Amyotrophic Lateral Sclerosis with the Enteric Nervous System

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Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease affecting motor neurons in the spinal cord, cerebral cortex, and medulla oblongata. Most patients present a clinical phenotype of classic ALS—with predominant atrophy, muscle weakness, and fasciculations—and survival of 3 to 5 years following diagnosis. There are two types of ALS: the familial form with genetic involvement, and the sporadic form with a multifactorial origin. ALS pathophysiology is characterized by involvement of multiple processes, including oxidative stress, glutamate excitotoxicity, and neuroinflammation. Moreover, it is proposed that conditioning risk factors affect ALS development—such as susceptibility to neurodegeneration in motor neurons, the intensity of performed physical activity, and intestinal dysbiosis with involvement of the enteric nervous system—which supports the existing theories of disease generation.

Keywords: amyotrophic lateral sclerosis ; superoxide dismutase (SOD1) ; neurodegeneration

1. Introduction: Relevant Clinical Features of ALS

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder that has devastating effects on motor neurons and exhibits vast disease heterogeneity at the clinical, genetic, and neuropathological levels [1][2]. The term “amyotrophic lateral sclerosis” was coined by the French neurologist Jean-Martin Charcot in the 19th century. “Amyotrophic” refers to the muscular atrophy that characterizes this disease, and “lateral sclerosis” to the scarring or hardening of tissues in the lateral part of the spinal cord. ALS involves the loss of lower motor neurons of the anterior horn of the spinal cord and brainstem nuclei, and the degeneration and loss of pyramidal neurons of the primary motor cortex and corticospinal tracts [1].

In Europe and North America, ALS incidence rates range from 1.5 to 2.7 cases per 100,000 population each year, while prevalence rates range from 2.7 to 7.4 per 100,000 population; however, there are significant geographical variations—for example, Portugal had an ALS prevalence of 10.3 in 2016 [3]. Within the age group with the highest risk of ALS development (45–75 years), the incidence is between 4–8 cases per 100,000 population [4]. To date, few published epidemiological studies describe ALS in Spain. A study including the population of Catalonia reported an incidence of 1.4 and a prevalence of 5.4 per 100,000 inhabitants, based on data collected up to 2011. Another study was conducted in Navarra using more recent data (collected up to 2018), and reported an incidence of 2.47 per 100,000 cases, and a prevalence of 6.64 per 100,000 individuals (95% CI: 4.52–8.45) [5].

ALS onset typically occurs in middle age, between 55–65 years of age. In most cases, it presents as progressive muscle atrophy and weakness. The prognosis is dismal, with a survival of 3–5 years from the onset of symptomatology. The most frequent complications are respiratory failure due to weakness of the thoracic musculature, and aspiration pneumonia due to dysphagia. About 20% of patients survive for 5 years after diagnosis, and 5% for 10 years or more. ALS is more frequent in males (2:1 male-to-female ratio). Compared with women, men have a higher risk of developing sporadic onset ALS, although this risk tends to equalize with increasing age. This progressive disease inexorably leads to death. Patients with genetic or bulbar involvement have a worse prognosis [4].

The specific cause of ALS is currently unknown, but it is believed that disease development is influenced by the association of a diversity of genes and environmental factors [6]. Up to 10% of ALS patients have at least one affected family member and are defined as patients with familial ALS (f-ALS), which has an earlier onset, starting around 40–60 years of age [1]. The remaining 90–95% of cases occur randomly, defined as sporadic ALS (sALS), which has a multifactorial origin [7][8].

Regarding the disease pathogenesis, it has been established that a complex interplay of molecular and cellular processes leads to neurodegeneration. Glutamate excitotoxicity induces cytoplasmic calcium accumulation and increased oxidative stress. Mutations in the *C9ORF72*, *FUS*, TDP-43, and SOD_1 genes lead to RNA dysregulation, which results in accumulation of intraneuronal aggregates and defective axonal transport. Additionally, microglia activation and neuroinflammation result in the secretion of proinflammatory cytokines and neurotoxicity, which also determine the neurodegeneration. All of these mechanisms, which will be further described throughout this research, constitute the neuropathological signature of ALS, characterized by the loss of neuromuscular connection, axonal retraction, and subsequent death of upper and lower motor neurons [7][8].

