UNIVERSITAT DE BARCELONA

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Final degree project

New hypothesis and treatment of Amyotrophic lateral sclerosis: a holistic point of view of the key evidence

Main subject: Pharmacology

Secondary subjects: Physiopathology and Biochemistry

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A l'équipe de Neurodialitics, spécialement à Bernard Renaud

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Abbreviations

AIS - Acute ischemic stroke
ALS - Amyotrophic lateral sclerosis
CNS - Central nervous system
CSF - Cerebrospinal fluid
DCTN1 - Dynactin subunit 1
DNA - Deoxyribonucleic acid
DNMT - DNA methyltransferase
ELA - Esclerosi lateral amiotròfica
ER - Endoplasmic reticulum
fALS - familial ALS
FUS - protein fused in sarcoma
iPSC - inhibit pluripotent stem cells
miRNA - micro RNA
mRNA - messenger RNA
MTNs - motor neurons
MSC - Mesenchymal Stromal Cells
NTF - neurotrophic factors
PrP - prion promoter
RBP - RNA-binding proteins
RNA - Ribonucleic acid
ROS - radical oxygen species
RTC - randomized controlled trials
sALS - sporadic ALS
SOD1 - superoxide dismutase 1
TK - tyrosine kinase
UAB - Universitat Autònoma de Barcelona
Unfolded-Protein Response (UPR
WFN - World Federation of Neurology

1. Abstract

The amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that affects the motor neurons (MTNs) of the central nervous system (CNS) causing the loss of enervation of these neurons with the muscles. ALS patients lose their mobility progressively due to the muscle enervation loss. At the end, internal muscles are affected by the constant loss of MTNs, and the patients die from respiratory depression due to the lack of MTNs enervating the smooth muscles allowing the lungs to function. Despite this fatal prognosis, the cure still unknown and there is no treatment allowing possible chronification. The only treatment that has shown a small survival elongation between 3 and 5 months is Riluzole, a blocker of glutamate receptor that avoids excitotoxic over stimulation in MTNs. Riluzole was approved in 1994 and since then, until 2017 with the approval of Edaravone, no more active compounds have been approved to deal with the disease. The fact that etiologic cause is not established yet, the molecular mechanisms, the animal models in which preclinical trials are developed, as well as the approach of clinical trials in humans, play an important role on the disease, which has represented a challenge to the scientific community for more than 150 years. Despite all the complications, the volume of new active compounds is at its highest point, and the new innovative therapies currently in phase II / III clinical trials provide new horizons for patients, relatives and researchers. In this report, we try to find the causes for this rugged therapeutic development behind ALS, scrutinizing all the possible defects and discussing possible solutions treat within the scientific community. At the same time, the report tries to convey an overview of the current knowledge about ALS, keeping the focus on biochemical processes and exclusively drug therapy of the disease.

Resum

L'esclerosi lateral amiotròfica (ELA) és una malaltia neurodegenerativa que afecta les motoneurones del sistema nerviós central provocant la pèrdua d'enervació d'aquestes neurones amb els músculs. Els pacients que la pateixen perden la mobilitat progressivament fins que els músculs interns es veuen afectats per la pèrdua constant de motoneurones fins que moren de depressió respiratòria per la falta d'enervació als músculs llisos que permeten el funcionament dels pulmons. Tot i aquesta prognosi fatal de la malaltia, encara no hi ha cura coneguda ni cap tractament que permeti cronificar-ne els efectes. L'únic tractament que ha demostrat un petit l'allargament entre 3 i 5 mesos de vida es tracta de Riluzole un bloquejador de l'estimulació del receptor de glutamat que evita una sobrestimulació excitotòxica en les motoneurones. Riluzole va ser aprovat l'any 1994 i des de llavors fins a 2017 amb l'aprovació d'Edaravone, no s'han aprovat més principis actius per fer front a la malaltia. Les causes etiològiques encara no establertes, els mecanismes moleculars patològics, els models animals en els quals es desenvolupen els assaigs preclínics, així com el plantejament dels assaigs clínics en humans juguen, cadascun, el seu paper en aquesta malaltia que representa tot un repte per a la comunitat científica des de fa més de 150 anys. Malgrat totes les complicacions, el volum de nous principis actius es troben al seu punt més alt, i les

noves teràpies innovadores actualment en fase II/III d'assaigs clínics proporcionen nous horitzons per a pacients, familiars i investigadors. En aquest treball s'intenta trobar les causes per aquest accidentat desenvolupament terapèutic darrere l'ELA, posant sota escrutini tots els possibles defectes i discutir possibles solucions debatudes dins la comunitat científica. Al mateix temps, transmetre una visió general del coneixement actual sobre l'ELA, mantenint el focus en els processos bioquímics i la teràpia exclusivament farmacològica de la malaltia.

2. Introduction

2.1. Natural history

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease causing a progressive death of the Motor Neurons (MTNs)[1]. This type of neuron whose cells bodies are located in the brain or the spinal cord, enervate the muscles and glands. The lack of MTNs results in the atrophy and degeneration of the muscle that supply, inducing a progressive control lost of the muscles that end with the death of the patient due to a respiratory failure between 3 and 5 years after the disease onset. Nonetheless, the life expectancy is variable and 10% of patients will survive ten years on average, and 5% will live 20 years or more[1].

ALS prognosis is dreadful. It starts before even the first symptoms are noticed. Approximately 80% of the MTNs are already lost at the time of the diagnosis[2]. This is a consequence of the processes of denervation and reinnervation that take place between the MTNs affected by the disease and those that are still healthy. The process of denervation is the loss of connection between MTNs and the motor endplates of the muscle, mostly due to the death of the first ones[3]. When a motor neuron dies, fibers that belong to healthy motor neurons develop "sprouts" and take over the neural control of denervated muscle fibers. This phenomenon is called the reinnervation process (see figure 1). As long as the balance between denervation and reinnervation is maintained, muscle weakness may not become clinically apparent.

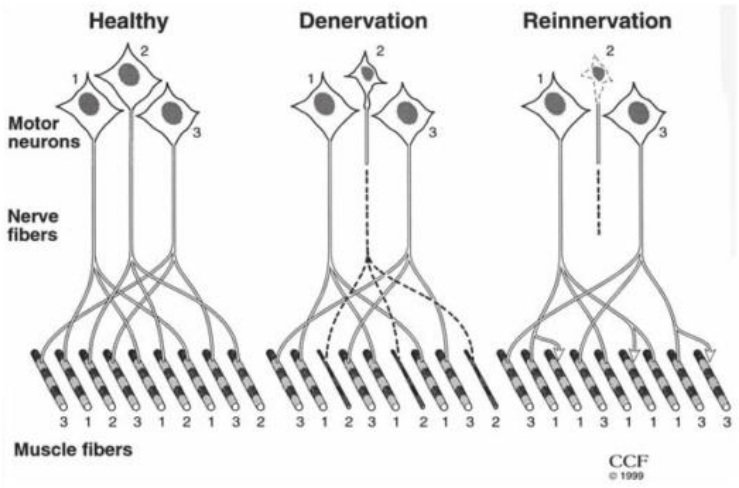

Figure 1. Example of denervation - reinnervation process[2].

