Pathway Interconnections in ALS Necessitate a Shift to Combination Therapies

Writing Assignment
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Summary for Layman: ALS and the Potential of Combination Therapies

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease where nerve cells responsible for controlling movement, known as motor neurons, gradually deteriorate and die. As these motor neurons degenerate, people with ALS experience increasing muscle weakness, loss of mobility, and eventually, difficulty breathing. The disease progresses rapidly, making ALS particularly devastating. Approximately 10% of ALS cases are inherited, caused by genetic mutations passed down through families. The remaining 90% are sporadic, meaning they occur without a known family history or specific genetic cause. In these sporadic cases, ALS likely results from a complex interplay of genetic predispositions, environmental exposures, and lifestyle factors that together trigger the disease unexpectedly.

Since no single test can confirm ALS, diagnosis is challenging, often causing delays that allow significant motor neuron damage to occur before the disease is recognised. Treating ALS is especially difficult, because nerve cell damage is usually irreversible and driven by several interconnected processes. Oxidative stress, a condition where highly reactive oxygen molecules damages cellular components and triggers the aggregation of abnormal proteins within cells. These protein aggregates contribute to mitochondrial dysfunction and inflammation. As mitochondria lose function, they produce release harmful molecules, further worsening oxidative stress and activating the immune response. This cascade intensifies excitotoxicity, where neurons become overstimulated by chemical signals, ultimately contributing to cell death.

Most current treatments focus on a single pathway, aiming for instance, to reduce protein clumping or limit excitotoxicity. However, these single-target approaches have not been able to halt or reverse ALS progression. Therefore, a combination of therapies that target multiple pathways simultaneously must be explored. By addressing several sources of cellular dysfunction together, these therapies have the potential to slow down or even interrupt the damaging cycle of that drives motor neuron loss.

Many promising therapeutic candidates for ALS have shown potential in preclinical studies, only to fail in clinical trials. While some of these treatments may have influenced specific pathways, the relentless progression of motor neuron degeneration could still have been drive by other processes. For many candidate therapies we may never know if these interventions had any biological impact, as clinical trials rely on validated outcome measures which in ALS research are typically broad measures like survival time or the ALS Functional Rating Scale-Revised, which reflects overall function, but does not capture changes in underlying cellular mechanisms.

To improve the precision of ALS research and treatment, biomarkers—biological indicators that reflect activity at the cellular level—are crucial. Biomarkers could enable researchers to track the effects of treatments of specific pathological pathways, helping to determine if a therapy is influencing underlying disease mechanisms, even if it doesn't immediately improve overall function. By including a panel of biomarkers in clinical trials, researchers can gain valuable insights into whether treatments effectively target protein aggregation, oxidative stress, mitochondrial dysfunction, excitotoxicity, or neuroinflammation. This approach not only improves our understanding of how potential therapies interact with ALS pathology but also guides the development of future combination therapies tailored to address multiple processes simultaneously.

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Glossary

4-HNE	4-hydroxynonenal
8-OHdG	8-hydroxy-2’-deoxyguanoside
ALS	Amyotrophic lateral sclerosis
ALSFRS-R	ALS functional rating scale revised
ASO	Antisense oligonucleotide
C9orf72	Chromosome 9 open reading frame 72
CAT	Catalase
CNS	Central nervous system
CSF	Cerebrospinal fluid
DAMP	Damage-associated molecular pattern
DPR	Dipeptide repeat
ER	Endoplasmic reticulum
ETC	Electron transport chain
fALS	Familial amyotrophic lateral sclerosis
FTD	Frontotemporal dementia
FUS	Fused in sarcoma
GPx	Glutathione peroxidase
GSH	Glutathione
GWAS	Genome-Wide Association Study
HRE	Hexanucleotide repeat expansion
HSP60	Heat shock protein 60
LMN	Lower motor neuron
MAMs	Mitochondria-associated ER membranes
MCU	Mitochondrial calcium uniporter
MDA	Malondialdehyde
mPTP	Mitochondrial permeability transition pore
mtDNA	Mitochondrial DNA
NLS	Nuclear localisation signal
NO	Nitric oxide
PUFAs	Polyunsaturated fatty acids
RNP	Ribonucleoprotein
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD1	Superoxide dismutase 1
sALS	Sporadic amyotrophic lateral sclerosis
TARDBP	TAR DNA-binding protein
TDP-43	TAR DNA-binding protein 43
TIMMDC1	Translocase of inner mitochondrial domain containing 1
UPR	Unfolded protein response
UMN	Upper motor neuron
VDAC1	Voltage-dependent anion channel 1

Introduction

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disorder characterized by the progressive loss of both upper motor neurons (UMNs) in the motor cortex and lower motor neurons (LMNs) in the spinal cord and brainstem.[1,2] The gradual failure of the neuromuscular system leads to increasing muscle weakness in both the upper and lower limbs and in the bulbar- and respiratory muscles. While the rate of progression of the disease varies, most patients die of neuromuscular respiratory failure within 2 to 5 years from the onset of symptoms.[2]

Although ALS is a relatively rare disease with a global incidence of around 2 per 100,000 person-years, with regional variations observed, more than half a million people have died from the disease in the past 80 years.[3] The prevalence of ALS in populations of European descent is estimated to be between 2.6 and 3.0 cases per 100,000 people, with lifetime risk approximations of 1 in 350 for men and 1 in 400 for women.[1] Incidence of ALS increase with age, peaking between 60 and 79 years.[4] Evidence suggests that the incidence of ALS is rising, which may be partly due to an ageing population as well as the improvement of clinical services supporting enhanced diagnostic capabilities.[2] Projections estimate that by 2040, approximately 400,000 people will be living with ALS globally.[3]

Around 10% of patients with ALS have a family history of the disease and so far, more than 30 different genes have been linked to these familial ALS (fALS) cases.[1–3] Though the remaining 90% of cases appear to be sporadic, systemic genetic testing has revealed that a large portion of the pathogenic mutations identified in fALS can also be detected as a genetic cause in many of sporadic ALS (sALS) cases.[2,3] Pathogenic mutations in the most prominent ALS-associated genes; chromosome 9 open reading frame 72 (*C9orf72*), TAR DNA-binding protein (*TARDBP*), superoxide dismutase 1 (*SOD1*), and fused in sarcoma (*FUS*), are responsible for around 60% of familial cases and roughly 10% of sporadic cases, equating to one in six ALS cases explained by these four genes.[3] Large genome-wide association studies (GWAS) of patients with apparent sALS indicate that the genetic underpinnings of ALS primarily consist of rare variants. Estimates suggest that the heritability of sALS could be as high as 50%. [2]

ALS is increasingly recognized as a genetically and clinically diverse neurodegenerative syndrome, characterized by a variety of pathophysiological pathways and distinct clinical subtypes.[1] As genetic testing becomes more widely implemented and candidate therapies become more targeted, the field is advancing towards a more precise molecular subclassification of ALS.[4] This evolving understanding has led to a reconsideration of the traditional classification into fALS and sALS, which is now seen as an overly simplistic distinction.[2]

ALS clinically presents when axonal connections fail and patients suffer from motor deficits that develop over the course of several weeks to months.[1,4] Any voluntary muscle can be impacted, leading to diverse clinical manifestations.[1] Dysfunction typically has focal onset, but muscle weakness is relentlessly progressive, usually spreading to adjacent anatomical areas, including across the body (contralaterally), towards the head (rostrally), and towards the lower body (caudally).[4] Interestingly, motor neurons in the Onuf's nucleus and oculomotor nuclei are not affected, preserving sphincter control and eye movement.[1] Two common phenotypes constitute more than half of all cases: spinal-onset ALS, characterized by limb movement weakness, and bulbar-onset ALS, marked by difficulties in speaking (dysarthria) and swallowing (dysphagia).[4]

In addition, nearly half of all ALS patients develop varying degrees of cognitive and/or behavioural dysfunctions, including impaired language fluency, compromised working memory, reduced inhibitory control, apathy, irritability, neglect of personal hygiene, and altered eating habits.[4] A strong association between ALS and frontotemporal dementia (FTD) is well-established, with about 15% of ALS patients meeting the diagnostic criteria for FTD. Similarly,

approximately 15% of behavioural variant FTD patients have ALS.[5] These conditions share genetic links, with the hexanucleotide repeat expansion (HRE) in *C9orf72* being the predominant genetic cause of both ALS and FTD among individuals of European descent.[5]

ALS is an unremittingly progressive condition, yet it displays considerable variability in progression, even among individuals carrying the same disease-inducing mutation. The diverse clinical manifestations and the differing rates at which the disease progresses severely complicate its diagnosis.[1] Currently, there is no definitive diagnostic test available to conclusively confirm ALS. ALS is diagnosed through a process of differential diagnosis, where more common diseases are systemically ruled out first, before considering ALS, leading to inevitable delays in diagnosis.[1,4] Typically, patients receive a diagnosis several months to more than a year following the emergence of the initial symptoms.[4] This delay is particularly detrimental as ALS progresses rapidly, and by the time of diagnosis, significant and often irreversible damage to motor neurons may have already occurred.[1]

In light of these challenges, identifying reliable biomarkers has become a top priority in ALS research. The development of diagnostic biomarkers could significantly shorten the current 9-12 months delay in diagnosis, enabling earlier treatment initiation when interventions for neurodegenerative disease are likely most effective.[6] Beyond facilitating earlier diagnosis, prognostic biomarkers that predict disease progression and severity can inform patient care and allow for more precise stratification in clinical trials, ensuring balanced groups of fast and slow progressors. Predictive biomarkers would further enhance trial design by identifying individuals more likely to respond to specific therapies, helping to prevent drug efficacy from being masked by non-responders.[7] With over 50 clinical trials for ALS failing to produce effective treatments, the need for biomarkers is critical to improve trial design and assess target engagement.[7,8]

