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REVIEW

Fluid-Based Biomarkers for Amyotrophic Lateral Sclerosis

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Abstract Amyotrophic lateral sclerosis (ALS) is a highly heterogeneous disease with no effective treatment. Drug development has been hampered by the lack of biomarkers that aid in early diagnosis, demonstrate target engagement, monitor disease progression, and can serve as surrogate endpoints to assess the efficacy of treatments. Fluid-based biomarkers may potentially address these issues. An ideal biomarker should exhibit high specificity and sensitivity for distinguishing ALS from control (appropriate disease mimics and other neurologic diseases) populations and monitor disease progression within individual patients. Significant progress has been made using cerebrospinal fluid, serum, and plasma in the search for ALS biomarkers, with urine and saliva biomarkers still in earlier stages of development. A few of these biomarker candidates have demonstrated use in patient stratification, predicting disease course (fast *vs* slow progression) and severity, or have been used in preclinical and clinical applications. However, while ALS biomarker discovery has seen tremendous advancements in the last decade, validating biomarkers and moving them towards the clinic remains more elusive. In this review, we highlight biomarkers that are moving towards clinical utility and the challenges that remain in order to implement biomarkers at all stages of the ALS drug development process.

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Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the degeneration of both upper and lower motor neurons (MN) in the brain and spinal cord [1, 2]. As the disease progresses, patients exhibit muscle atrophy and consequently lose respiratory function. Only one Food and Drug Administration-approved drug, riluzole, has been shown to extend a patient's lifespan for an average of 2 to 3 months [3–5]. Therefore, there is a critical need to gain further insight into the pathobiology of ALS to further aid in the development of more effective therapeutics.

One of the major challenges associated with ALS pathology stems from its inherent heterogeneity [6]. ALS is a complex disease associated with numerous pathologic mechanisms, including oxidative stress, mitochondrial dysfunction, axonal damage, microglial activation, inflammation, excitotoxicity, and protein aggregation [7–11]. Current diagnostic measures rely upon clinical examination and electrophysiological measurements [6, 12], which, in most cases, have not enabled early diagnosis where potential therapies would likely be most effective. One potential way to improve earlier diagnosis would be to utilize biomarkers specific to ALS that can be identified early in the disease process. In addition, there has been continuous failure of large, late-stage clinical trials [13–15]. This highlights the need to enhance preclinical (*in vitro* and *in vivo*) studies and ALS clinical trial design, for example through the inclusion of biomarkers throughout the drug development process [16, 17].

A biomarker is a characteristic that is objectively measured as an indicator of normal biological processes, pathogenic

processes, or pharmacological responses to therapeutic intervention [18–21]. To date, biomarker discovery efforts have been performed using various biofluids, including cerebrospinal fluid (CSF), serum, plasma, urine, and saliva [22]. However, the majority of studies have been performed on CSF, serum, and plasma. In this review, we discuss the current literature with regard to discovery and validation of fluid-based ALS biomarkers. While the list of biomarkers presented here is not exhaustive, we intend to discuss biomarkers that have been widely studied and highlight their demonstrated preclinical and clinical applications if available.

Biomarkers in CSF

The search for biomarkers related to ALS has been performed predominantly using CSF, owing to its intimate interaction with the central nervous system (CNS). In addition, because the CSF is in contact with the MN in the brain and spine, alterations in the biochemistry of the CSF could be indicative of neuronal injury and/or neurodegeneration, making the CSF an ideal target for biomarker discovery [23, 24].

Neurofilament Proteins

Neurofilaments are structural proteins of the cytoskeleton that are expressed in neurons and have been investigated as potential biomarkers for ALS and other neurologic diseases. Accumulation of neurofilament proteins has been linked to MN dysfunction [25–27]. Axonal injury releases these proteins into the CSF and subsequently to the blood. Owing to their relative stability in biofluids, detection of neurofilaments is relatively simple and can be performed via immunoassays, making these proteins potential biomarkers of the underlying pathology of ALS. Early studies identified increased phosphorylated neurofilament heavy chain (pNFH) in the CSF of patients with ALS when compared with healthy controls and other neurodegenerative diseases [28, 29]. Additional studies have demonstrated a utility for pNFH or a ratio of pNFH to complement C3 in the CSF as a diagnostic marker for ALS [30–33]. pNFH levels in the CSF or blood also have prognostic utility and can be used to assess the rate of disease progression and survival [30, 34], where disease progression is measured via the rate of decline of the ALS Functional Rating Scale-Revised (ALSFRS-R) score. Brettschneider et al. [28] observed decreased survival with high levels of pNFH in CSF; however, no correlation was found with the ALSFRS. Interestingly, higher CSF pNFH was also observed in patients with ALS who exhibited a predominant upper MN involvement, indicating potential use of pNFH in distinguishing upper and lower MN degeneration. In contrast to these findings, high levels of pNFH and neurofilament light chain (NFL) levels were correlated with central MN involvement [29]. While clinical examination is an effective method to distinguish upper MN and lower MN involvement, biomarkers could potentially be used to help monitor progression or to measure the effects of drugs specifically targeting MN within these areas. However, further studies are required to verify neurofilament or any other biomarker to distinguish involvement of upper *versus* lower MNs.

