Diagnosis, Pathogenesis and Therapeutic Targets in Amyotrophic Lateral Sclerosis

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Abstract: Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease of the motor system. The diagnosis is clinical, but additional investigations such as electromyography, transcranial magnetic stimulation and neuroimaging have demonstrated their usefulness in supporting diagnosis. Exhaustive research for the identification of molecular markers in the cerebrospinal fluid and plasma of ALS patients have been made; however, at present, there are no validated biomarkers for the disease.

Between 5 to 10% of the ALS cases have a positive familial history, up to now eleven genes have been identified as associated with the disease. The most studied gene encodes for copper, zinc superoxide dismutase enzyme. The identified abnormal genes potentially allow the generation of experimental cell and animal models to study the mechanisms of the disease and to test potential therapeutic compounds.

The pathological characteristics of ALS include protein aggregation, proteasome inhibition, impaired axonal transport, mitochondria damage and apoptosis, oxidative stress, glutamate induced excitotoxicity, neuroinflammation and transcriptional dysfunction. Many compounds targeted to one or more of these mechanisms have been tested in multiple clinical trials. Nonetheless, nowadays only one drug, riluzole, has demonstrated a positive effect in the disease progression, but a number of recent compounds are promising in ALS therapy.

Keywords: Amyotrophic lateral sclerosis, biomarkers, clinical trials, diagnosis, experimental models, pathogenesis, therapeutic compounds.

1. INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease of the motor system. In this disease there is a rapid degeneration of the cortical, brainstem and spinal motor neurons, with a heterogeneous clinical presentation and progression rate. ALS patients suffer from muscle weakness with a very high risk of death within the first 5 years of disease course. Death is mainly caused by respiratory complications. Overt frontotemporal dementia occurs in about 5-10% of patients, but when tested with appropriate tools 20-40% of them show impaired frontal executive function [1].

In general, it affects people late in life, with a mean age of disease onset between 55-60 years, but in 5% of patients the disease starts before the age of 30 years. The incidence is about 2-3 and the prevalence 7-8 per 100,000 in Europe [2]. Between 5 and 10% of the cases are familial (FALS) and several mutated genes have been identified as the cause of disease [3]. However, most cases (90-95%) are sporadic (SALS) without family history, only in a few cases mutated genes are identified [3]. Overall, genetic association and genome-wide association studies in patients with sporadic ALS have not established a simple genetic model for ALS.

ALS has several pathological features that include protein aggregation due to misfolding, impaired axonal transport, mitochondria damage and apoptosis, oxidative stress, glutamate induced excitotoxicity, neuroinflammation and transcriptional dysfunction among others. At present only Riluzole has shown to increase survival or the time to tracheotomy in ALS [4], in addition to its antiglutamatergic activity this drug has other less well known neuroprotective properties.

In this review, we will present the current methods used in diagnosis and monitoring of disease progression of ALS. A description of the genetics, pathological features of the disease and past as well as ongoing and future clinical trials will also be presented.

2. DIAGNOSIS AND MONITORING OF DISEASE PROGRESSION

The diagnosis of ALS is clinical as supported by the detection of upper motor neuron (UMN) and lower motor neuron (LMN) signs in the bulbar and limb territories, as defined in the revised El Escorial criteria [5]. The diagnosis should be sustained by disease progression. Motor neuron disease is a broader denomination to encompass patients without UMN signs (progressive muscular atrophy) or without LMN signs (primary lateral sclerosis), which are less frequent than the typical ALS. Manual muscle testing, muscle strength evaluation by dynamometry and functional scales are important to measure disease progression. The functional scale ALS-FRS is a sensitive and reliable score largely used in clinical trials [6], this scale is predictive of survival [7].

The most important investigation to rule-out other mimicking conditions and to confirm the diagnosis is the electromyography (EMG) [8]. A substantial loss of LMNs occurs before weakness and atrophy is clinically observed. The EMG is sensitive to detect LMN loss and thus it can identify widespread loss of motor units, the essential hallmark of this disorder, before it is clinically apparent. EMG criteria have been proposed to confirm clinical diagnosis. They are based on the presence of normal nerve conduction studies, diffuse loss of motor units and the observation of spontaneous activity (fibrillation and sharp-waves) in affected muscles. More recently, a new set of criteria has been proposed in which the importance of fasciculation potentials was underlined [8]. Furthermore, a number of neurophysiological methods were described to measure disease progression [9], in particular motor response amplitude or area, motor unit number estimation (MUNE) and the neurophysiological index (NI). A number of different and complex techniques were developed to calculate MUNE. NI is derived from a simple formula that includes the amplitude and the
molecules in the biological fluids of ALS patients [23-25]. For become surrogate markers, as meaning a measurement that Medical Research Council [23]. Some of those biomarkers may biomarker is "an objective measurement that acts as an indicator of and to measure objectively the effect of potential drugs. A therefore the identification of a molecular biomarker would be

The main role of neuroimaging in ALS is to exclude other diagnosis but a number of recent advances are promising regarding the identification of markers of UMN lesion. Magnetic resonance imaging (MRI) is able to recognize changes of the cortico-spinal tract in some patients [14]. Magnetic resonance spectroscopy can measure N-acetyl compounds (N-acetylaspartate and N-acetylaspartylglutamate) in the cortex, which are markers of neuronal integrity. This is usually expressed as ratios to creatine or choline [15], and these changes are correlated with the clinical stage (Wang et al, 2006). This technique can reveal abnormalities in the brainstem of ALS patients [16]. Diffusion tensor imaging detects the decreased fractional anisotropy of the corticospinal tract, which is an early change in ALS; the imaging abnormality is correlated with clinical UMN burden [17]. A novel method based on this principle – tract-based spatial statistics – permits to measure the corticospinal tract structure (tractography). This is a sensitive approach that can detect changes before disease onset [18]. The atrophy of the motor cortex and the white matter volume can be quantified using magnetic resonance voxel-based morphometry. A more severe involvement of the fronto-temporal area was observed in patients with associated dementia [19]. Functional imaging, in particular with functional MRI and PET have been investigated in ALS [20, 21], in general showing cortical hyperactivation during motor tasks that represents plastic adaptation following UMN loss. Although, these various methods are very exciting they did not prove their utility in measuring UMN degeneration over time.