Currently, ALS clinical trials primarily rely on functional outcome measures such as the ALS Functional Rating Scale Revised (ALSFRS-R). While the ALSFRS-R remains a valuable tool due to its standardized and easy-to-administer approach in measuring functional decline, it has inherent limitations in capturing the full complexity of ALS's underlying biological changes that drive disease progression.[7] Numerous candidate therapies targeting various mechanisms have been tested in ALS clinical trials, yet most have failed to meet their primary endpoints, and the reasons for these failures are often unclear.[7,8] Response biomarkers that indicate whether a drug engaged its target, along with pharmacodynamic biomarkers that measure the molecular or physiological activity of a therapeutic, can provide valuable insights, even if no immediate clinical improvements are observed.[8]

Despite years of research, only a few therapies have been approved for the treatment of ALS, primarily targeting a limited number of pathological pathways and extending life by only a few months. The development of effective therapies has been hindered by the complex and multifactorial nature of ALS, where multiple genetic, molecular, and environmental factors contribute to disease onset. Furthermore, multiple intricately linked pathological pathways drive motor neuron degeneration. This complexity suggests that single-target therapies are insufficient to significantly alter the disease course. Evidence from failed trials underscores the need for a more comprehensive approach that addresses multiple pathways simultaneously.

This review explores key pathways involved in ALS pathology—protein aggregation, oxidative stress, mitochondrial dysfunction, and inflammation—their mechanisms of motor neuron injury, and the intricate interconnections between these processes. These pathways not only independently contribute to motor neuron damage, but also act dynamically, where disruptions in one pathway, often exacerbate others, compounding neuronal injury. This interconnection underscores the insufficiency of monotherapies and highlights the potential of a multi-target approach through combination therapies to address the multifaceted nature of ALS.

Pathological Pathways in ALS

ALS is a multifaceted neurodegenerative disease marked by the progressive loss of motor neurons. Numerous pathways contribute to ALS pathology, including protein aggregation, oxidative stress, mitochondrial dysfunction, excitotoxicity, neuroinflammation, RNA metabolism dysfunction, nucleocytoplasmic transport defects, impaired autophagy, DNA damage, and hypermetabolism.[2,4] Although each pathway has its unique impact, they are intricately connected, forming a network of damaging processes that amplify neuronal injury. This review focusses on five primary pathways—protein aggregation, oxidative stress, mitochondrial dysfunction, excitotoxicity, and neuroinflammation—which are well established as key drivers of ALS pathology and directly linked to motor neuron degeneration.[2,9] These pathways interact dynamically, creating feedback loops that amplify damage across the nervous system.

Protein Aggregation

Motor neuron damage in ALS emanates from a complex interplay of multiple pathophysiological pathways. The heterogeneity of ALS indicates that these pathological pathways vary in prominence across patients.[2] Nevertheless, TAR DNA-binding protein 43 (TDP-43) aggregates or cytoplasmic inclusions are observed in neurons and glial cells in 97% of ALS patients. Notably, they are only absent in cases involving *SOD1* or *FUS* mutations, where these proteins form aggregates instead.[5] This universal presence of protein-aggregation makes proteinopathy a hallmark feature of ALS pathology.[3]

TDP-43 is a highly conserved and ubiquitously expressed RNA-binding protein, pivotal in several critical cellular functions, including the regulation of RNA metabolism, messenger RNA (mRNA) transport, microRNA (miRNA) maturation and stress granule formation.[10,11] Under physiological conditions, TDP-43 is predominantly localized in the nucleus, where it plays an essential role in RNA splicing and gene expression regulation.[11] However, TDP-43 shuttles between the nucleus and cytoplasm, a process mediated by active and passive transport, allowing it to fulfil cytoplasmic functions as well. [10]

In ALS, this delicate balance is disrupted, leading to the mislocalisation of TDP-43 from the nucleus to the cytoplasm, where it forms pathological aggregates. Several factors contribute to the aggregation process, including post-translational modifications such as phosphorylation and ubiquitination.[11] Phosphorylation of TDP-43's C-terminal domain, a pathological feature observed both in the cortex and spinal cord of ALS patients, promotes its oligomerization and fibrillization, enhancing its tendency to form insoluble cytoplasmic inclusions.[10,11] Additionally, ubiquitination facilitates the abnormal accumulation of TDP-43 by impairing its clearance via the proteasomal pathway.[11]

Mislocalisation of TDP-43 from the nucleus to the cytoplasm results in loss of its normal nuclear functions. One of its key nuclear roles is regulation of RNA splicing, particularly the repression of cryptic exon inclusions. Nuclear depletion of TDP-43 has been shown to cause inclusion of cryptic exons in key neuronal genes, including *STMN2* and *UNC13A*, leading to reduced expression of essential proteins involved in axonal maintenance and neurotransmitter release.[5] Moreover, genome-wide RNA immunoprecipitation techniques (CLIP-seq) identified more than 6000 mRNA targets that associate with TDP-43, accounting for nearly 30% of the entire transcriptome.[11] Disruption of TDP-43's ability to regulate these mRNA substrates contributes to widespread dysregulation of RNA metabolism, contributing to motor neuron dysfunction and degeneration in ALS.

In the cytoplasm, TDP-43 is involved in the regulation of mRNA transport and miRNA maturation by forming ribonucleoprotein (RNP) granules. These granules help facilitate the

transport of mRNA molecules and contribute to maintaining RNA homeostasis, ensuring proper RNA processing and stability.[10,11] Additionally, TDP-43 is an essential component of stress granules, dynamic structures that form in response to cellular stress, such as oxidative stress.[10] Stress granules temporarily sequester mRNA and RNA-binding proteins, effectively pausing translation to protect cells from damage during stress conditions.[2,10] TDP-43 plays a critical role in both the formation and stabilization of stress granules and also regulates the expression of nucleating proteins essential for stress granule assembly.[11]

In ALS, stress granule dynamics are disrupted by mutations in TDP-43, leading to the formation of larger, less mobile granules. These stress granules fail to disassemble properly, causing prolonged TDP-43 sequestration and aggregation. This impairs RNA homeostasis by trapping mRNAs, including nuclear encoded mitochondrial mRNAs and other regulatory proteins, disrupting their translation leading to further cellular stress.[10,11]

FUS, like TDP-43, is an RNA-binding protein essential for RNA metabolism, mRNA stability, and transport. Under normal condition, FUS is primarily localised in the nucleus, but in ALS, it mislocalises to the cytoplasm due to mutations affecting its nuclear localization signal (NLS). This mislocalisation leads to the formation of cytoplasmic aggregates, disrupting RNA homeostasis and contributing to motor neuron dysfunction. FUS also participates in stress granule dynamics, and its persistent aggregation in these granules further impairs cellular function.[12,13]

SOD1 normally functions to neutralise reactive oxygen species within cells, protecting them from oxidative damage. However, mutations in SOD1, which account for about 2% of ALS cases, cause the protein to misfold and aggregate. These aggregates accumulate in mitochondria, disrupting their function and contributing to oxidative stress.[2,11] Toferson, an antisense oligonucleotide (ASO) targeting SOD1 mRNA, reduces SOD1 protein levels and is the first therapy approved for patients with SOD1 mutations.[2,13]

While protein aggregation is a hallmark feature of ALS, it is increasingly viewed as a downstream consequence of other pathological processes, rather than an initial trigger. Upstream events, such as mitochondrial dysfunction, oxidative stress, and excitotoxicity, likely play a central role in driving the misfolding and aggregation of proteins like TDP-43, FUS, and SOD1, linking these pathways in a complex and interdependent manner.[9,11]

Protein aggregation in ALS is closely associated with oxidative stress, contributing to a reciprocal pathological relationship. Oxidative stress promotes the misfolding and aggregation of proteins, including TDP-43, SOD1, and FUS. Under oxidative conditions, TDP-43 undergoes modifications like acetylation and phosphorylation, impairing its RNA binding capabilities and promoting the formation of insoluble, hyperphosphorylated aggregates. These aggregates accumulate in the cytoplasm, sequestering various miRNAs and proteins, including nuclear genome-encoded mitochondrial proteins, further disrupting mitochondrial function and exacerbating oxidative stress.[11,14]

Mutations in SOD1 lead to its misfolding and aggregation, particularly under conditions of oxidative stress. These misfolded SOD1 aggregates accumulate in mitochondria, leading to mitochondrial dysfunction and increased reactive oxygen species production. The accumulation of oxidative damage in mitochondria further promotes the misfolding and aggregation of SOD1, establishing a cycle of oxidative stress and protein aggregation driving neuronal degeneration in ALS.[2,14]

TDP-43, FUS, and SOD1 aggregates are excreted into the extracellular space through mechanisms like microvesicle and exosome release, where they act as danger-associated molecular patterns (DAMPs).[11] These protein aggregates bind to pattern-recognition receptors (PRRs) on microglia, particularly Toll-like receptor 4 (TLR4), initiating a pro-inflammatory response. Once activated microglia release a range of inflammatory mediators, including tumour necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), nitric oxide (NO), and C-C

motif chemokine ligand 2 (CCL2), which amplify oxidative stress, exacerbate neuronal damage, and promote further protein misfolding.[15,16] Astrocytes become reactive in response to pro-inflammatory signals from microglia, adopting a neurotoxic phenotype, which impairs astrocytic functions. Additionally, the consistent uptake of misfolded proteins like TDP-43, FUS, and SOD1 induces ER stress within astrocytes, disrupting their homeostatic roles, resulting in impaired glutamate clearance, contributing to excitotoxicity.[15] The interplay of protein aggregation with other pathological pathways is illustrated in **figure 1**.

