Amyotrophic Lateral Sclerosis

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Abstract

Amyotrophic lateral sclerosis (ALS) is a rare neurodegenerative disorder, involving a progressive degeneration of upper and lower motor neurons, resulting in loss of motor function and eventual death. This scientific review will tackle the disease from multiple aspects so to shed light on both the clinical features and pathophysiological mechanisms of the disease. The clinical aspect will emphasize the different clinical phenotypes and touches upon the clinical overlap of frontotemporal dementia and ALS. The pathophysiology will focus on the genetic factors, contrasting the genetic architecture found in European ALS patients with that of the Maltese patients. The major pathological mechanisms involved in ALS will be mentioned. Additionally, the known environmental factors of ALS will be reviewed. Lastly, the therapeutic approaches will be discussed. In this section, riluzole is discussed, but emphasis is placed on the evolving, highly efficacious RNA-based therapy.

Introduction

Amyotrophic lateral sclerosis (ALS) is a chronic neurodegenerative disorder, characterized by a progressive degeneration of motor neurons, typically involving both upper and lower motor neurons, resulting in weakened motor function. Disease onset involves muscle weakness at a focal point and spreads immediately to affect numerous muscles. Therefore, the independence of the patient suddenly diminishes and death usually occurs after only 3-5 years, due to respiratory failure (following paralysis of the diaphragm), but survival varies from one individual to another (1).

Traditionally, ALS is classified into sporadic and familial. In familial ALS (fALS) there is history of ALS in a first- or second-degree relative and inheritance generally, but not exclusively, involves dominant traits (1). The remaining ALS patients are classified as sporadic, i.e., disease with no notable family history (2). Nevertheless, this does not mean that genes do not have a role in the development of sporadic ALS. In fact, around 10% of sALS cases show gene mutations that are common to those associated with fALS and the risk of first-degree relatives to suffer from ALS increases eight-fold, even with sALS (3,4).

ALS is the most frequent motor neuron disease (MND) in adults, with a median

prevalence of 5.40 per 100,000 population and an annual incidence rate of about 2.08 per 100,000 population, in European and European-descent populations (5). Both parameters increase with age; however, the risk of developing ALS rises until 50-75 years, whereby the risk then starts to decline. As a matter of fact, onset of disease occurs around the age of 65 years for sALS (3). Onset of ALS in early adulthood may indicate fALS (1). The lifetime risk for developing ALS is higher in males than in females, by a factor of 1.2 – 1.5 (6).

ALS incidence and prevalence in Malta follow the European statistics quite similarly, with a prevalence of 3.44 per 100,000 (as of 31st December 2018) and an annual incidence rate of 2.48 per 100,000 population (studied over a 2-year period, 2017-2018). The median onset of disease is 64 years for males and 59.5 years for females, as opposed to northern populations of the Mediterranean, where the age at onset of disease is higher in females. Males are also at a greater risk of developing ALS in Malta, across all age groups above the age of 49 (Fig. 1). As aforementioned, the risk of ALS increases with age. In Malta this is also the case up till the age of 79 years, from which the incidence decreases, for both males and females. As seen in figure 1, there are differences in the incidence peaks between males and females. The peak for males occurs in the 50-59 age group, whereas the peak in females occurs in the 70-79 age group (7).

A noteworthy remark of the Maltese population is that quite a significant number of cases, 12.5%, involve fALS (7). This number is relatively high when considering the 5% rate reported from the meta-analysis of various population-based registries (8). However, the frequency of fALS in the Maltese frequency is similar to that found in Liguria (northern Italy), which is 10% (9).

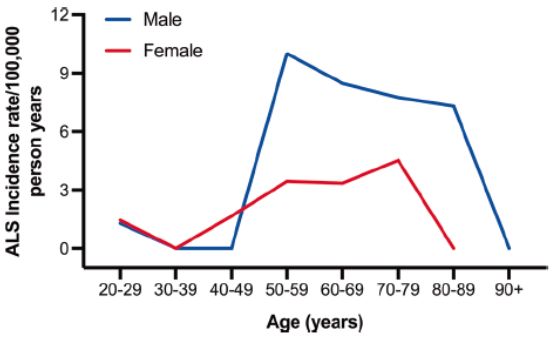

Figure 2: The ALS incidence rate in Malta with advancing age. Males (blue line) are at a higher risk of developing ALS than females, after the age of 49. The incidence of ALS increases with advancing age, until the age of 79, peaking at 50-59 years in males (blue) and 70-79 years in females (red).

Clinical Aspects of ALS

Symptoms and Clinical Phenotypes

ALS is considered clinically complex as there are many possible disease outcomes – a characteristic known as heterogeneity. Classification of clinical phenotypes can be done according to the motor neuron involved, i.e., upper motor neuron (UMN) and/or lower motor neuron (LMN), or according to the disease onset.

Degeneration of UMN presents with spasticity and weakness of muscles and the important clinical finding of hyperreflexia. Whereas degeneration of LMN leads to muscle weakness, atrophy, fasciculations and hyporeflexia/areflexia (10). Due to these pathological changes, patients with ALS often complain of fatigue and a reduced ability to carry out physical activity (11). Many ALS patients also present with clinically severe

weight loss. The cause of the weight loss is often related to the motor deficit in bulbar muscles, which leads to dysphagia. However, spinal-onset ALS also presents with weight loss and this shows that other factors such as hypermetabolism, reduced appetite and cachexia, that accompany ALS, also contribute to weight loss (12). Weight loss is important to tackle in management since reduced body mass index shows poorer prognosis (13).

Several motor neurons are affected in ALS, and this accounts for the heterogenous presentations. However, the oculomotor nuclei and Onuf's nucleus are spared and this is why eye movement and sphincters remain intact, until late in the disease (3).

