNA – Messenger RNA

ncRNA - Noncoding RNA

NF – Neurofilaments

NfL – Neurofilament light chain

NIV – Non-invasive ventilation

OPTN – Optineurin

PAM - Protospacer adjacent motif

Pol – RNA Polimerase

PMO - morpholino phosphorodiamidate ASO

pNfH – Phosphorylated neurofilament heavy chain

RCT – Randomized controlled trial

RISC - RNA-induced silencing complex

RNA – Ribonucleic acid

RNAi – RNA interference

sALS – Sporadic ALS

siRNA - Small interfering RNA

sgRNA – Single-guide RNA

shRNA – Short hairpin RNA

SOCS-1 - Suppressor of cytokine signalling 1

SOD1 – Superoxide Dismutase 1

TBK1- Tank-Binding Kinase 1

TDP-43 – TAR DNA Binding Protein 43 kDa

TNF-α – Tumor Necrosis Factor Alpha

tracrRNA - Transactivating RNA

UMN – Upper motor neuron

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1 Introduction

Amyotrophic Lateral Sclerosis (ALS) can be described as a progressive fatal neurodegenerative disorder that affects motor neurons (MNs) and consequently causes muscular atrophy. Generally, the manifestations start in one focal point and slowly and progressively spread to other regions of the central nervous system, resulting in many muscular complications. In 2- or 3-years form onset it often causes death by respiratory failure. These symptoms have a tendency to start in the late-adulthood as some aging-relates factors have been associated as one of the possible causes, but it can affect people in younger ages as well, as I will discuss ahead. Although ALS is mainly a motor neuron disease, nowadays it is known to have many extra-motor manifestations that can cause complications such as Fronto-Temporal Dementia (FTD) (1–3).

1.1 History framework

Although Jean-Martin Charcot is often credited as the first person to use the term "Amyotrophic lateral sclerosis" in the 1800's, the name of Charles Bell is also mentioned due to his findings concerning motor and sensory neurons. Charcot was able to collect and review various studies that were being made at the time, and separate ALS from similar disorders, by confirming evidence that linked muscular atrophy with corticospinal tract pathology, culminating in a publication about the subject in 1874. Jean-Martin Charcot also described the disorder as having three separate stages(1,4) .

1.2 Aetiology and Clinical Features

The main question that can be asked when dealing with ALS disease is "where does it begin?". There are many factors that can try to explain or even answer that question. ALS as a neurodegenerative condition can be caused by a combination of factors such as genetic factors, environmental factors and aging-related dysfunction.

ALS affects primarily upper motor neurons (UMNs), and lower motor neurons (LMNs), causing its loss in three distinct portions of the nervous system, the motor cortex (UMN), the brain stem nuclei, and the anterior horn of the spinal cord (LMN). That loss of neurons originates complications such as slow progressive muscle weakness (the primary clinical manifestation of ALS), muscle atrophy, fasciculations and muscle cramps. (5) 10% of ALS can derive from familial causes (genetic autosomal dominant inheritance pattern (fALS) or in 90% of the cases from a sporadic

cause (sALS) if there are no family history associated, although fALS remains the most studied today(5).

ALS starts typically in one focal point and tends to spread in a progressive way throughout the body. It can be classified as Spinal ALS if the onset is on the limbs (about 70% of the cases) or Bulbar-ALS if the symptoms involve speech and swallowing problems. (about 25% of the cases).

Spinal ALS can have an unilateral distal muscle weakness, with an upper limb onset or a lower limb onset. When the onset is in the upper limbs, normally it affects the dominant hand, causing split-hand syndrome where thenar muscles are more affected than hypothenar muscles, and finger extensors being more affected than flexors.

In the lower limb onset the most typical affected muscle is the tibial muscle, before the gastrocnemius muscle, and the hamstrings before the quadriceps muscles(5).

Bulbar ALS can be characterized by complications concerning speech and swallowing such as dysarthria, dysphagia and even dysphonia, but those symptoms can vary depending on the types of MNs involved (2,3,5). If UMNs are the main neurons involved, the dysfunction is spastic dysarthria, characterized by problems in the speech (slow, laboured, and distorted) and can be called Pseudobulbar Palsy. In Bulbar LMN disfunction, tongue wasting, fasciculations and weakness can be the first symptoms to appear, followed by flaccid dysarthria and dysphagia, and the term used is Bulbar Palsy (3,6).

Although these two classifications, based on the types of symptoms and MNs affected, are the most common, many other types of classifications can be made to help distinguish various subtypes of ALS and therefore help on the diagnosis, and prognostic of the disease.

1.2.1 Classifications and clinical types of ALS

Classification of ALS is not a simple and straightforward process, as a multifactorial disease with heterogenous causes, ALS can have many different clinical manifestations, so it can be classified in many different subtypes based on features such as, regional distribution involvement of the nervous system, genetic involvement and family history, motor manifestations, phenotypes based on the involvement of UMNs vs LMNs, extra-motor manifestations and pathological subtypes. Those types of

classifications often lead to overlapping terms and inconsistency on the definition of subtype (5,6).

Current ALS classifications at the time of diagnosis include the El Escorial Criteria and its revisions. Developed for research purposes by the Neurology Research Group on Motor Neuron Diseases, this criteria as the greatest agreement amongst experts to be used as a confirmatory diagnostic test for ALS. The original criteria is based in four categories that range from Suspected ALS, Possible ALS, Probable ALS and Definite ALS and considers manifestations in four regions, Brainstem, Cervical, Thoracic, and Lumbosacral. The review in the year 2000 proposed the substitution of "suspected ALS for "laboratory-supported probable ALS" and the investigation in 2008 by M.Carvalho, Dengler R, EinsenA, et al, suggested that "investigators and triallists should use the Awaji-Shima algorithm superimposed onto the El Escorial criteria, in selecting patients for research studies" due to the good results they had using electrophysiological data in the diagnosis of ALS, improving its sensitivity. The Awaji-Shima criteria eliminated the "laboratory-supported probable ALS" category, maintaining the others and was continuously studied, and reviewed in 2012 (7–10).

The classification with ICD coding system is also often used in hospital environments to compare mortality and morbidity from one another and with other countries. In the latest ICD review, (ICD-11) the World Health Organization includes ALS and other related complications with similar manifestations or even characterized as subtypes of ALS, such as Progressive Bulbar Palsy, Progressive Pseudobulbar Palsy, Progressive Muscular Atrophy, Primary Lateral Sclerosis, Amyotrophic Lateral Sclerosis-Plus, Monomelic Amyotrophy and other specified and unspecified motor neuron diseases in the same section, the 8B60 - "Motor Neuron Diseases or Related Disorders" section.

1.2.2 Extra-motor manifestations – FTD

ALS comes often associated with extra-motor manifestations such as changes in behaviour executive dysfunction and language problems. When these extra-motor manifestations become severe, it can match the clinical criteria of Fronto-Temporal-Dementia (FTD). FTD was thought to be an uncommon symptom of ALS, but the fact there were cases of patients with FTD developing ALS and familial clustering of both

disorders suggested that the two were somehow linked (11). The main complications associated with ALS-FTD involve executive function such as personality changes, language problems and behavioural problems. Some genetic mutations are more associated with ALS-FTD, TDP-43-positive inclusions are present in half of those patients and *C9orf72* hexanucleotide repeat expansion is also associated with an overlap between ALS and FTD (3,5).

1.3 Diagnostic

The Diagnostic for ALS usually comes with a delay up to a year after onset and can be a difficult task specially in patients with an early disease presentation, slow progression and a simultaneous central or peripheral nervous system disorder, because of the existence of some ALS mimicking syndromes that can cause 7 to 8% of misdiagnosis (5,12,13).

At the time, the diagnostic is based in observing clinical features such as the presence of UMN and LMN signs in patients with symptoms like muscle weakness and it relies in a physical examination, knowing the patients family history, electrodiagnostic testing like Electromyography (EMG), proposed by the 2000 revision of El Escorial Criteria, and neuroimaging (5,8).

The El Escorial Criteria and its subsequent revisions, are often used as an established criteria for the diagnosis of ALS, but recently there are some reviews being made to it, and some of its authors are proposing a new diagnostic criteria (14).

Biomarkers can also play an important role in the Diagnostic, Prognostic, tracking of the disease progression and the treatment process of ALS and some studies are being made in that direction. At the time there aren't any biomarkers that can be used solely to make a diagnostic or prognostic of ALS, but there are some candidates to it, and they can be divided in two groups, biological biomarkers and clinical biomarkers, according to K. Kadena and P. Vlamos's review (15).