Once in the extracellular space, TDP-43, SOD1, and FUS aggregates propagate pathology by inducing misfolding in neighbouring cells, contributing to a prion-like spread.[11,17] Their presence in the CSF provides a window into ALS progression, with phosphorylated TDP-43, and misfolded SOD1 and FUS, emerging as promising biomarkers. These CSF biomarkers not only reflect the accumulation of pathological aggregates but also offer potential for tracking disease progression and therapeutic response in ALS patients.[18,19]

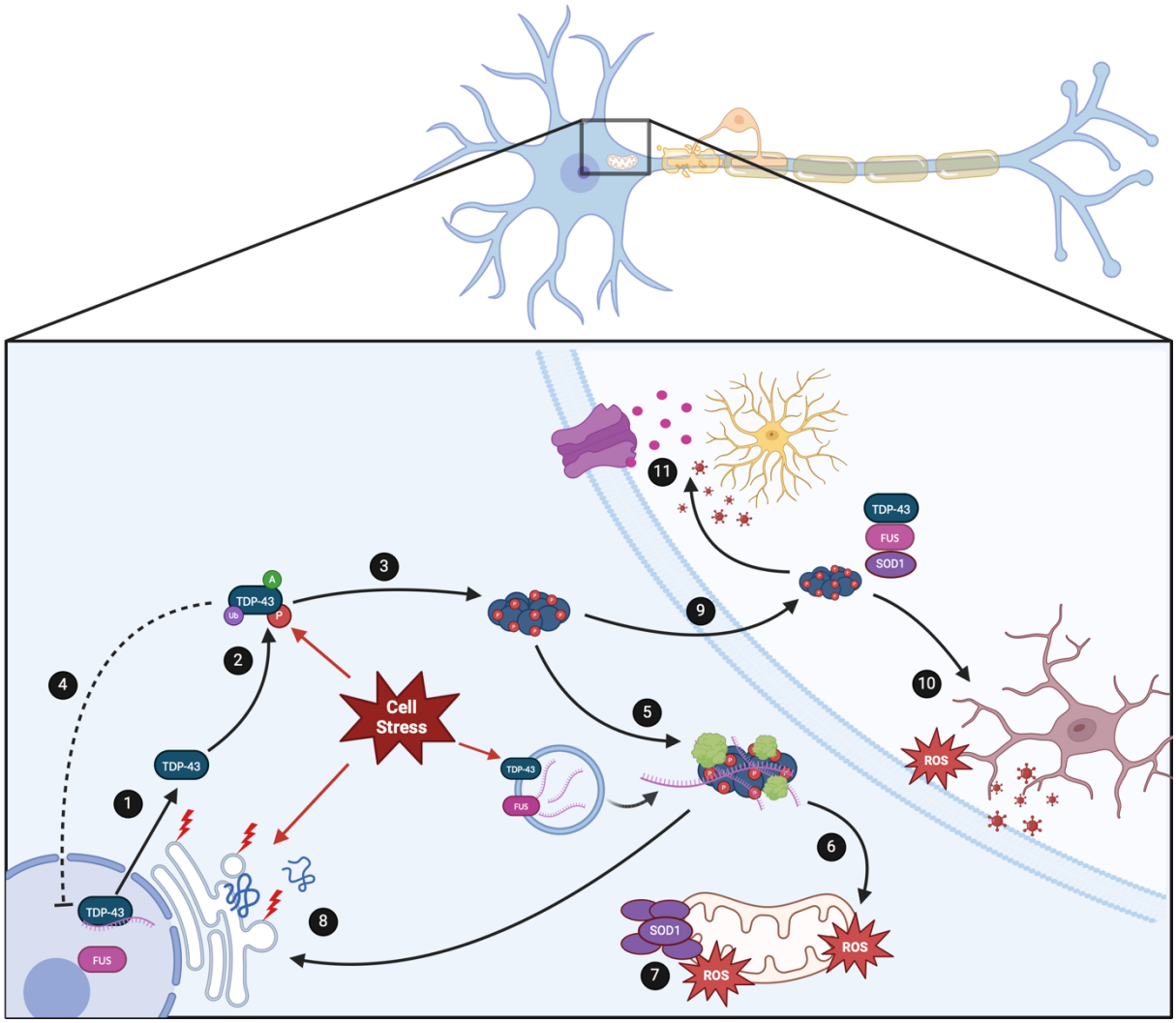

Figure 1: (1) In healthy cells, TDP-43 (and FUS) is primarily located in the nucleus, where it participates in RNA processing, with periodic shuttling to the cytoplasm for roles such as mRNA transport and stress granule formation. **(2)** In ALS, mutations and cellular stress, lead to mislocalisation of TDP-43 to the cytoplasm, where it is prone to misfolding and aggregation due to its prion-like domain. **(3)** Moreover, stress factors, induced by oxidative stress, inflammation, and mutations, trigger abnormal post-translations modifications (PTMs), such as phosphorylation and acetylation, which disrupt its structure, making it more prone to misfolding and aggregation. In an attempt to tag it for degradation, misfolded

TDP-43 is ubiquitinated, but instead accumulates in insoluble aggregates. **(4)** Mislocalisation of TDP-43 (and FUS) reduces their nuclear presence, leading to deficits in RNA processing for numerous genes, including those essential for neuronal health. **(5)** TDP-43 (and FUS) aggregates bind other proteins, stress granules, and sequester mRNAs, disrupting protein homeostasis and RNA metabolism. **(6)** Among bound proteins, are essential nuclear encoded mitochondrial proteins and RNAs, which disrupt mitochondrial function, leading to an increase in ROS production. **(7)** Also, in SOD1-related fALS, mutant SOD1 aggregates localise to mitochondria, disrupting their function, increasing ROS production. **(7)** Mutant SOD1 aggregates travel to the mitochondria, disrupting their function, increasing ROS production and contributing to oxidative stress. **(8)** Protein aggregates induce endoplasmic reticulum (ER) stress, activating the unfolded protein response (UPR), in an attempt to restore protein homeostasis. However, persistent UPR activation, when unresolved, leads to pro-apoptotic signalling pathways, ultimately triggering cell death. **(9)** Aggregated TDP-43, FUS, and SOD1 are released into the extracellular space, where they can be taken up by neighbouring cells, inducing protein misfolding and aggregation in a prion-like manner, propagating pathology. **(10)** These extracellular protein aggregates act as danger-associated molecular patterns (DAMPs), which are recognized by pattern recognition receptors on microglia (particularly Toll-like receptor 4 (TLR4)). This recognition activates microglia, triggering the release of pro-inflammatory cytokines and reactive oxygen and nitrogen species, contributing to further cellular stress and protein misfolding. **(11)** Signals from microglia and direct interactions with protein aggregates activate astrocytes, resulting in downregulation of EAAT2, the primary glutamate transporter in astrocytes, causing extracellular glutamate accumulation leading to excitotoxicity. *(Created in BioRender.com)*

Oxidative Stress

Substantial evidence shows that ALS is associated with elevated oxidative stress.[2] Oxidative stress results from an imbalance caused by excessive levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS), or the attenuated efficacy of antioxidant defence mechanisms.[2] ROS, characterised by their chemical reactivity, are molecules derived from oxygen, which has two unpaired electrons in its outer shell. Key examples of ROS include singlet oxygen, hydroxyl radicals, superoxide anion radicals, hydrogen peroxide, nitric oxide, and peroxynitrite anions, which are byproducts of both intracellular respiration and external reactions.[20] Exogenous sources include ionizing radiation, xenobiotics, viral and bacterial infections, smoking, alcohol consumption, poor diet.[21]

ROS and RNS play essential roles in regulating cellular homeostasis, influencing various processes such as redox signalling, immune defence, and protein folding. Many cellular organelles are equipped with mechanisms to scavenge and neutralise these reactive species, helping maintain redox balace within the cell.[20] This system includes antioxidants like glutathione, taurine, creatine, zinc, and vitamins E, C, and A. These work with enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) in an antioxidant defence mechanism.[14] However, once reactive species reach critical levels in cells, their noxious effects quickly eclipse their benign physiological functions as antioxidant countermeasures are overwhelmed.[20]

The central nervous system (CNS) is particularly vulnerable to oxidative stress due to several intrinsic factors. First, the CNS has a high metabolic rate, necessitating extensive energy production by mitochondria, which in turn generates large quantities of ROS. Second, neurons have a high content of polyunsaturated fatty acids (PUFAs) in their cell membranes, which are highly susceptible to lipid peroxidation. Lipid peroxidation disrupts membrane integrity, fluidity, and function, resulting in impaired cell signalling and increased cell death. Additionally, the CNS is enriched in redox-active metals such as iron and copper, which

catalyse the production of ROS, further exacerbation oxidative damage. Lastly, the CNS has relatively lower levels of antioxidant enzymes compared to other tissues, making it less equipped to neutralise ROS and maintain redox homeostasis. Despite these vulnerabilities, the antioxidant glutathione (GSH) is highly effective in neutralising ROS in neurons, when present in sufficient levels.[14]