The disease signs are initially exhibited at focal random regions of the body, such as masticatory muscles (bulbar) or thigh muscles. It is in this area where there is maximal degeneration of the UMN and LMN. Then, the symptoms at the same focal region worsen with time and they start to spread to contiguous areas of the body, not only to local regions (e.g., from arm to forearm), but also to neuroanatomically-linked regions (e.g., to contralateral side) (14). Since UMN and LMN have different somatotopic organization and spread distances, UMN and LMN deficits may appear discordant to each other, further adding to the complexity of the clinical presentation of ALS (15).

Apart from motor deficit, ALS commonly affects non-motor regions. Around half of ALS patients show cognition/behavioural impairment sometime during disease (14). A common manifestation of non-motor involvement is frontotemporal dementia, seen in 13% of incident cases (16). Other less common features of ALS, mostly associated with advanced disease, are extrapyramidal effects, supranuclear gaze palsy and autonomic nervous system involvement (10).

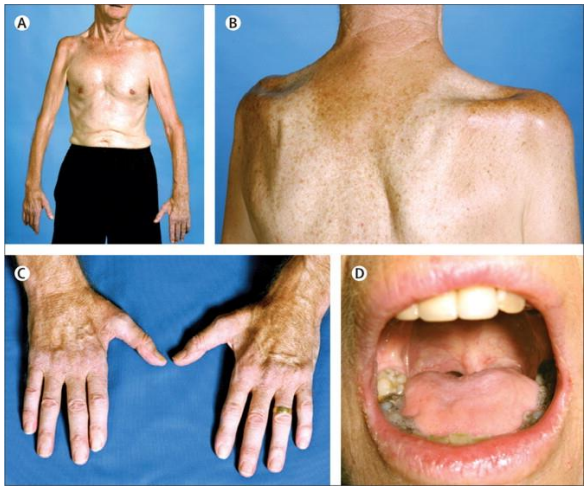

Figure 3: Clinical presentations of muscle atrophy in ALS. A: flail arm/man-in-a-barrel syndrome, with wasting of the proximal upper extremity muscles. **B**: muscle atrophy of the shoulder muscles. **C**: unilateral atrophy of the thenar muscles. **D**: atrophy of the tongue muscles and an absent elevation of the soft palate on vocalisation. Retrieved from Kiernan *et al.*, 2011.

Overlap of frontotemporal dementia and ALS

Recently, there has been much more research into frontotemporal dementia (FTD) and its overlap with ALS, both at the clinical and neuropathological level (17). Nowadays, it is believed that ALS and FTD are the two extremes of a spectrum of one disease, that is known as the MND-FTD continuum (Fig. 3) (3).

FTD, like ALS, is a progressive neurodegenerative disease that initiates at a focal point and eventually results in atrophy of the frontal and temporal lobes of the brain, due to the substantial loss of neurons (14).

The condition has two main subtypes: the behavioural variant and the primary progressive aphasias (which are further divided). FTD involves an immediate and devastating loss of independence as the individual starts showing progressive behavioural and cognitive decline, involving emotional instability, executive dysfunction and language deficit (18). Symptoms initiate at a relatively young age, most often in mid-life, and it is considered the second most common form of early-onset dementia under the age of 65 (17).

In conjunction with ALS, about 5-15% of patients have FTD, which is known as ALS-FTD. Whereas, up to half of ALS patients do not satisfy the diagnostic criteria for FTD, but still show behavioural or cognitive changes. This latter group of ALS patients can present with either behavioural impairment, executive dysfunction, or non-executive dysfunction, in addition to the ALS symptoms. Moreover, there are some individuals who are primarily diagnosed with FTD but develop motor neuron involvement later on and are known as FTD-MND (3).

It is the behavioural variant of FTD that mostly affects ALS patients. In fact, 10% of all ALS patients show behavioural symptoms. Most of such symptoms include apathy and loss of sympathy. When cognition is affected, aphasia, impaired social cognition, and executive dysregulation, are the most common symptoms encountered. Memory involvement is not a common occurrence (3).

Apart from the clinical overlap, ALS and FTD are linked at the molecular level. Many of the patients suffering from either ALS or FTD show TDP43 proteinopathy as a common feature. However, the hexanucleotide repeat expansion (HRE) mutation in the *C9orf72* gene, denoted as *C9orf72*$^{(GGGGCC)exp}$, was the discovery that truly linked ALS and FTD on a spectrum (10).

Survival is significantly affected when FTD signs accompany ALS. In fact, ALS-FTD patients have a survival of only 2.4 years from onset of disease, which is a year less than what is expected in typical ALS. Other than the poor prognosis, FTD often leads to the patient not complying to the care being undertaken and this puts a further strain on the healthcare workers and the patient's relatives. Cognitive decline will also interfere with the autonomy of the patient, and thus, may involve the need of medico-legal assistance due to ethical issues that may ensue (3).

The Pathogenesis of ALS

Genetic factors

Understanding the genetic aetiology of ALS is fundamental since it helps reveal the pathophysiological processes underlying the disease, which would allow for the development of more targeted therapy.

Mendelian inheritance is exhibited by some ALS genes, mostly in an autosomal dominant manner, characterized by high penetrance (19). Nonetheless, sALS does not show any family history and thus, this condition is far more complex genetically. Unlike many genetic disorders that depend on the presence of a large number of common genetic variants, studies on seemingly sporadic ALS cases show that the disease is mainly based on rare genetic variants that may be specific to families. These two features make the identification of

these rare variants more advanced and complicated (16).

Over 40 genes have been discovered to be associated with ALS thus far (Fig. 4). The first ALS gene to be discovered was the *SOD1* gene. There were a relatively few gene discoveries up until 2004, from which discoveries became much more frequent (Fig. 4) due to technological advances (20). Nowadays, the genetic factors accounting for two thirds of fALS cases and 10% of sALS cases are known (21).