One of those biological biomarkers include **Neurofilaments** (NF). Neurofilaments are part of the cytoskeleton that accumulate in cells and proximal axons when under pathological conditions. NF are a biomarker that is known for some time to be increased in cerebrospinal fluid (CSF) about 5-10 times in patients with ALS when

compared to healthy subjects (16). There has been a lot of studies concerning neurofilament levels in CSF, serum and plasma, and the most studied neurofilament subunits are neurofilament light chain (NfL) and phosphorylated neurofilament heavy chain (pNfH). Koen Poesen and Philip Van Damme reviewed several studies in the beginning of 2019 concerning those biomarkers and came with some conclusions about their diagnostic and prognostic value (17). They found that "diagnostic performance was found to be better in CSF compared to blood" (18,19), "The sensitivity and specificity for ALS was better for pNfH than for Nfl in studies comparing both neurofilament subunits" (18,20,21). They also found that NF can be a good biomarker to improve early diagnostic of the disease and to distinguish specific gene related ALS. Overall they found NF levels to be a valuable biomarker for the diagnostic, prognostic and monitoring of treatment of ALS and that "CSF pNfH levels seems to be the most accurate diagnostic marker, but both pNfH and NfL serum or plasma measurements perform good to predict survival and disease progression."

There are other biological biomarkers worth mentioning such as:

- Mutated genes and correspondent proteins – With TDP-43 playing an important role.
- miRNAs
- Inflammatory Mediators
- Cystatin C

Clinical Biomarkers include:

- Neuroimaging Biomarkers
- F-FDG positron emission tomography

1.4 Prognosis

Life expectancy in patients with ALS is very variable, ranging from 2 to 5 years from onset, typically dying from respiratory failure. Due to the large variability and heterogenous causes of the disease, there are some patients dying within a few months after onset, and others living for more than two decades (5,22,23). Some review papers say that "50% of patients die within 30 months of symptom onset and about 20% of patients survive between 5 years and 10 years after symptom onset" (3,24).

There is a big importance in having accurate prognostic indicators from a clinical perspective. Accurate prognosis can help patient stratification and optimise multidisciplinary care, planning interventions, advising patients on end-of-life decisions and more. There are some indicators that can be used to predict the evolution of the disease such as (5,23):

- Bulbar onset
- Diagnostic delay
- Functional decline (Measured by revised ALS Functional Rating Scale (ALSFRS-R))
- Loss of weight (body mass index)
- Presence of FTD
- Age of onset
- Forced vital capacity
- Genetic Factors
    - Ala5Val mutation in SOD1
    - *C9orf72* repeat expansion
    - P525L mutation in FUS

Although those indicators alone can help predict the disease evolution in some degree, there are a lot of studies considering a combination of prognostic factors and using various models and algorithms such as, machine learning (25) and boosting algorithms, random forest models, regression models, Uniform Manifold Approximation and Projection and more (23). Some prediction models have already been developed and externally validated (26) and can "help predict survival without tracheostomy and non-invasive ventilation for more than 23 h per day in European patients with ALS".

As in diagnostic, biomarkers can also play an important role in prognosis. NF have shown to good as a predictive value both in CSF and blood samples. This biomarker can be correlated with parameters of disease severity such as ALSFRS-R. Higher levels of NF showed to be unfavourable to patient survival, and patients with a long survival presented low levels of NFs (17).

Some ALS subtypes seem to be related with a better prognosis. LMN types of ALS such as Flail-limb variant and progressive muscular atrophy are characterized by a slower progression when compared to other forms of the disease (3).

1.5 Epidemiology

According to one of the latest (June 2020) clinical reviews of the disease, published by P.Masrori and P. Van Damme in the European Journal of Neurology (5) the estimated incidence of ALS is 1.75 per 100 000 persons per year in Europe and 4-8 per 100 000 persons per year for people in higher risk (45-75 years old). The prevalence of the disease is about 10-12 per 100 000 persons in Europe (27–29). They also noticed that the mean age at onset can vary from ALS subtypes, being around 58-63 years for sALS and 40-60 years for fALS (27), and that the cumulative lifetime risk for developing ALS varies according to sex, it is 1:350 in men and 1:400 in women (30,31), also men have greater risk of developing bulbar ALS than women, and the global sex ratio is 1.2-1.5 (32).

There are many recent papers studies that studied epidemiology from specific countries and regions of the world because incidence and prevalence varies depending on the studied population. Some of the most recent studies include countries and regions such as Tunisia, Colombia, Moscow (Russia), Latin America, Turkey, Korea and they all find similar results to what P.Masrori and P.Van Damme with some small differences that can be explained by methodological concerns and bias within the studies, and also differences in the population studied such as age and race for example, in Turkish and Tunisian studies male/female Ratio seems to be a little higher in around 2.0 but also with male predominance (33,34).

Overall, the world incidence of ALS seems to be estimated by 0,4-3.80 per 100 000 person per year and prevalence to be around 4.1 – 8.4 per 100 000 persons per year, although some studies can present different results (3,35–38).

1.6 Pathogenesis

There are a variety and a combination of neuropathological, genetic and molecular pathways that can lead to the loss of neuromuscular connection through

axonal retraction and consequent death of UMNs and LMNs. Protein aggregation in the cytoplasm, known as inclusion bodies, are the pathological hallmark in the disease. Some of the many molecular pathways implicated in the pathogenesis of ALS include (3,5,39):

- Failure of proteostasis
- Excitotoxicity induced by glutamate
- Neuroinflammation
- Mitochondrial dysfunction and oxidative stress
- Oligodendrocyte dysfunction
- Cytoskeletal disturbances and axonal transport system defects
- Disturbed RNA metabolism
- Nucleocytoplasmic transport deficits and impaired DNA repair
- Dysfunction of the sodium/potassium ion pump

1.6.1 Genetic involvement

Neuron death can be attributed to genetic factors, and more than 20 genes have been related with ALS. The first genetic factor to be related with ALS was a mutation in a gene that encodes for copper/zinc ion binding superoxide dismutase 1 (SOD1). This mutation is responsible for 20% of fALS and 1-2% of sALS (40,41). There's a lack consensus linking mutations in this gene to premature death of motor neurons and it is known that the harm that this mutation causes isn't due to loss of SOD1 function but rather by turning the enzyme prone to aggregation by inducing conformational instability and misfolding of SOD1 peptide. Those aggregates inhibit normal proteosome function and interfere with axonal transport systems, it also causes a toxic gain of function by generation of free radicals that lead to disturbance in important cellular functions and consequent cell injury and death (42,43).

The major component found in ubiquitinated cytoplasmic protein aggregates is a RNA and DNA binding protein named TDP-43, encoded by the TARDBP and FUS genes and present in more than 95% of ALS cases. As a DNA and RNA binding protein, TDP-43 is responsible for some genetic processes such as transcription, splicing, microRNA maturation and RNA transport. It is mainly localized is in the nucleus but also present in the cytoplasm and mitochondria, its mislocalization to the cytoplasm

and subsequent aggregation in truncated forms is a crucial process to block normal cell processes and loss of normal function of TDP-43 protein. Those mislocalizations occur predominantly in the brain cortex but are also present in the spinal cord. The toxicity caused in cells by aggregation of TDP-43 in the cytoplasm is suggested to be a process of loss-and-gain-of-function mechanisms. This protein mislocalization is considered to be more likely reversible than its aggregation, so understanding how this process works and is regulated at the cellular level, can help in therapy development (44,45).

There are other gene mutations identified as responsible for ALS, one of those is a hexanucleotide repeat expansion in chromosome 9 open reading frame 72 (*C9orf72*). This particular mutation is associated with ALS and FTD and is one of the main causes of fALS, responsible for around 40% of fALS (46). *C9orf72* pathogenesis is also a combination of gain-and-loss of functions. Gain of functions include accumulation and aggregation of dipeptide repeats (DPRs) and the formation of RNA foci that sequestrate RNA binding proteins. Loss of functions are less clear than gain of functions but they can include reduced expression of endogenous *C9orf72* mRNA (46,47) .

Other gene mutations and respective proteins worth mentioning include Fused in Sarcoma (FUS), a DNA/RNA binding protein involved in RNA metabolism and DNA repair. FUS mutations have been found in many other neurodegenerative diseases such as Frontotemporal lobar degeneration, the polyglutamine diseases (Huntington's disease, spinocerebellar ataxia, and dentatorubral-pallidoluysian atrophy). The pathological mechanism of FUS is its mislocalization to the cytoplasm forming stress granules. Some gain-of-functions include a gain of toxic function in the cytoplasm, sequestering important regulators or trigger abnormal signalling pathways to alter cell physiology and loss-of-functions include affected transcription, alternative splicing and affected DNA repair (48,49).

Various types of mutations in the gene that encodes TANK-binding kinase 1 (TBK1), a serine/threonine kinase involved in the regulation of essential cellular functions as selective autophagy and innate immunity, have been described as a potential cause for ALS/FTD by loss-of-function (50). Some of those include haploinsufficiency as splice site, frameshift and missense mutations (51,52). Although some studies linking some missense mutation variants with protein-protein interactions

and substrate-specific defects in innate immunity and autophagy pathways are showing unclear correlation with direct implications in ALS (51).