Once the process of reinnervation process is insufficient to mask the degeneration of the MTNs, the symptoms start to be noticeable. First, patients experiment with the muscle weakness. It can start with experiencing awkwardness when walking or if arms are the first affected, difficulty with tasks requiring manual dexterity. Then these first symptoms could degenerate to atrophy, and muscle spasms[4]. The parts of the body affected by early symptoms of ALS depend on which motor neurons in the body are damaged first (see figure 2)[5]. If upper MTNs, found in the brain, are affected first, the patients could develop the inability to swallow (Dysphagia), speaking or forming words (Dysarthria), control the salivation (Drooling), or develop an excessive production of saliva (Sialorrhea). Language and, executive dysfunction troubles with social cognition and verbal memory are the most commonly reported cognitive symptoms in ALS[6]. Sensory nerves and the autonomic nervous system generally remain unaffected, meaning the majority of people with ALS maintain hearing, sight touch, smell, and taste.

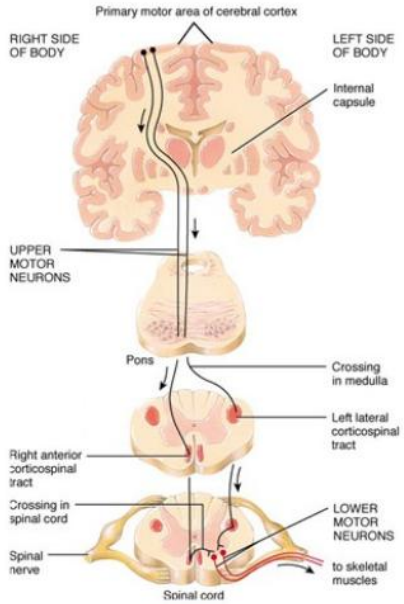

Figure 2. Diagram of the neural motor system[7].

ALS progression is not always a straight line. It is not uncommon to have periods lasting weeks to months where it is very little or no loss of function[1]. As the disease develops, patients experiment with spasticity, exaggerated reflexes (hyperreflexia) and an abnormal reflex commonly called Babinski's sign[6]. In the final stages, patients are unable to speak or swallow, have a lot of aspiration difficulties and painful laryngospasms and lose the ability to initiate and control all voluntary movement, although the muscles responsible for eye movement are usually spared until the final stages of the disorder (see figure 3). In the end, as it was said, patients die from respiratory failure.

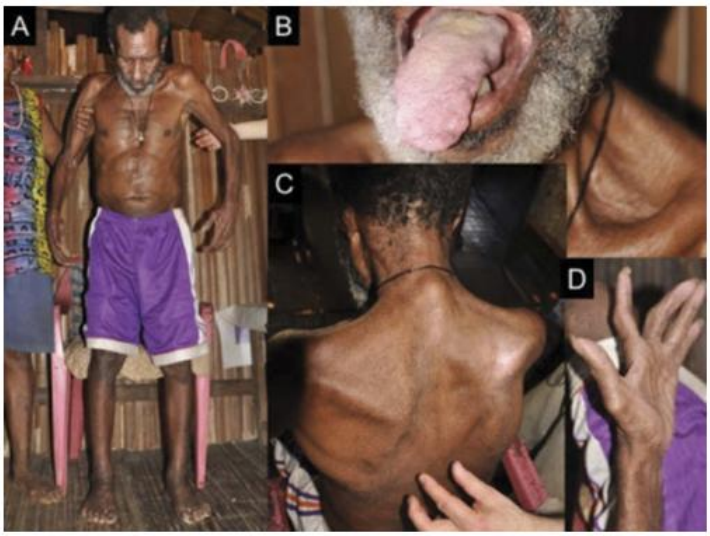

Figure 3. ALS patient. (A) The patient needs assistance from family members to stand. (B) Advanced atrophy of the tongue. (C) There is upper limb and truncal muscle atrophy with a positive Babinski sign. (D) Advanced thenar muscle atrophy[8].

2.2 Epidemiology

After all, since the French physician, Jean-Martin Charcot in 1874, establishing a complete clinical spectrum and specific examination features[9], ALS stills a big unknown for the neuroscientist community. However, the 2000s more and more researchers start to investigate about ALS spurred by ALS patients foundations, new founding directed to it and the intellectual challenge. More and more information has been actualized about the disease at the point of revolutionizing all known until now. For example, it has been proposed that ALS could be a conjunction of different diseases that express the same process of neurodegeneration. Recent evidence regarding the epigenetic mechanism of ALS and the wide variability between patients has lead researchers to this hypothesis. Therefore new hopes for familiar and patients are delivered every week.

In Catalonia ALS is speculated to be 400 people affected, being a total of 4000 people in all Spain[10]. On average, someone is diagnosed with or loses his life from ALS every 90 minutes[1]. The disease has an incidence of 1–5 per 100,000 inhabitants and a prevalence of 6 cases per 100,000 inhabitants, and affects adults between 50 and 75 years of age principally. It is important to clarify that ALS is not contagious and occurs throughout the world with no racial, ethnic, sex or socioeconomic boundaries[11].

2.3. Primary cause and primary mechanism

2.3.1 Genetic basis

The primary cause of this dreadful disease is still unknown. No environmental or physical cause has been linked directly. However, several genetic mutations have been linked to ALS development[12]. In roughly 10% of ALS cases, patients have one of these mutations, also found in other family members; these cases are called familial ALS

(fALS) cases. The other 90% of ALS cases have no familial relation, even though the cause could be a genetic mutation as well. These types of non-familiar related ALS cases are called sporadic ALS (sALS). It is estimated that almost 15% of sALS cases are caused by a genetic mutation[2]. The rest of sALS cases are not related to any genetic cause known yet. Since the mutation of superoxide dismutase 1 (SOD1) gene was linked to ALS pathogenesis in 1993[13], the theory of a genetical cause behind ALS pathogenesis is still being discussed spurred on the new gene linkage discoveries happening every week. Today, high numbers of mutations of SOD and other genes have been linked to ALS (consult all of them at alsresearchfoundation.com[7]). The most predominant ones in ALS pathogenesis are the followings:

1. Mutations in the superoxide dismutase-1 (SOD1) gene account for 20% of all fALS cases. The enzyme SOD1 normally functions as an antioxidant, helping to eliminate potentially damaging free radicals in cells throughout the body. The damage to this gene causes the production of a toxic SOD1 protein that damages motor neurons and surrounding cells in the nervous system, causing ALS[2].

2. The mutation of the TARDBP gene which codifies for the TDP-43 protein is responsible for 2-5% of fALS cases. TDP-43 is a nuclear protein involved in RNA transcription, splicing and transport. Under cell stress conditions, this protein moves to the cytoplasm, forming stress granules[14]. Stress granules contain non-translating mRNAs, translation initiation components, and many additional proteins affecting mRNA function. Moreover, stress granules have been proposed to affect mRNA translation and stability, as well as being linked to apoptosis and nuclear processes[15].

3. Abnormally expanded GGGGCC hexanucleotide intronic repeats in C9orf72 gene were identified as the most common genetic cause of fALS (40% of fALS cases)[16]. The first intron could be repeated to 100s or even 1,000s times[17]. Several cytotoxic mechanisms have been described for C9orf72. One of the most discussed is the possibility that these RNA repetitions were toxic *"per se"*. RNA hexanucleotides repeat form complex structures which result in transcriptional impediments and in the accumulation of abortive transcript that contain the hexanucleotide transcripts[18].