Several theoretical models have been proposed to explain the pathophysiological onset of ALS, and the underlying onset of molecular changes. The so-called “forward death” model proposes that ALS is primarily a disorder of motor neurons in the cortex, which monosynaptically connect with neurons in the anterior horn of the medulla and mediate the anterograde degeneration of motor neurons through glutamate excitotoxicity. Cortical hyperexcitability has been proposed as one key pathophysiological process, and a possible important diagnostic marker in early disease stages. On the other hand, the retrograde degeneration hypothesis proposes that ALS begins within the muscles or at the neuromuscular junction, when noxious factors induced by free radicals are retrogradely transported from the periphery to the neuronal body, where they exert toxic effects. Finally, there is a proposed hypothesis of mixed degeneration, with independent and simultaneous involvement of both upper and lower motor neurons [9].

The most common clinical form is known as spinal ALS, characterized by focal muscle weakness and atrophy, which tends to spread with disease progression. Within months to a few years, all striated muscles are affected, except the intrinsic eye muscles, sphincters, and heart muscle. Weakness most often begins in the distal muscles of the extremities —for example, with a loss of strength in either one hand or one foot. Additionally, due to upper motor neuron involvement, patients will present with spastic hypertonia, hyperreflexia, and Babinski’s sign. ALS does not involve sensory or autonomic system impairment [1][9].

About 25–30% of patients debut with a more specific clinical presentation of primarily bulbar involvement, exhibiting dysarthria, dysphagia, dysphonia, tongue twitching, or, more rarely, masseter weakness. Bulbar onset is more prevalent among females, is highly associated with cognitive compromise and altered emotional expression, and often directly correlates with depression. In spinal ALS, the disease evolution is also accompanied with bulbar disturbance, giving rise to joint alteration or oropharyngeal dysphagia due to cranial nerve involvement [9]. There is insufficient scientific evidence to diagnose the onset of the bulbar form in some patients [10].

There are other clinical phenotypes, such as primary lateral sclerosis, in which the affection is limited to the upper motor neurons. In most patients, symptoms begin in the bulbar muscles and arms, followed by leg involvement. It is controversial whether this phenotype truly includes lower motor neuron involvement, but the progression is slower and less aggressive compared with spinal ALS. On the other hand, progressive muscle atrophy can result from predominant involvement of lower motor neurons. This phenotype can begin in any region of the body, has a higher incidence among men, and generally exhibits a delayed onset. Approximately 30% of patients develop upper motor neuron symptoms within 18 months after disease onset [9][10][11].

Although motor symptoms are the most important, half of patients with ALS will suffer extra-motor manifestations to some degree. Behavioral changes or frontotemporal cognitive deficits occur in 35–40% of cases, and frontotemporal dementia (FTD) appears in 10%. FTD is characterized by degeneration of the anterior temporal and frontal lobes, clinically presenting as behavioral changes and impairments of executive functioning and language. ALS and FTD are now considered the two ends of a spectrum, due to the overlapping molecular mechanisms underlying both neurodegenerative disorders [1][11].

ALS is clinically diagnosed, and there is not yet any pathognomonic test. Complementary tests contribute to confirming diagnostic suspicion and to excluding other pathologies. Currently, establishing diagnostic certainty requires the involvement of both motor neurons, or involvement of the lower motor neurons in at least two of four regions (bulbar, cervical, thoracic, or lumbosacral), as well as the exclusion of other etiological conditions [12].