Conclusive diagnosis of ALS currently takes place approximately one year after the beginning of symptoms [22]. Therefore, the identification of a molecular biomarker would be useful for the earlier diagnosis of disease. Furthermore, such a biomarker would be extremely valuable in clinical trials to decrease the number of subjects included, to shorten the duration of the trial and to measure objectively the effect of potential drugs. A biomarker is "an objective measurement that acts as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" as defined by the UK Medical Research Council [23]. Some of those biomarkers may become surrogate markers, as meaning a measurement that indirectly indicates the effect of treatment on disease state.

Many groups have dedicated efforts to identify deregulated molecules in the biological fluids of ALS patients [23-25]. For example, among others, increased levels of glutamate [26], neurofilaments proteins [27], cystatin C [28, 29], modified transthyretin, peptide 7B2 [28], VGF and a 6.7 kDa fragment [29], or mediators of inflammation [30] have been identified in the cerebrospinal fluid (CSF) of ALS patients. More recently, decreased levels of vascular endothelial growth factor (VEGF) have been described in hypoxic patients [31] as well as decreased levels of erythropoietin in patients [32]. CSF is a promising biological fluid for the identification of potential biomarkers since it is in close contact with the brain and spinal cord motor neurons and glial cells. However, the collection of CSF is an invasive procedure with several ethical implications and it would be easier to use the plasma or the serum for that purpose. Indeed, there are a higher number of studies performed with blood and variations have been found in several proteins, but they were not validated.

There are several drawbacks in using the plasma or the serum due to their complex dynamic range of proteins and the high abundance of certain proteins, such as albumin or immunoglobulins, which dilute the potentially "interesting" molecules turning their detection and quantification difficult. Several techniques have been developed to overcome these problems such as the use of affinity resins to deplete the plasma from more abundant proteins [33] or specific mass spectrometry techniques [34]. Among many others, 4-hydroxy-2,3-nonenal [35], alpha2-macroglobulin and its proteolytic products [36], angiogenin [37] and insulin-like-growth factor [38], as well as variation of complement factors [39] have been tested in the blood. A predictive panel of biomarkers instead of only one molecule would probably be more useful for an accurate and reliable diagnosis. A large amount of work still has to be done to characterize sensitive and specific biomarkers of ALS with larger cohorts of patients and with standardized methodologies of collecting and storing the biological fluids [40, 41]. Currently, in the USA there is an initiative undergoing recruitment for a clinical trial for the validation of biomarkers in ALS (www.clinicaltrials.gov; NCT00677768).

For other neurodegenerative diseases there is also a large effort for the identification of biomarkers. In Alzheimer’s disease, Tau and Aβ are readily measured in the CSF by ELISA, and they are the most extensively studied biomarkers with the potential to become surrogate markers for the response of patients to novel therapies [42]. In Parkinson’s disease, alpha-synuclein in the CSF constitutes the most studied biomarker [43].

Analysis of the genome-wide data is also promising for the identification of biomarkers. Recent results revealed genome-wide significance for one SNP that is located at 19p13.3 and maps to a haplotype block within the boundaries of UNC13A, which regulates the release of neurotransmitters such as glutamate at neuromuscular synapses, and two additional SNPs located at chromosome 9p21.2, in a linkage region for familial ALS with frontotemporal dementia [44].

3. GENETICS OF ALS

Between 5 and 10% of the ALS cases are familial and they are predominantly autosomal dominant inherited. At this moment, mutations in eleven genes have been associated with the forms of familial ALS, ALS1 to ALS11. ALS 1 is associated with mutations in the gene that encodes the enzyme Cu/Zn superoxide dismutase or superoxide dismutase 1 (SOD1), which has been found in 1993 and has been widely studied [45]. It represents 1-2% of total ALS cases. SOD1 is a cytosolic homodimer of 153 amino acids. It catalyzes the dismutation of superoxide anion to hydrogen peroxide. More than 140 mutations have been identified (http://alsod.iop.kcl.ac.uk/index.aspx), some localized in the active site of the enzyme, but many of them do not affect the enzyme activity [46].

Mutations in the gene encoding alsin result in the recessive juvenile-onset form ALS2. Alsin is a 184 kDa protein that contains three putative guanine nucleotide exchange factor domain [47]. Alsine knock-out mouse predisposes neurons to oxidative stress [48], which exhibit age dependent neurologic deficits and altered endosome trafficking [49].

Mutations in the gene encoding senataxin result in another juvenile-onset form ALS4. Senataxin is a 303k Da DNA/RNA helicase domain that affects RNA processing [50].

Recently, mutations in the gene encoding the protein fused in sarcoma or translocation in liposarcoma (FUS/TLS) that result in ALS6 have been described. FUS/TLS is a DNA/RNA-binding protein that contains 526 amino acids and is localized in the nucleus
Mutations in the gene encoding the vesicle associated membrane protein (VAPB) cause ALS8. This protein is a 27.2 kDa homodimer that is bound to the endoplasmic reticulum (ER) membrane protein (VAPB) and regulates intracellular vesicular trafficking [55].

Mutations in the ANG gene that encodes angiogenin are associated with ALS9. Angiogenin is a 14.1 kDa protein with the function of neovascularization \textit{in vivo} [56].