4. Mutations in the DNA codifying the RNA-binding protein fused in sarcoma (FUS) have been identified as another protein involved in ALS[19]. FUS mutations are estimated to be responsible for 5% of fALS cases. FUS is a protein that has the ability to bind nucleic acids. Moreover, like TDP-43, FUS is mainly nuclear and under stress conditions form cytoplasmatic granules.

Even though, genetics plays a key role in the understanding ALS pathology, some patients present clinical heterogeneity showing different disease site and age onsets carrying the same mutation[20]. Also, sALS and fALS patients are clinically

indistinguishable and share the same pathophysiological mechanisms and disease development[21].

2.3.2 Epigenetical involvement

Several neurotoxic components had been proposed to be a subjacent cause to ALS, such as heavy metals (mainly aluminum, selenium, mercury and lead), sarín and cyclosarín, diets rich in β-methyl-amino-L-alanine, etc. Nonetheless, there exist mixed data on the topic, and no stable relation has been establishing between these neurotoxic agents as an ALS primary cause[22]. Nowadays these exposures are considered one of the several environmental factors that affect the curse of developing ALS, together with: age, genetic factors, and diet among others.

The fact that environmental factors and genetic factors cannot explain the inherent cause of ALS, has brought epigenetic as a potential convergence between them that could explain what remains unclear. Epigenetic is the structural adaptation of chromosomal regions so as to register, signal, or perpetuate altered activity states, and includes a variety of mechanism such as, methylation, histone remodeling, RNA editing and noncoding RNAs such as microRNAs[21]. The involvement of epigenetic mechanisms in disease development is strongly supported by studies that show disease discordance in monozygotic twins[23]

DNA methylation is a mechanism for gene regulation engaged by DNA methyltransferase (DNMT) catalyzed methyl group transfer to carbon-5 in cytosine residues[24]. The transcription of methylated DNA is disturbed due to methyl binding proteins that occupy the polymerase binding site or because activating transcription factors cannot bind to the methylated DNA. Methylation marks on DNA can be eliminated actively if the methyl group is removed through a series of enzymatic reactions. Interestingly, if the methylation occurs in the gene body or distal promoter regions, then the effect of the methylation is higher gene expression rather than gene silencing[23]

Recent genome-wide analyses have found differential gene methylation in human ALS[24]. These genes are engaged in calcium homeostasis, neurotransmission and oxidative stress pathways which could explain the pathophysiological mechanisms[23]. In addition, DNA methylation is increased in ALS independently of age onset, meaning that factor beyond the patient age could play a significant role. Hypermethylation of the C9ofr72 promoter plays an important role in neuroprotection and is associated with expanded hexanucleotide repeats. This leads to decreased promoter activity, which results in reduced gene expression and lower protein levels stopping the protein and RNA aggregates to form[23]. These hexanucleotide repeats in the C9orf72 gene also reduce gene expression due to histone acetylation. Histone acetylation occurs on lysine residues, where acetyl groups neutralize the lysine's positive charge, which leads to weaker interactions between histones and DNA and

enables gene transcription. The removal of acetylation marks reestablishes the positive charge on lysine residues and condenses the chromatin structure.

However, the most important epigenetically discovery was finding deregulated mature microRNAs on postmortem human spinal cord tissue[25] and leukocytes of sALS patient[23]. MiRNAs are evolutionarily conserved noncoding RNAs, about 22 nucleotides long, which can individually regulate several hundred targets via RNA-dependent posttranscriptional silencing mechanism[26]. A single miRNA can regulate several hundred mRNA targets via RNA-dependent post-transcriptional silencing mechanisms, and mRNA transcripts can be regulated by multiple miRNAs[21]. Global reduction of mature miRNAs and alterations in miRNA processing were identified in ALS patients expressing TDP-43 mutations, specifically deregulated in CSF and serum of SALS patients. However, the level between them were altered, suggesting an independent regulation of specific miRNAs in the two compartments and a generally low transition of miRNA across bloodcerebrospinal fluid barrier[27]

Epigenetic mechanisms are altered in both explained and unexplained ALS cases; however, it remains arguable whether these changes are a cause or consequence of the disease.

2.3.3. Molecular molecular mechanisms

Also, several molecular mechanisms have been discovered to produce neurodegenration in ALS patients. These mechanisms include: excitotoxicity, impaired proteostasis, disturbed RNA metabolism, cytoskeleton and axon-transport defects, Neuroinflammation, astrogliosis and mitochondrial dysfunction[12]. It is not clear what role they play or if they could be a relation between them in ALS pathogenesis. However, the same mechanism has been linked to several patients with different subjacent cases, even those with genetic and non-genetic primary causes as well as different patients with different genetic mutations[12] (see figure 4).

	Impaired proteostasis	Disturbed RNA metabolism and RBP's	Excitoxicity	Cytoskeleton and axonal transport defects	Mitochondrial dysfunction	Neuroinflamation and astrogliosis
C9orf72	x	x	x			
FUS	x	x	x			
SOD1	x		x	x	x	x
TARDBP	x	x			x	

Table 1. Molecular pathways related to the principal gene mutation affecting ALS[28].

2.3.3.1. Excitotoxicity

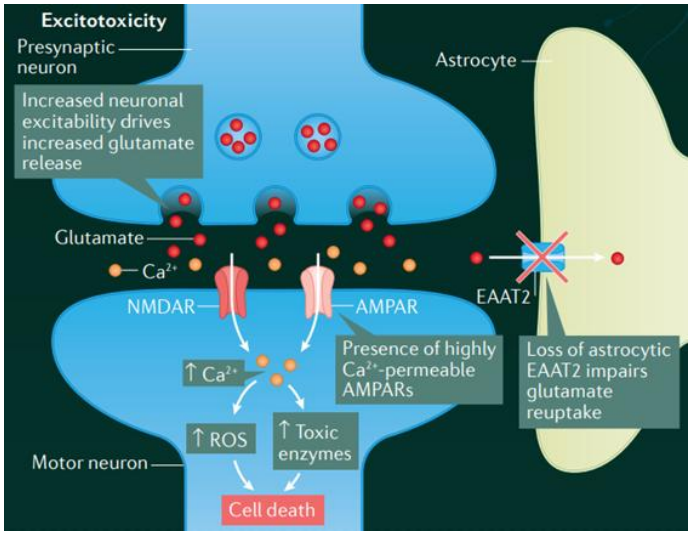

Figure 4. Excitotoxic mechanism developed in ALS neurons[28].

Excitotoxicity is the pathological process by which nerve cells are damaged or killed by excessive stimulation by neurotransmitters, mostly glutamate. When the glutamate concentration around the synaptic cleft cannot be decreased or reaches higher level, the neuron kills itself by apoptosis. An increment of the synaptic release of glutamate could be driven by over-activity of the presynaptic neuron or decreased clearance of glutamate (see figure 5)[12].

