INTERNATIONAL JOURNAL OF NOVEL RESEARCH AND DEVELOPMENT (IJNRD)
© 2026 IJNRD | Volume 11, Issue 8, August 2026 | ISSN: 2456-4184 | IJNRD.ORG

A REVIEW ON BLOOD DIAGNOSTIC BIOMARKERS IN AMYOTROPHIC LATERAL SCLEROSIS

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ABSTRACT:

Amyotrophic lateral sclerosis (ALS) is a severe, progressive neurodegenerative disease for which effective therapeutic options remain limited. The disorder primarily involves the degeneration of upper and lower motor neurons located in the brain, brainstem, and spinal cord, leading to gradual loss of motor function. A key pathological feature of ALS is the accumulation of abnormal proteins within neurons and supporting glial cells. In recent years, considerable research has focused on identifying biomarkers in cerebrospinal fluid, blood, and urine to improve early diagnosis, monitor disease progression, and assess treatment response. Among the proposed biomarkers, neurofilament proteins and glial fibrillary acidic protein (GFAP) are recognized as sensitive indicators of central nervous system injury, while serum creatinine and creatine kinase (CK) mainly reflect degeneration of peripheral nerves and skeletal muscles.

KEY WORDS: Amyotrophic, lateral sclerosis,biomarker,glial fibrillary, acidic proteinneurofilament ,lightchain ,TAR DNA-binding protein 43

1.INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder characterized by the progressive loss of upper and lower motor neurons, resulting in muscle weakness, paralysis, and death within 3–5 years of symptom onset. The disease usually begins in the spinal region (about two-thirds of patients) or the bulbar region (one-third of patients) before spreading to other areas. Around 25% of patients also experience frontotemporal lobe degeneration. ALS mainly affects older adults, with an average onset age of 65 years. Approximately 90% of cases are sporadic with no family history, while the remaining 10% are familial and inherited.

So far, over 40 genes have been linked to 11% of sporadic cases and nearly 70% of familial cases. Diagnosis of ALS entails the exclusion of mimic conditions in the presence of clinical evidence of spatiotemporal progression of upper motor neuron (UMN) involvement combined with clinical or electrophysiological evidence of progressive lower motor neuron (LMN) involvement . Despite the disease's fast progression, diagnostic delay ranges around 12 months, which is partly attributable to the disease's heterogeneity and a lack of a specific biomarker .

More than 40 genes have been associated with amyotrophic lateral sclerosis (ALS), accounting for approximately 11% of sporadic cases and nearly 70% of familial cases. The diagnosis of ALS is primarily based on identifying the progressive involvement of both upper motor neurons (UMNs) and lower motor neurons (LMNs), while carefully excluding other neurological disorders with similar clinical features. Clinical examination, supported by electrophysiological investigations, is essential for confirming disease progression over time.

Although ALS progresses rapidly, diagnosis is often delayed by about one year because of its clinical heterogeneity and the absence of a definitive disease-specific biomarker. Such delays can significantly reduce

the opportunity for early therapeutic intervention, particularly in a condition where the average survival is only three to five years after symptom onset. In addition to affecting patient outcomes, the prolonged diagnostic process imposes considerable financial costs, with diagnostic evaluations sometimes exceeding USD 20,000, while also contributing to substantial psychological stress for patients and their families.

To improve diagnosis and disease monitoring, numerous biofluid biomarkers have been explored in clinical studies. These biomarkers reflect various pathological mechanisms involved in ALS, including protein misfolding, neurodegeneration, neuroinflammation, and skeletal muscle denervation. This review focuses on quantitative biofluid biomarkers from a pathophysiological perspective, whereas imaging-based (structure-based) and functional biomarkers are not discussed.

2 NEUROFILAMENTS

Neurofilaments are among the most extensively studied biofluid biomarkers in neurodegenerative disorders. These neuron-specific intermediate filament proteins are essential components of the neuronal cytoskeleton and are particularly concentrated within large, myelinated axons. Damage to neurons and axons results in the release of neurofilaments into the extracellular space, from where they can be detected in cerebrospinal fluid (CSF). Among the different neurofilament subtypes, neurofilament light chain (NfL) and phosphorylated neurofilament heavy chain (pNfH) have gained considerable attention as reliable indicators of neuroaxonal injury and ongoing neuronal degeneration. In ALS, CSF concentrations of these biomarkers have been widely investigated for their potential roles in diagnosis, prognosis, disease-risk assessment, and monitoring treatment response. Recent advances in highly sensitive analytical methods have also enabled accurate measurement of neurofilaments in blood samples, including plasma and serum. Because blood collection is less invasive and more practical than CSF sampling, research evaluating circulating neurofilaments as accessible biomarkers for ALS diagnosis and disease monitoring has increased substantially.

2.1 NEUROFILAMENTS AS SUSCEPTIBILITY/RISK BIOMARKERS

Longitudinal measurement of neurofilament levels in cerebrospinal fluid (CSF) and blood has significant potential for monitoring individuals who carry ALS-associated genetic mutations but have not yet developed symptoms. Regular assessment of these biomarkers may help identify the transition from the presymptomatic to the symptomatic stage, thereby enabling earlier clinical intervention and supporting the development of more effective therapeutic strategies. One of the major obstacles in ALS management is the delay between symptom onset and diagnosis, which often postpones the initiation of treatment. Since the underlying neurodegenerative processes begin long before clinical manifestations become apparent, interventions are expected to produce greater benefits when started during the earliest stages of disease, or ideally before symptoms emerge. Consequently, neurofilaments are increasingly recognized as promising susceptibility and risk biomarkers because they may reflect disease activity before clinical onset and help estimate the timing of phenoconversion in individuals carrying pathogenic mutations such as **SOD1**, **C9orf72**, and **FUS**.

Evidence from longitudinal clinical studies supports this role of neurofilaments. In one investigation involving 84 individuals with a familial risk of ALS, researchers collected serial serum samples and clinical data before symptom onset, during the transition to symptomatic disease, and throughout the early stages of ALS. Elevated NfL levels were detected approximately 6–12 months before symptom onset in individuals carrying the **SOD1 A4V** mutation, about 3.5 years before symptom onset in carriers of the **C9orf72** repeat expansion, and nearly 2 years before disease onset in individuals with the **FUS c.521del6** mutation. These findings indicate that neurofilament biomarkers may provide valuable information for predicting disease onset and may support personalized monitoring strategies for genetically at-risk individuals.