A definitive diagnosis still requires a clinical history, neurological examination, and complementary tests. The most important test is the electromyogram (EMG), which may reveal evidence of a mild decrease in motor conduction, with reduced action potential amplitude, and acute or ongoing muscle denervation, as indicated by the presence of fibrillation potentials and fasciculations present in multiple muscles in the examined regions. Although EMG findings allow distinguishing neurogenic atrophies from muscle diseases, some myopathic processes can produce confusion with ALS,

such as chronic polymyositis, Pompe disease, or multifocal motor neuropathy with conduction blocks. Genetic testing has not traditionally been a routine part of evaluation for ALS. If a family history is present, genetic testing could be considered for presymptomatic diagnosis, although the indication for this test is controversial, as it is currently intended only for research trials [12].

Due to its multifactorial origin, there is not yet any effective or etiological treatment available for ALS. Advanced pharmacological trials focused on different mechanisms of action, such as methylcobalamin (to avoid oxidative stress), arimoclomol (directed to decrease autophagy), masitinib (a tyrosine kinase inhibitor for avoiding the neuroinflammation), tauroursodeoxycholic acid (an antiapoptotic agent that inhibits caspase-3), levosimendan (with action on troponin C), or gene therapy with tofersen (BIIB067) (to reduce protein level of SOD_1), are promising drugs that are under evaluation [13]. Currently, Rilutek® (with the glutaminergic inhibitor riluzole as the active ingredient) seems to extend the survival of ALS patients by about 3 months [13], and edaravone (a neuroprotective antioxidant and mitochondria-acting agent approved in several countries of Asia, as well as USA, Canada, and Switzerland), are the only available drugs that slightly delay the clinical course [13][14]. Based on the role of oxidative stress in ALS pathogenesis, antioxidant drugs have been tested to delay the onset of symptoms, such as vitamin E, vitamin C, carotenes, flavonoids, resveratrol, turmeric, and melatonin [13].

The cornerstone of disease management for ALS patients is still multidisciplinary care for symptomatology control, and supportive measures that improve quality of life. Among the symptoms, spasticity can be treated with baclofen, tizanidine, and muscle stretching. Muscle cramps may respond to magnesium supplements, gabapentin, or carbamazepine. Selective serotonin reuptake inhibitors, amitriptyline, and benzodiazepines may be used for emotional lability. Dietary changes may help to improve nutrition, and a gastrostomy tube is a frequent option for palliative treatment in patients with insufficient caloric intake, or when swallowing becomes dangerous.

One cause of mortality in ALS is respiratory failure due to the loss of motor neurons that innervate respiratory muscles, such as the diaphragm. In such cases of respiratory failure, non-invasive mechanical ventilation is the life-prolonging treatment of choice; additionally, it is sometimes necessary to perform a tracheotomy [15]. Treatments require individual assessments and frequent multidisciplinary interventions, and patients and their families must have an awareness of the conditions [1][16].

2. Etiology of Amyotrophic Lateral Sclerosis

2.1. Familiar Form

Familial ALS is hereditary, with predominance of autosomal dominant forms. Autosomal recessive or X-linked forms have also been described but constitute an insignificant minority of cases. The main risk factor for this type of ALS is a family history, and the developmental triggers are genetic factors. Mutations have been described in over 50 different genes. The most studied mutations are in the *superoxide dismutase* (*SOD_1*) gene, TDP-43 (RNA binding protein), *gene 9* (*C9ORF72*), and *fused protein in sarcoma* (*FUS*), which account for 75% of mutations in familial cases. The most frequent mutation is the *C9ORF72* gene mutation which is present in 45–50% of familial cases [2].