Other genes with implications in ALS and that account for 3% of rare genetic forms include ANG that encodes for angiogenin and is a hypoxia responsive gene that regulates RNA transcription (53), OPTN that encodes for optineurin, and more. This genetic study was made with ALS patients that tested negative for SOD1, FUS, TARDBP and *C9orf72* mutations, identified 160 variants in 117 out of 213 index cases and revealed that there is a very high number of genetic mutations that still need to be studied and discovered (54).

1.6.2 Glia Involvement

The Pathogenesis of ALS also includes glial cell involvement. Neurons make up only about a half of all Central Nervous System cell types, being glia cells the other major constituent of the Central Nervous System, so its involvement in the neurodegenerative process can play an important role.

Astrocytes and Microglia are known to contribute to neurodegeneration through mechanisms that lead to neuroinflammation including insufficient release of neurotrophic factors, secretion of neurotoxic mediators modulation of glutamate receptor expression (55). Some of those pro and anti-inflammatory chemokines and cytokines include TNF- α, IL-6, IL-10 and CC-chemokine ligand 2 (56).

Despite that, all cells of the nervous system are known to play a role in many processes that can be a part of the whole problem.

Autophagy dysfunction also plays an important role in neurodegenerative diseases and in ALS because autophagy is a crucial neurodegenerative-protective pathway. Some of the genetic mutations described in 1.6.1 can contribute to disfunctions in the autophagy process for example, OPTN is a known autophagy receptor, TBK1 is associated with autophagy regulation and initiation and TDP-43 has functions is autophagosome maturation. The main cell type with an affected autophagy process is microglia. TNF-α levels showed to be elevated in ALS patients, and studies have reported that an elevation of pro-inflammatory markers was observed concomitantly with a decreased in autophagic flux in microglia upon TNF- α exposure, suggesting that down regulation of autophagy on microglia leads to increased

neurotoxicity. In astrocytes the autophagy pathway seems to be affected by involvement of the supportive roles these types of cells are responsible for but either in astrocytes and in oligodendrocytes further research is needed to delineate a straight connection between autophagy and ALS (57).

Glia also play an important role in protein aggregation either by contributing to formation of protein aggregates or by get responses by those aggregates. These responses result in negative mechanisms such as, inflammatory and toxic factor release, synapse elimination or even positive mechanisms such as the formation of a glial barrier, the release of trophic factors and ectoenzymes and phagocytic clearance. (58)

Figure 1, adapted from a recent clinical review of the disease by P. Masrori and P. Van Damme, clearly demonstrates at a genetic and cellular level all the variables and pathways involved in pathogenesis of ALS and shows how Proteostasis, Autophagy, DNA metabolism and Cytoskeletal and axonal transport are affected (5).

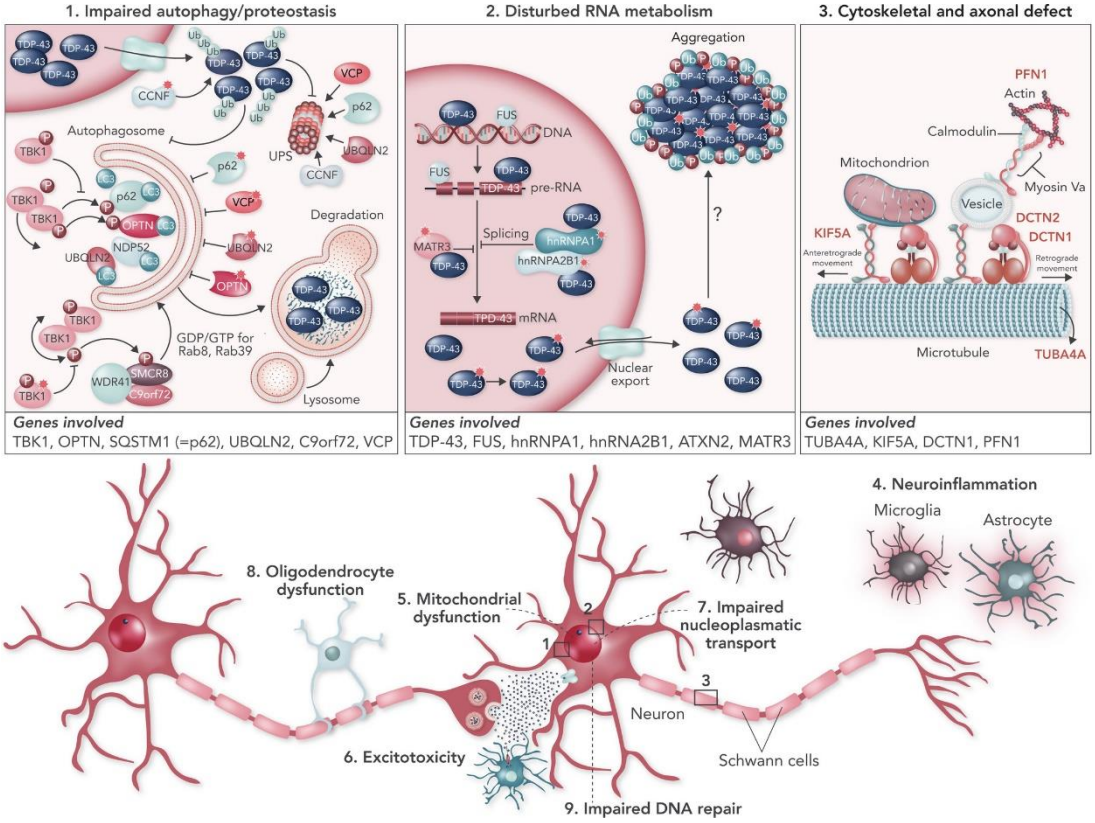

Figure 1 Pathogenic mechanisms involved in ALS

(1) Mutated genes affecting protein degradation pathways and that may contribute to TDP-43 accumulation. (2) Mutation genes that may affect RNA metabolism. (3) Mutations in genes that alter dynamics and axonal transport. Below those 3 images, a brief representation of the variety of mechanisms involved in pathogenesis. (1) TDP-43, TAR DNA Binding Protein 43 kDa; Ub, Ubiquitin; UPS, Ubiquitin-proteasome system; UBQLN2, Ubiquilin-2; CCNF, G2/mitotic-specific cyclin-F; VCP, valosin-containing protein; SQSTM1 (=p62), Sequestosome 1; OPTN, Optineurin; NDP52, nuclear dot protein 52 kDa; WDR41, WD Repeat Domain 41; *SMCR8*, Smith-Magenis syndrome chromosome region homolog; GDP, Guanosine diphosphate, GTP, Guanosine triphosphate; *C9orf72*, Chromosome 9 open reading frame 72; (2) FUS, Fused in Sarcoma; *hnRNP*, heterogenous nuclear ribonucleoproteins; DNA, Deoxyribonucleic acid ; RNA, Ribonucleic acid; mRNA, messanger RNA; (3) *PFN1*, profilin-1; *TUBA4A*, tubulin alpha-4A; *DCTN1*, dynactin 1; *KIF5A*, kinesin heavy chain isoform 5a. Reproduced from (5).

1.7 Risk Factors

There are only a few risk factors identified for ALS and they include:

- Genetic Factors – genotypes such as *UNC13A* and mediate repeat expansions in *ATXN2* (59,60)
- Age – Older age increases risk
- Sex – Male sex increases the risk
- Environmental risk factors – Smoking (61), High Body mass index, lack of exercise but also competitive sport activities such as professional football, trauma in particular head trauma, the use of private wells for drinking water, occupational and environmental exposure to metals, pesticides, β-methylamino-L-alanine, head injury and viral infections (62).

2 Treatment/Management

There is no cure for ALS so the main treatment remains a careful management of all the symptoms to improve survival and quality of life of patients. As shown in table 1, there are a lot of symptoms to take care of, and it often requires a multidisciplinary care and a team composed by physiotherapists, occupational therapists, respiratory physicians, gastroenterologists, speech therapists and social workers. The main complications to manage are respiratory and nutritional failures (63–65).

2.1 Respiratory Management

ALS progressive muscle weakness is the cause for respiratory symptoms such as dyspnoea and orthopnoea due to weakness of the diaphragm and accessory muscles of breathing. One way to improve life quality and survival of the patients is by using non-invasive ventilation (NIV) (66,67). The combination of the symptoms described above along with clinical measurements assure the guidelines for instituting this type of treatment. Measuring respiratory function is critical for monitoring ALS progression, and the main clinical measure to monitor respiratory function is vital capacity. There are other measurements such as overnight oximetry, partial pressure of carbon dioxide (PCO2), polysomnography, maximal inspiratory and expiratory pressure or sniff nasal pressure (68). Some patients with substantial bulbar impairment and sialorrhea might not tolerate non-invasive ventilation and there are other adjuvant mechanisms of treatment such as secretion suction or even tracheostomy with an mechanical insufflation-exsufflation system if patients no longer respond to NIV, although it isn't very used because of its high cost, proneness to infections and loss of quality of life (69).