Mutations in the TARDBP gene that encodes the trans-activating regulating-DNA binding protein (TDP-43) cause ALS10. TDP-43 is a 43 kDa nuclear protein [57, 58]. TDP-43 is a DNA and RNA-binding protein that regulates transcription and RNA splicing.

Mutations in the FIG4 gene that encodes phosphoinositide 5-phosphatase are associated with ALS11. This enzyme has a function in cellular signalling [59].

In addition to Mendelian inheritance mutations in other genes have been associated with increased risk of sporadic ALS [60]. One example is VEGF, which is a tumour secreted protein that regulates angiogenesis and which, in the nervous system, plays several roles including neuroprotection and neuroregeneration. Haplotypes of three genetic variations in the VEGF gene increased the risk of sporadic ALS in four European populations [61]. Furthermore, a recent meta-analysis of all the association studies on VEGF in ALS analysing over 7000 patients revealed that homozygous carriers of the -2578A risk allele had a significantly increased risk for ALS [62].

4. EXPERIMENTAL MODELS IN ALS

Although representing a reduced number of ALS cases the identification of disease associated mutations was crucial for the generation of experimental models to elucidate molecular and cellular mechanisms that, though caused by different mutations, share common features of ALS. The most studied gene has been SOD1 and several \textit{in vitro} and \textit{in vivo} models have been developed based on its mutations. Primary cultures of mouse or rat motor neurons have been used, however, they have several drawbacks since dissociation methods are restricted to embryonic tissues and, when isolated, they are resistant to genetic manipulation methods [63]. More recently, motor neurons derived from human embryonic stem cells overexpressing mutant SOD1 alone [64] or in coculture with human astrocytes [65] have been shown to constitute adequate models to study ALS.

As alternative to primary cultures, mammalian cell lines overexpressing SOD1 mutants, such as, the mouse motor neuron-like NSC-34 cell line [63, 66-72], mouse neuroblastoma Neuro2a cells [73-77], human neuroblastoma SH-SY5Y cells [78, 79] have been used in numerous studies. In addition, non-neuronal mammalian cells such as, human embryonic kidney cells [80, 81], simian COS-7 fibroblasts [82, 83], and mouse NIH3T3 fibroblasts [84] have been used. The baker’s yeast, \textit{Saccharomyces cerevisiae}, has been largely used [85-90].

Organisms have also been used as experimental models. For example, the worm \textit{Caenorhabditis elegans} has been used to study mutant SOD1 protein aggregation [76, 91, 92], oxidative stress [93] and also mutant VAPB [94]. Zebrafish (\textit{Danio rerio}) embryos overexpressing human SOD1 mutants had a specific motor axonopathy, and revealed the potential of VEGF for the treatment of ALS [95]. \textit{Drosophila melanogaster} has been used to uncover cell-autonomous injury by SOD1 to motor neurons \textit{in vivo}, as well as non-cell autonomous effects on glia [96].

After the discovery that SOD1 was mutated in familial ALS many mouse models expressing mutant human SOD1 have been developed [97]. These mouse models have been widely used in pre-clinical assays of therapeutic compounds, and, currently, more than 150 different potential therapeutic agents or strategies have been tested in transgenic ALS mouse models [97]. Other mouse models based on other genes different from SOD1 also emerged [98]. For example, the knock-out mouse of the neurofilament light chain [99], the mouse expressing mutant dynein heavy chain 1 gene [100], and, more recently, a mouse expressing a mutant form of human TDP-43, which showed a progressive and fatal neurodegenerative disease reminiscent of both ALS and frontotemporal lateral dementia [101].

The transgenic VEGF\textsubscript{926} mouse, in which the hypoxia responsive element (HRE) sequence in the VEGF promoter has been deleted, was unexpectedly found to suffer from motor neuron degeneration with neuropathological and clinical characteristics similar to those of ALS [102]. Decreased levels of VEGF were found in the neural tissue of this animal. This model was crucial in unveiling the important role of VEGF in ALS and its potential as a therapeutic agent.

Rat models of ALS expressing human SOD1 transgenes have also been developed for the study of ALS [97]. These have the advantage of their increased size and they have also been successfully used in several therapeutic strategies.

Recently, Awano and colleagues [103] described that the progression and distribution of lesions in canine degenerative myelopathy are similar to those reported for the UMN dominant onset form of ALS. They consistently contained cytoplasmic inclusions that stained with anti-SOD1 antibodies similar to those found in ALS patients and rodent models with SOD1 mutations. Sequencing of the gene encoding SOD1 revealed an E40K missense mutation in affected dogs.

These experimental models that mimic several characteristics of ALS are very useful in preclinical assays of potential therapeutic compounds to evaluate their effect in preventing the pathogenic mechanisms associated with the disease.

5. PATHOGENIC MECHANISMS AND DRUG TARGETS

Several cellular pathways have been shown to be deregulated in tissues of patients and cell models of ALS, which lead to motor neuron death. The sequence of pathogenic events is not clearly established and most of them are intimately correlated: protein aggregation due to misfolding that causes ER stress, proteasome inhibition and autophagy, impaired axonal transport, mitochondria damage and apoptosis, oxidative stress, glutamate induced excitotoxicity, neuroinflammation and transcriptional dysfunction are the most relevant pathogenic events underlying motor neuron death. Most of the recent therapeutic targets tested that have been tested aim to control the dysfunction of these pathogenic events (Table 1).