The first genetic alteration described was a mutation in the Cu/Zn-associated *superoxide dismutase* gene (*SOD_1*) on chromosome 21. About 20% of familial variants of ALS are related to this mutation, as well as 1–2% of the sporadic form [17]. The cytoplasmic enzyme SOD_1 has an antioxidant action and is heavily involved in the body's defense against harmful oxidative effects. SOD_1 is controlled through its sensitivity to oxygen pressure in tissues, which stimulates its activity upon physical exercise and various chemical compounds. Immunohistochemical analyses have demonstrated that SOD_1 is abundantly distributed in motor neurons, interneurons, and sensory neurons of the spinal cord. A mutation in this enzyme could induce neurodegeneration due to the accumulation of free radicals in motor neurons, causing their death. Alterations in this enzyme trigger cellular processes related to ALS pathogenesis, such as increased oxidative stress, neuroinflammation, and mitochondrial dysfunction, leading to alterations in the lipid layer of membranes, proteins, and DNA of motor neurons [18]. Patients with mutant *SOD_1* ALS exhibit more severe lower motor neuron degeneration compared with upper motor neuron degeneration. There appears to be a greater burden of mutated ubiquitinated SOD protein accumulation in the lower motor neurons, and greater axon loss.

In both sporadic ALS and familial ALS, low percentages of ubiquitinated TDP-43 inclusions have been detected in the cytoplasm of neurons. TDP-43 is a heterogeneous nuclear protein responsible for mRNA stability, processing, transport, and translation. Under normal conditions, TDP-43 is expressed in many tissues, including in the nuclei of neurons and glial cells. Mutations in this gene cause a loss of nuclear TDP-43, and formation of pathological aggregates in the cytoplasm, leading to neurodegeneration. Notably, this process is observed not only in ALS, but also in other

neurodegenerative diseases, such as Alzheimer's disease, Lewy body disease, and frontotemporal dementia, implying that this cytoplasmic accumulation may be related to aging and the associated functional loss [19].

Another genetic mechanism involved in ALS involves expanded short hexanucleotide sequence repeats (GGGGCC) in the non-coding region of the *C9ORF72* gene. This mutation has the characteristics of TDP-43 proteinopathy, and aggregates of p62 protein are also produced in the neuronal cytoplasm. The p62 protein is involved in both the proteasomal pathway and autophagy, and there has been growing interest in understanding how these pathways are involved in neurodegeneration. Most familial forms present with this type of mutation [20].

Genetic alteration in the gene for the FUS protein is detected in 3% of familial forms, and 1% of sporadic forms of ALS. This neuropathological subtype is characterized by basophilic inclusions in the cytoplasm of neurons of the motor cortex and spinal anterior horn. Although it is unknown how mutations in FUS cause motor neuron death, it may represent a loss of function of FUS in the nucleus, or an acquired toxic function of mutant proteins in the cytosol [2].

Other gene mutations include that of vascular endothelial growth factor (VEGF). The initial disease trigger could involve a variation in local blood flow, producing an untimely or misplaced vascular hypoperfusion event that triggers a molecular pathology mediated by "angioneurins", such as VEGF. In addition to its direct neuroprotective effect, VEGF also has an indirect effect that maintains blood flow in the spinal cord and brain at optimal levels. Reduced VEGF could lead to a worse response to hypoxia, and thereby to neuronal degeneration [21].

2.2. Sporadic Form

Sporadic ALS (90% of ALS cases) is not related to family history but appears randomly in patients. Since no specific triggers are known for this type of disease, it is considered to have a multifactorial origin involving the interaction of environmental factors on genetic, immunological, and neuronal susceptibility. In fact, the above-mentioned genetic factors (*SOD_1* and *C9ORF72*) have been described in 15% of sporadic ALS cases. In this setting, the genetic alterations do not trigger the disease, but rather represent susceptibility to interact with extrinsic factors in the generation of this aggressive disorder [22].

Advanced age, male sex, and family history have been established as verified risk factors. On the other hand, the toxicities of certain chemical substances contained in pesticides, metals, or cigarette smoke have been proposed as predisposing factors in the generation of neuronal damage and loss. Additionally, certain electromagnetic waves can lead to motor neuron death. Another theory involves viral infections—for example, by the human retrovirus K (HERV-K), also regulated by the TDP-43 protein, which induces cell toxicity [23].