2.2 Nutritional Management

There are a lot of symptoms that contribute to inadequate nutrition either in an early or advanced stage of the disease, for example limb weakness and dysphagia. Other complications such as depression, anxiety and gastrointestinal problems can lead to malnutrition. To guarantee a normal caloric intake there are a few methods that can be used and in the beginning it is very important to educate patients in safe swallowing techniques and a balanced nutrition. In advanced stages of the disease, when dysphagia

progresses and swallowing becomes a very difficult task, artificial feeding can be a useful method. Using enteric feeding such as percutaneous endoscopic gastrostomy (PEG) and radiologically insert gastrostomy are good options to complement inadequate oral feeding but in patients with vital capacity below 50% there has to be an additional care, because the respiratory symptoms can be exacerbated (70).

Recent studies on Integrative and Functional Medical Nutrition Therapy (IFMNT), a model of care that prioritizes a patient-centred approach and less of a diagnostic-centred approach in all aspects of treatment, suggest that nutrition is one of the key variables to understand ALS. Those studies include evaluation of a lot of protocol diets and nutrients, to specify their roles and physiological processes in ALS and other diseases, so that patient feeding becomes a methodical and organized process with therapeutical value (71).

Table 1 – Symptomatic care in Amyotrophic lateral sclerosis

Symptoms		*Pharmacological Care*	*Non-Pharmacological Care*
Constitutional	Insomnia	TCAs (i.e amitriptyline) Mirtrazapine Zolpidem Antihistamines Benzodiazepines	Comfort during sleep Noninvasive ventilation
	Fatigue	Modafinil	Improve sleep
	Weakness and disability		Orthotics (ankle foot orthosis, neck collars) Physiotherapy Adaptive aids (walking frame, wheelchair)
Psychological	Anxiety and Depression	SSRI Antidepressants Buspirone Mirtrazapine Benzodiazepines Trazodone	Psychological support and counselling
	Pseudobulbar affect (Emotional lability)	Dextromethorphan/quinidine TCAs SSRI antidepressants	Educate Patients and Caregivers
	Cognitive changes (FTD)	Antidepressants	Educate Patients and Caregivers
Otolaryngologic	Sialorrhea	Anticholinergic antidepressants and drugs (eg, amitriotyline and glycopyrronium bromide respectively)	Suction Irradiation of salivary glands Mouthcare products Botulinum toxin injections
	Laryngospasms	Benzodiazepines	Deep breathing Reassurance
	Dysphagia		Assessment by speech therapist and dietitian Safe swallowing techniques and modified diet Insertion of gastrostomy tube
Respiratory and Speech	Dysarthria		Assessment by speech pathologist Communication aids Educate family and caregivers
	Dyspnoea and poor cough	Morphine or Benzodiazepines	Ventilatory support Chest physiotherapy Suction machine Manually assisted coughing techniques
Gastrointestinal	Constipation	Docusate sodium Senna glycoside Bisacodyl Lactulose Magnesium citrate Polyethylene glycol	Increase hydration Dietary changes (Increase fibre intake Prune or apple juice)
Genitourinary	Urinary urgency	Oxybutynin Tolterodine Solifenacin Mirabegron	
	Erectile dysfunction	Sildenafil Citrate Tadalafil Vardenafil	
Musculoskeletal	Spasticity	Baclofen Tizanidine Benzodiazepines	Physical therapy Stretching exercises
	Cramps	Mexiletine Vitamin E	Physical therapy Massage

Adapted from (3,65).

2.3 Pharmacological Treatment

There has been a lot of phase II and III randomised controlled trials (RCTs) in the past decades concerning disease-modifying drugs, but more than 50 of them did not show positive results. Those results can be explained by three categories of reasons, the first one being issues regarding trial rationale and preclinical study results, the second being pharmacological issues, and the third being issues concerning clinical trial design and methodology. Two of those drugs however, have demonstrated survival benefits and reduced disease progression, the first one is Riluzole, an antagonist to glutamate release and the only drug approved by FDA and EMA the second is Edaravone, a free-radical scavenger, approved for ALS treatment in Japan, South Korea and USA (65,72,73).

2.3.1 Riluzole

Riluzole, an antagonist to glutamate release, is the only disease-modifying drug approved by EMA and FDA for the treatment of ALS, it was the first drug showing results in prolonging patients survival by 3 to 6 months, and it is available since the 1990's (74). Approved by two double-blind, placebo controlled trials, Riluzole is administered 50 mg twice a day in the form of tablets or liquid (75). New Real-World Evidence Studies are proving that Riluzole is an effective drug in extending patient survival, and some studies even showed that it may be extended by 6 to 19 months instead of the initial 2 to 3 months reported in the initial RCTs (76,77). Some of Riluzole side effects include nausea, diarrhoea, fatigue, dizziness and liver problems.

2.3.2 Edaravone

Edaravone, also known as radicava, is a free radical scavenger that has been approved for ALS treatment in Japan, South Korea, and USA. Edaravone has showed in phase III, double-blinded trials that it can have a "significant smaller decline of ALSFRS-R score compared with placebo" during a 6 month treatment period in which it was administered 60 mg intravenous edaravone in 4 week cycles (2 weeks on and 2 weeks off) (78). Some critics have been made to this study, concerning its short duration, small size, selected patients and lack of data on survival, suggesting that pros and cons should be weighted when initiating an individual treatment with edaravone,

and it should be taken into account how closely patients match the trial clinical criteria (79).

Recent studies (released in October 2020) are relating Edaravone use in US Veterans with documented or probable ALS with an increase of acute all—cause hospitalizations when compared to riluzole-only treatment. Although this findings need to be carefully evaluated in real-world settings, and may have some inherent methodologic limitations, this pharmacovigilance evaluation showed that edaravone should be more carefully considered in ALS therapy (80).

2.3.3 Masitinib

Masitinib, an oral tyrosine kinase inhibitor, demonstrated promising preclinical results in ALS rat models. Having immunomodulatory properties, through targeting of microglia, macrophage and mast cell activity, in central and peripheral nervous systems (81), it was submitted to a randomized clinical trial, where it demonstrated significant benefit in ALSFRS-R over placebo in patients with typical disease progression, when using 4.5 mg/kg/day as an add-on therapy to riluzole (82).

There was a proposition for Masitinib to enter the market by the commercial name of Alsitek, but in 2018 the European Medicines Agency adopted a “negative opinion, recommending the refusal of the marketing authorisation for the medical product Alsitek, intended for the treatment of amyotrophic lateral sclerosis (ALS)”, arguing that the study did not show that Alsitek is effective at slowing down progression of the disease and problems with the patient representation (83).

2.3.4 Dextromethorphan/quinidine

Dextromethorphan/quinidine was approved in 2011 by the FDA to be administrated in cases of emotional lability (pseudobulbar affect), a condition characterized by spontaneous episodes of crying or laughing in people with ALS and other neurological conditions. Some randomized trials have shown positive effects of this drug in symptoms related to pseudobulbar affect such as speech and swallowing and improved ability to handle oral secretions (84–86).

3 New approaches in treatment – Biological and Technical Advances in Therapies

3.1 Phase II and III clinical trials

Nowadays there are still a lot of trials being made concerning the development of disease-modifying drugs, but only a few are proving to be promising. Some of the main concerns in finding new therapy ideas are, the need to define a specific target with crucial roles in the pathophysiology of ALS, making experiments with molecules that can have actions in various pathophysiological mechanisms, the need to develop biomarkers to ensure that the tested molecules are being engaged in the correct target, the look for more sensitive biomarkers, able to predict clinical response at new treatments, and the need to innovative clinical trial designs that can accelerate the drug development process. Andrea Barp, Francesca Gerardi, Andrea Lizio, Valeria Ada Sansone & Christian Lunetta reviewed a series of recent phase II and III clinical trials and presented their perspective in a few of these problems. To develop new therapeutic strategies, research goals and scientific rationale must be considered, so that all the known complex and multi pathophysiological mechanisms (described in 1.6) responsible for neurodegeneration, can be targeted. A lot phase II that are being made about new drugs to treat ALS, concerning those pathophysiological targets (73):

Therapies targeting autophagy and neuroinflammation:

- Rapamycin
- Colchicine
- RNS60
- IL-2 and T lymphocytes
- IC14

Therapies targeting oxidative stress:

- Vitamin E
- Inosine

- Deferiprone

Therapies targeting mitochondrial dysfunction:

- Copper complex diacetylbis(N(4)-methilthiosemicarbazonato copper (II) – CuATSM

Therapies targeting excitotoxicity:

- Perampanel
- Memantine
- AstroRx

Therapies targeting neuroprotection:

- RhEPO
- AMX0035

Therapies targeting impaired proteostasis:

- L-serine

Therapies targeting axonal transport defects:

- Isochinoline derivate – Fasudil

Therapies targeting endogenous retroviruses:

- Triumeq

Therapies targeting multiple mechanisms:

- Metformin

Therapies targeting symptoms:

- Lacosamide
- Ranolazine

Phase III clinical trials include:

- Antisense oligonucleotides (ASOs)
- Masitinib
- Tauroursodeoxycholic acid
- Ibudilast (MN-166)
- Levosimendan

- Ravulizumab
- High dose methylcobalamin

3.2 Gene silencing

3.2.1 Antisense Oligonucleotides (ASOs)

Treatment with ASOs is one of the most promising in the next few years. ASOs are short (8-50 bp) single-stranded nucleotide sequences that bind to RNA sequences (pre-mRNA and mRNA), modulating gene expression and altering processes such as splicing. Nevertheless, there are multiple mechanisms by which ASOs can interfere with RNA function, and that depends on the chemical modifications, the position the modifications are incorporated into the oligonucleotide, and where on the target RNA the oligonucleotide binds. ASOs mechanisms are described in detail in Fig.2.