Genetic factors involved generally exhibit missense substitutions, whereby there is a single nucleotide variant (SNV) that results in a different amino acid in the product. However, the *C9orf72* gene shows a large expansion of the GGGGCC hexanucleotide repeat in the first intron and not a missense substitution. weakness. Whereas, when there are 27-33 CAG repeats, the susceptibility of developing ALS is increased (19).

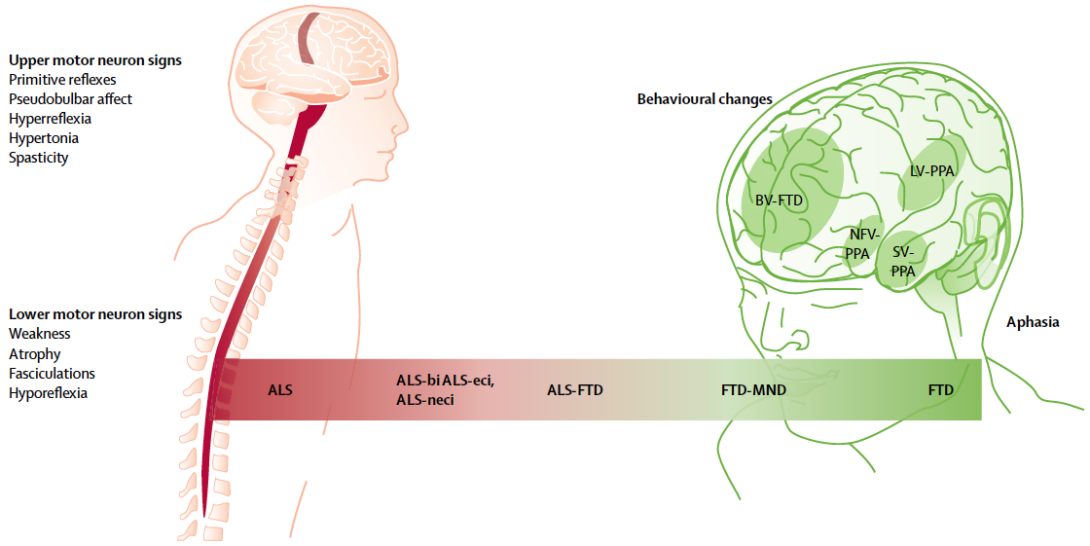

Figure 4: ALS and FTD as extremes on a spectrum & the clinical phenotypes in between. ALS-bi denotes behavioural changes, ALS-eci denotes executive dysfunction and ALS-neci denotes non-executive dysfunction with other forms of cognitive impairment (such as memory impairment). Retrieved from van Es Ma *et al.*, 2017.

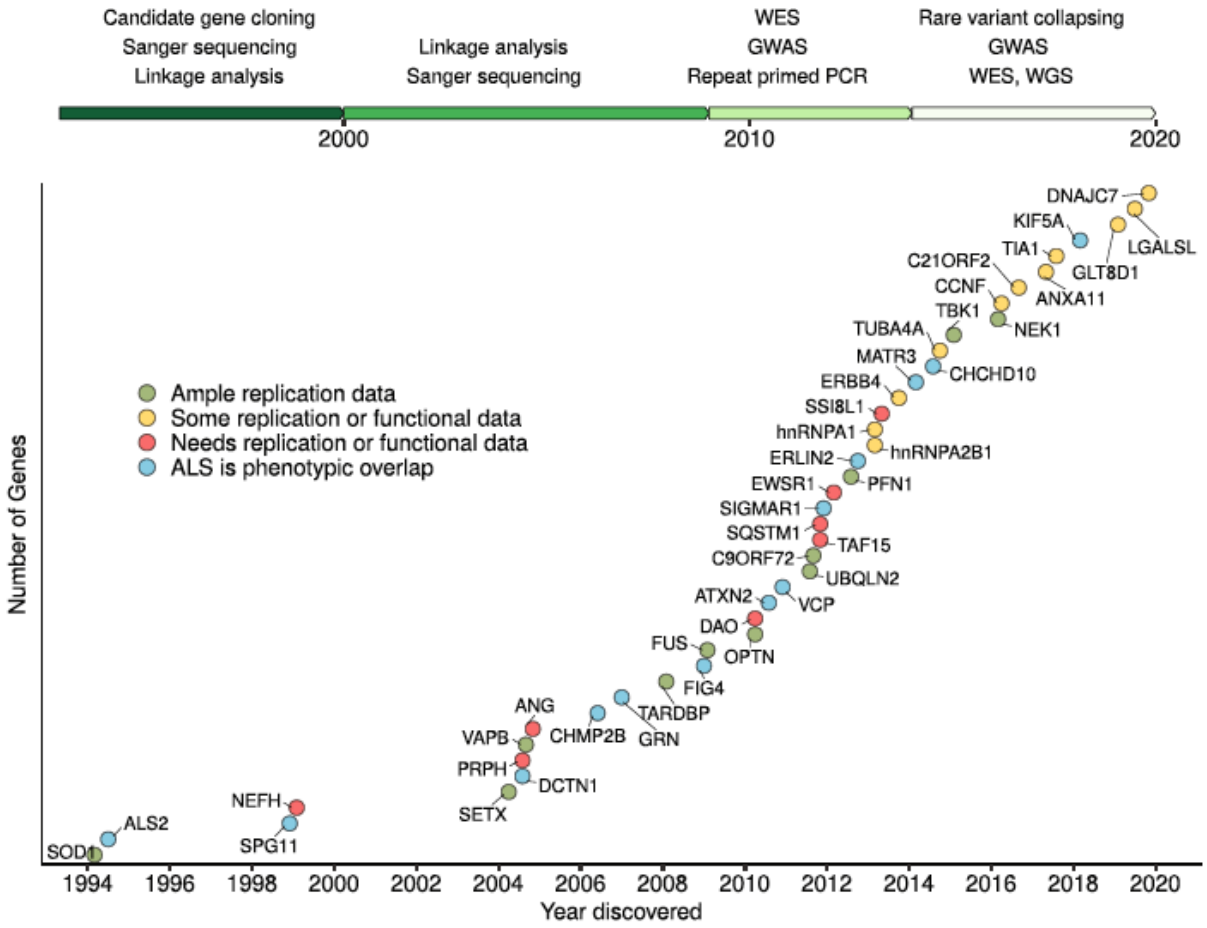

Figure 5: ALS gene discovery and its evolution. From the first gene discovery in 1994, there was a lag due to lack of advanced and efficient gene sequencing technology. This graph is showing 43 ALS genes that were discovered. Abbreviations: GWAS: genome-wide association studies; WES: whole-exome sequencing; WGS: whole-genome sequencing. Retrieved from Belbasis *et al.*, 2016.