Current research raises the possibility of physical activity as a risk factor, as it has been found that many ALS patients [24] [25] have certain professions in common, such as being firefighters, military personnel, and athletes. It is also being investigated whether gut microbiota may be related to the onset of ALS, and whether involvement of the enteric nervous system via the gut–brain axis may trigger the disease [26].

3. Pathogenic Risk Factors Involved in ALS

3.1. Neurodegeneration

Neurodegeneration is defined as a set of defective processes that lead to misfolded protein aggregates in the cytoplasm of motor neurons, generating oxidative and inflammatory damage, and leading to their death [27]. Due to the peculiar characteristics of motor neurons, ALS involves more rapid and progressive neurodegeneration than is observed in other neuron types. Motor neurons are large cytoskeletal cells that require high metabolic and mitochondrial activity. They are also characterized by the presence of easily oxidizable polyunsaturated fatty acids in their membrane [2]. Their high energy requirement and membrane characteristics make motor neurons particularly susceptible to the consequences of aberrant free radical accumulation. Free radicals cause lipid peroxidation, protein modifications, mitochondrial dysfunction, and DNA alterations in motor neurons, which lead to further accumulation of these toxic molecules, aggravating the progression of ALS neurodegeneration. Excess free radicals within the cell come from the mitochondria (via the respiratory chain), the endoplasmic reticulum, and peroxisomes. A portion of the free radicals is generated by enzymes, such as NADPH oxidase (NOX). These enzymes are found in motor neurons and neuroglia and are responsible for transferring electrons to oxygen and generating free radicals. Free radicals generated in the mitochondrial chain can induce the production of free radicals in NOX and vice versa, such that both mechanisms are potentiated, perpetuating neuronal damage. Moreover, these enzymes are regulated by SOD_1, which is altered in both familial and sporadic types of ALS, thereby maintaining elevated oxidative stress [28]. This type of enzyme also exists in glia cells, which explains the

role of neuroinflammation and how astrocytes can also generate free radicals that condition neurodegeneration. The NOX enzyme exists in several isoforms. Mice with ALS exhibit increased NOX type 2 enzyme, and its inhibition leads to an improvement of symptom progression [29][30].

In sporadic forms of ALS, oxidative stress can be generated by the interaction between several extrinsic factors, such as smoking or exposure to metals, which increase the prooxidant pathways, leading to accumulation of free radicals. This process, together with the susceptibility of motor neurons and their low capacity for renewal, causes motor neurons to be damaged and die, generating the symptoms and progression of this disease.

Neurodegeneration can start at any point of the pyramidal pathway. It can occur simultaneously in both upper and lower motor neurons, following the theory of mixed disease generation, which maintains that the disease appears as an independent process and originates in all motor neurons at the same time.

3.2. Relationship of Physical Activity Intensity and Muscle Metabolism

Van den Berg's research group compared the lifestyles of 1557 individuals diagnosed with ALS in Europe versus 2922 healthy individuals [31]. Their findings showed that individuals diagnosed with ALS were more likely to have participated in intense exercise, with individuals who exercised more having an up to 26% higher risk of developing ALS compared with less-active individuals [31][32]. It has also been noted that certain professions, such as firefighters, soccer players [33], or military personnel [34], may be predisposed to ALS. An increased risk of ALS was even found with higher levels of leisure-time physical activity [35]. In this sense, the lack of association with occupational physical activity strengthens the hypothesis that a genetic profile or lifestyle that promotes physical fitness increases susceptibility to ALS rather than physical activity per se [35]. On the other hand, several articles analyze how the intensity of performed physical activity can induce oxidative stress initiated in the muscle fibers, which has repercussions on neurodegeneration of the motor neurons responsible for their innervation.