2.3.3.2. Impaired proteostasis

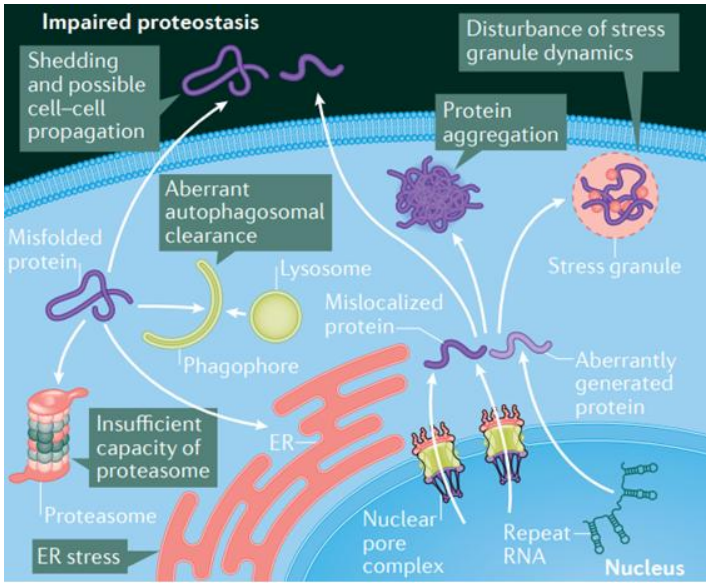

Figure 5. Miss folding proteins and aberrant protein generation mechanism developed in ALS neurons[28].

One of the principal mechanisms of ALS pathogenesis is the excessive formation of protein aggregates. These aggregations are formed by misfolded proteins and RNA

components due to problems in the RNA or DNA functioning, or a lack of activity by the chaperones. The formation of these aggregates has been linked to patients with TARDBP and FUS mutations. These accumulations of misfolded proteins active the endoplasmic reticulum (ER) stress response. ER-resident chaperons recognize the accumulation of these proteins and activate the Unfolded-Protein Response (UPR) which degraded these proteins (see figure 6). Although, prolonged UPR activation can trigger apoptotic signaling[30]. Also, the protein aggregates can cross the plasmatic membrane, spreading to other motor neurons or non-neural cells. This type of prion-like transmission has been postulated as the method of transmission inside the CNS. However, has been demonstrated that the neurodegenerative disease cannot infect other humans in the same way that other prion diseases do[31].

2.3.3.3. Disturbed RNA metabolism and RBP's

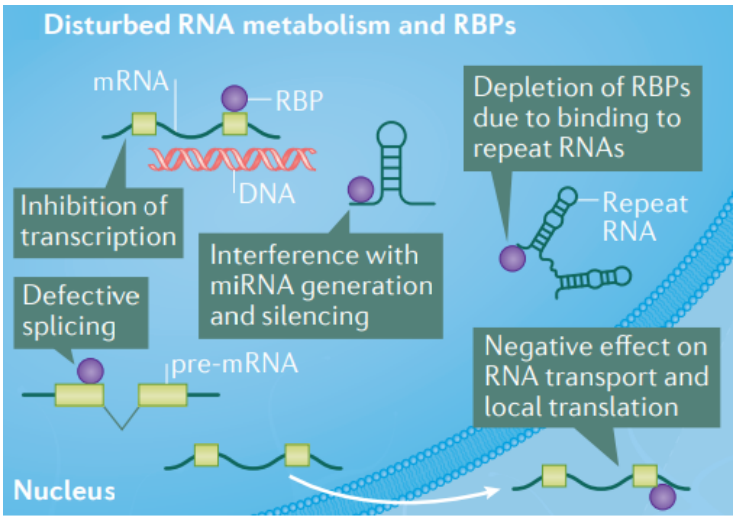

Figure 6. Defective RNA processing mechanism developed in ALS neurons[28] .

RNA processing alterations are strongly related to the protein aggregation and other pathophysiological mechanisms in ALS. The pathological processing in RNA is due to an incorrect functioning of the RNA-binding proteins (RBP). RNA-binding proteins are proteins that bind to the double or singlestranded RNA in cells and participate in forming ribonucleoprotein complexes. In ALS patients, the RBP control the inhibition of the transcription of several genes, like TARDP. Interfere with mRNA generation and silencing, produce defective splicing and have a negative effect on RNA transport and local translation (see figure 7)[32]. Also, this RNA malfunction could be produced by oxidative agents that have been shown to be increased in CSF and serum from ALS patients[2].

2.3.3.4. Cytoskeleton and axon-transport defects

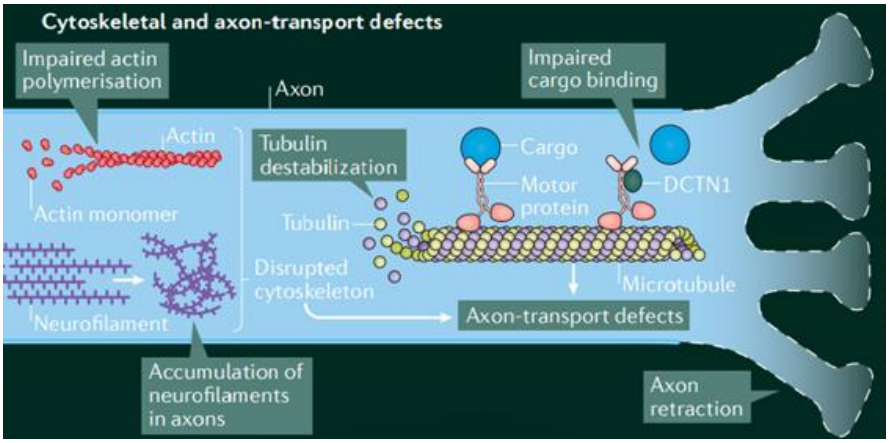

Figure 7. Axonal defects developed in ALS neurons[28].

One of the main findings supporting axonal transport deficits contributing to neurodegeneration is the axonal and cell body accumulation of organelles and other proteins observed in human neurodegenerative diseases, mainly accumulation of neurofilament and impaired proteins. However, axonal transport deficits may evolve independently from MN degeneration. It has been demonstrated that axons can survive despite long-lasting transport deficits[12]. Another strong hypothesis is the perturbation of the Dynein/dynactin complex. This complex is involved in axon maintenance, removal of damaged organelles, vesicles, misfolded and aggregated proteins from axons to the cell body. Many point mutations in the gene encoding for dynactin were found in sporadic and familial cases of ALS. The largest dynactin subunit (DCTN1) which mediates dynein–dynactin interaction is a critical component of the whole dynein/dynactin complex. The mutations on the genes expressing this subunit show accelerated motor neuron degeneration in humans and mice which resembled ALS (see figure 8)[33].

2.3.3.5. Mitochondrial dysfunction

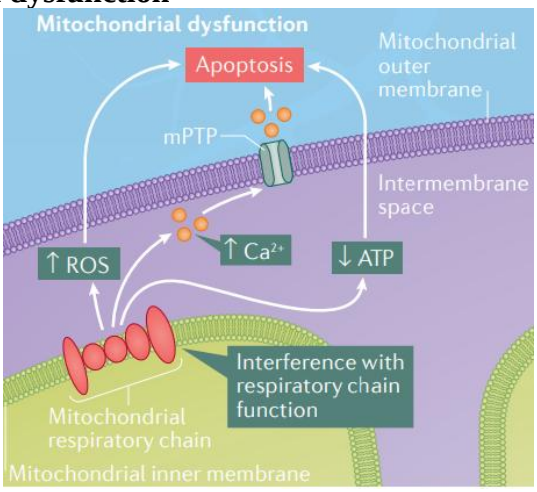

Figure 8. Mitochondrial dysfunction developed in ALS neurons[28].