5.1. Protein Aggregation and ER Stress

The hallmark of familial and sporadic ALS is protein misfolding and aggregation in the cytoplasm of motor neurons as well as glial cells [104]. Protein aggregation due to misfolding is described in other neurodegenerative diseases, such as Alzheimer’s and Parkinson’s diseases [105]. In ALS, three types of aggregates have been found: ubiquitinated inclusions, Bunina bodies and hylaine conglomerate inclusions. Ubiquitinated inclusions are found in the LMN of the spinal cord and brainstem and tend to occur in all patients with SALS. One of their protein constituents is the recently identified protein TDP-43 [106]. Bunina bodies are small eosinophilic intraneuronal inclusions in some LMN and appear to be specific for the disease [107]. Hylaine conglomerate inclusions...
### Table 1. Previous Clinical Trials

<table>
<thead>
<tr>
<th>Compound</th>
<th>Putative Target</th>
<th>Results/Progress</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin E</td>
<td>Oxidative stress</td>
<td>Negative</td>
<td>[148, 149]</td>
</tr>
<tr>
<td>N-acetyl-L-cysteine L-methionine</td>
<td>Oxidative stress</td>
<td>Negative</td>
<td>[147]</td>
</tr>
<tr>
<td>BDNF</td>
<td>Neurotrophic factor</td>
<td>Negative</td>
<td>[220]</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Neurotrophic factor</td>
<td>Negative</td>
<td>[219]</td>
</tr>
<tr>
<td>CNTF</td>
<td>Neurotrophic factor</td>
<td>Negative</td>
<td>[221]</td>
</tr>
<tr>
<td>Xaliproden</td>
<td>Neurotrophic factor</td>
<td>Negative</td>
<td>[222]</td>
</tr>
<tr>
<td>Branched-chain amino acids</td>
<td>Neurotrophic factor</td>
<td>Negative</td>
<td>[171]</td>
</tr>
<tr>
<td>Riluzole</td>
<td>Glutamate metabolism</td>
<td>Survival &gt; Survival</td>
<td>[157], [232]</td>
</tr>
<tr>
<td>Gabapentin</td>
<td>Anti-glutamate</td>
<td>Negative</td>
<td>[168]</td>
</tr>
<tr>
<td>Topiramate</td>
<td>Anti-glutamate</td>
<td>Negative</td>
<td>[170]</td>
</tr>
<tr>
<td>Lamotrigine</td>
<td>Anti-glutamate</td>
<td>Negative</td>
<td>[169]</td>
</tr>
<tr>
<td>Memantine</td>
<td>Anti-glutamate</td>
<td>Negative</td>
<td>de Carvalho, unpublished</td>
</tr>
<tr>
<td>Nimodipine</td>
<td>Excitotoxicity Calcium regulation</td>
<td>Negative</td>
<td>[175]</td>
</tr>
<tr>
<td>Verapamil</td>
<td>Excitotoxicity Calcium regulation</td>
<td>Negative</td>
<td>[174]</td>
</tr>
<tr>
<td>Dextromethanpharn</td>
<td>Excitotoxicity</td>
<td>Negative</td>
<td>[167]</td>
</tr>
<tr>
<td>Celecoxib</td>
<td>Excitotoxicity</td>
<td>Negative</td>
<td>[187]</td>
</tr>
<tr>
<td>Creatine</td>
<td>Mitochondrial enhancer Antioxidant</td>
<td>Negative</td>
<td>[152, 153]</td>
</tr>
<tr>
<td>Pentoxifylline</td>
<td>Neurotrophic factor</td>
<td>Negative</td>
<td>[223]</td>
</tr>
<tr>
<td>Minocycline</td>
<td>Anti-apoptosis</td>
<td>Negative</td>
<td>[139]</td>
</tr>
<tr>
<td>TCH346</td>
<td>Anti-apoptosis</td>
<td>Negative</td>
<td>[140]</td>
</tr>
<tr>
<td>Glatiramer acetate</td>
<td>Neuroinflammation</td>
<td>Negative</td>
<td>[233]</td>
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<tr>
<td>Interferon beta 1A</td>
<td>Anti-apoptosis</td>
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<td>[188]</td>
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<td>[189]</td>
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<tr>
<td>Tamoxifan</td>
<td>Immune modulation Neuroinflammation</td>
<td>Negative</td>
<td>Brooks, personal communication</td>
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<td>Selegline</td>
<td>Antioxidant</td>
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<td>[150]</td>
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<td>Anti-inflammatory Mitochondrial enhancer</td>
<td>Negative</td>
<td>[151]</td>
</tr>
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<td>Histone deacetylase inhibition</td>
<td>Negative</td>
<td>[194]</td>
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<tr>
<td>Sodium phenylbutyrate</td>
<td>Histone deacetylase inhibition</td>
<td>Negative</td>
<td>[196]</td>
</tr>
<tr>
<td>Minocycline/Creatine</td>
<td>Neuroinflammation Cellular energy deregulation</td>
<td>No clear benefit Safe and tolerable</td>
<td>[154]</td>
</tr>
<tr>
<td>Celecoxib/Creatine</td>
<td>Anti-inflammatory Cellular energy deregulation</td>
<td>No clear benefit Safe and tolerable</td>
<td>[154]</td>
</tr>
<tr>
<td>Recombinant human granulocyte-colony stimulating factor</td>
<td>Anti-apoptotic Neurotrophic</td>
<td>Negative</td>
<td>[141]</td>
</tr>
<tr>
<td>Levetiracetam</td>
<td>Histone deacetylase inhibition Modulates calcium channels Increases release of BDNF</td>
<td>Improvement on spasticity</td>
<td>[195]</td>
</tr>
<tr>
<td>Arimoclomol</td>
<td>Hsp induction</td>
<td>Tolerable</td>
<td>[117]</td>
</tr>
<tr>
<td>Lithium</td>
<td>Neuroprotection Autophagy inducer</td>
<td>Positive efficacy?</td>
<td>[125]</td>
</tr>
</tbody>
</table>
contain phosphorylated and non-phosphorylated neurofilament subunits but they are not specific for the disease [1, 104].