The effects that such gene variations have on ALS are varied and include inducing motor neuron degeneration, increasing patient susceptibility and/or affecting the rate of progression. A typical example involves the ATXN2 gene. An expansion of the CAG trinucleotide repeat in the coding sequence of the ATXN2 can have two outcomes, depending on the number of repeats involved in the expansion. When there are more than *34* CAG repeats, spinocerebellar ataxia type 2 is caused, which can mimic ALS as it may present with motor weakness. Whereas, when there are 27-33 CAG repeats, the susceptibility of developing ALS is increased (19).

The major gene variants exhibited in ALS involve the *SOD1*, *TARDBP*, *C9orf72* and *FUS* genes. Their frequency differs from one population to the other, however, in the European population, the *C9orf72* gene is most commonly involved in ALS, followed by *SOD1*, *TARDBP* and *FUS* gene involvement, as seen in figure 5 (22).

While variation in the above four genes is most seen in ALS, geographic isolation can result in an altered frequency due to natural

consequences observed in population genetics, such as the Founder effect.

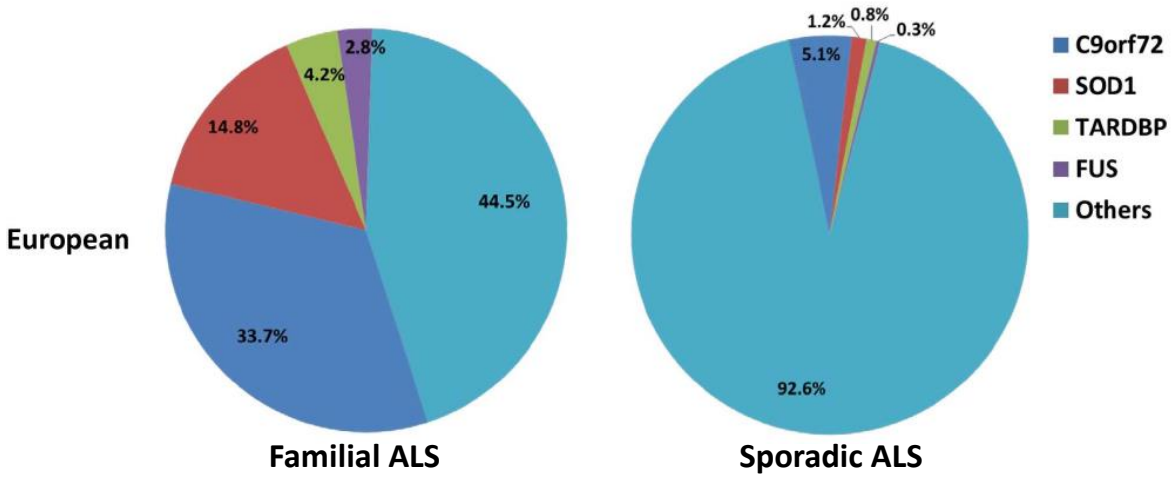

Figure 6: The genetic architecture of familial and sporadic ALS in the European population. The two pie charts are depicting the frequency of ALS cases according to the gene involved. In both fALS and sALS, the main contributing genes, sorted from most common to least common, are *C9orf72*, *SOD1*, *TARDBP* and *FUS* genes *(22)*.

This is the case in Malta, whereby, a recent study revealed that the damaging variants in *C9orf72*, *SOD1*, *TARDBP* and *FUS* genes were absent in the Maltese ALS patients. Instead, the genes *ALS2*, *SCFD1*, *DCTN1*, *SPG11*, *ERBB4*, *DAO*, *NIPA1*, *ATXN2* and *SETX*, which are considered as 'minor', were involved in ALS. This discovery has highly significant implications as it means that advances in therapy targeting the European population may not be suitable for the Maltese patients (7).

Environmental and lifestyle factors

A considerable portion of sALS cases cannot be explained by genetic factors and these are attributed to environmental factors and/or to yet-unidentified factors (1).

While genetic knowledge regarding ALS is developing at a relatively fast pace due to the availability of more-advanced technology, environmental factors cannot be clearly studied due to several reasons, e.g., it is difficult and may be inaccurate to recall all the exposures in one's life.

Regardless, there are still some environmental and lifestyle factors that are suspected to be associated with ALS. A highly probable environmental/lifestyle factor is cigarette smoking, which may account for earlier onset of the disease. An evident decrease in ALS risk is observed upon increasing the time-since-quitting (in years), in fact, the risk is halved within 5 years of quitting. There are many proposed mechanisms stating why cigarette smoking increases ALS risk, including the exposure to oxidizing chemicals, lead and formaldehyde (23).