Mitochondrial dysfunction has been implicated as playing a role in MTNs death in ALS. Fragmentation of mitochondria and changes in mitochondrial morphology and expression of fusion/fission proteins are well described in ALS and have pronounced effects on normal mitochondrial function. In addition, it has been proved that neural mitochondria calcium buffering capacity is altered prior to symptoms onset in the brain and spinal cord of SOD1 mice[12]. Also, Impairment of calcium buffering in MTNs could increase their susceptibility to the altered calcium homeostasis associated with glutamate-mediated excitotoxicity. Mitochondria from ALS patients have impaired Ca^{2+} homeostasis and an increased production of reactive oxygen species (ROS). Besides mitochondrial could have the serious impact, causing apoptosis of the MTNs (see figure 9)[28]. Several small molecules have been developed targeting mitochondrial dysfunction such as Cu(II)ATSM and Rasagiline being in phase I/II clinical trials.

2.3.3.6. Neuroinflamation and astrogliosis

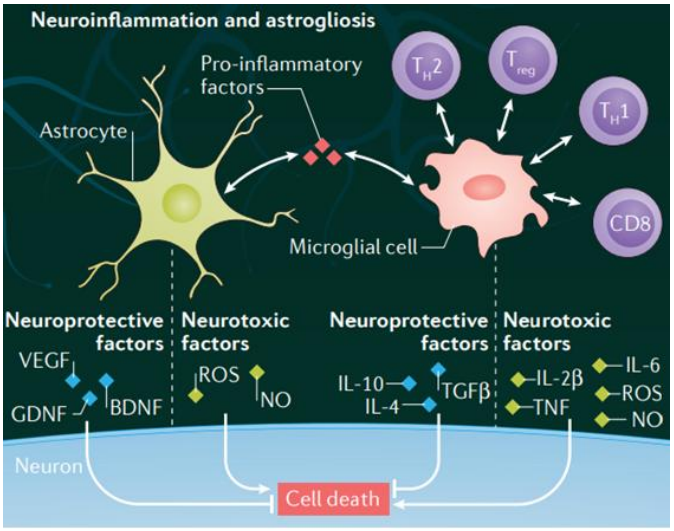

Figure 9. Neuroinflamation processes developed in ALS neurons[28].

Neuroinflammation is a common pathological event of neurodegenerative disorders. In ALS, MTNs damage leads to the activation of microglia, astrocytes and the complement system, further contributing to neurodegeneration[34]. It is accepted that neighboring glial cells have a crucial role in the MN degeneration occurring in ALS, modulating ALS progression (see figure 10)[28]. Astrocytes also play a key role in secreting inflammatory mediators, such as prostaglandin E2, leukotriene B4, and nitric oxide both at basal (non-activated) and activated conditions. Several small molecules and antibodies are being researched in order to improve ALS pathogenesis. The drug NP001 and the antibody Tocilizumab are actually in phase II and a possible treatment for ALS.

2.3.4. Rodent models

For the past twenty years the SOD1 mouse model, mainly the SOD1G93A mouse model, has been used to characterize the basic biology of ALS as well as to explore specific benefits of potential therapies with questionable success. These mice overexpress human SOD1 under control of the human SOD1 promoter and regulatory elements, at similar (SOD1G85R) or increased levels (SOD1G93A) (see figure 11)[35].

Mutation carrier	Promotor	Disease onset (months)	Survival (months)	Paralysis	Aggregation	Motor neuron loss
G93A	Human SOD1	3	4	Yes		Yes
G37R	Human SOD1	3.5-6	7	Yes	SOD1 accumulation in axons	Yes
G85R	Human SOD1	8	8.5	Yes	astrocyte SOD1 inclusions	40% MN loss
G86R	Mouse sodl	3-4 (line M1)	4	Yes	NA	Yes

Table 2. Most commonly used SOD1 mice to study ALS[35].

A major discrepancy between the SOD1 mouse model and patients is that in the majority of ALS patients appears to involve aberrant RNA metabolism. Also some patients present inclusions ubiquitinated, phosphorylated and comprised cleaved C-terminal TDP-43 fragments. In order to solve these trammels a TDP-43 mouse model was required. Thus, TDP-43 transgenic mice were generated that overexpress either mutant or wild type TDP-43 expressed under control of the prion promotor (PrP)[35].

Related to other mutation producing ALS, some C9ORF72 knockout or transgenic mouse models have been generated. Thus far, they have mainly contributed to unraveling the physiological function of C9ORF72 rather than to reproduce ALS symptomatology[36].

2.4. Diagnosis

Due to these difficulties already mentioned, ALS diagnosis remains complex procedure and only can be accomplished correctly when the later stages of the disease manifest. It also depends on the exclusion of other diseases with similar clinic expression through electrophysiological studies, clinical examination and the natural history of the patient. It is thought that approximately 22% of ALS deaths happen before a proper diagnosis[37]. To improve the diagnosis, the ALS research community proposed the El Escorial ALS diagnosis criteria, a group of characters and evidence that establish a guide for physicians to diagnose ALS cases correctly[38]. This criterion is under revision constantly, the last being the revision of Awaji[39]. Besides, there is an absence of specific

disease markers that could be used to get an early diagnose, due to ALS intra and inter variability between patients[12].

2.5. Current treatments

Currently, there is no cure or drugs available for ALS and only 3 approved drugs to treat ALS: Riluzole, Edaravone and Nuedexta^{TM} being Riluzole the only one that increases life expectancy between 2 and 3 months[40]. Edevarone and Nuedexta^{TM} only are available to treat a specific spectrum of the ALS patients (see figure 12). This lack of effective drugs causes the treatment guides to focus only on symptomatic treatment and physical therapy, trying to reduce a series of symptoms that difficult the everyday life of the patients.

Nowadays, and due to the lack of biomarkers, the rate of ALS progression can be measured using an outcome measure called the "ALS Functional Rating Scale Revised (ALSFRS-R)", a 12-item instrument administered as a clinical interview or self-reported questionnaire[41] it is the most commonly used outcome measure in clinical trials and is used by doctors to track disease progression[42].

DRUG NAME ▾	COMPANY	TYPE	MECHANISM TYPE	STATUS FOR ALS IN THE U.S.
Edaravone	Mitsubishi Tanabe Pharma Corporation	Small Molecule	Anitoxidant	FDA Approved
Nuedexta	Avanir Pharmaceuticals	Small Molecule	Sigma-1 receptor (S1R) and NMDA receptor agonist	FDA Approved
Riluzole (Rilutek)	Covis Pharma	Small molecule	Glutamate Excitotoxicity Blocker	FDA Approved

Table 3. Table with the current drug approved by the FDA to treat ALS[2].

2.5.1. Edaravone

Edaravone has been a recent success in the ALS new drug approval history. It was approved by the Food and Drug American administration on 5th May 2017[43]. The antioxidant mechanism of Edaravone seems to increase prostacyclin production, decrease lipoxygenase metabolism of arachidonic acid by trapping hydroxyl radicals, and inhibit alloxan-induced lipid peroxidation and quench active oxygen[54]. Edaravone show difficulties during its trials, because a first phase III clinical trial was performed didn't show a significant variance between the placebo and Edaravone treatment group[44]. It was not until a post-hoc clinical trial based on the first one but with more restrictive patient condition (patients with a shorter disease duration and a larger vital capacity) for the enrolment showed statistical significant improvements.

2.5.2. Riluzole

Riluzole is the oldest drug approved for the ALS treatment. Its use has now been well-established as consistently extending lifespan. At low concentrations (<1-10μM), riluzole suppress the persistent Na^+ current and enhance the calcium-dependent K^+ canals. In higher concentration (20–100 μM), the inhibition affects the voltage-dependent A-type or delayed rectifier K^+ currents, which can modulate neural excitability, and the voltage-dependent Ca^{2+} channels[45].