2.2 NEUROFILAMENTS AS RESPONSE MARKERS

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Evidence from longitudinal clinical studies supports this role of neurofilaments. In one investigation involving 84 individuals with a familial risk of ALS, researchers collected serial serum samples and clinical data before symptom onset, during the transition to symptomatic disease, and throughout the early stages of ALS. Participants who eventually developed clinical symptoms showed a marked increase in serum neurofilament light chain (NfL) approximately one year before symptom onset, with concentrations continuing to rise during the months following disease manifestation. Follow-up research that included additional individuals who converted to symptomatic ALS demonstrated similar presymptomatic elevations in both NfL and phosphorylated neurofilament heavy chain (pNfH). Furthermore, the timing of these increases appeared to vary according to the underlying genetic mutation. Elevated NfL levels were detected approximately 6–12 months before symptom onset in individuals carrying the **SOD1 A4V** mutation, about 3.5 years before symptom onset in carriers of the **C9orf72** repeat expansion, and nearly 2 years before disease onset in individuals with the **FUS c.521del6** mutation. These findings indicate that neurofilament biomarkers may provide valuable information for predicting disease onset and may support personalized monitoring strategies for genetically at-risk individuals.

2.3 LIMITATIONS AND FUTURE PERSPECTIVES OF NEUROFILAMENT PROTEIN BIOMARKERS

Overall, current evidence indicates that neurofilament proteins measured in cerebrospinal fluid (CSF) and blood have considerable value across several biomarker applications in ALS. Despite their clinical usefulness, these biomarkers also have important limitations. Elevated neurofilament concentrations primarily reflect ongoing neuronal and axonal damage, a feature common to several neurodegenerative and neurological disorders. Therefore, although neurofilaments are highly sensitive indicators of neuroaxonal injury, they lack the disease specificity required to establish a definitive diagnosis of ALS when used alone.

Longitudinal studies have demonstrated that neurofilament concentrations begin to rise before the appearance of clinical symptoms in individuals carrying pathogenic ALS-associated mutations, suggesting their usefulness for identifying those at risk of developing the disease. Interestingly, the interval between biomarker elevation and symptom onset appears to differ according to the underlying genetic mutation. It has been proposed that individuals with slower disease progression experience a longer presymptomatic phase, whereas those with rapidly progressive disease transition more quickly to symptomatic ALS. Although this hypothesis requires further validation, research in presymptomatic mutation carriers remains challenging because such studies involve relatively small patient populations and require prolonged follow-up periods. Nevertheless, monitoring neurofilament levels in genetically at-risk individuals offers an opportunity to detect impending disease onset and facilitates the recruitment of suitable participants into preventive clinical trials before symptoms develop.

Among the various clinical applications of neurofilaments, their greatest strength appears to lie in disease prognosis. Numerous studies have demonstrated their ability to predict disease progression and survival, although some variability exists between published findings. These inconsistencies may result from differences in study design, sample size, biological specimens analyzed (CSF, plasma, or serum), the

neurofilament subtype measured (NfL or phosphorylated neurofilament heavy chain [pNfH]), analytical techniques, and assay performance. Future efforts should focus on standardizing laboratory methods, identifying the most reliable neurofilament subtype and assay platform, establishing normal reference ranges, and recognizing clinical factors that may influence biomarker concentrations, including chronic kidney disease and acute neuroaxonal injury. Furthermore, integrating neurofilament measurements with genetic information, clinical assessments, neuroimaging findings, digital health technologies, and other molecular biomarkers—supported by advanced machine learning approaches—may substantially improve the accuracy of ALS diagnosis, prognostic evaluation, patient stratification, and therapeutic decision-making.

2.4. SOD1 AS A PHARMACODYNAMIC MARK

Reduction of superoxide dismutase 1 (SOD1) expression has emerged as a promising therapeutic strategy for patients with SOD1-associated ALS because the abnormal misfolding and aggregation of mutant SOD1 protein are believed to play a central role in disease pathogenesis. Preclinical studies using transgenic rodent models expressing the human **SOD1 G93A** mutation, as well as non-human primates, have demonstrated that SOD1-targeted antisense oligonucleotides (ASOs) effectively lower SOD1 messenger RNA (mRNA) and protein levels within the central nervous system (CNS). These molecular changes were accompanied by delayed symptom onset and slower disease progression in experimental models. Furthermore, treatment with ASOs produced a reduction in cerebrospinal fluid (CSF) SOD1 concentrations, indicating that CSF SOD1 may serve as a useful pharmacodynamic biomarker for assessing therapeutic target engagement. This concept is further supported by findings from the **VALOR** clinical trial, in which administration of **tofersen** resulted in significant decreases in CSF SOD1 levels.

Earlier investigations involving patients with sporadic ALS (sALS) did not identify a clear relationship between CSF SOD1 concentrations and clinical measures of disease severity or progression. However, ongoing follow-up studies of participants enrolled in the tofersen extension trial may provide additional insight into this relationship. Specifically, it will be important to determine whether greater reductions in CSF SOD1 following treatment are associated with improved clinical outcomes in patients with SOD1-related ALS. Confirmation of such an association would strengthen the evidence supporting CSF SOD1 as a surrogate endpoint biomarker capable of predicting therapeutic efficacy and clinical benefit.

2.5 DIPEPTIDE REPEAT PROTEINS AS PHARMACODYNAMIC MARKERS

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As mentioned above, lowering SOD1 is being investigated as a therapeutic for SOD1 ALS given that the pathological misfolding and aggregation of mutant SOD1 are believed to underlie its toxicity [1713]. In preclinical studies using rodents expressing human SOD1 G93A or using non-human primates, SOD1-targeting ASOs decreased SOD1 mRNA and protein in the central nervous system (CNS), delayed symptom onset, and/or slowed disease progression. The ASO additionally decreased CSF SOD1 in rats and non-human primates suggesting that CSF SOD1 has utility as a pharmacodynamic marker [1914]. The use of CSF SOD1 as a marker of target engagement is supported by data from the aforementioned VALOR study showing that tofersen administration decreases CSF SOD1. Though a former study of patients with sALS found no association between CSF SOD1 and disease characteristics , it will be of great interest to examine, once the tofersen extension phase is complete, whether larger decreases in CSF SOD1 associate with greater improvements in clinical end-points in patients with SOD1 ALS. In this manner, information may be gleaned as to whether CSF SOD1 shows potential as a surrogate endpoint biomarker to predict clinical benefit. remove plagiarism