Research has consistently demonstrated the critical role of oxidative stress in the pathophysiology of ALS, particularly by highlighting altered biomarker profiles in various biological samples. Specifically, elevated levels of malondialdehyde (MDA) modified protein, nuclear DNA 8-hydroxy-2'-deoxyguanoside (8-OHdG), and lipid peroxidation products have been identified in the urine, cerebrospinal fluid (CSF), and blood of ALS patients.[14] Moreover, postmortem ALS spinal cord tissue show increased protein carbonyls, 8-OHdG, MDA, 4-hydroxynonenal (4-HNE) conjugates, and nitrotyrosine products, all markers that reflect the extent of oxidative damage contributing to motor neuron degeneration. [20] Additionally, compromised antioxidant defences, as evidenced by reduced levels of catalase, glutathione, glutathione reductase, and glucose-6-phosphate dehydrogenase in patients' erythrocytes, point to a systemic oxidative stress that may significantly exacerbate the vulnerability of motor neurons to oxidative damage.[20]

Mutations in key ALS-related genes such as *SOD1*, *C9orf72*, and *FUS* significantly contribute to the oxidative stress observed in ALS. [2] SOD1 is a crucial Cu-Zn metalloprotein distributed across the nucleus, cytosol, peroxisomes, and mitochondria. It plays a pivotal role in cellular antioxidant defence by catalysing the conversion of superoxide radicals into hydrogen peroxide and oxygen, crucial for maintaining oxidative balance within cells.[14] In ALS, more than 200 mutations have been identified in coding and noncoding regions in the *SOD1* gene, affecting SOD1's activity in different manners. The harmful effects of *SOD1* mutations in ALS are thought to arise from a toxic gain-of-function rather than altered enzymatic activity, or from an initial misfolding that leads to further gain of function within the cytosol and organelles, resulting in impaired nuclear protection. Mutant SOD1 mediates these toxic effects either by generating cytotoxic amounts of hydrogen peroxide, reacting with nitric oxide to form large amounts of peroxynitrite, or by forming toxic aggregates due to decreased stability of SOD1 monomers and dimers.[20] Furthermore, mutated SOD1 may exploit hydrogen peroxide, a byproduct of its reactions, as a substrate for peroxidase activity, thereby producing hydroxyl radicals. In oxidative stress conditions, these mutant proteins are more prone to misfolding and form aggregates, intensifying the damage to neuronal cells.[20]

Mutations in the *C9orf72* gene, induce oxidative stress by selectively interacting with mitochondrial ribosomal proteins. This interaction leads to elevated mitochondrial membrane potential and increased ROS production, thereby fostering oxidative stress and contributing to neuronal damage in ALS.[20] In addition to genetic mutations, protein dysfunctions significantly influence ALS pathophysiology through mechanisms related to oxidative stress. For example, loss-of-function mutations in *FUS* lead to DNA strand breaks, heightening neuronal sensitivity to oxidative stress, suggesting that the normal role of FUS includes protective mechanisms against such stress.[20]

Concurrently, TDP-43 undergoes pathological changes under oxidative conditions. Chronic oxidative stress has been found to trigger the phosphorylation of TDP-43 by GADD34. Furthermore, cell-based assays show that oxidative stress acts as a signalling cue that promotes acetylation of TDP-43, contributing to the formation of aggregates. This acetylation not only disrupts TDP-43's ability to bind RNA but also facilitates the buildup on insoluble, hyperphosphorylated TDP-43 species, which are similar to the pathological inclusions observed in the CNS of ALS patients. These TDP-43 inclusions accumulate in the cytoplasm and sequester various miRNAs and proteins, including nuclear genome-encoded mitochondrial proteins, thereby disrupting mitochondrial function and further exacerbating oxidative stress.[2]

Mitochondria are the predominant ROS and RNS producing cellular organelles, mostly as by-products of ATP generation via oxidative phosphorylation and the electron transport chain. Neurons, characterized by their high metabolic demand, contain a high number of mitochondria to meet their energy needs. ROS directly damages proteins and lipids, thereby impairing the bioenergetic functions of mitochondria. Also, it has devastating impacts on mitochondrial DNA, leading to the inactivation of promotors and downregulation of mitochondrial gene expression. This accumulation of mitochondrial damage may lead to perturbed mitochondrial protein production, alterations in membrane permeability and calcium homeostasis, and an increase in lipid oxidation and protein carbonylation. [14]

In ALS, oxidative stress and inflammation form a self-amplifying cycle that accelerates motor neuron damage. ROS increase the expression of pro-inflammatory genes, setting of a cascade where neuroinflammation, in turn, stimulates ROS production. In physiological conditions, inflammation serves a protective role. However, in ALS, redox imbalances initiate inflammatory processes that drive the release of pro-inflammatory molecules and neopeptides, which further damage motor neurons. Key inflammatory mediators, including IL-1β, IL-6, interferons, and tumour necrosis factor, intensify ROS generation in non-phagocytic cells through the activation of NADPH oxidase (NOX). This vicious cycle of oxidative stress and neuroinflammation aggravates cellular damage, accelerating motor neuron degeneration.[22]

Mitochondrial Dysfunction

Mitochondrial dysfunction is recognized as a fundamental part in the pathophysiology of ALS.[2] One of the first changes seen in ALS patients' Bunina bodies and motor neurons are aggregated and structurally altered mitochondria, with a vacuolated and swollen appearance.[23] Additionally, animal and cell models of ALS consistently indicate the presence of morphologically abnormal mitochondria.[2,23] Interestingly, early disease stages in *in vivo* ALS models revealed fragmentation of the mitochondrial network and structural damage to mitochondria, suggesting that alterations to the morphology of mitochondria might occur as an upstream source of degeneration.[23]

In ALS, mitochondrial dysfunction presents through several pathways, including disrupted oxidative phosphorylation, excessive ROS production, diminished calcium buffering capacity, perturbed mitophagy, and altered mitochondrial structure and dynamics.[2] Moreover, apart from RNA toxicity, mitochondrial dysfunction seems connected, either directly or indirectly, to all proposed pathological pathways in ALS, including loss of protein homeostasis, excitotoxicity, and defective axonal transport.[23]

Physiologically, mitochondria are essential for cellular survival and metabolism, playing crucial roles in ATP production through oxidative phosphorylation, phospholipid synthesis, cellular calcium regulation, and apoptosis.[23] Neurons are highly active and have high metabolic demands, consuming 20% of the resting ATP production of the body, despite constituting only 2% of its mass.[23] Unsurprisingly, impairment in mitochondrial function is linked to many neurodegenerative diseases.[9] Moreover, as neurons are long-lived cells enduring throughout an individual's life, they are more vulnerable to cumulative damage from mitochondrial dysfunction.[23] Mitophagy normally clears defective mitochondria, but its efficiency decreases with age. This decline results in and increased buildup of malfunctioning mitochondria, causing higher levels of ROS production, and an increase in apoptosis.[24]

Mutations in various ALS-associated genes have been identified to affect mitochondrial function through diverse mechanisms.[2] Several key proteins linked to both fALS and sALS, including TDP-43, SOD1, FUS, C9orf72, and the C9orf72 GGGGCC repeat expansion-associated dipeptide repeat (DPR) protein, have been shown to directly interact with mitochondria.[23] Also notable is CHCHD10, a protein localized at the contact sites between the

inner and outer mitochondrial membranes. Mutations in CHCHD10 disrupt mitochondrial cristae and significantly alter mitochondrial morphology.[23] Mutations in the *SOD1* gene lead to the aggregation of mutant SOD1 protein in the mitochondrial intermembrane space where it significantly impairs the activity of the electron transport chain (ETC) complexes.[2] Aggregates of SOD1 are also suspected of interfering with the activity of voltage-dependent anion channel 1 (VDAC1), which exchanges ATP, ADP, and other respiratory substrates across the outer mitochondrial membrane.[23]

Mutations in *C9orf72* also significantly impact mitochondrial dynamics; the dipeptide repeat protein (DPR) poly-GR, associated with *C9orf72*-mutant ALS, binds to ATP5A1, a component of mitochondrial complex V, leading to its degradation.[23] Wild-type C9orf72 protein localizes to the inner mitochondrial membrane, where it stabilizes the translocase of inner mitochondrial domain containing 1 (TIMMDC1) protein, essential for the proper assembly of the oxidative phosphorylation complex. Impairment of mitochondrial complex I function has been observed in neurons derived from patients with *C9orf72* mutations.[2]

TDP-43 is indicated to play a role in preserving mitochondrial homeostasis through mitochondrial transcripts processing. However, mutations disrupt this regulatory function contributing to mitochondrial dysfunction.[2] Additionally, the cytoplasmic aggregation of TDP-43 sequesters essential mitochondrial miRNAs and proteins, encoded in the nuclear genome, further exacerbating mitochondrial dysfunction and oxidative stress.[10] Lastly, both wild-type FUS and its mutant form, FUS P525L, associate with mitochondrial chaperone heat shock protein 60 (HSP60). Accumulation of FUS in mitochondria was shown to increase ROS generation, and its overexpression is correlated with a reduction in ATP production.[12]