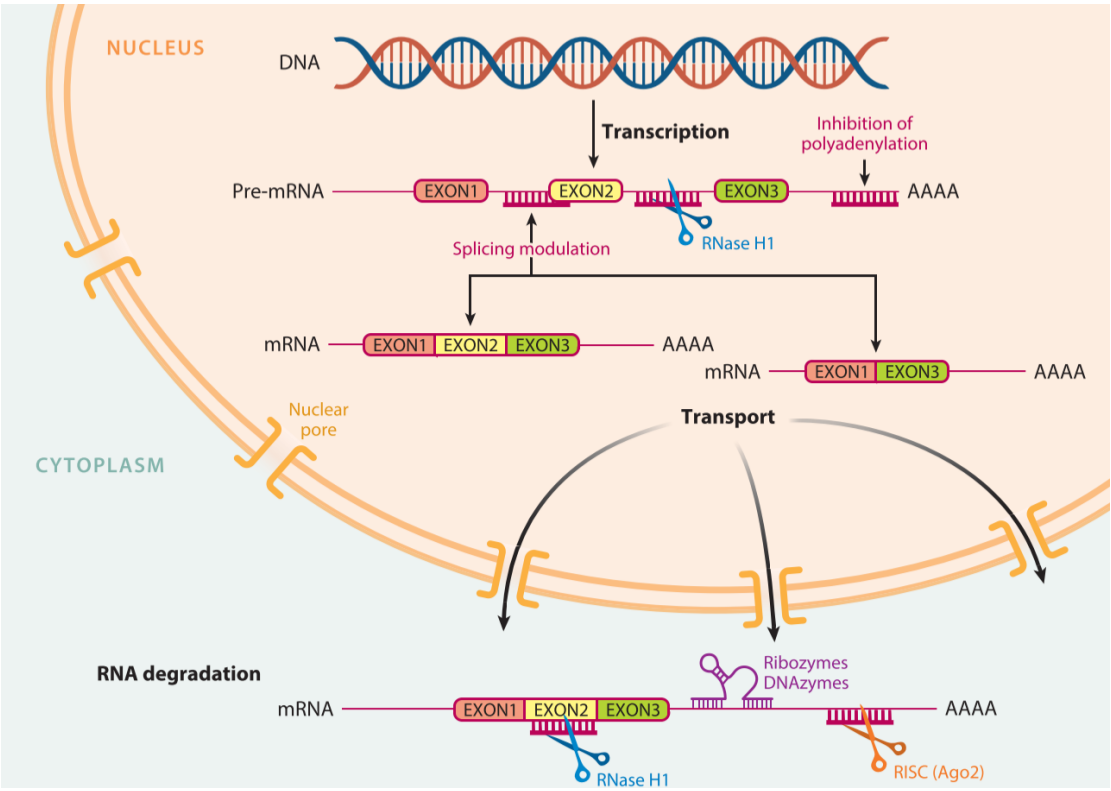

Figure 2 - Mechanisms of action of antisense oligonucleotides (ASOs).

ASOs can modulate pre-mRNA function in the nucleus or mature RNA in the cytoplasm. RNase H1 can degrade RNA in the nucleus and in the cytoplasm and RISC complex (Ago2) or ribozymes or DNAzymes can degrade RNA only in the cytoplasm. ASOs can also modulate RNA function by nondegradative mechanisms such as splicing or polyadenylation modulation in the nucleus. ASO, antisense oligonucleotide; pre-mRNA, precursor mRNA; mRNA, micro RNA; RISC, RNA-induced silencing complex. Adapted from (90).

Altering the structure of ASOs can give them increase stability and resistance to nucleases, for example in the case of morpholino phosphorodiamidate ASOs (PMOs). ASOs have a reduced biodistribution and bioavailability and cannot cross the blood-brain barrier, so the choice of an appropriate delivery site and method is very important for reaching the target sited and get the desired affects. The most effective way of delivering ASOs to his target is injecting them directly into the CNS (87–90).

There is already one approved antisense drug on the market, nurinersen, for the treatment of spinal muscular atrophy and that can help to transpose ASOs therapies to other diseases. Specifically in ALS there are currently therapies in development targeting SOD1 and C9orf72 transcripts (90).

3.2.1.1 Experimental trials

3.2.1.1.1 SOD1

Over the last years there has been some studies and trials concerning ASOs. One of the first studies, by Smith and colleagues, demonstrated that cerebroventricular injections of an ASO named ISIS 3336111 targeting SOD1 reduced SOD1 mRNA through the activity of RNase H enzyme in the brain and spinal cord of SOD1 rats and significantly slowed down disease progression, increasing their mean survival by 10 days (91). One of the main concerns was the loss-of-function effects that the knockdown of SOD1 may have in ALS progression. After this study, a phase 1, randomised placebo-controlled clinical trial was made in patients with SOD1 familial ALS. The ASO ISIS 333611 was delivered intrathecally to CSF and overall the study demonstrated that it was well tolerated and there were no serious adverse effects registered (92). Although these results were promising, it was anticipated that the drug would be administered as a continuous infusion using implantable pumps and at the same time, some studies demonstrated that intrathecal bolus injection was more effective than continuous infusion (93). This work on ISIS 333611 was halted, and an investigation on a more potent backup drug started being done (94).

There were studies made with subclasses of ASOs, in particular morpholino-oligonucleotides (Mos) targeting SOD1 both in rodent and human fALS-derived induced Pluripotent Stem Cells (iPSCs) that demonstrated that Mos silenced SOD1 expression by up to 80% both *in vitro* and *in vivo.* MO were administered by local and

systemic injections and improved neuromuscular function and the survival of mice. It also increased the number of MN and axons in the spinal cords and ventral spinal roots, reducing astrogliosis and activated microglia (95).

Given these findings of safety profiles, an open-label trial was initiated with the objectives to evaluate the long-term safety and tolerability of this ASO in participants with ALS and confirmed SOD1 mutation and also evaluate pharmacokinetic, pharmacodynamic and efficacy of Tofersen (BIIB067) in the same type of patients.

This study is already taking place, it started in the 20th of January of 2016 and the estimated study completion date is in the 6th of July of 2021, with the title "An Extension Study to Assess the Long-Term Safety, Tolerability, Pharmacokinetics, and Effect on Disease Progression of BIIB067 Administered to Previously Treated Adults With Amyotrophic Lateral Sclerosis Caused by Superoxide Dismutase 1 Mutation " it has an estimated enrolment of 183 patients from different locations in the United States, Canada, some European countries and Japan (NCT02623699 at www.clinicaltrials.gov).

This study has three different parts, Part A and B are already completed (single ascending dose and multiple ascending doses respectively), and part C is ongoing. Part B results are already available, a multiple ascending-dose trial that evaluates the safety, pharmacokinetics, and pharmacodynamics of tofersen in adults with ALS. Primary outcomes of this study include incidence of adverse events and serious adverse events, abnormalities in clinical assessments and vital signs, physical examination including cranial nerves, coordination and cerebral function, reflexes, motor function and Mini-Mental state Examination. Secondary outcome was the change from baseline in the SOD1 protein concentration in CSF at day 85. Concerning safety, all 50 patients were submitted to safety analysis and all of them reported adverse effects including head ache (16 participants), procedural pain (16 patients), post-lumbar puncture syndrome (13 patients) and falls (13 patients) most of them due to lumbar puncture. There were also some serious adverse events with five patients who received tofersen and two who received placebo. Three deaths occurred, one in the placebo group, one in the 20-mg dose group and one in the 60-mg group, both during follow-up and due to respiratory failure. Positive results in this trial include the reduction from baseline in the total CSF SOD1 concentration, it was 3% in placebo groups and 36% in the group that received the highest dose (100 mg) of tofersen. With cessation of the 100 mg dose of tofersen,

smaller decreases in the SOD1 concentration in CSF, NfL and pNfH were observed, as well as greater decreases in the ALSFRS-R score. Although the objective of the study wasn't to measure any clinical or biological effects beyond reduction of SOD1 concentration in CSF, some evidence of a disease slow down and increase in the ALSDR-R score, slow vital capacity were observed. Some limitations to this study are the small number of participants, the short duration of treatment and follow-up, the exploratory nature of the efficacy outcomes, and the post hoc methods for defining the fast-progression sub-group as compared with the other subgroup (96).