G4C2 hexanucleotide repeat expansions in the C9orf72 gene are the most common known genetic cause of ALS and FTD [2115]. These bidirectionally transcribed expansions cause the accumulation of G4C2 or G2C4 repeat RNA, and the production of five aggregation-prone dipeptide repeat (DPR) proteins. Nuclear foci formed of the repeat RNA and DPR proteins are thought to contribute to disease pathogenesis, as is loss of C9orf72 function. Discovery of these traits specific to C9orf72-associated ALS and FTD (c9ALS/FTD) brought to light new therapeutic targets, chief among them, repeat RNA and DPR proteins formed thereof. This quickly prompted early investigations to identify approaches to neutralize or degrade the repeat RNA, and to establish means to monitor whether the therapeutic strategies being tested have the intended outcomes. With the latter in mind, and with the expectation that lowering repeat RNA would attenuate DPR protein production, an immunoassay was developed to measure poly(GP) DPR proteins in preclinical models and in CSF from C9orf72 repeat expansion carriers . This study engendered a number of subsequent investigations on the prognostic and pharmacodynamic potential of poly(GP) and other DPR proteins. Poly(GP), poly(GA) and poly(GR) were detected in CSF from asymptomatic and symptomatic C9orf72 repeat expansion carriers, with levels being generally similar between these two groups, but were not detected in CSF from individuals with no C9orf72 repeat expansio. Levels of CSF DPR proteins in symptomatic C9orf72 expansion carriers did not associate with clinical traits such as age at disease onset, ALSFRS-R score, rate of decline of ALSFRS-R score or survival, nor with NfL concentrations. Despite their lack of prognostic utility, ample evidence from preclinical models supports the use of CSF DPR proteins as pharmacodynamic biomarkers. For example, treating c9ALS/FTD mouse models with an ASO targeting G4C2 repeat-containing transcripts resulted in sustained decreases in brain, spinal cord and CSF DPR proteins [2716]. What is more, repeated intrathecal delivery of a G4C2 repeat-targeting ASO to a C9orf72 repeat expansion carrier significantly decreased CSF poly(GP), poly(GA) and poly(GR) [28,29]. The data above support the use of DPR proteins as preclinical and clinical pharmacodynamic biomarkers for therapeutic approaches targeting G4C2 repeat RNA. In March 2022, Biogen and Ionis Pharmaceuticals announced that BIIB078, an investigational ASO for C9orf72-associated ALS that degrades G4C2 expansion-containing mRNA, did not show clinical benefit in their phase 1 clinical trial (NCT03626012), which has since been discontinued. Whether BIIB078 decreased CSF DPR

proteins in trial participants has not been publicly reported. This information, along with data on DPR protein levels from similar clinical trials (e.g., the Wave Life Sciences NCT04931862 trial evaluating intrathecal WVE-004, an ASO targeting repeat-containing pre-mRNA variants in patients with c9ALS/FTD), would facilitate the interpretation of trial results and inform whether alternative therapeutic approaches for c9ALS/FTD should be considered. remove plagarism

Hexanucleotide (G4C2) repeat expansions in the **C9orf72** gene represent the most common inherited genetic abnormality associated with both amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). These repeat expansions are transcribed in both directions, leading to the accumulation of abnormal repeat RNA molecules and the production of several aggregation-prone dipeptide repeat (DPR) proteins through repeat-associated non-AUG (RAN) translation. The formation of RNA nuclear foci together with the accumulation of DPR proteins, combined with reduced normal C9orf72 protein function, are considered key contributors to disease pathogenesis. Recognition of these disease-specific molecular mechanisms has created new opportunities for targeted therapeutic development, particularly strategies aimed at reducing toxic repeat RNA transcripts and the DPR proteins derived from them.

To evaluate the effectiveness of these emerging therapies, sensitive assays have been developed to quantify DPR proteins, especially poly(glycine–proline) [poly(GP)], in experimental models and cerebrospinal fluid (CSF) samples from individuals carrying **C9orf72** repeat expansions. These assays have enabled extensive investigation of DPR proteins as potential biomarkers for disease monitoring and treatment response. Studies have shown that poly(GP), poly(glycine–alanine) [poly(GA)], and poly(glycine–arginine) [poly(GR)] are detectable in the CSF of both presymptomatic and symptomatic mutation carriers, whereas these proteins are absent in individuals without the **C9orf72** expansion. Furthermore, DPR protein concentrations are generally comparable between asymptomatic and symptomatic carriers, indicating that their levels do not closely reflect disease stage.

Although CSF DPR proteins demonstrate high specificity for **C9orf72**-associated ALS and FTD, current evidence suggests that they have limited prognostic value. Their concentrations have not shown consistent relationships with important clinical measures such as age at symptom onset, disease severity assessed using the ALS Functional Rating Scale–Revised (ALSFRS-R), rate of functional decline, overall survival, or neurofilament light chain (NfL) concentrations. Consequently, these biomarkers appear to have greater utility for monitoring biological responses to therapy than for predicting disease progression.

Collectively, available evidence supports the use of CSF DPR proteins as pharmacodynamic biomarkers in both preclinical and clinical studies evaluating therapies directed against **C9orf72** repeat RNA. However, translating these biological effects into meaningful clinical improvement remains challenging. For example, the investigational ASO **BIIB078**, developed to reduce mutant **C9orf72** transcripts, failed to demonstrate clinical benefit in a Phase I trial involving patients with **C9orf72**-associated ALS, leading to discontinuation of its development. To date, the effects of BIIB078 on CSF DPR protein concentrations have not been publicly disclosed. Reporting these biomarker data, together with findings from other ongoing therapeutic studies targeting **C9orf72** repeat expansions, would improve understanding of treatment mechanisms and help guide the future development of more effective therapies for **C9orf72**-associated ALS and FTD.

3. TDP-43-RELATED FLUID BIOMARKERS

Hexanucleotide (G4C2) repeat expansions in the **C9orf72** gene represent the most common inherited genetic abnormality associated with both amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). These repeat expansions are transcribed in both directions, leading to the accumulation of abnormal repeat RNA molecules and the production of several aggregation-prone dipeptide repeat (DPR) proteins through repeat-associated non-AUG (RAN) translation. The formation of RNA nuclear foci together with the accumulation of DPR proteins, combined with reduced normal C9orf72 protein function, are considered key contributors to disease pathogenesis. Recognition of these disease-specific molecular mechanisms has created new opportunities for targeted therapeutic development, particularly strategies aimed at reducing toxic repeat RNA transcripts and the DPR proteins derived from them.