Many studies have also examined levels of NFL in ALS and control groups [35–38]. Similar to pNFH, increased levels of NFL were correlated with rate of progression, as monitored by the rate of decline in ALSFRS-R scores, as well as upper MN involvement [36, 39]. A rapid time to generation (time of symptom spreading from bulbar or spinal localization to both) has also been noted with increased CSF NFL [37]. Diffusion tensor imaging was recently used to evaluate the correlation between increased CSF NFL levels and white matter damage, where decreased fractional anisotropy and increased radial diffusivity were observed within the corticospinal tracts of patients with ALS but not in healthy controls, reflecting the degeneration of axons and subsequent release of NFL into the CSF [39].

An important question is whether levels of pNFH or NFL change during disease progression within individual patients. Longitudinal blood and CSF studies focusing on levels of NFL were recently evaluated from patients with slow, intermediate, and fast progressing ALS, as defined by the ALSFRS-R rate of decline [38]. In the CSF, NFL levels exhibited a modest 1.6% increase/month in slow-progressing ALS, whereas those considered as intermediate ALS progressors exhibited little change over a 15-month period. However, patients with fast-progressing ALS exhibited a 3.3% increase/month over a 6-month period. The reason for modest increases in slow progressors but no increase for intermediate progressors is unclear, though may be owing to the small numbers of patients in each group and is worthy of further examination in a much larger patient population. A recent study has also shown increases in CSF NFL correlated with decreases in ALSFRS-R scores [40]. However, no differences between fast-, slow-, and intermediate-progressing ALS was observed with NFL, pNFH, Tau, or pTau [40]. While these results suggest a prognostic biomarker utility of NFL, prior studies have noted inherent instability of NFL in CSF and its susceptibility to protease degradation [41, 42]. Therefore, caution may be exercised with studies involving NFL as a fluid-based biomarker.

Recent longitudinal studies examining pNFH in CSF or blood show relatively stable levels over time, though levels of pNFH in serum appeared to increase at early stages of the disease [34, 43]. Weydt et al. measured levels of pNFH and NFL in CSF and blood of symptomatic and asymptomatic carriers of disease causing mutations to determine if either biomarker could be detected in symptomatic individuals [44]. While elevated levels were observed in symptomatic

mutation carriers, neither pNFH nor NFL were detected at elevated levels in asymptomatic ALS mutation carriers. It should be noted that one caveat of this study is that most of the asymptomatic mutation carriers harbored the *C9orf72* repeat expansion. However, *C9orf72* does not have 100% penetrance and therefore some of these individuals may never develop ALS and thus no changes in pNFH or NFL would be detected, which is consistent with the results from this study. A study in the superoxide dismutase 1 (SOD1) transgenic mouse model of ALS also indicated that pNFH levels increase near or at the time of symptom onset. From current data, we believe that pNFH and NFL levels may significantly increase in patient biofluids only near or upon symptom onset in patients with ALS.

Biomarkers of Inflammation

A common feature among many neurodegenerative diseases is glial activation accompanied by increased levels of inflammatory mediators, potentiating neuroinflammation and cell death [45, 46]. Recent proteomic analyses identified 248 proteins in the CSF from both healthy controls and patients with ALS that exhibited enrichment in biologic roles related to the complement cascade and acute inflammation [47]. Additionally, studies in our group using unbiased proteomic analyses of CSF also identified that acute inflammatory responses and complement activation were among the top pathways altered in the CSF of patients with ALS when compared with healthy controls and other neurologic diseases [10]. Therefore, inflammatory mediators present potential biomarkers for ALS. Inflammatory mediators that have been measured in the CSF of ALS and control patients are summarized in Table 1.