A phase III, randomized, double-blind placebo controlled clinical trial is being made currently, where safety and efficacy of tofersen are being evaluated. (NCT02623699 at www.clinicaltrials.gov) and its long-term extension study (NCT03070119).

Antisense oligonucleotides can also function as potent inhibitors of miRNAs (anti-miRNAs) as will be discussed ahead.

3.2.2 RNA interference (RNAi)

RNA interference therapy is based on the function of the RNA-induced silencing complex (RISC), a ribonucleoprotein complex responsible for gene expression regulation, mRNA degradation and inhibition. This complex works due to the interaction of noncoding RNAs (ncRNAs), which represent more than 85% of the transcribed human genome with complementary mRNA sequences, forming double stranded RNAs (dsRNA). ncRNA include sequences originated endogenously, known as miRNA and sequences originated exogenously, small interfering RNA (siRNAs) and short hairpin RNA (shRNAs).

These ncRNA recognize the complementary mRNA target sequence of the RISC and once the target is recognized, Argonaute (AGO) effector proteins achieve silencing through translation blocking in case of partial complementarity or mRNA degradation in case om complete complementarity (97).

Considering such mechanism, there is ways to silence target mRNAs by inducing the transcription of miRNAs or shRNA that get processed via that pathway, for example through viral vectors that penetrate the nucleus.

These engineered miRNAs and shRNAs are commonly transcribed from genes by RNA polymerase II (Pol II) or less frequently by RNA polymerase III (Pol III). Pol II is responsible for the synthesis of precursors for small RNAs, Poll III can synthesize small RNAs but also transcribe housekeeping genes with abundant expression (98).

There are some approved RNAi-based therapies on the market, the example of patisiran, an siRNA used in the treatment of amyloidosis (99). RNAi present a poor stability, susceptibility to the action of serum nucleases, and the possibility to silence nontarget mRNAs (97). Some ways to try to reduce these limitations include chemical modifications of the original RNA structure such as modifications of the sugar moiety of siRNAs and backbone modifications such as the replacement with boranophosphate, to improve stability and resist nucleases action (100).

3.2.2.1 Experimental trials

3.2.2.1.1 shRNAs/siRNAs

Several studies have demonstrated good results concerning the effectiveness of shRNAs and siRNAs in several variables. Tested in different viral vectors such as adeno-associated viruses (AAV), lentiviruses (LVs) and adenoviruses (AVs), shRNAs and siRNAs showed results in retardation of disease onset and progression rate, specific and efficient reduction of SOD1 expression consequently improving survival of vulnerable motor neurons and motor performance in tested animals (101–104).

shRNAs and siRNAs therapy after onset trials done in non-human primates have also shown good results in treating glia associated complications such as astrogliosis and microgliosis as well as preservation of spinal alpha-motor neurons, interneurons and neuromuscular junctions, showing once more a slowdown in disease progression and significantly extended survival, setting the stage for AAV9-mediated therapy in human clinical trials (105). However, different studies were delivered in different postnatal stages and using different delivery methods, suggesting some correlation between age of treatment and outcomes and delivery method and outcomes. Earlier silencing seemed to result in increased survival, and intramuscular and intravenous injection of AAV6 resulted in no clinical benefit (103,105–107) (108).

Some problems can be identified in these types of studies. Because shRNAs are driven by strong Pol III promoters, an overload can occur in the RNAi machinery and cause cell toxicity. Maczuga et al. studied this and showed that using a Pol II promoter

leads to reduced toxicity compared to Pol III-driven shRNAs, but overall shRNAs were still less effective than miRNAs (109).

The fact that shRNA target both wild-type and mutant genes can explain the high silencing efficacy of the shRNAs used. This problem can be overcome by gene editing and gene replacement, such as Xia and colleagues did in their study, where mutant SOD1 gene was inhibited by shRNAs and wild-type gene was replaced by designed RNAi-resistant wild-type SOD1 by inserting silent mutations in that way avoiding the excessive target gene silencing and RISC saturation (110). Another problem identified is the lack of an *in vivo* biomarker, to track the therapeutic response. hSOD1 protein from CSF can help to overcome this problem. A study showed that hSOD1 taken from CSF after treatment with AAV9-shRNA was 63% decreased compared to untreated controls (111).

3.2.2.1.2 miRNA

miRNAs have lower risk of toxicity, exhibit a safer immunogenic profile and produce fewer off-target effects compared to shRNA (112).

Silencing efficacy in miRNAs can be enhanced by tailoring vectors. Vector-derived genes can transcribe artificial RNAs by Pol II or Poll III, opening the possibility to use Pol II tissue specific promoters. miRNAs can also be chained to transcribe multiple miRNAs from the same promoter, this is called combinatorial RNAi and allows simultaneous targeting of different mRNAs by joining different miRNA sequences, enhancing silencing efficacy (113).

Various studies have been made considering these properties (114,115). Those studies used different vectors such as AAV6, AAV9, AAVrh10 with different delivery methods such as intravenous and intrathecal. The results demonstrated that some vectors performed better than others, for example AAVrh10 and AAV9 performed much better than AAV1, 2 and 8 in achieving widespread transduction of motor neurons, astrocytes and oligodendrocytes in the spinal cord and brain stem. Overall, the studies successfully silenced SOD1 mutations either in motor neurons and in glia cells, and in both cases there were improvements in several parameters such as the number of spinal motor neurons, the diameter of ventral root axons, the extent of neuroinflammation in spinal cord, increasing median survival in 50% and preservation of motor function (116).

Other studies showed complete rescue of neuromuscular function in ALS after neonatal intracerebroventricular injection of AAV-miRNA targeting SOD1, and demonstrated that selecting the cell types to which silencing clues are targeted is important to obtain high therapeutic efficacy and that motor neurons are crucial therapeutic targets compared to astrocytes and microglia. Motor neurons protection showed to be highest when SOD1 is manly silenced in motor neurons following injection of AAV vectors in both new-born and adult mice, with 83-92% of motor neurons still present in end-stage mice treated at birth. Despite that, the expression of AAV-miR-SOD1 in astrocytes lead to the survival of 54-62% of motor neurons and to normalization of compound muscle action potential, supporting the critical role played by SOD1 expression in astrocytes in the decline of neuromuscular function (117).

After that, a study was made to investigate how these results translated to primates (cynomolgus macaques - *Macaca fascicularis*) and to study safety-profiles using AAVrh.10 harbouring an artificial miRNA. The AAV was delivered to the spinal cord by preimplantation of a catheter and placement of the subject with head down at 30º during intrathecal infusion. Different promoters were compared during the experiment (Pol II and Pol III).

The study results demonstrated that the large volume (5ml) intrathecal delivery of the respective vector in non-human primates was safe and well tolerated by the macaques up to 92 days after administration. The study was made with the presence of Green Fluorescent Protein (GFP), resulting in a mild liver toxicity as expected (118), and without GFP where the liver toxicity didn't occur, suggesting the evidence of any RNAi mediated toxicity given the high vector load per cell, either expressed by Pol II or Pol III promoter. Another concern about the study was the possibility of off-target toxicity because of the processing of mature miRNA targeting SOD1. The maturation process could lead to higher abundance of the complementary sequence instead of the guide strand that targets SOD, however, the study demonstrated good results in that matter having obtained an accurate processing with a favourable guide/passenger strand ratio. The study also showed that silencing of SOD1 gene, measured in motor neurons, was more enhanced by use of Pol III promoters (H1 and U6) than by the Pol II promoter (CB). Poll II obtained a 93% silencing at the lumbar section. They also noticed a spatial difference between lumbar and cervical cord that become less evident after long periods of follow-up, with lumbar section presenting a greater silencing.

Based in these results, the FDA approved a pilot phase I clinical trial in humans for an investigational drug application (IND #17179). This trial will try to clarify safety profile in humans and some important limitations such as the fact that cellular immune response characterized by IFN-$^{\gamma}$-secreting T cells cannot be precisely reproduced in animals.

3.2.2.2 miRNAs as targets

miRNAs can also be potential targets because of their capacity to modulate apoptosis and regulated necrosis, in particular necroptosis, both mechanisms that play a significant role in the progressive death of MNs in ALS (Fig.3). In some cases, miRNAs expression profile is dysregulated, and it consequently affects RISC, originating several disorders.