Recently, a mutant of the DNA/RNA-binding protein FUS/TLS protein has also been found to form aggregates in ALS [51, 52]. However, the protein most widely studied that forms aggregates in ALS is mutant SOD1. SOD1 aggregation has been observed in tissues from ALS patients, transgenic mice, and in cell culture model systems [104, 108, 109]. The observation that the knock-out mouse SOD1 does not present symptoms of ALS [110] and that many of the ALS associated SOD1 mutants are active [46], support the notion that mutant SOD1 induced toxicity is probably due to a gain of toxic function possibly related to misfolding. In general, the majority of SOD1 patients with short disease duration at diagnosis have mutations that show a high propensity to form aggregates [111].

Mutant SOD1 aggregation is a multistep pathway of sequential protein monomerisation, deaggregation and oligomerisation but the toxic biochemical species, whether monomeric, multimeric, soluble, insoluble or disulfide reduced, is still unknown [97, 112].

ER stress is triggered by the accumulation of misfolded proteins within the ER lumen [113], and it can activate two adaptive pathways: the conserved ER to nucleus signaling pathway called the unfolded protein response, and the ER-associated protein degradation pathway. The unfolded protein response is known to reduce global protein synthesis and to induce the synthesis of chaperones [114]. The latter eliminates misfolded or unassembled proteins from the ER by a quality control system within the ER lumen, which also involves the cytosolic ubiquitin-proteasome system. Mutant SOD1 has been found to induce ER stress but to specifically impair ER-associated protein degradation [113]. On the other hand, an inhibitor of ER stress delayed the formation of insoluble aggregates of the mutant SOD1 and suppressed cell death [73], thus suggesting a role for ER stress in cellular aggregate formation.

The presence of high amounts of misfolded proteins may also interact and saturate cellular chaperones. Indeed, mutant SOD1 was found to directly interact with Hsp70, Hsp40, Hsp27 and alpha beta crystallin [84]. Furthermore, overexpression of Hsp70 reduced the accumulation of mutant-specific insoluble SOD1 [115]. In conclusion, strategies to promote protein folding in ALS appear as good candidates to decrease aggregate formation, cell injury and death.

Arimoclomol induces heat shock protein expression and, therefore, increases the levels of chaperones, which aid in protein folding and help to prevent protein aggregation. Arimoclomol has been found to extend survival by 5 weeks of mutant SOD1 mouse [116]. It has been found to be safe in humans [117]. A phase II-III trial including familial cases with SOD1 mutation is currently recruiting patients (www.clinicaltrials.gov).

5.2. Proteasome Inhibition and Autophagy

In eukaryotic cells, there are two main systems for the degradation of cytoplasmic proteins: the ubiquitin-proteasome system and autophagy. The ubiquitin-proteasome system is the main intracellular proteolytic system that accounts for most of the selective intracellular protein degradation and mainly degrades short-lived damaged and misfolded proteins [118]. Expression of mutant SOD1 leads to proteasomal inhibition that may result in the accumulation of mutant SOD1 and motor neuron death [119, 120].

Autophagy is less selective and is involved in the degradation of long-lived proteins and organelles [121]. In vivo, autophagy was found to be activated in the spinal cord of SOD1G93A mice [122, 123]. Accordingly, mutant SOD1 was shown to be degraded by macroautophagy in experimental cell models [124].

Autophagic degradation has also been implicated in the removal of aggregated proteins associated with pathological conditions such as Alzheimer’s, Parkinson’s, Huntington’s and prion diseases [121]. Lithium in low doses is a well-known autophagy inducer that clears misfolded proteins and abnormal mitochondria, as well as suppresses glial proliferation. An exploratory clinical trial in mice and patients suggested a positive effect [125]. However, a recent large phase III clinical trial in Canada and U.S with lithium carbonate was stopped because the interim analysis showed no efficacy for lithium treatment.

5.3. Impaired Axonal Transport

Motor neurons may have axons of more than one meter long, therefore, axonal transport is crucial for the functionality of those neurons. Axonal transport occurs in anterograde and retrograde manners and it is promoted by molecular motors of the kinesin and dynein families, respectively. Mutations in the p50Glud subunit of dynactin from the dynein/dynactin complex have been reported in ALS patients [126]. Furthermore, several transgenic mouse models with mutated genes required for anterograde or retrograde transport (e.g., the Crail or the Loa mice) exhibited motor neuron degeneration [98].

Neurofilament and peripherin proteins are two types of intermediate filaments detected in axonal inclusions of ALS patients [127, 128]. Knock-out mice for intermediate filament proteins, neurofilament proteins, peripherin or u-interenixin, did not develop severe motor neuron disease [98]. On the other hand, overexpression of neurofilament subunits led to the accumulation of neurofilaments in cell bodies, and aggregation of NF-heavy-chain causes motor neurons atrophy and axonal transport dysfunction but without motor neuron death [129]. Overexpression of peripherin in mice caused death of motor neurons during aging [98].

To our knowledge no drug has been tested to act on this particular mechanism.

5.4. Mitochondria Damage and Apoptosis

Numerous studies have focused on the role of mitochondria in the pathogenesis of ALS, based on reports of morphological (vacuolated and diluted mitochondria with disorganized cristae and membranes and paracrystalline inclusions) and biochemical (defects in the respiratory chain complexes I and IV and elevated levels of mitochondrial calcium) mitochondrial abnormalities in human, mouse and cellular models of ALS [63, 130]. Mitochondrial dysfunction may cause motor neuron death by producing inadequate levels of ATP, increasing the generation of reactive oxygen species, by enhancing calcium-mediated excitotoxicity, or by triggering apoptosis.

Although most SOD1 is localized in the cytosol, a fraction of mutant SOD1 is associated with the mitochondria [131], where it could create membranous pores, to aggregate, as well as to impair the entrance of functional proteins. Furthermore, the anti-apoptotic factor Bcl-2 was found to be entrapped by large SOD1 aggregates which could lead to apoptosis [77].