Mitochondria are particularly vulnerable to damage induced by ROS with mtDNA being especially susceptible by the absence of histones and limited repair mechanisms. Damage to mtDNA, proteins, and lipids can significantly impair mitochondrial function and has been implicated in the aging process.[23] Oxidative stress intertwines with protein aggregation, worsening ALS's pathophysiology. Misfolded SOD1 not only disrupts mitochondrial function but also boosts superoxide production, fostering a vicious cycle of mitochondrial damage and stress that promotes further SOD1 misfolding and damage to mitochondria.[20,23] Oxidative damage also plays a role in the aggregation of TDP-43, through acetylation, cysteine oxidation, and disulphide bond formation. Experiments in COS-7 cells showed that 4-hydroxynonenal (4-HNE), a product of lipid peroxidation, causes phosphorylation of TDP-43, which becomes insoluble and partially localized in the cytosol.[23]

Mitochondria are crucial organelles in maintaining calcium homeostasis in neurons, which is important for functions such as neurotransmitter release. Studies in SOD1 G93A transgenic mice show early declines in mitochondrial calcium loading capacity in the CNS, hinting at a potential causal relationship with ALS.[23] A key factor is the breakdown in communication between the endoplasmic reticulum (ER) and mitochondria, specifically at mitochondria-associated ER membranes (MAMs), which links around 5-20% of mitochondria closely to the ER.[24] Dysregulation of calcium homeostasis, possibly by defects in MAMs, is postulated to be a primary driver of motor neuron death in ALS.[23,24] Defects in these domains have been reported to culminate in axonal degeneration, by perturbing calcium homeostasis, ER function and autophagy, and mitochondrial dynamics.[2]

The loss of calcium homeostasis is intricately linked with other ALS-associated toxicities, including oxidative stress, loss of protein homeostasis, and axonal transport deficits.[23,24] Motor neurons have high levels of calcium permeable NMDA receptors, which makes them especially prone to excitotoxicity by excessive calcium influx.[23] They also depend more on mitochondrial calcium buffering, because their cytosolic buffering capacity is diminished by low expression of calcium buffering proteins such as calbindin and parvalbumin.[2] Calcium overload in mitochondria, often resulting from increased permeability

of NMDA receptors, disrupts oxidative phosphorylation boosts ROS production and augments oxidative stress.[23] Additionally, overload of mitochondrial calcium is postulated to deplete calcium in the ER, causing ER stress and protein misfolding.[24]

Furthermore, mitochondrial calcium exerts roles in ATP production, by regulating rate limiting enzymes in the Krebs cycle and oxidative phosphorylation. A reduction in ATP levels, commonly observed in ALS, directly impairs axonal transport by compromising the activity of molecular motor proteins, such as kinesins and dyneins, which depend on ATP to transport mitochondria, vesicles and other cellular components. Elevated cytosolic calcium can also disturb this transport by interfering with mitochondrial kinesin-1 receptor Miro1.[23] Additionally, elevated cytosolic calcium levels activate calpain, which cleaves TDP-43, resulting in the generation of aggregation-prone fragments, exacerbating TDP-43 pathology in ALS.[23]

Ultimately, chronic overload of mitochondrial calcium and protein misfolding can both induce apoptosis.[9] Excess calcium triggers the opening of the mitochondrial permeability transition pore (mPTP), leading to mitochondrial outer membrane permeabilization and the release apoptogenic factor cytochrome c into the cytosol, initiating the intrinsic apoptotic pathway by activating caspase-9.[25] Moreover, protein misfolding induces ER stress, activating the unfolded protein response (UPR), which, if unresolved, triggers pro-apoptotic pathways through CHOP and caspase-12 activation.[9] **Figure 2** illustrates a schematic from the crosstalk of oxidative stress and mitochondrial dysfunction with other pathways.

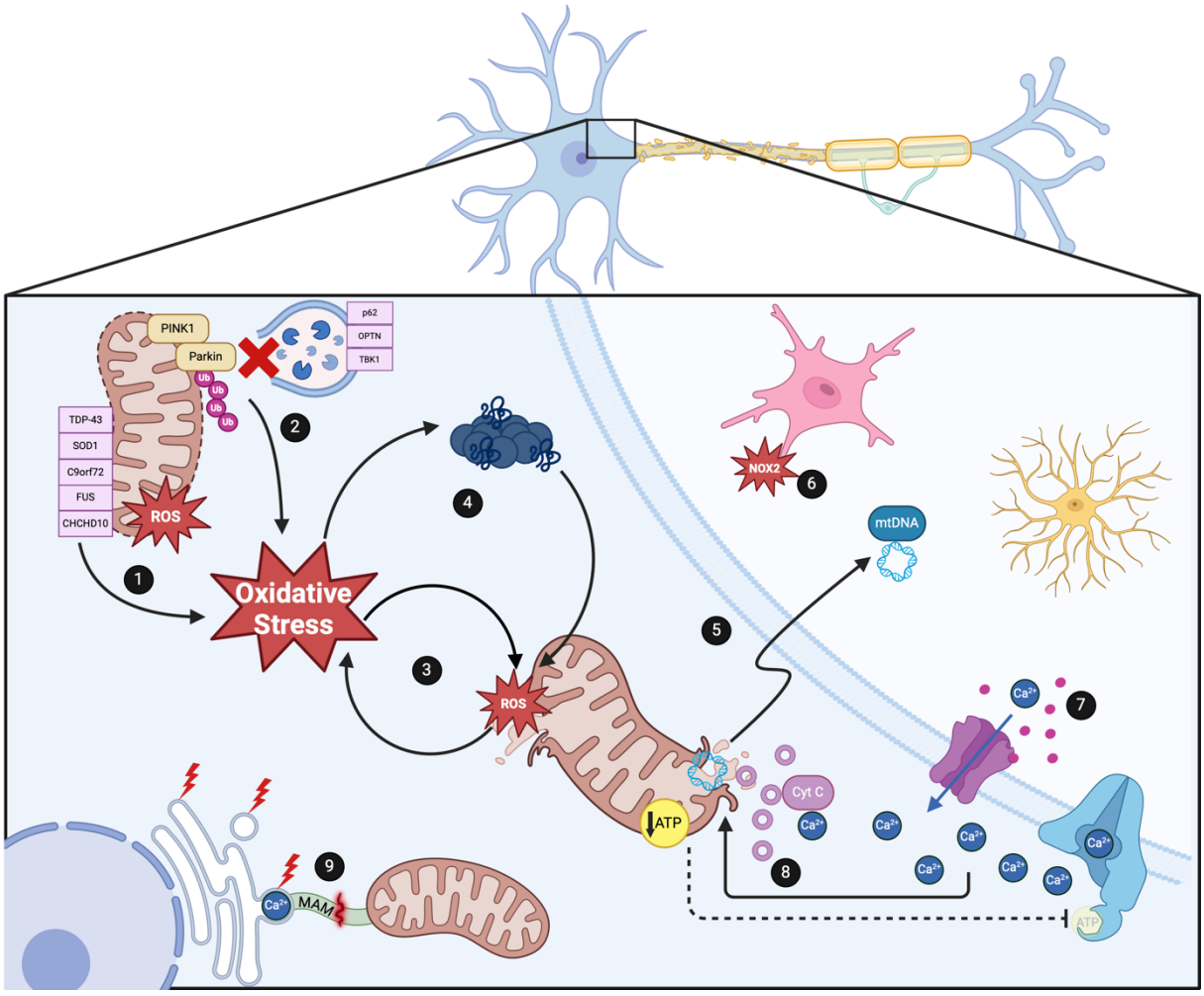

Figure 2: (1) Mutations in ALS-associated proteins, including TDP-43, SOD1, C9orf72, FUS, and CHCD10, disrupt mitochondrial function and induce excess ROS production, contributing to oxidative stress.[17] **(2)** Mitophagy, the process clearing defective mitochondria, is impaired in ALS. In healthy cells, PINK1 recruits Parkin to tag damaged mitochondria with ubiquitin for degradation by autophagosomes. Mutations in mitophagy-related proteins, including p62, OPTN, and TBK1, are associated with earlier disease onset in fALS, highlighting the importance of mitophagy. TDP-43 and FUS regulate Parkin expression and loss of nuclear function of TDP-43 has been linked to reduced Parkin protein levels, impairing mitophagy and contributing to an increase in intracellular ROS.[23] **(3)** Mitochondria are particularly vulnerable to oxidative stress, which damages mitochondrial DNA (mtDNA), proteins, and lipids, triggering a cycle of increased ROS production and mitochondrial dysfunction.[17]**(4)** Mutations and oxidative stress drive aggregation of TDP-43, SOD1, and FUS. These aggregates sequester mitophagy proteins and essential nuclear encoded mitochondrial proteins and RNAs, disrupting mitochondrial function, amplifying oxidative stress, and accelerating further protein aggregation in a self-perpetuating cycle.[17] **(5)** Oxidative damage to mitochondria induces leakage of mtDNA into the cytosol or release through mitochondrial derived vesicles (MDV). Additionally, TDP-43 promotes opening of the mitochondrial permeability transition pore (mPTP), causing leakage of mtDNA and cytochrome c, and other molecules, into the cytosol.[26,27]**(6)** Neuronal cells release most mtDNA via exosome vesicles, which neighbouring microglia and astrocytes take up through endocytosis. Resembling bacterial DNA, mtDNA acts as a DAMP by binding to endosomal Toll-like receptor 9 (TLR9) and activating the cytosolic cGAS/STING pathway, the primary cytosolic DNA sensor in these microglia and astrocytes. This activation promotes microglial polarisation toward to pro-inflammatory M1 phenotype, contributing to a neuroinflammatory environment and an increase in ROS production.[26,27] **(7)** Pro-inflammatory cytokines, including IL-1α, IL-1β, and TNF-α, drive astrocytes to a reactive state. In this activated state, astrocytes release pro-inflammatory cytokines like IL-6 and TNF-α, amplifying neuroinflammation.[15] They also reduce glutamate uptake, mainly through EAAT2 downregulation, leading to excessive extracellular glutamate. This imbalance induces excitotoxicity in neighbouring neurons, increasing calcium influx and contributing to neuronal damage.[28] **(8)** Mitochondrial dysfunction leads to reduced ATP production, impairing ATP-dependent calcium pumps, compromising the cell's ability to regulate calcium levels. Concurrently, excitotoxicity-driven calcium influx causes intracellular calcium overload. This excess calcium boosts ROS production, exacerbating oxidative stress, and opens the mPTP, releasing pro-apoptotic factors.[25] **(9)** The ER is highly dependent on mitochondrial function for maintaining calcium homeostasis, ATP supply, and lipid synthesis. Approximately 20% of mitochondrial surface connects to the ER at mitochondria-associated membranes (MAMs), enabling calcium critical calcium and phospholipid transfer. Mutations in MAM-related genes (e.g. SIGMAR1 and VAPB) are linked to fALS, underscoring the importance of the exchange. Mitochondrial dysfunction reduces ATP, impairs ER calcium regulation, disrupts protein folding, and triggers ER stress. Prolonged ER stress activates the UPR, which, if unresolved, leads to apoptosis.[24,29,30] *(Created in BioRender.com)*