Physical activity is a debated lifestyle factor, however a large study confirmed that there is an independent linear relationship between physical activity and ALS risk. While

strenuous physical activity carried out by professional athletes increases the risk substantially, recreational physical activity, of moderate intensity, also increases the risk (24). This risk explains why ALS is referred to as 'Lou Gehrig's disease' in the United States. Lou Gehrig was a professional baseball player who developed ALS in his thirties, and this sparked an interest in the possible association between professional athleticism and ALS. An emphasis is now being placed on those sports that involve repetitive head trauma, due to the risk of developing chronic traumatic encephalopathy. The latter results in frontotemporal atrophy and deposition of tau protein (characteristic of Alzheimer's and Parkinson's diseases) and of TDP43 protein (characteristic of ALS) (1).

Military service is another probable risk factor of ALS. While certain studies do not attest, one study that is of a higher quality revealed that veterans show an ALS risk that is 1.3 times greater than that of their civilian counterparts. This increase in risk may be due to involvement of vigorous physical activity, trauma and exposure to smoke, lead and pesticides (25).

Lead is also associated with increased ALS risk. It is one of the few environmental factors that is greatly supported in its causal link to ALS, via several studies (26–28). An interesting study, was a questionnaire-based case-control study, which revealed that even participating in hobbies that involve lead is associated with an increased ALS risk (29). The pathophysiology behind this environmental factor is still unclear, however it is known that lead increases free radical formation, causes perioxidative damage to cell walls, leads to aggregation of insoluble TDP43 and induces neuronal cell death (30).

Air pollution was also observed to increase the risk of ALS. A population-based case-control study conducted in the Netherlands revealed that traffic-related air pollutants, specifically $PM_{2.5}$ absorbance and the nitrogen oxides, contributed to the increased ALS risk (31). An important source of traffic-related air pollution is diesel exhaust and its association with ALS has been revealed in various occupational studies. Another study in 2015, found a potential association between ambient air pollution, specifically aromatic solvents, and ALS (32).

Lastly, pesticides are also considered an environmental risk factor of ALS. Indeed, persons working within the agricultural sector who are exposed to agricultural chemicals (mostly pesticides) long-term were at an increased risk of developing ALS. This was especially true when the work duration amounted to or exceeded 10 years. From the pesticides, fungicides were found to have a higher association with ALS. Of note, pesticide usage in homes was not associated with an increased ALS risk (33).

The pathology of ALS

ALS involves the degeneration of motor neurons at multiple sites of the nervous system: the motor cortex of the brain (UMN), the corticospinal tract (UMN), the motor nuclei in the brainstem (LMN) and the anterior horn cells in the spinal cord (LMN). Degeneration of the UMN in the lateral corticospinal tract leads to scarring (19). While LMN degeneration results in skeletal muscle atrophy and features of denervation and reinnervation (16).

As neuron degeneration progresses, molecular neuropathology develops. This involves the presence of dense and round, or skein-like protein aggregates, known as inclusions, in the cytoplasm of the motor neurons (14). These cytoplasmic inclusions are often ubiquitinated and the hallmark feature of ALS neuropathology is the deposition of ubiquitinated TDP43 protein, which is encoded by *TARDBP* gene. This is a recurring feature, seen in most ALS cases (both familial and sporadic), except those cases in which the *SOD1* and the *FUS* genes are mutated as these show SOD1 and FUS protein inclusions, respectively. Thus, TDP43 function is considered important in the pathogenesis of ALS (3). Those ALS cases involving the *C9orf72* gene variant exhibit an atypical combination of inclusions consisting of abundant p62 positive, TDP43 negative ubiquitinated intranuclear inclusions, especially found in the cerebellum and hippocampus (14).

Apart from these molecular findings in motor neurons, non-neural cells also show pathology. This is manifested as astrogliosis, spongiosis and microgliosis. It is a combination of neural and non-neural cell pathology that results in ALS (19).

The cellular pathogenic mechanisms of ALS

In this review, focus is made on five major mechanisms involved in ALS, namely protein homeostasis, ribonucleic acid (RNA) metabolism, axonal transport, glutamate-mediated excitotoxicity, and mitochondrial dysfunction.

Aberrant protein homeostasis

Considering that protein aggregates (inclusions) are a classic pathological feature of ALS, protein homeostasis is a major mechanism in the pathogenesis of ALS. The basis of this mechanism lies in the nature of the translated product of the damaging gene variant, wherein the protein produced can be misfolded, mislocalised, or aberrant (16). In this abnormal form, it has a number of downstream effects, including a direct effect on autophagy and the ubiquitin-proteasome system (UPS), the two major pathways by which proteins are cleared in the cell (19).

When these protein clearance pathways are disrupted, there is an accumulation of various proteins which has toxic effects. Mutated TDP43, SOD1 and FUS proteins are the three major proteins to misfold and form aggregates. These protein aggregates become prion-like via self-assembly and propagation to other cells, resembling how ALS initiates at a focal point and then spreads to other areas. This prion-like spread has become a hallmark feature in many neurodegenerative diseases, including Alzheimer's disease and Parkinson's disease (15,19,34).

Impaired RNA metabolism

Like in all cells, messenger RNA (mRNA) is produced in the nucleus and is transported to the cytoplasm, where translation occurs. However, in neurons, once the mRNA is in the cytoplasm it can be transported to distal parts, into the axonal compartment (16).

The TDP43, FUS and hnRNP A1 proteins all form part of the heterogenous nuclear ribonucleoproteins (hnRNP) family and when

mutated, they leave the nucleus and are mislocalised in the cytoplasm. In this manner, the proteins can no longer bind to RNA, and this has drastic effects on RNA processing, since one RNA-binding protein normally binds to many RNA targets. Thus, the normal function of the nucleus is significantly impaired, e.g., mutant TDP43 protein disrupts alternative splicing (19,35).

The *C9orf72* gene greatly contributes to this mechanism. The HREs are key in causing disease and this is done via multiple pathways, involving both loss of function and gain of function (Fig. 6B) (36).

Additionally, the generation of microRNAs (miRNAs) is reduced when there are damaging variants affecting TDP43 and FUS proteins. miRNA are non-coding RNAs that have a role in silencing gene expression and maintaining neuromuscular junctions (19). Due to the reduced levels of miRNAs in ALS patients, they are considered as promising biomarkers, which could help improve the patients' quality of life though more-targeted therapy (37).