Evidence has suggested that the ultimate mechanism of neuronal damage in human ALS occurs through programmed cell death, resembling apoptosis [130, 132]. Biochemical markers of apoptosis have been detected in the terminal stages of human patients and in models of ALS [133-136]. Caspase-3 activation in glial cells proteolytically inactivates the glutamate transporter EAAT2 [137], predisposing nearby neurons to excitotoxicity. Moreover, inhibition of caspase-1 [136] and increased expression of Bel-2 [138] prolonged the life of a transgenic mouse model.

Minocycline is known to inhibit the proapoptotic release of mitochondrial cytochrome c and to reduce the activity of caspase enzymes and of the p-38 mitogen activated protein kinase. However, a recent phase III trial showed that ALS patients taking...
this drug had a poorer outcome [139]. TCH346 exerts antiapoptotic effects by binding to glyceraldehyde 3-phosphate dehydrogenase and blocking the apoptotic pathway in which this enzyme is involved. A recent phase II/III trial showed that disease progression was not changed in patients taking this drug [140]. Recombinant human granulocyte colony-stimulating factor has relevant anti-apoptotic and neurotrophic effects, but a recent phase III controlled study was negative regarding efficacy [141].

R (+) pramipexole (KNS-760704) is an enantiomer of pramipexole that blocks mitochondrial transition pore. A phase II trial with this compound was encouraging and new trials are underway [142]. Tro-19622 is another drug which inhibits the mitochondrial transition pore, in addition to act as noncompetitve glutamate antagonist [143]. A phase II-III trial is recruiting patients in Europe (www.clinicaltrials.gov).

5.5. Oxidative Stress

There is a large body of evidence for the presence of oxidative damage in ALS [144]. For example, increased levels of 3-nitrotyrosine, a marker of oxidative stress mediated by peroxinitrite, were observed in both sporadic and SOD1 familial ALS patients [145]. Oxidative stress is the result of unregulated production of reactive oxygen species, such as hydrogen peroxide, peroxynitrite, superoxide and hydroxyl radicals. Reactive oxygen species are by-products of the mitochondrial respiratory chain and, to a lower extent, of other cellular oxidative enzymes, including xanthine oxidase in the cytoplasm and the P450 system in the ER. The discovery of mutations in SOD1, an anti-oxidant enzyme, causative of ALS has supported the relevance of oxidative stress in the disease. It was hypothesized that toxicity might be caused by aberrant copper chemistry yielding nitration of tyrosine residues and production of toxic hydroxyl radicals [146]. Alternatively, it was also hypothesized that SOD1 mutants could yield the production of toxic peroxynitrite [144].

Acetylsyete protects motor neurons from free radical damage, but a small phase III trial was negative [147]. The antioxidant vitamin E (alpha-tocopherol) has been shown to slow down the onset and progression of paralysis in transgenic SOD1 mice, but two large phase III clinical trials were negative [148, 149]. Selegline hydrochloride is a monoamine oxidase-B inhibitor with antioxidant properties; this drug was evaluated in a large phase III clinical trial with no positive results [150].

High-dose of coenzyme Q10 (a component of the mitochondrial electron transport chain and a potent antioxidant) was negative in a randomized, placebo-controlled study [151]. Creatine enhances mitochondrial function and acts as an antioxidant, increasing survival of the SOD1 mouse model when given before disease onset. Two recent trials with creatine were negative [152, 153]. A more complex association trial with high doses of creatine (20 g/day), associating creatine + celecoxib vs creatine + minocycline vs historical controls suggested that the first association would deserve a further conventional phase III trial [154]. Edaravone (MCI-186) is a free radical scavenger which blocks the mitochondrial transition pore and up-regulates bcl-2 expression [155]. A phase III trial is underway (www.clinicaltrials.gov). AEOL-10150 is an antioxidant small-molecule, analogous to the catalytic site of superoxide dismutase [156], which has been tested in the mice model and in patients (phase I/II trials). More developments are expected.

5.6. Excitotoxicity

Glutamate induced excitotoxicity is a major mechanism underlying motor neuron death in ALS is well-recognized and is supported by a large body of evidence. First, the only approved drug for ALS, riluzole, interferes with glutamate release [157]. Second, there are several reports that showed increased levels of glutamate in the CSF of patients with ALS [26, 158, 159]. In part this was due to decreased glutamate transport in the brain and spinal cord in ALS [160] caused by the selective loss of glial glutamate transporter GLT-1 (EAAT2) that was found in ALS patients [161].

Glutamate stimulation of AMPA receptors provides another possible mechanism causing selective death of motor neurons in ALS. Supporting this view were the observations that AMPA receptor antagonists prolonged the survival of the mutant SOD1 mouse [162, 163]. Motor neurons are particularly susceptible to deregulated high levels of calcium since they have a low expression of calcium-binding proteins and, therefore, a diminished capacity relatively to other neurons to buffer calcium [164]. Moreover, they have a high number of calcium permeable AMPA receptors [165]. In healthy conditions, the AMPA receptors that contain at least one GluR2 subunit have a low permeability to calcium. However, when there is incomplete editing of the GluR2 pre-mRNA, which has been reported in spinal motor neurons of ALS patients, there is increased calcium permeability, therefore, leading to cell death [166].

Previous trials with anti-glutamatergic drugs, as dextromethorphan [167], gabapentin [168], lamotrigine [169] and topiramate were negative [170]. Memantine phase II/III trial was finished and it did not modify the primary or the secondary outcome measurements (de Carvalho et al., unpublished).