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Strong evidence from preclinical studies supports the role of DPR proteins as pharmacodynamic biomarkers. In mouse models of **C9orf72**-associated ALS/FTD, administration of antisense oligonucleotides (ASOs) directed against G4C2 repeat-containing RNA produced sustained reductions in DPR protein levels within the brain, spinal cord, and CSF. Similar findings have been reported in human studies, where repeated intrathecal administration of G4C2 repeat-targeting ASOs resulted in significant decreases in CSF concentrations of poly(GP), poly(GA), and poly(GR). These observations indicate that CSF DPR proteins are useful indicators of therapeutic target engagement and biological activity.

Collectively, available evidence supports the use of CSF DPR proteins as pharmacodynamic biomarkers in both preclinical and clinical studies evaluating therapies directed against **C9orf72** repeat RNA. However, translating these biological effects into meaningful clinical improvement remains challenging. For example, the investigational ASO **BIIB078**, developed to reduce mutant **C9orf72** transcripts, failed to demonstrate clinical benefit in a Phase I trial involving patients with **C9orf72**-associated ALS, leading to discontinuation of its development. To date, the effects of BIIB078 on CSF DPR protein concentrations have not been publicly disclosed. Reporting these biomarker data, together with findings from other ongoing therapeutic studies targeting **C9orf72** repeat expansions, would improve understanding of treatment mechanisms and help guide the future development of more effective therapies for **C9orf72**-associated ALS and FTD.

3.1 OTHER APPROACHES TO TDP-43 BIOMARKER DEVELOPMENT

Most investigations evaluating TAR DNA-binding protein 43 (TDP-43) as a fluid biomarker have relied on conventional enzyme-linked immunosorbent assay (ELISA) techniques to measure its concentration in cerebrospinal fluid (CSF) or plasma. However, several researchers have explored alternative analytical approaches to improve the detection of disease-specific TDP-43 species. One such strategy combined atomic force microscopy with a biopanning technique to isolate single-chain antibody fragments (scFvs) that selectively recognized TDP-43 variants present in ALS brain tissue but showed minimal binding to tissues obtained from individuals with frontotemporal dementia (FTD) or healthy controls. Using these antibody fragments, investigators developed a specialized ELISA capable of detecting disease-associated TDP-43 forms in plasma. Nine scFvs consistently reacted with plasma samples from patients with sporadic ALS, whereas no reactivity was observed in samples collected from healthy individuals. Interestingly, the antibody-binding patterns varied among ALS patients, suggesting that TDP-43 pathology may differ between individuals and that distinct molecular subtypes of the disease may exist. These findings highlight the potential value of personalized TDP-43 biomarker profiles, although additional research is required to determine their clinical significance.

Beyond measuring the concentration of TDP-43, investigators have also examined structural changes in the protein. One study employed immuno-infrared sensor technology to evaluate the secondary structural conformation and misfolding of TDP-43 in CSF rather than its absolute levels. This approach successfully

distinguished patients with ALS from those with Parkinson's disease, achieving a sensitivity of 89% and a specificity of 77%. It also differentiated ALS from other neurological disorders with a sensitivity of 89% and a specificity of 83%. These findings suggest that assessing the structural properties of misfolded TDP-43 may provide greater diagnostic value than measuring total protein concentration alone.

3.2 LIMITATIONS OF CURRENT TDP-43-RELATED BIOMARKERS

Several studies have investigated the potential of TAR DNA-binding protein 43 (TDP-43), including its concentration, structural alterations, and autoantibody responses in cerebrospinal fluid (CSF) and blood, as biomarkers for amyotrophic lateral sclerosis (ALS). Because abnormal TDP-43 aggregation is a defining pathological feature of most ALS cases, it has attracted considerable interest as a candidate biomarker. However, the translation of these findings into clinical practice has been limited by several methodological and biological challenges.

Further uncertainty arises from the limited understanding of how TDP-43 is transported between the CNS, CSF, and peripheral blood. This incomplete knowledge complicates the interpretation of biofluid measurements and restricts their current clinical application. Moreover, reported associations between plasma TDP-43 concentrations and clinical outcomes have been inconsistent. For example, higher plasma TDP-43 levels have been linked to better functional performance, as reflected by improved ALS Functional Rating Scale–Revised (ALSFRS-R) scores and slow vital capacity (SVC), while simultaneously correlating with less favorable values on the SI index. These conflicting findings suggest that the relationship between circulating TDP-43 and disease severity is complex and requires further investigation before TDP-43 can be established as a reliable biomarker for ALS.

4. PATHOPHYSIOLOGY OF AMYOTROPHIC LATERAL SCLEROSIS

Although the exact pathophysiological mechanisms underlying amyotrophic lateral sclerosis (ALS) have not been completely elucidated, advances in genetic research have identified several mutations that contribute to disease development. Among the most extensively investigated ALS-associated genes are **C9orf72**, **SOD1 (Cu/Zn superoxide dismutase 1)**, **TARDBP**, and **FUS**. Together, mutations in these genes account for approximately 48% of familial ALS cases and about 5% of sporadic cases. Each mutation contributes to neurodegeneration through distinct but often overlapping molecular pathways.

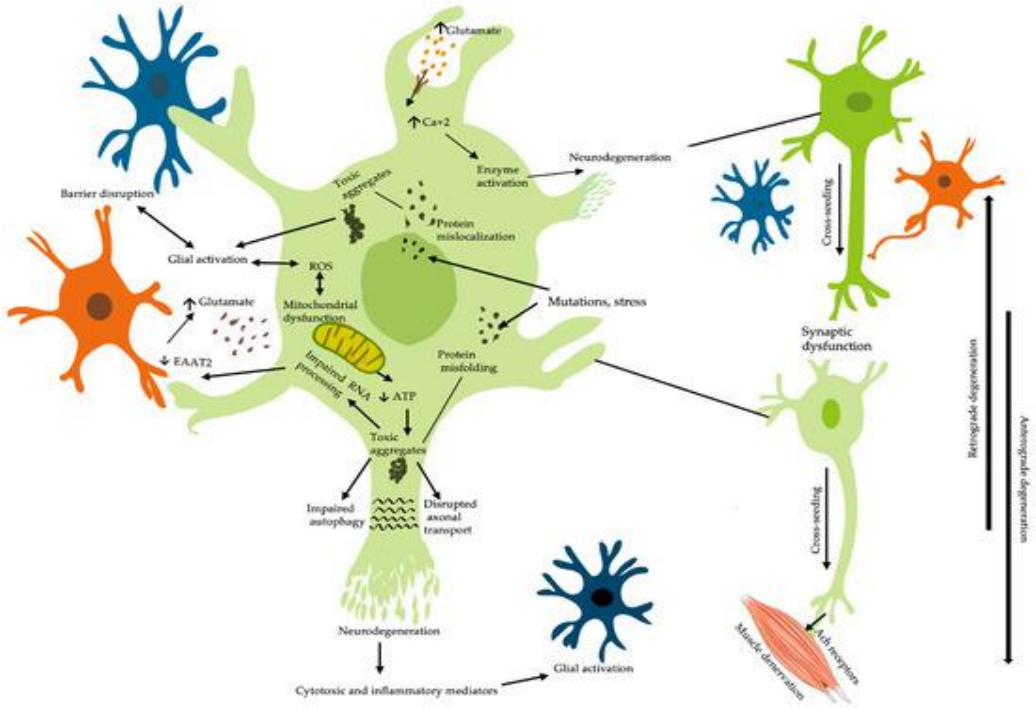
Mutations in **TARDBP** and **FUS** promote abnormal folding and cytoplasmic aggregation of the RNA-binding proteins TDP-43 and FUS, respectively. Expansion mutations in the **C9orf72** gene result in the accumulation of toxic RNA foci and dipeptide repeat proteins, while also promoting TDP-43 pathology. In contrast, mutations in **SOD1** produce unstable mutant SOD1 proteins that aggregate within motor neurons, disrupt mitochondrial function, and enhance oxidative stress. Additional ALS-associated genes, including **TUBA4A** and **PFN1**, have been implicated in cytoskeletal instability and impaired axonal transport, highlighting the diverse molecular mechanisms involved in disease progression.