2.5.3. Nuedexta

Pseudobulbar affect (PBA), or emotional incontinence, is a type of emotional disturbance characterized by uncontrollable episodes of crying and/or laughing, or other emotional displays, produced most of the time in inappropriate contexts. PBA affects 25% of ALS patients, mostly when the upper MTNs are affected[46]. In 2013, the European Medical Agency approved Nuedexta™. This drug contains two active compounds, the Dextromethorphan and Quinidine sulfate. Even though, the exact way dextromethorphan works in PBA is unclear, it is thought that it may bind to NMDA receptors (impairing the glutamate action) as well as on 5-HT1A receptors. The role of the quinidine is to prevent Dextromethorphan from being broken down early in the body.

2.6. Objectives

This report attempts to explain the ALS disease from its hypothetical causes to the actual treatments and new therapeutic strategies in current clinical trials. Thus, the main scope of the report is Pharmacology, because the research about the current, future and possible future treatment of ALS and the explanation of why there is no treatment to cure or chronify the disease past two centuries since its discovery, are the main objectives to treat in this report. However, due to the overview nature of the report, other scopes appear in it, being Physiopathology and Biochemistry the more prominent ones. They are going to be discussed further when talking about the disease mechanisms. Besides, other scopes are treated in the report, but with less depth such as: toxicology or public health.

This principal objective could be extended in 4 concrete objectives:

1. Understand the pathophysiology of the disease.

2. Discuss the efficacy and safety of the current treatments.

3. Expose the trends in ALS drug discovery.

4. Display current drugs under clinical trials.

3. Methodology and methods

This report is a continuation of investigation realized in the Neurodialitics research unit, belonging to the University of Lyon, in 2016. At that time, first started by proposing a scheme in which the report is going to be based, clarifying the process and madding clear every step. Then, the research information began with the ALS patient foundations principally the American, Spanish and Catalan. These pages have easy information mostly directed to patients and familiars that do not have biology and pharmacological knowledge. Therefore, provide an excellent first source of information going through the basics of the disease, natural history, and actual treatments.

At that point, realizing the insufficient actual ALS treatment, and the poor prognosis and natural history of this disease, decided to research more indepth information regarding pathophysiological mechanism, drug discovery linked to these mechanisms and the actual clinical trials taken place. Mainly reviews, articles and some books provided that information. Most of them being free–access found in the main databases (Pubmed, Scopus, Researchgate and Medline). Besides, this information was complemented by the University of Barcelona and Lyon databases. The articles were written mostly in English, but some were found written in Spanish and French, and with that form a first raw database. Regarding the pathophysiological mechanism, the review of Xavier Navarro and Renzo Mancuso[12] two researchers from the Universitat Autònoma de Barcelona (UAB) is one of the most complete and actual reviews of this topic. This review constituted a first start and background for the report. Similarly, on drug discovery information, the review by the Deloanch et al.[48] conducted by the group of Mahmoud Kiaei was also considered a primary stand point. This group of researchers from the University of Arkansas for Medical Science specialized in neurodegenerative disease and focused in ALS with several previous works on the topic. Then, realizing that these two reviews were written in 2015, the research was focused on finding new information from 2016 and forward. Also, for the last part of the report, regarding current ALS clinical trials, the webpage clinical.gov was proven a useful resource. This webpage is provided by the U.S. National Library of Medicine and publicly funded. Collecting all the information regarding all the clinical trials from phase I to phase III that are currently running or have been finished.

Developing this report, new information comes out almost every day. Thanks to my tutor, once the research was based on Barcelona, could reorientate the report and stay on track while all these information was coming. This continuous flow of new information made the realization of this report exciting and interesting. Also, it made sure that a substantial methodological treat of the information was applied to make a proper final report.

4. Results

4.1. Drugs in Phase I/II

Nearly 50 clinical trials for disease-modifying treatments have been undertaken in the past half-century. The FDA approval rate of investigational compounds from the time they first entered clinical trials is about 16% for trials initiated by pharmaceutical companies.23 RCTs (of 18 different drugs) completed in the past decade 22–47 were progressively better organized than previous trials, but had recurrent and similar issues. Six different hypothetical, pathogenic targets underlie the rationale for these RCTs, which were undertaken on the basis of the hypothetical therapeutic targets and evidence derived from superoxide dismutase (SOD1) transgenic mouse models, previous studies in human beings, or both[64]. Since then, a smaller number of pathogenic targets have been tried. Taking as a representation the drugs that nowadays are in phase I and II (see figure 13), we observe that they have been reduced to the protection of the Mithocondrial, Neural hyperexcitability and Immunomodulation

Drug name	Company	Type	Mechanism Type	Status for ALS
AMX0035	Amylyx Pharmaceuticals	Small Molecule	Immunomodulation	Phase I
Cu(II) AT SM	ProCypra Therapeutics	Small Molecule	Mitochondrial protectant	Phase I
Ibudilast	MediciNova	Small Molecule	Immunomodulation	Phase II
Mexitil	Boehringer Ingelheim	Small Molecule	Neural hyperexcitability	Phase II
NP001	Teva Pharmaceuticals	Small Molecule	Immunomodulation	Phase II
Rasagiline	Teva Pharmaceuticals	Small Molecule	Mitochondrial protectant	Phase II
Retigabine	GlaxoSmithKline	Small Molecule	Neural hyperexcitability	Phase II
TW001	Treeway	Small Molecule	Antiioxidant	Phase I
Tocilizumab	Genentech	Protein Biologic	Immunomodulation	Phase II

Table 4. Table with the current drug in phase I/II clinical trials[2].

4.1.1.Cu(II)ATSM and Rasagiline

As mitochondrial protector we can found Cu(II) ATSM and Rasagiline. Cu(II)ATSM is a bisthiosemicarbazone which selectively delivers copper to cells with impaired mitochondrial electron transport chains, a characteristic of ALS and many other neurodegenerative diseases. In both SOD1 mouse models of ALS and in ALS patients, scientists have found alterations in copper homeostasis, providing a rationale for testing this candidate in ALS. In a series of publications in mouse models of ALS, Cu(II)ATSM improved motor function and prolonged survival[7]. Rasagiline is an inhibitor of type B monoamine oxidase (MAO-B). It is thought to help protect neurons by bolstering mitochondria by suppressing Ca(2+) efflux through the mitochondrial permeability transition pore (Wu et al., 2015). A phase II clinical trial is ongoing.

4.1.2.Mexiletine and Retigabine

As neural excitability protectors we can found Mexiletine and Ratigabine. Mexiletine is a sodium channel blocker that may reduce neuronal hyperexcitability by reducing sodium ion influx (the persistent sodium current). A phase II study is currently recruiting. The study aims to determine whether mexiletine may slow progression of ALS, and/or reduce muscle cramps and twitching in the disease[7]. Retigabine is a Kv7 potassium channel activator that may reduce neuronal hyperexcitability by helping neurons return to the resting state upon firing. The phase 2 clinical trial of retigabine in ALS is ongoing.

4.1.3.Immunomodulators

As said before, neuroinflammation is a common pathological event of neurodegenerative disorders. It is accepted the activation of microglia, astrocytes and the complement system, play a key role contributing to neurodegeneration[34]. Immunomodulators are one of the principal focus on ALS research and we can found several drug in clinical trials.