Overall, trends from these studies show increases and decreases in the level of proinflammatory and immune response mediators, suggesting that inflammatory pathways in ALS are independently regulated by many factors. While reasons for these observations need clarification, they demonstrate that cytokines and chemokines may play a role in the pathogenesis of ALS. For example, NSC-34 cells treated with CSF from patients with ALS exhibited decreased cell viability and increased lactate dehydrogenase activity [48], suggesting that factors within or released to the CSF contribute to neurodegeneration. Further analyses in the same study also showed that Chit-1 increased expression in microglia upon exposure to CSF from patients with ALS, suggesting its role in microglial activation. A recent study has also shown a positive correlation between CSF and serum interferon (IFN)-γ levels and both correlated with disease progression but not with ALSFRS-R scores [57]. Levels of interleukin (IL)-6 also correlated with hypoxic severity [54], suggesting that cytokine levels are dependent on oxygenation, which highlights a link between hypoxia and inflammation in ALS.

Table 1 Inflammatory mediators detected in the cerebrospinal fluid of patients with amyotrophic lateral sclerosis (ALS)

Biomarker	Key finding	Reference(s)
Chit-1	↑ levels and ↑ activity	[48]
	↑ levels	[49]
Prostaglandin E2	NC	[50]
	↑ levels	[51, 52]
VEGF	↑ levels	[53]
	↓ levels	[50]
IL-6	↑ levels	[53, 54]
GM-CSF	↑ levels	[53]
IL-2	↑ levels	[53]
IL-15	↑ levels	[53]
IL-17	↑ levels	[53]
MIP-1β	↑ levels	[53]
FGF	↑ levels	[53]
G-CSF	↑ levels	[53]
MIP-1α	↑ levels	[53]
MCP-1	↑ levels	[53, 55]
IL-10	↓ levels	[53]
IFN-γ	↓ levels	[53]
IL-8	↑ levels	[55]
Angiogenin	↓ levels	[50]
Angiopoietin 2	NC	[50]
Follistatin	↓ levels	[56]
IL-1α	↓ levels	[56]
Kallikrein-5	↓ levels	[56]

↓ and ↑ represent decreased and increased levels in patients with ALS *vs* controls respectively

Chit-1 = chitotriosidase-1; NC = no change observed; VEGF = vascular endothelial growth factor; IL = interleukin; GM-CSF = granulocyte macrophage colony-stimulating factor; MIP = macrophage inflammatory protein; FGF = fibroblast growth factor; G-CSF = granulocyte colony-stimulating factor; MCP = monocyte chemoattractant protein 1; IFN = interferon

In addition to extracellular cytokines and chemokines as biomarkers for ALS, the presence of activated inflammatory cell types can also serve as a potential biomarkers. Peripheral monocytes and regulatory T cells enter the CNS and can also modulate inflammation during ALS and play key roles in regulating disease progression [58, 59]. The point of entry of these cells is believed to be either the blood–brain barrier or the choroid plexus [60]. A combination of integrin receptors and inflammatory signaling molecules [IFN-γ, C-X-C motif chemokine 10, tumor necrosis factor (TNF)-α] regulate trafficking of monocytes across the blood–brain barrier and leukocytes across the choroid plexus to gain access to the CSF for distribution within the parenchyma [60, 61]. Further studies of peripheral immune cell trafficking into the CSF and entry into the brain and spinal cord tissue could potentially be explored using flow-assisted cell sorting in order to define the cell types

involved. These experiments could yield new insights into the pathogenesis and progression of ALS, as well as reveal the intricate intercellular signaling pathways that will allow for the identification of novel therapeutic biomarkers.

C9orf72 Dipeptide Repeat Proteins

The *C9orf72* repeat expansion is the most common genetic cause of familial ALS (fALS) and frontotemporal lobar degeneration (FTLD) [62, 63]. Nuclear export of mRNA containing this repeat expansion can result in non-ATG-mediated translation that has been detected in multiple neurodegenerative diseases [64]. Non-ATG-mediated translation of the *C9orf72* repeat expansion yields 5 dipeptide repeat proteins (DPRs) detected in patients with ALS and FTLD [65, 66]. While these DPRs can be exchanged between cells and have been shown to induce neurotoxic effects in model systems [67, 68], the presence of DPR pathology does not correlate with clinicopathologic measures of disease [69]. However, a recent study has also shown that DRPs can induce spontaneous formation and persistence of stress granules [70], which are cytoplasmic bodies that consist of proteins and mRNA that function to limit translation initiation and are a feature of ALS pathobiology [71]. While monitoring DPR pathology within CNS tissue during disease may be difficult, DPRs have been detected in CSF and blood and therefore could be monitored within these biofluids during disease progression and in clinical trials. Therefore, DPRs could be excellent biomarkers for tracking ALS pathology in response to therapeutic intervention. Previous studies have generated immunoassays for DPRs in tissue extracts and more recently in CSF [72, 73]. Currently, these DPRs have only been detected in patients with ALS or FTLD with the *C9orf72* repeat expansion. Further clinical studies will be required and are currently under development to validate this biomarker for future use in C9-based therapies.