Historically, ALS has been viewed as a protein misfolding disorder in which abnormal accumulation of mislocalized proteins represents a central pathological feature. This concept is supported by postmortem studies demonstrating a close relationship between motor neuron degeneration and the presence of intracellular protein inclusions. Protein aggregation may arise from genetic mutations, oxidative damage, cellular stress, impaired protein homeostasis, or cross-seeding mechanisms that promote the misfolding of additional proteins. The resulting protein deposits interfere with essential neuronal functions, including axonal transport, mitochondrial energy production, and cellular stress-response pathways, ultimately contributing to neuronal dysfunction and death.

5. BIOMARKERS IN AMYOTROPHIC LATERAL SCLEROSIS

An ideal biomarker for an ND would need to have the following characteristics. The biomarker should be stable in the fluid from which it is measured. The biomarker should be sensitive to ongoing injury and preferably specific to the underlying pathology. Its release into the surrounding cerebrospinal fluid (CSF) or

through the BBB into the plasma should be passive and, therefore, reflective of the extent of pathology [20]. The biomarker should be easily isolated and analyzed, and its collection should be minimally invasive for patients. CSF has the advantage of being in direct contact with the CNS, but its use is limited by the invasiveness of lumbar puncture.On the other hand, blood sampling is less invasive, but its analysis is ultimately more complex as it contains a variety of proteins. Such biomarkers can be diagnostic, prognostic, predictive, or pharmacodynamic. Di agnostic biomarkers can distinguish those affected by the disease from those that are not. Diagnostic biomarkers can expedite diagnosis, therapeutic administration, and enrollment in early disease clinical trials. Predictive biomarkers determine which patient will likely experience an outcome in response to a clinical intervention. Validation of a predictive biomarker therefore requires a controlled trial including patients with and without the biomarker [21].



5.1. BIOMARKERS RELATED TO PROTEINOPATHY

Amyotrophic lateral sclerosis (ALS) is recognized as a neurodegenerative proteinopathy characterized by the accumulation of insoluble protein aggregates within affected motor neurons. The major pathological inclusions consist of abnormal forms of TDP-43, FUS, and SOD1 proteins, which arise as a result of protein misfolding or abnormal cellular localization. These aggregated proteins can propagate between neighboring motor neurons through a self-perpetuating, prion-like mechanism, contributing to the progressive spread of neurodegeneration throughout the central nervous system. The anatomical progression of neuronal loss closely parallels the distribution and accumulation of these pathological protein deposits..

Under normal physiological conditions, TDP-43 is a 414-amino-acid DNA- and RNA-binding protein that is highly expressed in the central nervous system. It plays essential roles in regulating gene expression, RNA splicing, RNA transport, mRNA stability, and other post-transcriptional processes required for neuronal function. The C-terminal glycine-rich domain of TDP-43 contains most disease-associated mutations and is critical for protein–protein interactions, cellular stress responses, and the assembly of intracellular RNA granules. Structural alterations within this region promote abnormal protein aggregation, ultimately contributing to motor neuron dysfunction and disease progression in ALS.

5.2. BIOMARKERS RELATED TO NEURODEGENERATION

The progressive degeneration of both upper motor neurons (UMNs) and lower motor neurons (LMNs) is the defining pathological hallmark of amyotrophic lateral sclerosis (ALS). Loss of these neurons results in degeneration of the corticospinal tracts, gliosis within the lateral columns of the spinal cord, and gradual paralysis of skeletal muscles. Disease onset is typically focal, with pathology subsequently spreading to anatomically connected motor neuron populations. Although the precise mechanism responsible for disease propagation remains unclear, it has been proposed that neurodegeneration may occur through anterograde transmission from the primary motor cortex to lower motor neurons, retrograde spread from LMNs to the cortex, or a combination of both mechanisms. Neuropathological studies have demonstrated a greater burden of protein inclusions within LMNs than UMNs, whereas transcranial magnetic stimulation studies suggest that cortical dysfunction may precede clinically detectable lower motor neuron impairment.

Neuron-specific enolase (NSE) is a neuronal glycolytic enzyme predominantly expressed in neurons and other neuroectoderm-derived cells. Neuronal injury results in the passive release of NSE into the cerebrospinal fluid (CSF), making its concentration a useful indicator of neuronal damage. In patients with ALS, CSF NSE levels can be quantified using electrochemiluminescence immunoassays (ECLIA). Elevated CSF NSE concentrations have shown the ability to distinguish ALS from conditions such as cervical spondylotic myelopathy and other neurological disorders with good diagnostic performance. Although plasma NSE has not been extensively investigated in ALS, increased circulating levels have been reported in several other neurological diseases, indicating its potential relevance as a marker of neuronal injury.

Among all proposed biomarkers for ALS, neurofilament light chain (NfL) and phosphorylated neurofilament heavy chain (pNfH) have received the greatest attention. Neurofilaments are major structural proteins of the neuronal cytoskeleton and consist primarily of neurofilament light, medium, and heavy chains together with α-internexin in the central nervous system and peripherin in the peripheral nervous system. These proteins are highly abundant in large pyramidal neurons of the motor cortex, including Betz cells, as well as in heavily myelinated axons, where they provide mechanical stability and maintain axonal architecture. Injury to motor neurons causes the release of neurofilaments into the extracellular space, allowing their detection in cerebrospinal fluid and blood. Consequently, NfL and pNfH have become the most extensively studied fluid biomarkers for diagnosing ALS, monitoring disease progression, predicting prognosis, and evaluating therapeutic responses.