Several miRNAs dysregulations were demonstrated to have a negative impact in patients with ALS either by triggering mechanisms of intrinsic apoptosis, extrinsic apoptosis, or necrosis.

MicroRNA(miR)-155 and miR-29a are two possible targets because of their involvement and overexpression in those mechanisms, and some pre-clinical trials were made in that sense (119,120). The pre-clinical trial concerning oligonucleotide-based miR-155 inhibitors (anti-miR-155) therapy showed good results, slowing down disease progression, extending mice survival by 10 days and disease duration by 15 days (38%) comparing to control. miR-155 was targeted through antisense oligonucleotides, more specifically anti-let-7, injecting them into cerebral lateral ventricle in mice. Anti-let-7 targets were depressed through the brain and spinal cord, being the first study to demonstrate widespread inhibition of miRNA in the spinal cord (119).

miR-155 also showed to be a major inflammation regulator (121) and to have neuroprotective effects (122). Those properties suggest that miR-155 are involved in neuroinflammatory processes, and instability process in glial cells. Some studies already confirmed that influence in glial cells by helping in the production of inflammatory mediators and microglia activation, nitric oxide production and targeting suppressor of cytokine signalling 1 (SOCS-1) protein, a key inhibitor of the inflammatory process and suggested that in chronic inflammation context, miR-155 inhibition can have neuroprotective effects (123). However, recent studies

demonstrated that miR-155 can have a beneficial effect in inflammation and even act as an anti-inflammatory factor (124,125).

Taking these results in consideration it can be said that miR-155 is a good example of the complex role that miRNA plays in ALS and the opportunity they held in understanding the disease and developing new combined therapies.

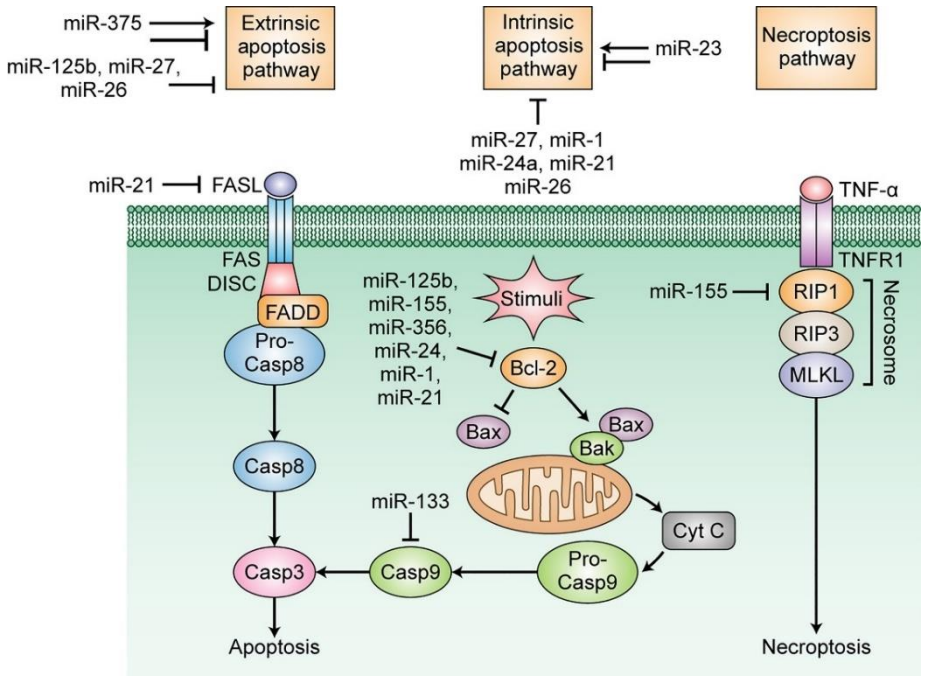

Figure 3 - Influence of mi-RNAs on cell death pathways implicated in motor neuron death in ALS.

Extrinsic apoptosis. Death-inducing signaling complex (DISC) is formed when Fas ligand (FASL) activates the cognate FAS death receptor. DISC interacts with Fas-associated protein with death domain (FADD) inducing the recruitment of pro-caspase-8 and dissociation from DISC. When activated caspase-8 initiates caspase reaction leading to apoptosis through caspase-3. Inhibition of this pathway can be achieved by silencing of p53 by miR-375, miR-125b and miR-27a and targeting FasL with miR-21. (B) Intrinsic apoptosis. BH3-only proteins activated by cytotoxic stimuli silence Bcl-2, allowing Bax and Bac to dimerize and make the mitochondrial membrane permeable, allowing cytochrome *c* release into the cytoplasm. Cytochrome *c* (Cyt *c*) then activates caspase-9 cascade by binding to apoptotic protease activator factor-1 (Apaf-1) and forming the apoptosome. miR-125b, miR-155, miR-365, miR-24, miR-1 and miR-21 promote apoptosis by Bcl-2 silencing. miR-133a inhibits apoptosis binding into Casp9. (C) Necroptosis. Interaction of all the components of the necrosome, receptor-interacting protein 1 and 3 (RIP1 and RIP3), mixed lineage kinase domain like pseudokinase (MLKL) causes cell membrane rupture. miR-155 inhibits necroptosis by silencing RIP1. miR, Micro RNA; TNF, Tumour necrosis factor; TNFR1, Tumour necrosis factor receptor 1. Reproduced from (126).

3.2.3 CRISPR/Cas9

CRISPR stands for clustered regularly short palindromic repeats and this cluster is normally associated with protein-9 nuclease (Cas9). This system is a genome editing tool that functions as a defence mechanism in bacteria against foreign nucleic acids such as plasmids and bacteriophages (127). This system creates double-strand breaks (DSBs) in target sites in the genome that allow precise genome editing. In bacteria, there are two constructs responsible for viral DNA recognition, CRISP targeting RNA (crRNA) and transactivating RNA (tracrRNA). Cas9 is a gene editing protein that derives from *Streptococcus pyrogenes.*

CRISPR/Cas9 is a combination of fused crRNA and tracrRNA into a single guide RNA (sgRNA), responsible for targeting DNA sequences, and the gene protein editor, Cas9. In order to reduce off-target effects, not abolishing them completely, sgRNA has two different recognition sequences, one recognizes the complementary target sequences and the other, a protospacer adjacent motif (PAM), matches with the complementary PAM at the 3' end of the target sequence, to initiate cleavage. There are two DNA repair mechanisms as represented in Fig.4 (128).

CRISPR-Cas9 system have produced two innovative treatments approved for cancer therapy, based on the modification of autologous T-cells, CAR-T (chimeric antigen receptor engineered T cells) and TCR-T (T cell receptor engineered T cells) (129).

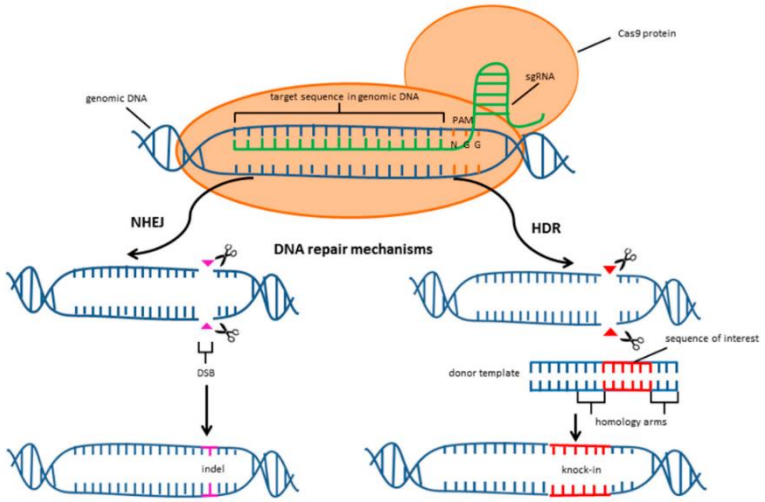

Figure 4 CRISPR/Cas9 mechanism of cleavage of genomic DNA and two major repair pathways.

NHEJ, non-homologous end joining; HDR, homology-directed repair. Reproduced from (128).

3.2.3.1 Experimental trials

CRISPR/Cas9 is being used in the last few years, but there are not a lot of studies testing its efficiency and safety as a treatment for ALS. One of those few studies was a test made *in vitro* on targeting FUS and SOD1 of iPSCs from fibroblasts of familial ALS patients (130). Only one *in vivo* study has been made in SOD1 mouse models, through intravenous administration of an AAV9 vector containing a sgRNA targeting the mutant SOD1 gene and encoding the Cas9 nuclease (131). This study demonstrated beneficial results such as delay in disease onset, improved muscle function and extended survival in mice, however it was not able to slow down disease progression once the disease was established. Authors speculated that this result was due to secondary degeneration caused by inefficient SOD1 disruption in astrocytes and consequent abolishment of motor neurons support.