Altered axonal cytoskeletal dynamics

The axonal compartment of the neuron depends on the cell body for biosynthesis and therefore, components are shuttled between the cell body and the terminal end of the axon via the cytoskeleton. There are two modes of transport: anterograde (from cell body to axon terminal) and retrograde (from axon terminal to cell body). The cytoskeleton is comprised of microtubules, and they form a network onto which motor proteins, e.g., dynein, move.

The proteins dynactin subunit 1 (encoded by *DCTN1*), profilin-1 (encoded by *PFN1*) and tubulin α-4A chain (encoded by *TUBA4A*) are essential in maintaining cytoskeletal dynamics. Therefore, damaging variants in these genes, as can be seen in ALS, has a significant impact on axonal transport.

In addition, *SOD1* variation is known to slow down both anterograde (38) and retrograde (39,40) transport before neurodegeneration initiates. Apart from *SOD1*, those genes encoding the RNA-binding proteins have a contribution in this transport, since RNA processing in the axonal compartment requires the delivery of the required components (e.g., ribosomes) from the cell body (19).

There also exists a relationship between *EPHA4* expression and axonal extension, wherein reduced expression of *EPHA4* is associated with greater axonal extension (1). Finally, the *NEFH* and *PRPH* genes, which are involved in the maintenance of neurofilaments, are also implicated in ALS, but they are seen in a smaller number of cases (16,41,42).

Glutamate-mediated excitotoxicity

This is one of the oldest mechanisms to be proposed in the pathophysiology of the neurodegeneration involved in ALS (43). This phenomenon is characterized by excess glutamate (Glu) neurotransmitter in the synaptic cleft, resulting in a prolonged activation of Glu receptors, which causes neurotoxicity. Glu-mediated excitotoxicity is implicated in other diseases, including Alzheimer's disease (44).

There are various Glu receptors. The inotropic Glu receptors (iGluR) are predominantly involved in the Glu-mediated excitotoxicity, with the NMDA receptor (NMDAR), a subtype of iGluR, being the key player (44).

Calcium plays a pivotal role in the molecular mechanism of Glu-mediated neurotoxicity – hence, why NMDAR is the key receptor due to its high calcium permeability (45). Prolonged activation of NMDAR results in a drastic increase in intracellular calcium concentration and this has many downstream effects that are cytotoxic. To decrease the effects of the drastic increase in calcium, the mitochondria capture some of the calcium (46). Being a cation, excessive uptake of calcium results in depolarization of the mitochondrial membrane and this has adverse effects on the production of ATP, leading to a low-energy state, with an abundance of ROS (45). Nonetheless, the pro-oxidant enzyme nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase is the greatest contributor to reactive oxygen species (ROS) production following excessive Glu exposure (47).

Astrocytes are intrinsically involved in this process as they regulate the Glu levels in the synaptic cleft by maintaining its synthesis and clearing and recycling it via the glutamate-glutamine cycle. Glu uptake by the astrocyte is mediated by the excitatory amino acid transporter 2 (EAAT2), which transports Glu from the synaptic cleft to the astrocyte and is sodium-dependent. In ALS, there is astrocyte-mediated downregulation in the production of EAAT2, encoded by *SLC1A2*, thereby resulting in less Glu uptake (44). It has recently become known that membralin, a protein residing in the endoplasmic reticulum membrane, also induces EAAT2 downregulation, via the TNF-α/TNF receptor 1/nuclear factor κB pathway (48). Apart from this, ALS causes astrocytes to release neurotoxic factors, which exacerbate neurodegeneration. Also, astrocytes release less neurotrophic factors, which normally function in neuronal growth and development (44).

Mitochondrial dysfunction

Mitochondria are of great importance to neurons which have relatively high metabolic requirements.