Ibudilast is an orally bioavailable, centrally acting phosphodiesterase inhibitor that attenuates glial cell activation, at least in part, by reducing the effect of proinflammatory factors. MN-166 may minimize the production of pro-inflammatory cytokines[7].

AMX0035 a proprietary combination of two drugs, TUDCA and sodium phenylbutyrate, that acts by reducing cell death and neuronal inflammation in response to oxidative insult. The phase II clinical trial launched in June 2017[7].

NP001 is a small molecule regulator of macrophage activation. It is thought to restore macrophages to their neuroprotective state and reduce inflammation seen in ALS. Treatment of ALS mouse models with NP001 extended survival. In the Phase IIa safety study, administration of a high dose of NP001 (2mg/kg) over a 6 month period was associated with a slowing of disease progression in 27% of patients, approximately 2.5

times greater than the percentage in patients on placebo (10%). A Phase II study was completed in 2012. A confirmatory Phase II study is ongoing. Neuraltus is establishing a managed access program for ALS patients in Europe[7].

Tocilizumab may reduce neuroinflammation by lowering levels of the pro-inflammatory cytokine IL-6. The drug, which is a monoclonal antibody, competitively inhibits the binding of interleukin-6 (IL-6) to its receptor. A phase II clinical trial is ongoing[7].

4.2. Current drugs in Phase III clinical trials.

The lack of new drugs approved during the past 2 decades has not intimidates researcher that keep trying to find a new drug that could improve ALS actual pathogenesis. Even though, only few drugs from the preclinical trials arrive at a phase III, in this report is discussed the possible new drugs being currently in phase III clinical trial (see figure 15).

DRUG NAME ▾	COMPANY	TYPE	MECHANISM TYPE	STATUS FOR ALS IN THE U.S.
Arimoclomol (Orph-001)	Orphazyme	Small molecule	Proteostasis, Protein misfolding modulator	Phase II/III
Masitinib	AB Science	Small Molecule	Immunomodulation	Phase III
NurOwn	BrainStorm Cell Therapeutics	Stem Cell Therapy	Neuroprotection	Phase III

Table 5. Table with the current drug in phase III clinical trials[2]

4.2.1. Arimoclomol

Arimoclomol acts stimulating the cells' heat shock response, the protective system involved in maintaining proper protein folder. However, it acts as a "*smart drug*" to co-induce the heat shock response only in cells already under stress[49]. This ability to focus the damaged cells, suggest that this approach may have therapeutic value in neurodegenerative disorders, in which neurons are under prolonged stress. Arimoclomol have been tried in a phase II randomized, double-blind, placebo-controlled trial that included 36 patients with rapidly progressive ALS due to mutations in the SOD1 gene. This clinical trial has been the first one including only certain types of ALS patients[50]. In previous trials, Arimoclomol has proved to be beneficial in the SOD1 ALS patients, even when administered after symptom onset. In all phase I and phase II clinical trials to date, arimoclomol has been found to be safe and well tolerated[51].

4.2.2. Masitinib

Masitinib is a tyrosine kinase (TK) enzyme inhibitor developed by the French company AB Science[52]. The activation of TK is central to the ability of immunoglobulin E to transmit downstream signaling events required for the regulation of mast cell activation[53]. An inhibition of TK could inactive the mast cells and macrophages in the nervous system and be able of controlling microgliosis, neuroinflamation, and the emergence/expansion of aberrant glia cells. In preclinical studies, Masitinib significantly prolonged survival when delivered after paralysis onset in SOD1 rats[54].

These facts encouraged the Masitinib clinical trials, starting a phase III clinical trial in December 2015. The study was a blinded, placebo-controlled, 3-treatment arms (randomization 1:1:1), testing Masitinib 4.5mg/kg/day + Riluzole, Masitinib 3mg/kg/day + Riluzole and Placebo + Riluzole. The treatment duration was 48 weeks, and a total of 392 patients were enrolled in the study, a 2/3 in Europe and 1/3 outside Europe (Argentina and Canada)[55].

Masitinib orally administered at 4.5 mg/kg/day as an add-on to Riluzole demonstrated a significant therapeutic benefit with acceptable safety in ALS patients with a baseline ALSFRS-R progression rate of <1.1 points / month (see figure 16). Also, Significant benefit on key secondary endpoint the PFS, which is defined as the earliest event between ALSFRS-R deterioration of more than 9 points or death[56].

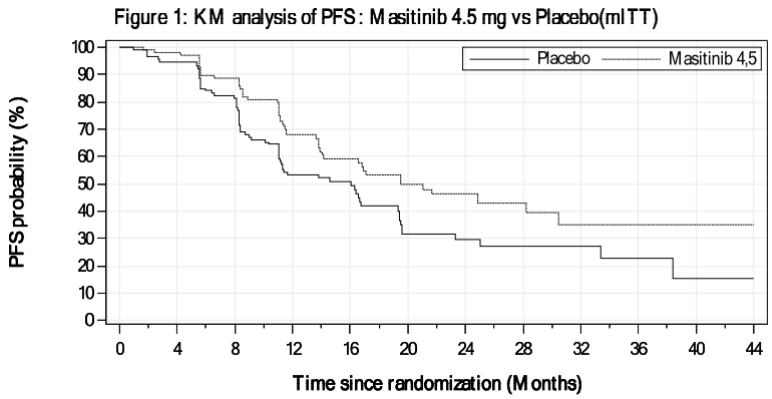

Figure 10. Graphic showing the PFS comparison between Masitinib and Placebo patient group [56].

However, these results were insufficient to The Committee for Medicinal Products for Human Use (CHMP) of the European Medicine Agency (EMA), adopting a negative opinion for the marketing authorization of Masitinib in the treatment of adult patients with Amyotrophic Lateral Sclerosis. In the near future, AB Science will provide additional data through a reexamination procedure[57]

4.6.3. NurOwn

MSC-NTF cells are Mesenchymal Stromal Cells (MSC) induced to express high levels of neurotrophic factors (NTFs) using a culture-medium based approach. These MSC-NTF cells were found to alter the miRNA proportions in the human DOD1 transgenic mouse model of ALS. To elucidate the potential of MSC-NTF in ALS treatment a preclinical study was made to determine whether the miRNA profile could provide a tool for MSC-NTF cell characterization and to distinguish them from the matched MSC from which they are derived. Principal component analysis revealed two distinct clusters based on cell type (MSC and MSC-NTFs). Nineteen miRNAs were found to be upregulated and 22 miRNAs were downregulated in MSC-NTF cells relative to the MSC cells of origin. In an analysis of the mRNA targets, three mRNA targets of hsa-miR-132-3p (HN-1, RASA1 and KLH-L11) were found to be significantly downregulated[58].

After this success, a phase II clinical trial has been finished recently with success[59]. The study evaluate the safety, tolerability and therapeutic effects of transplantation of escalating doses of autologous cultured mesenchymal bone marrow stoma cells secreting neurotrophic factors (MSC-NTF), in patients with amyotrophic lateral sclerosis (ALS). The study was conducted in Israel at Hadassah Medical Center and three medical centers in the United States at Mayo clinic, MGH (Massachusetts General Hospital) and UMass (University of Massachusetts). The trial was 48 patient randomized, double-blind with placebo-controlled groups. The trial revealed a statistically significant improvement in the rate of ALSFRS-R progression (see figure 17) for those with complete follow-up. The results suggest that IT and IM administration of MSC-NTF cells in patients with ALS is safe and provide indications of possible clinical benefits, to be confirmed in upcoming clinical trials[60].