Excitotoxicity

Excitotoxicity in ALS is driven by the overactivation of postsynaptic glutamate receptors. Extended stimulation by synaptic glutamate induces prolonged neuronal firing, causing a rise in intracellular calcium which exerts downstream neurotoxic effects.[2] Motor neurons possess cell-specific characteristics, including a high presence of calcium-permeable NMDA receptors and reduced expression of the calcium buffering proteins calbindin and parvalbumin, which increases their susceptibility to excitotoxic damage.[2]

Glutamate-mediated excitotoxicity and neuronal hyperexcitability are considered key factors in ALS's aetiology.[9] Both animal studies and analysis of CNS tissue from ALS patients have demonstrated a decreased expression of GLT1-EAAT2, the primary glutamate re-uptake transporter.[2] Furthermore, mutations in *C9orf72* in iPSC-derived motor neurons have been shown to enhance excitotoxicity mediated by calcium-permeable NMDA receptors and impair mitochondrial calcium-buffering capabilities.[2] In mice, overexpression of the mutant *SOD1-G93A* gene leads to early disruptions in mitochondrial calcium homeostasis and its subsequent motor neuron degeneration mimics human disease progression.[25]

Mitochondria play a crucial role in regulating calcium homeostasis; they can take up enormous amounts of calcium quickly through the mitochondrial calcium uniporter (MCU), a high-capacity low affinity uptake system, and then gradually release it back into the cytosol. Underscoring their importance in managing calcium fluxes in neurons, is CNS specific expression of the MICU3 isoform, which gives the MCU a higher affinity for calcium than in any other cell type.[25] Furthermore, mitochondrial function is essential for powering ATP-dependent calcium pumps located on the plasma membrane, where they expel intracellular calcium, and within the ER, where they sequester calcium from the cytosol.[25]

Calcium influences numerous mitochondrial functions, such as activating rate-limiting metabolic enzymes to promote ATP production, enhancing buffering capacity through feedback mechanisms to avert neuronal toxicity, and regulating mitochondrial transport (anterograde/retrograde).[23] Synaptic function heavily relies on precise regulation of mitochondrial function and energy supply, essential for maintaining calcium homeostasis and ionic balance, which depends on membrane transporters to restore ion gradients after neuronal signalling. The absence of mitochondria near synapses leads to increased hyperexcitability due to the lack of homeostatic balancing mechanisms.[25]

Moreover, prolonged exposure to high levels of glutamate also contribute to oxidative stress by impairing the uptake of cysteine by the glutamate-cysteine antiporter. Cysteine is a crucial substrate for the production of GSH, the most important neuronal antioxidant.[2]

Given that excitotoxicity arises from the overstimulation of glutamate receptors, it is plausible to hypothesise that elevated levels of extracellular glutamate may contribute to ALS progression. Glutamate levels in the CSF have been investigated as a potential biomarker for excitotoxicity in ALS, with some studies demonstrating elevated levels in ALS patients compared to healthy controls. However, other studies found similar levels of glutamate in the CSF of ALS patients and healthy controls.[6] Nonetheless, glutamate concentrations in serum were found to be decreased after a 6-month treatment with riluzole, demonstrating its utility as a serum biomarker for ALS in response to drug intervention.[6] Riluzole, the first FDA-approved therapy for ALS, has been shown in population studies to increase survival by 6 to 19 months. Its mechanism of action includes blocking presynaptic voltage-gated sodium channels, reducing glutamate release into the synaptic cleft, and thereby mitigating excitotoxicity.[2]

Neuroinflammation

Neuroinflammation is a critical component of ALS pathogenesis, contributing to the progressive degeneration of motor neurons.[2] In ALS, microglia and astrocytes, which are normally involved in neuronal support and regulation, become chronically activated. This activation shifts them from protective roles to pro-inflammatory states, leading to the release of cytokines and chemokines that exacerbate neuronal damage and drive disease progression.[15]

Microglia, the primary immune cells of the CNS, initially adopt a neuroprotective (M2) phenotype in ALS. In this state, they secrete anti-inflammatory factors like IL-4, IL-10, and TGF-β, which promote tissue repair and motor neuron survival. However, as the disease progresses, oxidative stress and the accumulation of misfolded proteins, such as TDP-43 and mutant SOD1, trigger a shift to the neurotoxic (M1) phenotype.[15,16]In this M1 state, microglia release pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, perpetuating inflammation.[16]

Astrocytes, which normally maintain homeostasis in the CNS, become reactive in ALS. This reactivity is driven by signals such as IL-1α, TNF-α, and C1q, released by activated microglia.[22] Reactive astrocytes lose their ability to regulate glutamate levels and instead release pro-inflammatory mediators like NO and prostaglandin E2, which amplify the inflammatory environment. In particular, astrocytes expressing mutant SOD1 release toxic soluble factors that are selectively damaging to motor neurons, further acceleration neurodegeneration.[22]

ROS are central to the inflammatory cascade in ALS. ROS activate inflammatory pathways, such as the NF-κB pathway, in microglia, leading to the production pro-inflammatory cytokines.[2,22] This increase in ROS not only amplifies oxidative stress but also damages neuronal proteins, lipids, and DNA, particularly mitochondrial DNA (mtDNA). The release of damaged mtDNA into the cytosol acts as a damage-associated molecular pattern (DAMP), further activating microglia and perpetuating inflammation.[15,16]

Moreover, inflammatory cytokines, especially TNF-α and IL-1β, impair mitochondrial function by increasing ROS production, which further damages mitochondrial components.[15] Damaged mitochondria release mtDNA, which triggers the cGAS-STING pathway, a key regulator of the inflammatory response. This pathway amplifies the inflammatory loop, linking mitochondrial dysfunction to neuroinflammation.[16]

In addition, activated microglia and astrocytes contribute to excitotoxicity by releasing pro-inflammatory cytokines, which enhance excitatory signalling of glutamate by reducing the efficiency of the glutamate transporter EAAT2. This results in excessive activation of NMDA receptors on motor neurons, leading to calcium overload, oxidative stress, and further neuronal injury, creating a self-perpetuating cycle.[2,22]

Inflammatory biomarkers could aid in understanding the progression of ALS and. Elevated levels of pro-inflammatory cytokines, such as IL-6, IL-8, and TNF-α, are consistently observed in both plasma and CSF of ALS patients.[6,7] Theses cytokines not only contribute to the inflammatory profile of the disease but have also been correlated with disease progression, making them potential prognostic biomarkers.[6]

Moreover, studies have demonstrated that inflammatory chemokines, including MCP-1, MIP-1β, and IL-17, are significantly elevated in ALS, especially in the CSF, emphasizing their potential as biomarkers.[6]

Additionally, alterations in immune cell populations, such as increased neutrophil-to-lymphocyte ratio, can indicate peripheral inflammation.[7]

Interestingly, while pro-inflammatory cytokines correlate with disease severity, TGF-β1, which has anti-inflammatory properties, is also elevated in ALS, suggesting a complex interplay of inflammatory pathways that may influence disease progression.[6]

Discussion

This review aims to elucidate key ALS pathological contributing to motor degeneration, including protein aggregation, oxidative stress, mitochondrial dysfunction, and neuroinflammation. These interconnected pathways form a complex, self-reinforcing network that amplifies neuronal injury, highlighting the inadequacy of single-target pathway therapies. Given ALS's multifactorial nature, a shift towards multi-target therapies is necessary to address multiple pathological processes concurrently, supported by pathway-specific biomarkers. To assess therapeutic impact. By targeting ALS pathology on multiple fronts, combination therapies offer a promising strategy to slow disease progression and improve clinical outcomes.

Findings

The findings of this review underscore the complex interplay among ALS pathological pathways, where protein aggregation, oxidative stress, mitochondrial dysfunction, excitotoxicity, and neuroinflammation converge to drive motor neuron degeneration. Protein aggregation, central to ALS pathology, results from the misfolding and cytoplasmic accumulation of TDP-43, SOD1, and FUS. These aggregated proteins disrupt mitochondrial function, increase ROS production, and release inflammatory signals that activate microglia, thereby worsening oxidative stress, fuelling neuroinflammation, and disrupting calcium homeostasis, to ultimately amplify excitotoxicity and neuronal damage.