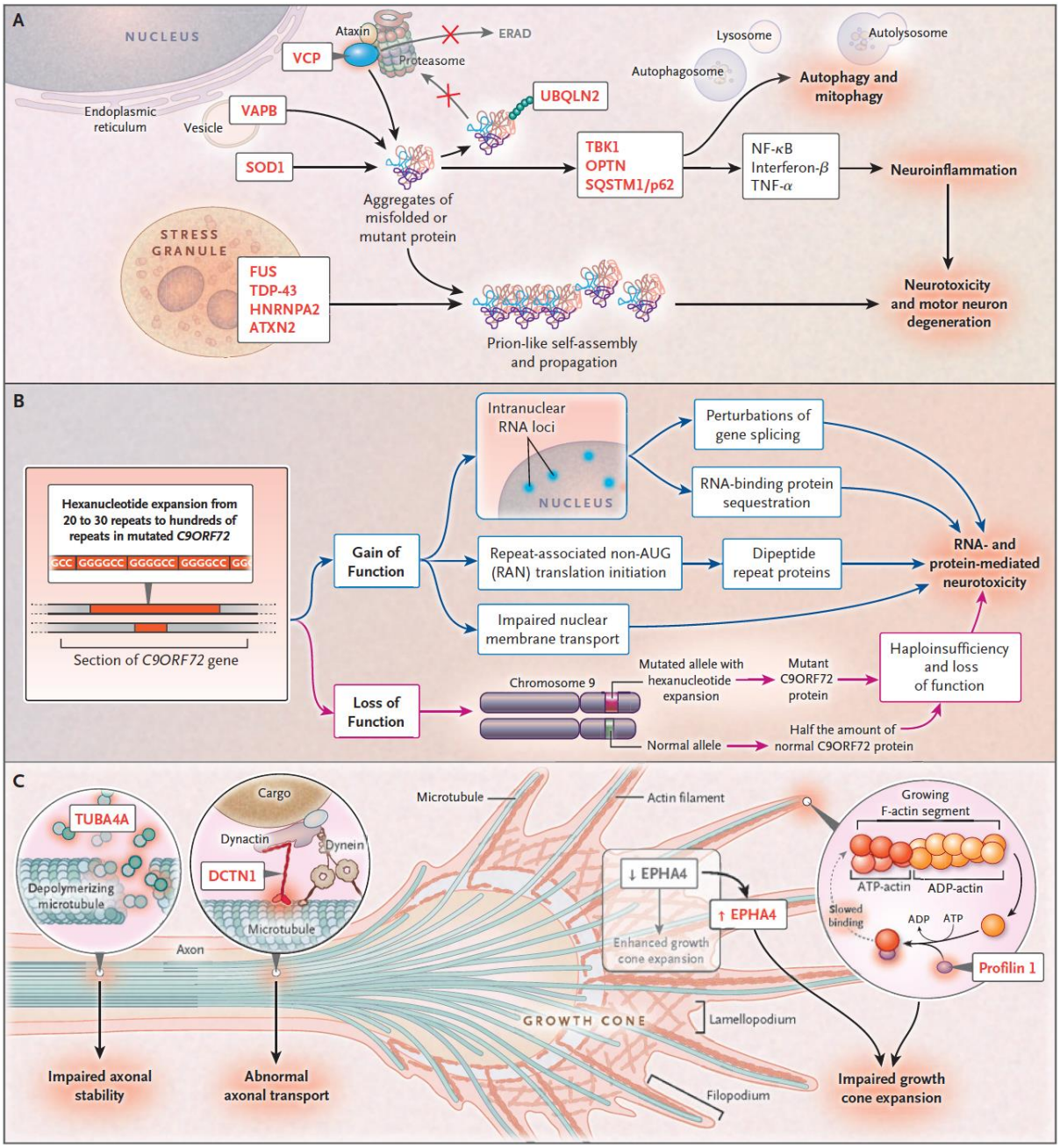

Figure 7: Protein homeostasis, RNA metabolism and axonal cytoskeletal dynamics, and their implications in ALS. Panel A concerns altered protein homeostasis, through disruption of protein clearance via endoplasmic reticulum-associated degradation (ERAD), the ubiquitin-proteasome system and autophagy. Moreover, there is the prion-like spread of misfolded protein. Panel B concerns disrupted RNA metabolism, specifically the mechanisms of the *C9orf72* gene variant. The expansion of the intronic hexanucleotide repeat has several gain-of-function effects, including the formation of RNA foci, formation of dipeptide repeat proteins and altered nucleocytoplasmic transport, all of which are toxic to the neuron. In addition, there can be loss of function, where the decreased levels of C9orf72 protein are toxic to the neuron. Panel C concerns impaired cytoskeletal dynamics, particularly the effects of gene variants in *TUBA4A*, *DCTN1*, *EPHA4* and *PFN1* (profilin 1). There is disruption of axonal transport, cytoskeletal integrity and axonal expansion. Retrieved from Brown *et al.*, 2017.

These organelles are important not only as producers of ATP via oxidative phosphorylation, but also for their role in apoptosis and as calcium buffering organelles (49,50). One must keep in mind that neurons are post-mitotic (i.e., long-lived cells) and thus, they are more vulnerable to the cumulative damage of mitochondrial dysfunction (50,51). There are many facets to ALS-associated mitochondrial dysfunction, including, impaired oxidative phosphorylation, defective calcium homeostasis, activation of apoptosis and oxidative stress via production of ROS.

In the past, mitochondrial dysfunction in ALS was mostly linked with *SOD1* mutations – however, evidence has been emerging that other familial ALS causes, particularly mutations in *TARDBP* and *C9orf72* also contribute to both functional and morphological defects in mitochondria (36).

An integral part of inducing mitochondrial damage is the direct interaction of ALS-associated proteins with the mitochondria itself. For example, mutant SOD1 protein interferes with mitochondrial function by localising to the intermembrane space, where it aggregates and reduces the activity of the electron transport chain (ETC). Additionally, this mutant protein interferes with the exchange of ATP, ADP, and others across the outer mitochondrial membrane via disruption of voltage-dependent anion channel 1 (VDAC1). TDP43 disrupts mitochondrial function via a different mechanism: it targets mRNA and impairs the transcription of components of complex I of the ETC. Furthermore, ALS mutant FUS protein is associated with increased ROS production and reduced ATP production (49). In a recent study, it was shown that C9orf72 has a role as a mitochondrial-inner-membrane-associated protein that stabilises a component of complex I of the electron transport chain and thus, helps regulate oxidative phosphorylation. Therefore, haploinsufficiency and loss of function of *C9orf72* in ALS resulted in a reduction in complex I activity (52). Overall, in all cases there is a trend of decreased ETC activity, decreased ATP production and increased oxidative stress. Considering the high energy requirements of neurons, ATP depletion may thus, trigger neuron degeneration (49).

Mitochondrial dysfunction in ALS also involves apoptosis, an important mechanism by which a damaged cell is removed from the environment in a controlled manner. The mechanism is regulated by the mitochondria via pro-apoptotic and anti-apoptotic proteins of the Bcl-2 family. Pro-apoptotic signalling in ALS is mostly an indirect consequence of other toxic events. However, it was discovered that mutant SOD1 protein directly affects apoptotic signalling by sequestering the anti-apoptotic protein Bcl-2, resulting in a pro-apoptotic state (53).

Mitochondria have the essential role of buffering surges of calcium (Ca^{2+}) within excited cells and hence, these organelles are particularly important in excitable cells such as motor neurons. Ca^{2+} buffering and uptake into the mitochondria is a finely tuned event that is of great significance not just for normal mitochondrial function, but for overall cellular homeostasis (36,50). Of note, Ca^{2+} uptake depends on the mitochondrial membrane potential, which in turn depends on mitochondrial respiration via the electron transport chain (36).