5.3. BIOMARKERS RELATED TO NEUROINFLAMMATION

Neuroinflammation is recognized as a major pathological mechanism contributing to the development and progression of amyotrophic lateral sclerosis (ALS). The importance of inflammatory pathways has been reinforced by the identification of mutations in **TANK-binding kinase 1 (TBK1)**, a gene associated with both ALS and frontotemporal dementia (FTD). In addition to neurons, glial cells in ALS accumulate abnormal protein aggregates similar to those observed in affected motor neurons. These pathological inclusions stimulate glial activation through signaling pathways involving chromogranin A and chromogranin B. Furthermore, degenerating neurons release cytotoxic molecules and inflammatory mediators that further enhance microglial activation.

Once activated, microglia promote the activation of astrocytes and contribute to disruption of the blood–brain barrier (BBB). Damage to the BBB permits circulating pro-inflammatory cytokines and immune mediators to enter the central nervous system, thereby amplifying the local inflammatory response. Activated microglia also produce additional inflammatory cytokines, creating a self-sustaining positive feedback loop that perpetuates neuroinflammation. Although this immune response may initially serve a protective role by maintaining tissue homeostasis, prolonged activation alters microglial function, reducing their normal motility and phagocytic capacity while increasing their neurotoxic effects. This persistent inflammatory environment accelerates motor neuron degeneration and disease progression.

5.4. BIOMARKERS RELATED TO BLOOD–BRAIN BARRIER

Disruption of the blood–brain barrier (BBB) is considered an important feature of neuroinflammation in amyotrophic lateral sclerosis (ALS). It has been proposed that inflammatory processes compromise BBB integrity, permitting peripheral immune cells and inflammatory mediators to enter the central nervous system (CNS). This infiltration further amplifies neuroinflammation through a self-reinforcing feedback mechanism, thereby accelerating neuronal damage and disease progression. BBB dysfunction has been reported in several neuroinflammatory neurodegenerative disorders, including ALS.

Neuropathological examination of postmortem ALS tissue has revealed multiple structural abnormalities consistent with BBB impairment, including degeneration of endothelial cells, leakage of capillaries, and perivascular edema. Increased deposition of hemoglobin around CNS blood vessels has also been demonstrated using glycophorin A immunostaining, providing evidence of erythrocyte extravasation. Experimental studies in ALS animal models indicate that BBB breakdown and red blood cell leakage occur before the appearance of muscle weakness, suggesting that vascular dysfunction may represent an early event in disease development.

5.5. BIOMARKERS RELATED TO SYANPTOPATHY

Synaptic dysfunction, commonly referred to as **synaptopathy**, is considered one of the earliest pathological events in amyotrophic lateral sclerosis (ALS), often occurring before significant motor neuron degeneration becomes apparent. Evidence from experimental models indicates that abnormalities in synaptic structure and communication precede neuronal loss, while postmortem studies of ALS patients have demonstrated a marked reduction in dendritic spine density within corticospinal motor neurons. Although excitotoxicity is regarded as a major contributor to synaptopathy, multiple interacting mechanisms are believed to participate, including the accumulation of misfolded proteins, mitochondrial impairment at distal axon terminals, defective protein homeostasis, and chronic activation of microglia. In turn, synaptic dysfunction further aggravates these pathological processes by promoting mitochondrial damage, impaired proteostasis, and degeneration of the neuromuscular junction.

Excessive glutamate signaling causes sustained calcium influx into motor neurons, triggering a cascade of calcium-dependent pathways that ultimately lead to neuronal injury and death. Motor neurons are particularly vulnerable to this process because they possess relatively low concentrations of calcium-binding proteins and therefore have limited capacity to buffer intracellular calcium. Calcium overload disrupts mitochondrial function, reduces cellular energy production, and activates degradative enzymes such as calpains, which damage essential structural and functional proteins within neurons. Collectively, these events contribute to progressive motor neuron degeneration.

The clinical importance of glutamate excitotoxicity is reflected in the mechanism of action of **riluzole**, the first medication approved worldwide for the treatment of ALS. Riluzole reduces glutamate-mediated neuronal toxicity by suppressing presynaptic glutamate release, inhibiting persistent voltage-gated sodium currents, and decreasing neuronal hyperexcitability. Although its therapeutic effect is modest, riluzole has been shown to modestly slow disease progression and prolong survival in patients with ALS.

5.6. BIOMARKERS RELATED TO OXIDATIVE STRESS

Oxidative stress is widely recognized as a major contributor to the pathogenesis of amyotrophic lateral sclerosis (ALS) and has been consistently demonstrated in both experimental models and postmortem studies. Its importance is further supported by the observation that familial ALS associated with **SOD1** mutations is closely linked to abnormal oxidative damage. Excessive production of reactive oxygen species (ROS) disrupts normal cellular homeostasis and promotes neuronal injury through several interconnected mechanisms.

One consequence of oxidative stress is the aggregation and inactivation of mitochondrial aconitase within the mitochondrial matrix, leading to impaired mitochondrial function. Dysfunctional mitochondria generate additional ROS, creating a self-perpetuating cycle that further intensifies oxidative damage. At the same time,

elevated ROS levels reduce the ability of astrocytes to remove glutamate from the synaptic cleft, resulting in excessive extracellular glutamate accumulation and glutamate-mediated excitotoxicity.

Persistent glutamate stimulation increases intracellular calcium concentrations in motor neurons. Because damaged mitochondria have a diminished capacity to buffer calcium and produce adenosine triphosphate (ATP), calcium overload becomes progressively more severe. This combination of mitochondrial dysfunction and calcium dysregulation interferes with the interaction between kinesin-1 motor proteins and microtubules, compromising axonal transport and destabilizing the neuronal cytoskeleton. As efficient axonal transport is essential for neuronal survival, disruption of this process contributes significantly to motor neuron degeneration.

In addition to its direct effects on neurons, oxidative stress also promotes neuroinflammation by activating glial cells. Activated microglia and astrocytes release pro-inflammatory cytokines and additional reactive oxygen species, thereby amplifying inflammatory signaling and oxidative damage within the central nervous system. These interconnected processes establish a vicious cycle in which oxidative stress, mitochondrial dysfunction, excitotoxicity, impaired axonal transport, and neuroinflammation collectively accelerate the progression of ALS.