One of the issues raised in this study is the lack specificity of sgRNA used, because it was unable to select between mutant and wild-type human SOD1. This lack of specificity can lead to off-target effects and excessive SOD1 silencing.

Other *in vitro* studies were made targeting the *C9orf72* promoter. Investigators in this study were able to delete part of the *C9orf72* promoter, responsible for the repeat expansions of RNA species that translate toxic proteins and diminish levels of those proteins but also the levels of *C9orf72* proteins. However other studies demonstrated that the absence of those proteins did not cause neurodegeneration. So, their proof-of-concept study suggests that "CRISPR/Ca9-based targeting of the promoter region to eliminate sense repeat RNA and its toxic translation products, may be a potentially useful therapeutic approach for *C9orf72*-ALS/FTD, especially before significant accumulation of DPR proteins" (132).

3.3 Stem Cell treatments

It is known that glial cells have an important role in the pathogenesis of ALS and that surrounding neurons with a healthy environment glia is a crucial step in trying to stop motor neuron death. Knowing that, stem cell transplantation has been had as a promising therapeutic strategy in ALS treatment. Stem Cell transplantation can have different approaches depending on the aimed ALS aspects, including either the replacement of lost cells, or the protection of motor neurons from degeneration or toxic

microenvironment. Various phase I and II clinical trials have been done in that direction, using different stem cells such as granulocyte-colony stimulating factor-induced peripheral blood stem cells, mesenchymal stem cells and non-neural progenitor cells (133–135). However, there are critical variables that can determine the effectiveness and feasibility of a therapy with stem cells. Those include the need to identify the most effective therapeutic cell source (mesenchymal stem cells, immune or neural stem cells), the definition of the optimal injection site (cortical area, spinal cord or muscles), a suitable administration protocol (local or systemic injection) and the analysis of therapeutic mechanisms (136). Elena Abati et al. (136) recently reviewed a series of papers concerning clinical trials with stem cells, where those variables were taken into consideration to try to determine the best potential therapy with stem cells for each specific case of the disease. With a lot of up to date studies reviewed, the authors concluded that although stem cell therapy produced a lot of good results at preclinical level in terms of their regenerative capacity and safety, their clinical use is still limited to early clinical trials. This is due to limitations such as economic factors, lack of risk/benefit ratio data of every translational study, regulatory and ethical issues such as “therapeutic misconception”, manufacturing practices issues, the doubt about the site of stem cell administration and its implications in treatment efficacy.

Stem cell therapy trials are overall presenting positive results in ALS progression rate, amelioration of toxic microenvironment and contributing to a lot of discoveries and confirmations of pathological mechanisms, but all the different variables to take in consideration still demand further research.

Some of the most promising therapeutics being studied today concerning Stem Cells are Ropinirole (ROPI), retigabine and bosutinib (137). Those are iPSC-based drug screens and are now under clinical trials for safety and effectiveness. Ropinirole is a dopamine D2 and D3 receptor agonist used as an antiparkinsonian drug, retigabine is a neuronal Kv7 channel opener used as an antiepileptic drug and bosutinib is a dual Src/Abl tyrosine kinase inhibitor use as an anti-chronic myelocytic leukaemia (CML) drug. Table 2 explains the mechanisms involved in these therapies and specifies the targeted ALS subtype.

Table 2 – Potential mechanism and Targeted ALS subtypes of the Three Therapeutic Candidates for ALS Developed by iPSC Drug Discovery

	Potential mechanism	*Targeted ALS subtype*
Ropinirole	Inhibition of FUS and TDP-43, suppression of oxidative stress, improving of mitochondrial function.	Most of sporadic TDP-43 mutation FUS mutation NOT SOD1 mutation
Retigabine	Inhibition of motor neuron excitability, decreasing of endoplasmic reticulum stress pathway	SOD1 mutation C9orf72 mutation FUS mutation
Bosutinib	Inducing autophagy Inhibition of mutated genes	SOD1 mutation TDP-43 mutation C9orf72 mutation A part of sporadic

TDP-43, TAR DNA Binding Protein 43 kDa; FUS, Fused in sarcoma; SOD1, superoxide dismutase 1; *C9orf72*, Chromosome 9 open reading frame 72. Adapted from (137).

The potential anti-ALS mechanism of ROPI is independent of antioxidant activity, rescue of mitochondria, reduction of stress granules and abnormal proteins (TDP-43 and FUS).

Ongoing clinical trial protocols for these potential drugs include the ROPALS randomized, double-blind, placebo controlled, single-centre and open label phase I/IIa trial for ROPI(138). A phase II pharmacodynamic trial for Retigabine (NCT02450552) in www.clinicaltrials.gov) and a Phase I dose escalation study for Bosutinib. (Unique ID issued by UMIN: UMIN000036295).

3.4 New approaches during SARS-CoV-2 pandemic

Coronavirus disease 2019 pandemic and consequent social restriction containment measures has influenced the lives of ALS patients. With the rearrangements of resources and spaces, a lot of consultations have been postponed or converted in teleconsultations, which influenced ALS patients as well. Giving the high probability of this disease persistence in a recent future and a risk of a lost follow-up, new measures need to be taken to guarantee ALS patient follow-up.

Telemedicine has been the go-to, to replace face-to-face visits and it is important to establish a good service and ways to monitor the progression of the disease and

manage complications as soon as possible. There are two main variables that should be monitored, ALSFRS-R, as it is the most used scale to evaluate ALS progression and neurological examination. Some studies showed the possibility to do an online self-administered version of ALSFRS-R (139).

American Academy of Neurology recently published recommendations to improve telemedicine service (140).

Overall, measures such as audio-video link neurological examination; monitorization through the use of sensors with accelerometers for motor activity assessment and heart rate variability detection; invasive and non-invasive ventilation monitorization through videoconferencing or home based self-monitoring; bulbar function monitorization through the analysis of recordings of patients reading; nutritional status can be monitored through certified and tested mobile applications for example "Nu Planit" application that helps patients monitor their food habits and weight measurements and adapt their diet according to the results; psychological support can be given through videoconference. Some limitations of these tools are the high costs of some of the equipment needed and he lack of validation of some methods and instruments (141).

4 Conclusions

ALS/FTD still remains a lethal disease nowadays, and a lot of efforts are being made to try to develop new strategies for possible drug treatments and improved therapies that give patients a better life quality and a longer life as well. The knowledge about the disease status is being renovated almost each day with the appearance of new studies almost every month with potential to develop a new treatment.

Although therapy for ALS still remains a symptomatic management and with only two disease-modifying drugs approved on the market, steps are being taken with the objective of improving patients' lives either through new ways of managing symptoms and complications associated with the disease progression, such as nutrition and respiratory methods, for example the Functional Medical Nutrition Therapy approach that tries to view patient nutrition as an integrated part of the therapy.

In the drug development field, there are also a lot of Phase II and III clinical trials trying to apply drugs that are already on the market and function as therapies for other neurodegenerative diseases and even other types of disorders, to ALS symptom treatment for example the case of Memantine, Colchicine and Metformin. There are also innovative ideas of approaching therapies for ALS, and clinical trials already testing those ideas. Antisense oligonucleotide therapy is a good example of both, trying to implement therapies that are already on the market for other diseases (Nurinersen), at the same time studies as being made to translate some of the ideas of mechanisms to the treatment of ALS. Those types of studies also open doors for the continuous understanding of the disease's mechanisms. The case tests being made with miRNA, either using miRNA as the therapy or the therapeutical target are tests that lead to a lot of new understandings in how the pathological process of the disease is organized and how interactions in pathological mechanisms occur. For example, discoveries concerning the inflammatory process associates with mi-RNAs in various types of neuron and glial cells.

Some investigations concerning CRISPR/Cas9 are still in an early phase, but the results *in vitro* seem to be very promising, with neurodegeneration being rescued and slowed down in most of the cases.

Cell transplant and stem cell therapy is a long-time study, and the main limitation is trying to figure what combination of cells and delivery methods work the best in producing a specific response. Despite of that, there are also some advanced phase II clinical trials based on therapies with induced pluripotent stem cells, as well as other preclinical results showing good results with the repurposing of drugs, such as ropinirole, retigabine and rosutinib, already being used to treat other diseases.

In the state that we live nowadays, under the pandemic of COVID-19, some measures needed to be taken so that patients with ALS did not lose their regular follow-up of the disease. That follow-up is very important to slow down disease progression and to adopt better lifestyle mechanisms for patients and care owners. Some of the methods being applied today showed to be successful, under the possible conditions, although ALS patients lives still are very harmed with this pandemic.

Despite of all the novel discoveries and new clinical trials in therapeutics for ALS, a lot of the disease pathogenic mechanisms are still to discover so that we can find a cure. Until then we need to try to do our best to improve patients' lives by managing symptoms.

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