Three branched-chain amino acids, L-leucine, L-valine and L-isoleucine, activate glutamic-dehydrogenase and were tried as therapy in ALS on the basis that they would reduce glutamate levels and so would retard disease progression. Reports were subsequently published of an alternative glutamate-modifying therapy, the amino acid L-threonine: its administration was proposed to increase concentrations of the inhibitory amino acid glycine. However, amino acids trials were negative in ALS [171].

Ceftriaxone is a promising drug as it increases the expression of EAAT2/GLT1 activity (astroglial glutamate transporter) [172]. A phase III trial is running at this time. Talampanel is a noncompetitive modulator of AMPA receptors [173] that prolongs SOD1 mice survival. A phase II trial is recruiting patients (www.clinicaltrials.gov).

Another approach is to block calcium channels to decrease one of the downstream effects of glutamate excitotoxicity. Verapamil was tried in a phase II trial [174] and nimodipine was tested in a phase III trial [175], but both were negative.

Riluzole is the only approved drug that has been shown a positive significant effect in prolonging life in ALS [4]. The mechanism of action of this drug is complex and involves interference with NMDA receptor, inhibition of glutamate release, increased extracellular glutamate up-take, inhibition of G-protein-dependent processes and stabilization of the inactivated state of voltage-dependent sodium channels.

5.7. Neuroinflammation

Even if it is not yet clear whether inflammation plays a crucial role in motor neuron degeneration, evidence in humans indicates that microglial, astrocytic and dendritic cell activation is one of the earliest microscopic manifestations in ALS patients [176-178] and in transgenic mutant SOD1 mice [179-182]. Furthermore, lowering microglial mutant SOD1 expression significantly extended the survival of the transgenic SOD1 mouse, by slowing disease progression after onset [183]. However, in the transgenic mouse SOD1, no impact was observed on MN degeneration [184]. Inflammatory associated molecules, such as cyclooxygenase-2, were increased in the spinal cord of ALS patients and in mutant SOD1 mouse [185]. A significant increase in CSF MCP-1 level was also seen in ALS patients [186].

Celecoxib is a selective cyclooxygenase-2 inhibitor non-steroidal anti-inflammatory drug that has been shown to be
beneficial in preclinical testing. A large double-blind, placebo-controlled, clinical trial associating riluzole with celecoxib was negative regarding the rate of change in upper extremity motor function measured by the maximum voluntary isometric contraction strength [187]. It has been proposed that interferons might affect the progression of ALS by interfering with putative immune mechanisms involved in the pathogenesis of the disease. However, a pilot phase III trial testing recombinant interferon beta (IFNbeta)-1a was negative [188].

Tamoxifen may be neuroprotective in ALS because of its ability to inhibit protein kinase C, which mediates inflammation in spinal cords of patients with ALS. Tamoxifen extended survival in a mouse model and a phase II study was promising (Brooks et al., unpublished). On the other hand, thalidomide suppresses tumor necrosis factor-α and has anti-inflammatory properties. A phase II study was negative [189] and its cardiac toxicity would prevent its use in larger clinical trials [190]. ONO-2506 is an enantiomer of the valproic acid that has anti-glutamate and anti-inflammatory effects. A phase III has recently finished, but no results are available. Glutamine acetate has anti-inflammatory, anti-glutamatergic and growth factor stimulating effects, a recent phase III trial was negative [191].

5.8. Transcriptional Dysfunction

Transcriptional dysfunction has been implicated as being important in the pathogenesis of many degenerative diseases [192]. Histone acetylation promotes gene transcription by facilitating access to DNA for the transcriptional protein complexes. In this way histone deacetylase inhibitors, as valproic acid, increases gene transcription [193]. In addition, valproic acid has antioxidant, anti-apoptotic (increasing bcl-2 anti-apoptotic protein) and anti-glutamatergic properties. A recent large phase III trial showed no effect in survival or functional outcome of ALS patients [194]. Levetiracetam in addition to inhibit histone deacetylase, modulates calcium channels, increases the release of brain-derived neurotrophic factor, and a phase II study testing its benefit in cramps and spasticity had some positive results [195]. Sodium phenylbutyrate that has histone deacetylase inhibition activity was well tolerated in a phase II trial [196].

5.9. Motor Neuron Death and Neuroprotective Factors

Even if some authors showed that neuron-specific expression of mutant SOD1 was sufficient to induce ALS in transgenic mice [197, 198], there are many reports supporting the concept that the death of the motor neuron is non-cell autonomous and depends, at least in part, on the contribution from surrounding glia and possibly other cell types. For example, expression of mutant SOD1 in motor neurons [199, 200] or astrocytes [201] in isolation was insufficient to induce neurodegeneration. Furthermore, analysis of chimeric mice with mixed populations of cells expressing either endogenous or transgenic mutant SOD1 showed that motor neurons expressing mutant SOD1 did not degenerate if they were adjacent to large numbers of normal supporting cells. Reciprocally, normal motor neuron surrounded by mutant-expressing non-neuronal cells, such as astrocytes or microglia, showed reduced survival [65, 202-206]. More recently, it has been proposed that a non-cell autonomous component associated with pathogenesis also exists in several other neurodegenerative diseases, such as Parkinson's disease, Huntington's disease, spinocerebellar ataxias, prion diseases and Alzheimer's disease and tauopathies [207].

The molecular basis for transmission of toxicity remains to be elucidated however, one of the possible mechanisms could be associated with SOD1 secretion. There is emerging evidence that SOD1 can be secreted via a brefeldin-A-sensitive pathway [208], and in association with chomogranins also via the ER-Golgi pathway [86]. Additionally, mutant SOD1 has been found to be secreted in association with exosomes [209]. Exosomes are secretory vesicles and in prion diseases they were found to carry abnormal scrapie prion protein and to be infectious to other cells [210]. Similarly, in Alzheimer's disease exosomes were found to contain β-amyloid peptides [211].