5.7. BIOMARKERS RELATED TO ABERRANT RNA PROCESSING

Abnormal RNA metabolism has emerged as a fundamental mechanism underlying the development and progression of amyotrophic lateral sclerosis (ALS). Many of the proteins associated with ALS pathogenesis participate directly in RNA processing, highlighting the importance of RNA homeostasis in maintaining motor neuron function. Among these, TAR DNA-binding protein 43 (TDP-43) is the most extensively studied and plays a central role in regulating multiple aspects of RNA metabolism, including RNA splicing, transport, stability, and microRNA (miRNA) biogenesis. These processes are essential for controlling gene expression and preserving the functional integrity and survival of mature motor neurons.

Genetic mutations associated with ALS, including those affecting **SOD1**, **C9orf72**, and **FUS**, further disrupt normal RNA metabolism through both direct and indirect mechanisms. In addition, the accumulation of abnormal protein aggregates interferes with several stages of RNA processing, including splicing, 5′ capping, polyadenylation, intracellular transport, and RNA localization. These pathological protein inclusions also impair the formation and dynamics of stress granules, resulting in defective miRNA biogenesis and altered post-transcriptional regulation of gene expression. Collectively, these findings demonstrate that dysregulated RNA processing and impaired miRNA function are central molecular events in ALS pathogenesis and contribute significantly to motor neuron degeneration.

5.8. BIOMARKERS RELATED TO ABERRANT RNA PROCESSING

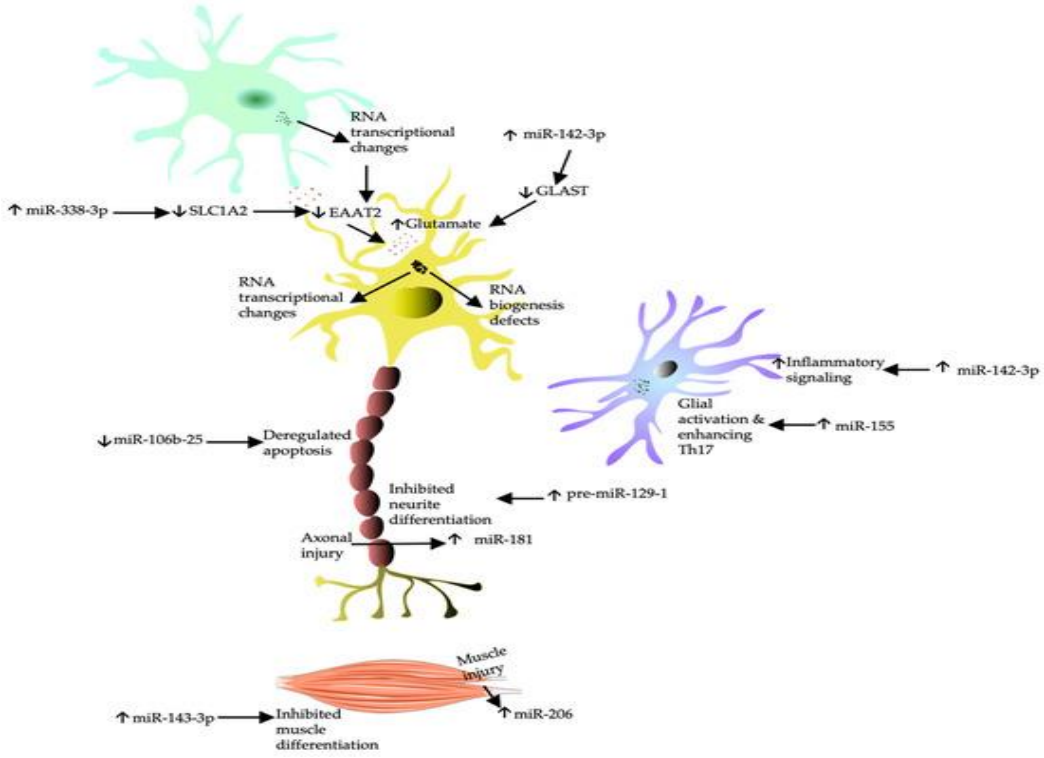
Disruption of RNA metabolism is recognized as a key molecular mechanism in the pathogenesis of amyotrophic lateral sclerosis (ALS). A considerable number of ALS-associated proteins are involved in regulating RNA processing, emphasizing the importance of maintaining RNA homeostasis for normal neuronal function. Among these proteins, TAR DNA-binding protein 43 (TDP-43) plays a central role by controlling multiple aspects of RNA metabolism, including RNA splicing, transport, stability, and microRNA (miRNA) maturation. Both messenger RNAs and miRNAs act as essential epigenetic regulators that maintain transcriptome plasticity, which is critical for the survival and normal function of mature motor neurons.

Mutations in major ALS-related genes, including **SOD1**, **C9orf72**, and **FUS**, disrupt RNA metabolism through a variety of direct and indirect mechanisms. Furthermore, the accumulation of pathogenic protein aggregates interferes with several post-transcriptional processes, such as RNA splicing, 5′ capping, polyadenylation, and intracellular RNA transport. These aggregates also disturb the formation and function of stress granules, leading to impaired miRNA biogenesis and dysregulation of gene expression. Collectively, these molecular abnormalities highlight the crucial contribution of defective RNA processing and altered miRNA biology to the initiation and progression of motor neuron degeneration in ALS.

6. BIOMARKERS RELATED TO MUSCLE CHANGES

The neuromuscular junction is highly susceptible to oxidative stress and intracellular calcium imbalance, making it one of the earliest sites affected in amyotrophic lateral sclerosis (ALS). Excessive oxidative damage and calcium overload impair presynaptic nerve terminals, leading to defective neurotransmitter release and early synaptic dysfunction. Disturbances in the synthesis, storage, and release of acetylcholine (ACh) at presynaptic terminals are thought to contribute to degeneration of distal motor axons and progressive disruption of neuromuscular transmission.

Muscle-specific microRNAs (myomiRs), including **miR-1, miR-206, miR-133a, miR-133b, and miR-27a**, play essential roles in skeletal muscle growth, differentiation, regeneration, and maintenance. Because their expression reflects muscle integrity, these myomiRs have been proposed as biomarkers of residual muscle mass and regenerative capacity in ALS. Among them, **miR-206** and **miR-133b** are believed to exert neuroprotective effects by promoting the repair and re-establishment of functional neuromuscular synapses following injury. In contrast, other microRNAs, such as **miR-143-3p** and **miR-374b**, negatively regulate muscle regeneration by inhibiting myoblast differentiation, potentially contributing to progressive muscle wasting during disease progression.