Metabolic Biomarkers

While there is evidence of numerous pathologic mechanisms associated with ALS, excitoxicity continues to be one of the accepted mediators of disease progression and MN death and has been studied extensively [74–76]. Excitoxicity is a result of overstimulation of glutamate receptors, thereby increasing intracellular calcium levels leading to increased cell death. Therefore, it is reasonable to hypothesize that increased levels of extracellular glutamate could contribute to the progression of ALS. This has been addressed in previous studies where increased levels of glutamate were observed in the CSF of patients with ALS compared with controls [77–82]. In contrast to these findings, others have also demonstrated similar glutamate levels in CSF from patients with ALS and controls [83, 84]. Interestingly, another study shows lower levels of glutamate in the CSF that fails to correlate to disease progression or decreased MN cell death [85]. However, measurements of CSF concentrations of glutamate in response to therapeutics remains to be explored and therefore further investigation is warranted.

Mass spectrometry-based metabolomics has been used to discover metabolic signatures in patients with ALS. Wuolikainen et al. examined the CSF of patients with ALS with mutations in the SOD1 gene and identified distinct metabolic profiles for particular *SOD1* mutations, with the main differentiating metabolites being arginine, lysine, ornithine, serine, threonine, and pyroglutamic acid [86]. A recent study from the same group combining multiple mass spectrometric methodologies found increased creatine and decreased creatinine levels in the CSF of patients with ALS when compared with controls [87]. While the large phase III clinical trial of dexpramipexole failed to exhibit efficacy, a post hoc analysis found that creatinine loss correlated with disease progression and this loss was reduced in patients treated with dexpramipexole [88]. This result supports continued investigation of patient creatine and creatinine in the blood and CSF as potential prognostic biomarkers for ALS.

TAR DNA-Binding Protein of 43 kDa

TAR DNA-binding protein of 43 kDa (TDP-43) is a core component of ubiquitinated cytoplasmic inclusions in sporadic ALS (sALS), many forms of fALS, and FTLD [89–91]. In postmortem studies, TDP-43 inclusions are detected in approximately 97% of patients with ALS. Based on these findings, investigators have pursued TDP-43 as a biofluid biomarker for ALS [92–95]. If changes can be reliably detected in blood or CSF, TDP-43 could be a valuable biomarker for the majority of patients with ALS. Overall, these studies identified increased levels of TDP-43 in CSF from patients with ALS compared with a variety of neurologically diseased and nondiseased controls. Additionally, increased TDP-43 levels were also observed among patients who were examined within 10 months of disease onset, suggesting that TDP-43 might be useful as a prognostic indicator for early stages of ALS [93]. However, the absolute levels of TDP-43 measured in CSF varies across the studies, suggesting that the TDP-43 immunoassays are inconsistent for measuring the protein within CSF or blood samples. One challenge may be that as TDP-43 is a self-aggregating protein, it may self-assemble and generate structures that limit access of the detection antibody when present in biofluids. In addition, TDP-43 has been shown to be subject to alternative splicing causing truncations of the N- or C-terminus, leading to splice variants linked to ALS [96]. TDP-43 has been detected as 2 distinct species via Western blot: a 45-kDa (phosphorylated form) and 28-kDa isoform [95]. However, the 28-kDa isoform was determined to be nonspecific binding to IgG light chain, indicating antibody cross-reactivity. While interest remains high to monitor

levels of TDP-43 in CSF or blood as a biomarker, significant improvements in TDP-43 assay methodologies are necessary in order to attain this goal.

Cystatin C

Previous efforts in our group, as well as others, identified cystatin C as a potential biomarker in the CSF of patients with ALS [49, 97–100]. Cystatin C is a cysteine protease inhibitor that is involved in extracellular matrix (ECM) regulation, as well as a variety of CNS diseases [101, 102]. Within the context of ALS, cystatin C has been identified as a component of bunina bodies, which are small eosinophilic inclusions in the lower MN [103]. Additionally, a reduction in cystatin C levels in spinal MN and astrocytes has also been correlated with the formation of TDP-43 inclusions [104]. Using surface-enhanced laser desorption/ionization, reduced levels of cystatin C were observed in CSF from patients with ALS as compared with control subjects (healthy and diseased) [97]. Levels of cystatin C positively correlated with survival time for patients with limb onset but did not correlate with the disease duration [98]. A longitudinal study was also performed on CSF and plasma over a 1 to 2-year time period [105]. In this study, analyses of cystatin C levels in the CSF showed that fast and slow progressors exhibited trends towards decreased and increased levels over time, respectively. However, only the trend in the slow progressors was significant. In contrast to these findings, plasma levels of cystatin C were significantly higher in patients with ALS as compared with healthy controls [105]. While protein levels can provide insight into the physiology and pathology, protein levels do not necessarily correlate with enzymatic activity, highlighting a significant limitation to the studies performed to date. To address this, one study measured activity of cystatin C; however, no difference in activity was determined between ALS and control groups [106]. Therefore, further studies are needed in order to determine how decreased cystatin C levels relate to ALS pathogenesis and disease progression and to determine the use of cystatin C as a biomarker for ALS.