An immunization approach using recombinant metal-free human SOD1 mutant purified from Escherichia coli prolonged the life span of the transgenic mutant SOD1G93R mouse [212]. Therefore, immunization could be considered as a potential strategy in the therapy of ALS.

In addition, the neurodegenerative diseases, including ALS, may be considered as multisystem disorders. For example, the SOD1G93A mouse exhibited in the asymptomatic phase reduced adipose tissue accumulation, increased energy expenditure, and skeletal muscle hypermetabolism. Compensating this energetic imbalance with a highly energetic diet extended mean survival by 20% [213].

To decrease motor neuron degeneration several compounds with neurotrophic and neuroprotective properties have been tested. A number of trials have been performed with growth factors over the last 15 years in ALS.

The decreased levels of VEGF observed in ALS suggested the possibility that the administration of VEGF could have a beneficial effect in the disease. In fact, the SOD1G93A mouse after intramuscular injection of a lentivirus vector encoding human VEGF showed improved motor performance and increased survival [214]. Intracerebroventricular delivery of VEGF also delayed onset and prolonged life expectancy in the SOD1G93A rat [215]. Crossing between the SOD1G93A mouse and a mouse overexpressing VEGF in neurons exhibited delayed motor neuron loss and prolonged survival [216]. Unfortunately, previous clinical trials with systemic delivery of a neurotrophic factor did not show beneficial effects possibly due to its incapacity to cross the blood-brain barrier or to the rapid clearing from blood stream [217].

Recombinant human insulin-like growth factor I (rhIGF-I) was tested in two large trials, and overall the results were not clearly positive [218]. A longer and more recent trial over 2 years did not demonstrate a beneficial effect [219]. Other trials with growth factors were negative, including brain derived neurotrophic factor (BDNF) [220] and human ciliary neurotrophic factor (rhCNTF) [221]. Gliarial cell-derived neurotrophic factor (GDNF) was not appropriately tested in ALS. Negative results could reflect poor drug delivery, small dosage or incorrect therapeutic strategy.

Xaliproden is a novel small peptide with both neurotrophic and neuroprotective properties and good CNS penetration. Recently, two phase III trials were negative for the primary outcome (survival) but modest benefit towards vital capacity deterioration was detected [222]. Pentoxifylline increases cellular cyclic AMP and GMP, which are considered to function as neuroprotective agents in degenerating neurons. However, a European phase III trial was negative [223].

5.10. Gene Therapies in ALS

RNAi is a pathway within living cells that originates double-stranded RNA molecules as microRNAs or small interfering RNA molecules, which promote post-transcriptional gene silencing [224]. This strategy has been largely used for the manipulation of gene expression in the laboratory and has application in several diseases including neurodegenerative diseases. In ALS, it protected mutant SOD1 expressing neuroblastoma cells [225]. Furthermore, intramuscular or intraspinal injection of viral vectors that produced RNAi-mediated silencing of mutant SOD1 led to decreased motor neuron death and delay in the disease onset [226, 227]. These results are promising for a possible therapeutic strategy.

On the other hand, antisense technology has also been used with success in several ALS models [228]. For example, the SOD1G93A mouse treated with antisense peptide nucleic acid
directed against GluR3 component of the AMPA receptor showed a significant reduction in GluR3 protein levels in the lumbar spinal cord [229]. This may be a novel strategy for controlling excitotoxic lesion of motor neurons.

6. REASONS FOR NEGATIVE CLINICAL TRIALS IN ALS

Except for riluzole, all previous clinical trials in ALS have been negative. The first trials were based on concepts of disease pathogenesis that derived from laboratory or pathological investigation. For example, evidence in favor of glutamate excess in the CNS or inflammatory changes in the central nervous system stimulated trials with branch-chain amino acids or immunsuppressive drugs, respectively. The identification of SOD1 mutations and the development of transgenic mice have initiated a phase of extensive screening of a large number of drugs using the animal model. However, the transgenic mice do not represent most ALS patients, who have a sporadic form of the disease. Indeed, a large number of drugs were effective in this model but proved ineffective in patients. Nowadays, the identification of the specific ubiquitinated-inclusions are positive for TDP-43 points out that a new animal model should be used to screen a number of promising compounds.

We are not necessarily close to understand the mechanisms of disease onset. Although many authors claim that the disease onset is at the cortical level [12], there is good evidence that the presence of NOGO A (an inhibitor of axonal growth not normally present at the cortical level [12], there is good evidence that the presence of disease onset. Although many authors claim that the disease onset is

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new animal model should be used to screen a number of promising

compounds.

Novel clinical trials designs are being described, in order to test new drugs with less time and economical effort. The primary outcome have been the survival time or the functional outcome in most trials, which can be insensitive measurements for screening new drugs. The use of sensitive biomarkers, as the neurophysiological measurements, might represent a different strategy to test a large number of drugs with less patients’ inconvenience. The identification of sensitive and reliable molecular biomarkers would be great advance in future clinical trials.

7. CONCLUSIONS

ALS is a fatal neurodegenerative disease with a generally rapid progression that shares several pathological features with other neurodegenerative diseases. In vitro and in vivo experimental models have been developed and many pre-clinical and clinical trials have been performed. However, at present three crucial questions are still open: understanding the etiology of the disease, identification of molecular biomarkers, and finding of effective therapeutic agents. Progress towards solving these questions will undoubtedly contribute to a better understanding of ALS and increase patients’ survival. It may also contribute to the understanding of other neurodegenerative diseases.

ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ALS</td>
<td>Amyotrophic Lateral Sclerosis</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>LMN</td>
<td>Lower motor neuron</td>
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<td>SOD1</td>
<td>Superoxide dismutase 1</td>
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<td>UMN</td>
<td>Upper motor neuron</td>
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VEGF = Vascular endothelial growth factor

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