Oxidative stress plays a pivotal role in ALS pathology by intensifying protein misfolding, mitochondrial dysfunction, and neuroinflammation. Elevated ROS levels lead to modifications in TDP-43, SOD1, and FUS, increasing their aggregation propensity. Oxidative damage to mitochondria also reduces energy production and calcium buffering, thereby heightening excitotoxicity. Additionally, ROS activate microglia, reinforcing a cycle of neuroinflammation.

Mitochondrial dysfunction is a crucial driver of ALS pathology, contributing to energy deficits, disrupted calcium homeostasis, and elevated oxidative stress. Damaged mitochondria generate excessive ROS, which exacerbate protein misfolding and aggregation. The resulting energy deficits impair cellular repair processes, while impaired calcium buffering increases neuronal vulnerability to excitotoxicity. Additionally, mitochondrial damage triggers the release of mtDNA and apoptotic factors into the cytoplasm, activating inflammatory pathways like the cGAS-STING pathway, which further amplifies neuroinflammation and contributes to cell death.

Implications for treatment and research

The complexity of ALS pathology, characterised by the interconnection of multiple pathological pathways, poses significant challenges for treatment. Traditional monotherapies, aimed at targeting a single pathway, have consistently failed to produce meaningful clinical outcomes. It can be argued that this is largely due to the interconnected pathways. For instance, attempting to reduce oxidative stress without addressing mitochondrial dysfunction, may lead to limited results, as mitochondria are a significant source of ROS.

Similarly, targeting excitotoxicity alone may be insufficient if inflammation and protein aggregation continue to contribute to motor neuron damage. Thus, these interactions necessitate a shift in therapeutic strategies towards combination therapies that can concurrently modulate multiple pathological processes. This approach offers the potential not only to slow disease progression but also to mitigate the cascading effects of neurodegeneration, providing a more comprehensive and effective treatment paradigm for ALS.

The need for biomarkers

A critical hurdle in ALS clinical trials is the lack of reliable biomarkers, which hampers the ability to detect subtle therapeutic effects. Most ALS clinical trials fail despite preclinical evidence suggesting the treatment has potential, largely because traditional outcome measures, such as survival or the ALSFRS-R scale, do not capture early or pathway-specific effects of the drug. Without biomarkers, it is challenging to assess whether a therapy is influencing underlying disease mechanisms, like oxidative stress, protein aggregation, or neuroinflammation, which may not directly translate into clinical improvements.

The use of biomarkers could prevent the premature dismissal of therapies that don't meet primary outcome measures, preserving data on their effects within specific ALS pathways. This approach allows for a more comprehensive evaluation of biological efficacy, potentially identifying therapies that may later be valuable as part of a combination strategy. Additionally, incorporating biomarkers into clinical trials creates opportunities to adapt trials dynamically in response to biomarker data. When a response is observed, dosage and frequency can be optimized; if no effects are detected, trials can be ended prematurely, conserving resources and minimising patient exposure to ineffective therapies.

Hurdles of biomarker implementation

One of the main challenges in implementing biomarkers in ALS clinical trials is the lack of standardisation and validation across studies. Many biomarkers, while promising in preclinical or early-phase trials, have not been consistently validated in larger clinical settings, limiting their utility and reliability.

Additionally, integrating biomarkers into clinical trial designs is hindered by a lack of standardised protocols, complicating their use as reliable outcome measures. Without consistent guidelines, different trials measure and interpret biomarkers in various ways, leading to variability that undermines their validity as dependable indicators of therapeutic impact. This inconsistency also complicates regulatory approval, as biomarkers must undergo stringent validation to demonstrate reproducibility and comparability across studies.

Biomarker panel

Incorporating a panel of biomarkers as secondary outcome measures in ALS clinical trials could accelerate their validation and offer deeper insights into the biological mechanisms affected by candidate therapies. While these biomarkers may not yet be fully accepted as primary endpoints, their consistent use across multiple trials would help create a robust dataset, revealing correlations with disease progression and therapeutic efficacy. In addition, as artificial intelligence advances, its role in future biomedical research is inevitable, especially for complex diseases like ALS. Yet, realising its potential depends on extensive data for machine learning, which can be gathered now in ALS trials to expedite its use for diagnosis and to predict disease progression and therapeutic efficacy.

Furthermore, incorporating a standard set of biomarkers as secondary outcome measures in ALS trials could provide crucial data for designing combination therapies. By showing which pathological pathways are engaged by a therapy and which remain unaddressed, biomarkers guide the selection of additional treatments to target specific pathways. Ultimately, the validation of pathway specific biomarkers could optimize therapy combinations, enhancing the likelihood of successfully slowing or halting disease progression. **The appendix** offers a preliminary list of pathway-specific biomarkers.

Conclusion

In conclusion, the intricate network of pathologic pathways implicated in ALS necessitates a comprehensive therapeutic strategy. Given that singular pathway modulation

often results in compensatory mechanisms that perpetuate neurodegeneration, a multitarget approach or combination therapies emerge as imperative. By concurrently addressing multiple pathways, these strategies hold the potential to effectively disrupt the cascade of neurodegenerative events in ALS, offering a more promising avenue for altering the course of the disease.

Appendix: Preliminary Pathway-Specific Biomarker Panel for ALS

This appendix outlines a preliminary set of pathway-specific biomarkers that, pending further validation, hold potential for measuring therapeutic impact across key ALS pathways. Each biomarker is selected based on current preclinical and early-phase trial findings, with suggested utility in reflecting effects on protein aggregation, oxidative stress, mitochondrial dysfunction, excitotoxicity, and neuroinflammation.

A. Protein Aggregation Biomarkers

Biomarker	Measured in	Indicator of	Response Function
Phosphorylated TDP-43 (pTDP-43)	CSF	Aggregated and phosphorylated TDP-43 in the cytoplasm of neurons and glial cells	Reflects ALS-specific proteinopathy; changes in levels may indicate disease progression and therapeutic impact.[19,31]
SOD1	CSF	Misfolded and aggregated SOD1	Indicates pathological SOD1 accumalution; changes may reflect disease severity and response to therapy.[6]
FUS	CSF	Mislocalised and aggregated FUS	Reflects ALS related FUS-proteinopathy; alterations can indicate disease progression and potential treatment effects.[31]
Neurofilament light chain (NfL)	CSF, Blood	Axonal degeneration	Elevated levels correlate with neurodegeneration; useful for monitoring disease progression and treatment efficacy.[8,32,33]

Phosphorylated TDP-43 (pTDP-43) is a hallmark of ALS-related proteinopathy, with specific phosphorylation at serines 409/410 distinguishing pathological forms from normal TDP-43. This biomarker, measured in the CSF, provides a readout of TDP-43 aggregation and mislocalisation in the central nervous system.[19,31]

SOD1 has been validated in as pharmacodynamic biomarker in ALS. Toferson, a SOD1-targeting antisense nucleotide (ASO) has been shown to SOD1 in the CSF of SOD1-fALS patients in the VALOR trial [NCT02623699].[6]

FUS, similar to TDP-43, mislocalises and aggregates in the cytoplasm in ALS, from where it is excreted into the CSF, making it a potential biomarker for tracking disease progression and therapeutic impact.[31]

Neurofilament light chain (NfL) is a structural protein of the neuronal cytoskeleton, released into the CSF when axonal damage occurs.[6] In an FDA study, NfL levels have shown a positive correlation with validated clinical endpoints, including ALSFRS-R score, disease progression

(DP) slope, and mortality.[32] Rather than tracking a specific pathological pathway, NfL is a general marker for neuronal injury.[31]Importantly, NfL serves as a promising biomarker for measuring phenoconversion in asymptomatic individuals with fALS-associated mutations. Longitudinal measurements of NfL in the CSF and blood can signal impending symptom onset, with studies indicating that NfL levels begin to rise months to years before clinical symptoms begin. Research on at-risk individuals has shown that serum NfL levels increase around 12 months before symptoms onset in *SOD1* mutation carriers, up to 3.5 years before phenoconversion in *C9orf72* mutation carriers, and approximately 2 years for *FUS* mutation carriers.[8]

B. Oxidative Stress Biomarkers

Biomarker	Measured in	Indicator of	Response Function
8-hydroxy-2′-deoxyguanosine (8-OHdG)	Urine, CSF, Blood	Oxidative DNA damage	A decrease in levels indicates reduced DNA oxidation and successful oxidative stress mitigation.[6,34–36]
Malondialdehyde (MDA)	Blood, Urine	Lipid peroxidation	Lower levels reflect reduced lipid peroxidation and improved cellular membrane integrity.[20,34,37,38]
4-hydroxynonenal (4-HNE)	Blood, Tissue samples	Lipid peroxidation	Reduction reflects lower oxidative lipid damage.[20,34,38]
3-nitrotyrosine	CSF, Blood	Nitrosative stress	Decreased levels indicate reduced nitrosative stress and protection from RNS damage.[20,36]
GSH/GSSG ratio	Blood,	Cellular antioxidant capacity	An increase in ratio reflects improved antioxidant response and redox balance.[34,37]
Catalase	Erythrocytes	Antioxidant capacity	Elevated levels suggest enhanced antioxidant capacity.[20]

Because reactive oxygen species (ROS) are highly short-lived, directly measuring their concentrations is nearly impossible. Instead, oxidation products—biomarkers resulting from oxidative modification of proteins, lipids, and DNA—are measured to quantify oxidative stress levels. These stable byproducts offer an indirect but reliable measure of cumulative oxidative damage.[34]