Skeletal muscle fibers are classified into fast-twitch (type IIa, IIb, and IIx) or slow-twitch (type I) muscle fibers, according to their functional and metabolic properties. In ALS, the motor neurons that innervate type IIb muscle fibers—i.e., the motor neurons that innervate rapidly fatiguing fibers responsible for anaerobic burst activity—are most vulnerable to the disease process [21].

The *SOD$_1$*-mutated transgenic mouse model displays reduced contraction and loss of motor units in hindlimb muscles containing a high percentage (>90%) of type II muscle fibers. This corresponds to other findings showing that motor neurons innervating type II muscle fibers degenerate before slower fibers are affected [36]. Another study in *SOD$_1$*-mutant mice reported altered muscle performance in both slow- and fast-twitch muscles, suggesting that muscle fiber vulnerability is a consequence of the type of motor neuron that innervates those muscle fibers, rather than the muscle metabolism [32].

The motor neurons most vulnerable to ALS pathogenesis are those that innervate muscle fibers with anaerobic metabolism. These muscle fibers are responsible for rapid contraction, fatigue more quickly, and are specialized for use in intense exercise—for example, in a 400 m sprint. This metabolism requires the attainment of a lot of energy, in the absence of oxygen, which is achieved using the anaerobic pathway of glucose degradation, producing energy and lactic acid. Lactic acid is mostly eliminated or oxidized by the muscles. Excess lactic acid can induce the transformation of a free radical that is not very harmful (superoxide radical) into another much more harmful free radical (perhydroxyl), due to the interaction of the superoxide radical with protons derived from lactic acid [37]. Individuals who perform intense physical exercise are more susceptible to rapid generation of lactate, which leads to increased oxidative stress, causing neurodegeneration and neuronal death in the neuromuscular unit and, consequently, in the motor neurons. A large number of studies show that free radicals play important roles as mediators of the muscle damage and inflammation produced due to strenuous exercise. Additionally, excess lactate can induce mitochondrial dysfunction in motor neurons, which generates greater oxidative stress [34]. These findings support that ALS generation would present a retrograde transmission, with its initial origin at the level of the motor unit. In this setting, the damage would begin in the lower motor neurons and progress to the upper motor neurons, which is how the onset of symptoms occurs in most cases of classical ALS. In early disease, the only symptoms are muscle weakness and atrophy, possibly reflecting damage in the lower motor neurons that innervate this type of muscle fibers. Progression of the disease leads to symptoms of upper motor neuron damage, such as spasticity and hyperreflexia.

The hypothesis that the intensity of performed physical activity may be a trigger for ALS is also supported by the fact that 52% of clinically validated ALS-related genes are differentially expressed after acute exercise, including the *C9ORF72* gene [38]. *C9ORF72* gene expression is downregulated during physical exercise, which could act synergistically by increasing toxicity in motor neurons. Genes linked to fibroblast growth factor (FGF) and nerve growth factor (NGF)

signaling may also be altered. FGFs are highly expressed in motor neurons, and FGF secretion can be stimulated by oxidative stress, hypoxia, and hypovolemia, to induce astrocyte activation. Both produced by astrocytes, NGF and FGF have been shown to trigger motor neuron apoptosis under specific conditions in vitro, and this signaling has been implicated in ALS pathophysiology [38]. In fact, the progressive neuronal degeneration may be due to prolonged stimulation with FGF-1 or SOD-mediated oxidative stress in astrocytes [39]. It is possible that physical activity may be an ALS-triggering factor in patients with genetic susceptibility. For example, among individuals with a genetic alteration in *C9ORF72*, the ALS phenotype may be less aggressive in patients with a previous history of low physical activity, compared with patients having a history of strenuous physical activity [38]. Proposing that ALS may originate from the generation of oxidative stress in the neuromuscular junction, as a consequence of intense physical activity, allows researchers to develop recommendations for individuals genetically susceptible to ALS development, and to provide advice regarding their lifestyles and the type of physical exercise they should perform, with the aim of reducing their disease progression.

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