It has recently emerged that Ca^{2+} miscommunication between the endoplasmic

reticulum (ER) and mitochondria is a major contributor to calcium mishandling in ALS. Normally, the membranes of the ER and mitochondria are closely connected via several protein complexes on the outer mitochondrial membrane. It was discovered that these complexes were disrupted in SOD-1, TDP43 and FUS-related ALS. Specifically, in the TDP43 and FUS models, this miscommunication led to a consequent rise in cytosolic Ca^{2+}, that may activate cellular death pathways (54,55).

Therapy in ALS

Thus far, there is no therapy that has been proven to significantly benefit ALS patients. In Europe there is only the drug riluzole, that has been approved by the European Medicines Agency (EMA) for use in ALS patients, and its overall therapeutic benefit is questioned till this day. This is why symptomatic treatment (e.g. managing pain, sialorrhea and muscle spasticity) is given a lot of importance in the management of ALS (3,11,16).

Nonetheless, there are encouraging studies underway regarding the availability of much more effective and gene-targeted therapy that are RNA-based.

The neuroprotective agent: Riluzole

Riluzole is a synthetic benzothiazole (2-amino 6-(trifluoromethoxy)benzothiazole) that acts as a neuroprotective agent in the therapy of ALS (56).

Out of the randomized clinical trials (RCT) performed, it is shown that riluzole 100mg improves survival in ALS patients by about three months (57). Riluzole has short-term benefit as was shown in a retrospective study, whereby the mortality rate was reduced by 23% at six months from diagnosis and by 15% at twelve months from diagnosis, but this beneficial effect was lost within 18 months. This study also showed that bulbar-onset ALS patients benefitted more from riluzole than spinal-onset ALS. In addition, patients diagnosed with suspected or possible ALS (according to the El Escorial criteria) showed a 16% reduction in mortality rate at twelve months from diagnosis (58). Riluzole is well-tolerated and adverse drug reactions are not frequent. Elevated liver function tests, nausea and asthenia (lack of energy) are the commonest side-effects encountered (59).

RNA-targeted therapy in ALS

RNA-targeting therapeutics are evolving to be a major drug category and this enthusiasm is associated with the fact that this therapy is highly specific, when compared to the traditional drug categories of small molecules and proteins. Additionally, and importantly, these drugs are being studied for their use as a promising, efficient, and safe cure for ALS.

RNA-targeted therapy involves the use of synthetic oligonucleotides (a short chain of nucleic acids) and is based on the canonical Watson-Crick base pairing, exploiting, and targeting normal cellular components, such as endogenous nucleases. However, it is not this simple – structural and chemical modifications are required to enhance their effectiveness in the clinical setting.

When tackling the structure, one can either opt for antisense oligonucleotides (ASOs), which are single-stranded; or small interfering RNA (siRNA), which are double-stranded.

The emerging ASO in the therapy of ALS

Currently, there are undergoing clinical trials for an ASO therapy that targets the *SOD1* gene, which is one of the commonest genes to be affected in European ALS patients.

As previously mentioned, a damaging variant in *SOD1* gene results in misfolded aggregates of SOD1 protein, suggesting gain-of-function toxicity. Thus, lowering SOD1 protein levels is beneficial. This is exploited in the novel *SOD1*-targeting ASO: BIIB067, now known as Tofersen, which is an intrathecally administered ASO (60). The drug induces the degradation of *SOD1* mRNA by RNase H activity, thereby reducing the synthesis of SOD1 protein (61). Additionally, when tofersen was tested on mice expressing the $hSOD1^{G93A}$, a partial recovery in motor neuron function was exhibited (62).

A 28-week phase 3 randomised trial was conducted on tofersen, wherein the efficacy and safety were analysed in adults with *SOD1* ALS. It was discovered, that tofersen reduced concentrations of SOD1 in CSF, a marker of target engagement; and of plasma neurofilament light chains (NfL), a marker of neuronal degeneration. However, at 28 weeks, there was no significant difference in the change from baseline in the ALS Functional Rating Scale–Revised (ALSFRS-R) score between tofersen and the placebo group. These results suggest that early initiation of the drug may be more beneficial in delaying the onset of clinically manifest ALS. Thus, the ATLAS study is currently underway to evaluate the impact of tofersen when initiated in clinically presymptomatic adults with a confirmed *SOD1* mutation (63).

Also, tofersen was associated with mild to moderate adverse events, mostly associated with the disease progression of ALS and side effects of lumbar puncture. Nevertheless, 7% of the participants who received tofersen had serious neurological adverse events, including myelitis, meningitis and lumbar radiculopathy (64).

One must keep in mind that the *SOD1* gene is not damaged in the Maltese ALS cohort (7). This renders the drug ineffective for Maltese patients and this underscores the importance of the ongoing research concerning the unique genetic architecture in Maltese ALS patients. This research paves the way for the manufacture of efficient RNA-based therapy that is suited for the Maltese patients.

Conclusion

ALS is a devastating disease because of many aspects – it deprives the patients of their independence, it has poor prognosis, it can present in many forms and thus, is difficult to diagnose and it puts a severe strain on the healthcare system.

Clearly, there has been a dramatic advancement in the understanding of the genetic factors that are involved in ALS in the recent years. This will certainly continue to evolve, decoding more ALS genes as the vigorous research continues. Nevertheless, more research is required on the

environmental factors involved in the disease pathogenesis, since, as of now, such information is scarce. To do this, studies on ALS patients and on healthy controls are required. Several of such studies are already taking place, including Project MinE, which is an international ground-breaking study on ALS and healthy patient cohorts.

Thanks to the strong will and dedication of scientists, the manufacture of highly promising and efficacious cure is underway and should be available in the near future.

Declarations

Conflict of interest: N.A.

Ethical statement: N.A.

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