Mesure	Baseline	Change per month			% Improvement	
		Run-in	3 Months	6 Months	3 Months	6 Months
ALSFRS-R						
Phase 1 / 2 (n=6)	24.8	-1,56	-0,98	-0.28	37%	82%
Phase 2a (n=14)	39,9	-1,41	-0,78	-0,60	45%	57%
Pooled per protocol (n=15)		-1,2	-0,6			50%

Table 6. Results on phase I and II clinical trial administration of MSC-NTF[60].

5 . Discussion

Amyotrophic lateral sclerosis is a dreadful neurodegenerative disease affecting the life of the patients and their families even previous the diagnosis. The loss of lower MTNs affecting limbs and upper MTNs producing PBA, produce a disabling disease that difficult the everyday life to patients and caregivers. ALS progress tireless since its onset, auguring a fatal final for which still no cure yet.

ALS is spread all across the globe without difference of location, gender and socioeconomic status. Except for particular cases in history such as the indigenous tribes of Guam Island and the Gulf War US veterans (which are being studied carefully).

The linkage of some ALS cases to genetic mutations[13] supposes a great improvement in ALS research, especially the C9orf72 intron hexanucleotide repetition discovering that allowed to explain almost 70% of fALS cases and 10% of sALS cases[16].Even though, the most predominant genes (which have been explained in this report) have a clear relation with ALS, their mechanism still not fully understood. For example, the fact that a loss function mechanism could not be involved in SOD1 mutation is not clear. In addition, more data is needed to elucidate if C9orf72 mutations affect RNA processes as well as creates RNA misfolded aggregation, and to fully understand the prion-like mechanism of transmission in patients with TDP-43 and FUS mutations[12]. Besides, improvements in nucleotide sequencing and drop in sequencing cost will permit large-scale exome/genome sequencing, helping to generate new candidates that could improve the ALS genetic understanding[61].

An epigenetic subjacent cause has been proposed to explain these genetic discrepancies and involvements in ALS onset. Epigenetic mechanisms are altered in both explained and unexplained cases; however, it remains arguable whether these changes are a cause or consequence of the disease. For example, DNA methylation and altered histone modifications around expanded hexanucleotide repeats in C9orf72 are most likely a consequence, as the expansion itself is a cause of the disease. On the other hand, the role of altered miRNA expression is less apparent; it may be a cause of the disease, leading to altered gene expression, or it may be a consequence of some yet unknown cause[23].

Several pathophysiological mechanisms still not fully understood. Perturbation of axonal transport, especially in SOD1, may increase inflammatory and excitotoxic mediators, altering mitochondria or damaging transport cargos, despite the misfolded tubulin and protein accumulation. Showing that it is not clear if one mechanism triggers the others or a subjacent unknown cause triggers one or several of these mechanisms. Also, with the same mechanism linked to several patients with different subjacent cases difficult to establish a coherent linear mechanism. However, Mitochondria dysfunction, the accumulation of oxidative species and involvement of glial cells and other immune cells seems to play a principal role in ALS together with excitatory mechanism. So far,

thus are the mechanisms that have responded to treatment targeting them, despite the lack of clear data[12].

One of the major problems associated with the development of effective ALS treatments is the inadequacy of the animal models. Indeed, despite the wide use of mouse models for ALS in biomedical research, they would mimic only partial aspects of human pathology, often leading to a consistent lack of concordance between the preclinical and clinical studies. Therefore, a better understanding of the similarities and differences between the animal and human ALS pathophysiology is of fundamental importance to the rational identification of therapeutic interventions capable of stopping or at least effectively modifying the course of ALS[62]. Besides, the utilization of just a single-disease model for research is not without risks by combining the use of several model systems and capitalized on the strength of each organism to screen large chemical libraries in the search for potential therapeutic drugs for ALS[63]. More epigenetic understanding also could bring the possibility of the epigenetic mouse model that could bring a new scope on the limited library of models that researcher dispose of in the actuality. In addition, a research program with the objective of reprogramming ALS patients' fibroblasts into induced pluripotent stem cells (iPSC) appears as a promising opportunity to develop sALS models. These cells have been differentiated into ALS-relevant cell subtypes including MN and atrocities, among others[12].This type of cells are being also tested as a possible treatment without reprogramming them (see part 4.2.3. Nurown).

Possibly the main difficult in the advance of finding a cure for ALS it is behind the clinical trials. Even though, the latest success of Edaravone, approved last year to treat a specific type of ALS patients (see part 4.2.1), more than 50 randomized controlled trials (RTC) had been performed in the past half-century with negative results[64]. The research community has proposed several answers trying to explain these negatives results. First of all, the heterogeneous, pathogenesis of ALS remains mostly unknown. This fact merged with the rapidly fatal and so far intractable nature of the disease, produces the research community tends to welcome new ideas or hypotheses[64]. Secondly, therapeutic approaches in animal models are usually applied prior to clinical onset of the disease. Despite this strategy might offer better results in preclinical studies and could be relevant for fALS cases, it cannot be replicated in human sALS[12]. Also there is the possibility that the data obtained from the Phase II RTC is being potentially misleading since a lot of these compounds accomplish to succeed in this RTC after failing in phase III RTC.

These enrolment problems could be solved by restricting the conditions for the patients. The Edaravone clinical trial reflects a growing trend in ALS studies to try to divide study populations into subgroups that might be more likely to benefit from compounds clinical sub grouping. Different therapeutic compounds may have efficacy in other ALS subgroups besides those chosen in this development programmed. However, identifying those specific subgroups remains a challenge which will perhaps be addressed through emerging works in 'precision medicine[43]

Regarding new developments, the recent focus on mitochondrial dysfunction, neural excitatory and immunomodulation could be a temporary trend because no recent key development in ALS has been reached lately. Obviously, these clinical trials are started based on new pathophysiological information, but that do not mean a possible success could be expected, if we take the past 50 years of research into consideration. However new approaches to clinical trials, as said earlier, and drug development could lead to new approved drugs. Examples of that are Ariclomol with its "smart drug" approach or NurOwn starting a steam cells treatment. These new approaches could signify new hopes for ALS development, still to be confirmed in their respective phase III clinical trials.

Lastly, the most pressing need in the field is for biomarkers that will be most relevant to therapy development. Discovery and early development of such biomarkers will appropriately utilize samples housed within established repositories. However, the development and clinical validation of biological fluid-based pharmacodynamic and disease progression biomarkers will require prospective as well as standardized protocols for biological specimen collection, processing, and storage[65].

6. Conclusions

1.ALS is a complex disease with the genetic, epigenetic and several pathophysiological mechanism involvements that difficult a complete understanding about it.

2.The only treatment available that improves the survival expentacy is Riluzole. Neudexta and Edaravone only act in a small range of ALS patients, they lack an effective treatment at the moment.

3.Mitochondrial dysfunction, Neural excitatory and immunomodulation are the predominant therapies under investigation right now. However, further development could be needed to bring those hypotheses into actual treatments.

4.New drugs with new innovative approaches, far from current mechanism focus, could be success in the next years, establishing the actual trend of more biological treatments in front of more chemical ones.

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