Several studies have investigated the clinical significance of muscle-specific microRNAs (myomiRs) as potential biomarkers for amyotrophic lateral sclerosis (ALS). In one study involving fourteen patients with ALS, serum levels of **miR-206** and **miR-133** were significantly elevated, whereas **miR-27a** expression was reduced compared with healthy controls. Patients with bulbar-onset ALS exhibited significantly lower serum levels of **miR-133a**, **miR-133b**, and **miR-206** than those with spinal-onset disease. These findings were accompanied by more severe muscle fiber atrophy in the bulbar-onset group, suggesting that differences in myomiR expression may reflect the more rapid disease progression typically observed in this phenotype.

However, subsequent longitudinal investigations have produced mixed findings regarding the prognostic value of these biomarkers. One study reported that circulating levels of **miR-143-3p** and **miR-374b-5p**, but not **miR-206**, were associated with disease progression, raising questions about the usefulness of miR-206 as a prognostic indicator. The same investigation found no significant differences in the expression of these microRNAs between patients receiving riluzole therapy and those who were untreated. During follow-up, **miR-143-3p** expression gradually increased, whereas **miR-374b-5p** levels declined over time. Experimental

studies using C2C12 myoblast cultures demonstrated that overexpression of both microRNAs suppresses muscle cell differentiation. The contrasting expression patterns observed in patients therefore remain difficult to explain. It has been proposed that the reduction in **miR-374b-5p** represents a compensatory mechanism aimed at enhancing muscle regeneration by promoting myoblast differentiation. In contrast, the persistent elevation of **miR-143-3p** may simply reflect ongoing muscle denervation, although this explanation remains uncertain because its expression has been shown to correlate negatively with serum creatine kinase concentrations.

Despite inconsistent evidence regarding its prognostic value and limited disease specificity, **miR-206** remains one of the most consistently reported microRNAs in ALS research. As a regulator of histone deacetylase 4 (HDAC4), miR-206 is highly expressed in skeletal muscle and is believed to enter the circulation following muscle injury. Owing to its reproducible detection across multiple studies, miR-206 continues to be considered a promising candidate biomarker that may assist in the diagnosis of ALS.

The expression of circulating myomiRs may also be influenced by therapeutic interventions. A preliminary study involving eighteen ALS patients found that six weeks of moderate aerobic exercise resulted in reduced circulating levels of several myomiRs, accompanied by a significant improvement in Amyotrophic Lateral Sclerosis Functional Rating Scale–Revised (ALSFRS-R) scores. These findings suggest that alterations in myomiR expression may reflect muscle recovery and adaptation following exercise. However, interpretation of these results is limited by the absence of a control group and the possibility that concurrent lifestyle, nutritional, or therapeutic factors influenced microRNA expression. Other investigations have likewise reported reduced expression of muscle-related microRNAs, including **miR-27a**. Nevertheless, little information is currently available regarding myomiR expression in presymptomatic mutation carriers or in disorders that clinically mimic ALS.

7.FUTURE OF BIOMARKERS

Amyotrophic lateral sclerosis (ALS) is a complex, multifactorial neurodegenerative disorder that cannot be explained by a single pathogenic mechanism. In addition to progressive motor neuron degeneration, the disease involves numerous non-neuronal processes, including neuroinflammation, metabolic dysregulation, glial dysfunction, and immune activation. These diverse pathological pathways have broadened the search for biomarkers that can improve diagnosis, predict disease progression, and monitor therapeutic responses.

Among the inflammatory biomarkers under investigation, cerebrospinal fluid (CSF) chitinases have shown considerable promise. Increased concentrations of **CHIT1**, **CHI3L1**, and **CHI3L2** have been associated with accelerated disease progression, greater cognitive impairment, and reduced survival in patients with ALS, suggesting that these proteins reflect the degree of neuroinflammatory activity. Another glial-derived biomarker, **S100 calcium-binding protein B (S100B)**, has also demonstrated prognostic potential. Lower CSF S100B concentrations have been linked to longer survival, whereas serum S100B appears to provide only limited prognostic information when compared with established biomarkers such as neurofilament light chain (NfL).

Because ALS pathology evolves over time and involves multiple biological pathways, a single biomarker is unlikely to capture the full complexity of the disease. Instead, a combination of complementary biomarkers is expected to provide a more comprehensive assessment of disease mechanisms, clinical progression, and therapeutic response. Such a multimodal biomarker panel would ideally integrate both fluid-based and non-fluid biomarkers, combining established indicators with newly identified candidates to improve individualized patient evaluation and facilitate clinical translation.

Extracellular vesicles, particularly **exosomes**, have recently emerged as promising sources of novel biomarkers. These membrane-bound vesicles can be isolated from biological fluids such as CSF and blood and contain proteins, nucleic acids, lipids, and other bioactive molecules that reflect the physiological state of their cells of origin. Several studies have identified disease-associated molecular signatures within exosomes from patients with ALS. For example, neuron-derived extracellular vesicles isolated from plasma have been reported to contain numerous differentially expressed microRNAs (miRNAs) compared with healthy

individuals. In addition, alterations in the lipid composition of plasma extracellular vesicles have been observed in ALS, indicating potential value for both diagnosis and prognosis. An important advantage of exosome-based biomarkers is their ability to identify molecules originating from specific cell populations, thereby providing greater insight into disease mechanisms than conventional biofluid analyses.

CONCLUSION

Amyotrophic lateral sclerosis (ALS) is a complex, non-cell-autonomous neurodegenerative disorder involving multiple interconnected pathological processes that affect the entire neuromuscular system. Disease progression is driven not only by motor neuron degeneration but also by the interactions of various cell types, including interneurons, microglia, astrocytes, oligodendrocytes, and skeletal muscle cells, through diverse molecular and genetic mechanisms. This biological complexity, together with the marked clinical and genetic heterogeneity of ALS, presents considerable challenges for the discovery and validation of reliable biomarkers.

Numerous biomarkers associated with the underlying pathophysiology of ALS have been identified in biological fluids and can be measured using established laboratory techniques. However, direct comparison of biomarker performance across studies remains difficult because of substantial differences in study design, sample size, patient selection, control populations, analytical methods, and outcome measures. These inconsistencies emphasize the need for standardized protocols governing sample collection, processing, assay methodology, data analysis, and reporting. The establishment of uniform guidelines, similar to those adopted for other neurological disorders, would improve the reproducibility, comparability, and clinical applicability of ALS biomarker research.

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