8-hydroxy-2′-deoxyguanosine (8-OHdG) is formed when hydroxyl radicals interact with guanine bases in DNA, leading to mutations and impaired DNA function. 8-OHdG levels can measure the effectiveness of therapies aimed at reducing oxidative damage.[33–35]

MDA and **4-HNE** are by products of lipid peroxidation, which are formed when ROS attack PUFAs in cell membranes.[20,34,38]

3-nitrotyrosine is a biomarker of protein damage due to nitrosative stress. It is formed when tyrosines is nitrated by reactive nitrogen species (RNS), such as peroxynitrite, and nitrogen dioxide.[36]

The **GSH/GSSG ratio**, of reduced glutathione (GSH) to oxidized glutathione (GSSG), is a key indicator of the cell's oxidative state, with low ratio's indicating high oxidative stress.[34]

Catalase is an enzyme that decomposes hydrogen peroxide into water and oxygen, directly reducing ROS. Its activity can be measured in patients' erythrocytes and reflects the antioxidant capacity.[20]

C. Mitochondrial Dysfunction Biomarkers

Biomarker	Measured in	Indicator of	Response Function
Mitochondrial DNA (mtDNA)	CSF, Blood	Mitochondrial damage	Elevated levels indicate mitochondria damage and dysfunction.[26,27,39,40]
Lactate/Pyruvate ratio	CSF, Blood	Shift to anaerobic metabolism	High ratio suggests mitochondrial dysfunction, normalisation indicates restored function.[41]
Mitochondrial Membrane Potential	Erythrocytes, Fibroblasts	Mitochondrial membrane integrity and function	Reduced potential reflects membrane dysfunction, improvement suggests therapeutic benefit.[40]
Oxidized Cardiolipin	Blood, CSF	Mitochondrial oxidative stress, and damage	Reduced levels indicate reduced oxidative stress in mitochondria.[40]
Phosphatidylethanolamine (PE) levels	Blood, Tissue	Mitochondrial membrane integrity	Levels provide insight into the structural integrity of mitochondrial membranes.[42]

Elevated levels of **mtDNA** in CSF and blood serve as an indicator of mitochondrial damage, as mtDNA is typically released into biofluids following mitochondrial disruption. In ALS, mtDNA levels correlate with disease progression, making it a potential marker for mitochondrial health. Reductions during treatment could indicate stabilized mitochondrial function.[39,40]

The Lactate/Pyruvate ratio reflects the balance between aerobic and anaerobic metabolism, with higher values indicating mitochondrial dysfunction and increased reliance on anaerobic pathways. In ALS, and elevated ratio highlights metabolic stress, and normalization may signal restored mitochondrial efficiency.[41]

Mitochondrial membrane potential is essential for ATP production and a reduction reflects compromised mitochondrial integrity in ALS. Improvements in this potential suggest a stabilization in mitochondrial function.[40]

Oxidized Cardiolipin: Cardiolipin is a mitochondria-specific phospholipid and crucial for mitochondrial structure and function, by stabilizing respiratory chain complexes. Due to its unique fatty acid composition, cardiolipin is highly susceptible to oxidation. When oxidized, cardiolipin undergoes structural changes impairing mitochondrial integrity, disrupting ATP synthesis and promoting mitochondrial degradation. Monitoring oxidized cardiolipin levels offers a pharmacodynamic marker for mitochondrial oxidative stress.[40]

Phospatidylethanolamine (PE) is a major phospholipid primarily synthesized in mitochondria and plays a crucial role in maintaining mitochondrial membrane structure and function. In ALS, PE levels decrease with disease progression. Reduction in PE is thought to be associated with mitochondrial dysfunction, as it negatively affects oxidative phosphorylation and ATP production.[42]

D. Excitotoxicity Biomarkers

Biomarker	Measured in	Indicator of	Response Function
Glutamate	Serum	Excess glutamate	Decreased levels indicate response to treatment.[33]
S100B	CSF	Astrocyte reactivity and impaired glutamate regulation	Elevated levels reflect astrocytic contribution to excitotoxicity.[8]

Research indicates that serum **glutamate** levels are more reliable indicators of excitotoxicity in ALS then CSF glutamate, as CSF levels have shown inconsistency across studies. Serum glutamate, however, has proven responsive to treatment, with reductions observed after six months of riluzole therapy.[33]

S100B serves as a biomarker reflecting astrocytic contributions to excitotoxicity. In ALS, astrocytes release S100B in response to cellular stress and reactive inflammatory states. Elevated levels of S100B indicate heightened astrocyte reactivity, which correlates with excitotoxicity due to impaired extracellular glutamate regulation by astrocytes.[8]

E. Inflammation Biomarkers

Biomarker	Measured in	Indicator of	Response Function
CHIT1 (Chitotriodase)	CSF,	Microglial activation	Decrease indicates response to anti-inflammatory treatment.[7,43–45]
CHI3L (YKL-40)	CSF, Blood	Astrocyte activation and tissue remodeling	Reductions suggest effective anti-inflammatory response.[43,44]
IL-6	CSF, Blood	Systemic and neuroinflammation	Reduced levels indicate a decrease in systemic inflammation and neuroinflammation.[33,44]
IL-8	CSF	Immune cell recruitment	Reduced levels suggest reduced neutrophil recruitment and inflammation.[43,44]
IL-2	CSF, Blood	T-cell activation and proliferation	Reduced levels indicate decreased T-cell response.[44]
TNF-α	CSF, Blood	Neuroinflammation and cell death	Decreased levels reflect reduced neuroinflammation.[33,44]
CCL2/MCP-1	CSF, Blood	Monocyte and macrophage recruitment	Reductions indicate therapeutic impact on immune cell recruitment and inflammation.[43,44]
IFN-γ	CSF, Blood	Macrophage activation and immune response	Decreased levels suggest reduced T-cell and macrophage-driven inflammation.[44]
TGF-β	CSF, Blood	Immune regulation and tissue repair	Adjusted levels indicate modulation of inflammation pathways.[44]
CRP (C-reactive protein)	Blood	Systemic inflammation, microglial activation	Decreased levels reflect reduced systemic inflammation.[7,44,45]

sCD14	CSF, Blood	Monocyte activation	Lower levels indicate reduced monocyte activation and systemic inflammation.[43]

CHIT1 is associated with microglial activation in ALS, and its expression levels correlate with disease progression and respiratory function decline. As a pharmacodynamic marker, a decrease in CHIT1 could indicate a response to anti-inflammatory treatments aimed at reducing microglial activation.[7,43]

CHI3L1, primarily excreted by reactive astrocytes, is involved in tissue remodelling and immune activation. Its levels are notably elevated in ALS, correlating with disease progression and cognitive decline. CHI3L1 can serve as a pharmacodynamic biomarker, with treatment-induced reductions reflecting therapeutic efficacy in modulating astrocytic activity.[43,44]

IL-6 is a pro-inflammatory cytokine linked to systemic and CNS inflammation, with levels correlating to disease severity and respiratory decline in ALS. A phase 2 trial of the IL-6 receptor blocker tocilizumab in ALS indicated that decreased IL-6 levels may serve as a pharmacodynamic marker, demonstrating anti-inflammatory target engagement in therapeutic interventions aimed at cytokine reduction.[33,44]

IL-8 uniquely elevates in ALS compared to other neurodegenerative diseases and is associated with neutrophil recruitment. Tracking IL-8 levels as a pharmacodynamic marker could provide insights into the efficacy of treatments that aim to attenuate neutrophil-driven inflammation in ALS.[43,44]

IL-2 is involved in T-cell activation and proliferation, with elevated levels in ALS associated with muscular dystrophy severity. Decreases in IL-2 after intervention may indicate effective modulation of T-cell responses.[44]

TNF-α, predominantly produced by activated microglia and astrocytes, plays a dual role in neuroinflammation, with neuroprotective or neurotoxic effects depending on receptor activation pathways. In ALS, TNF-α elevations are associated with neurotoxic inflammation, and reductions could act as a pharmacodynamic measure of the therapeutic impact on neuroinflammatory pathways.[33,44]

CCL2/MCP-1 recruits immune cells, including T cells and macrophages to the CNS, exacerbating ALS-related neuroinflammation and blood-brain barrier disruption. It levels correlate with rapid disease progression. A reduction in CCL2 could reflect decreased immune cell infiltration and inflammatory modulation, indication the efficacy of interventions targeting monocyte/macrophage recruitment.[43,44]

IFN-γ is produced by microglia and peripheral immune cells. IFN-γ drives macrophage activation and inflammatory immune responses. It levels correlate with ALS progression, with reductions suggesting attenuated T cell and macrophage activation.[44]

TGF-β is involved in immune regulation and tissue repair, with elevated levels linked to muscle weakness and disease progression in ALS. Monitoring TGF-β levels offers insights into the modulation of immune responses and repair mechanisms.[44]

CRP, a systemic inflammatory marker, is associated with increased blood-brain barrier permeability and microglial activation. In ALS, CRP levels rise as the disease progresses. A reduction in CRP could reflect an intervention's systemic anti-inflammatory effects, particularly treatments targeting peripheral inflammation and microglial reactivity.[7,45]

sCD14, elevated in ALS, sCD14 reflects monocyte activation and correlates with disease progression speed. As a pharmacodynamic marker, sCD14 reduction may indicate effective modulation of monocyte-driven inflammation.[43]

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