MicroRNA

MicroRNAs (MiRNAs) are 20 to 25 nucleotide-long noncoding transcripts that regulate biological processes via mRNA cleavage or translational repression of mRNA [107, 108]. During canonical miRNA biogenesis [109], miRNAs are synthesized from primary miRNAs, which are transcribed in the nucleus. Primary mRNAs are processed into pre-miRNAs by Drosha and subsequently exported to the cytoplasm. Pre-miRNAs are finally processed by the Dicer complex, resulting in the formation of mature miRNA, which form RNA-induced silencing complexes. There are over 1000 miRNAs in humans and they are typically measured using either RNA sequencing, microarray, Nanostring, or real-time quantitative polymerase chain reaction methodologies. Alterations in miRNA regulation has been implicated in a wide variety of CNS disorders [110–112]. Defects in miRNA biogenesis, for example via knockout of Dicer, has been correlated with decreased motor activity and survival, muscle atrophy, denervation, spinal cord sclerosis, and axonopathy in mice [113]. Additionally, previous studies have demonstrated decreased miRNA expression in the ventral lumbar spinal cord from patients with ALS [114], and also in spinal MN with ALS-causing mutations [115]. These results highlight the potential utility of miRNAs as biomarkers for ALS. Profiling of miRNAs in CSF, serum, and plasma of patients with ALS has been performed in few studies (Table 2), indicating the need for continued investigations and large validation studies for potential miRNA biomarkers.

Overall, similar to the results in tissues from patients with ALS, most miRNAs levels are decreased in the CSF and serum of patients with ALS when compared with healthy and diseased controls. miRNA181a-5p [116], miRNA-143-5p [117], miRNA-574-5p [117], and miRNA-338-3p [118] are the only 4 that have been found to be increased in CSF from patients with ALS. While the physiological roles of most of these miRNAs still remains to be defined within the context of ALS, expression of miRNA-143-5p and miRNA-574-5p was shown to be decreased in lymphoblastoid cell lines (LCL)

Table 2 MicroRNAs (miRNAs) differentially expressed in the cerebrospinal fluid (CSF), serum, and plasma

miRNA	CSF	Serum	Plasma	Reference
miRNA-15b-5p	↓	NA	NA	[116]
miRNA21-5p	↓	NA	NA	
miRNA195-5p	↓	NA	NA	
miRNA148a-3p	↓	NA	NA	
Let7b-5p	↓	NA	NA	
miRNA181a-5p	↑	NA	NA	
Let7a-5p	↓	NA	NA	
Let7f-5p	↓	NA	NA	
miRNA-132-5p	↓	↓	NA	[117]
miRNA-132-3p	↓	↓	NA	
miRNA-143-5p	↑	↓	NA	
miRNA-143-3p	↓	↓	NA	
miRNA-574-5p	↑	NC	NA	
miRNA-338-3p	↑	↑	NA	[118]
miRNA-1234-3p	NA	↓	NA	[119]
miRNA-1825	NA	↓	NA	
miRNA-4649-5p	NA	NA	↑	[120]
miRNA-4299	NA	NA	↓	

↓ and ↑ represent decreased and increased levels in patients with ALS *vs* controls, respectively

NA = miRNA was not measured in the biofluid; NC = no change observed

expressing ALS mutant forms of *TDP-43*, *C9orf72*, and *FUS*, as well as in sALS-derived LCLs. miRNA-143-5p expression was also decreased in *SOD1* mutant LCLs. These results suggest a link between levels of miRNAs and the different forms of ALS (familial and sporadic), making these viable biomarkers. One challenge with the use of miRNAs as biomarkers stems from the fact that 1 miRNA acts on multiple downstream targets. Therefore, the effects of most miRNAs require in-depth studies to elucidate which downstream targets or pathways are activated or inhibited. Clarifying these targets would provide a means for validation for specific miRNAs as potential ALS prognostic or diagnostic biomarkers. In addition, multiple studies often identify different or overlapping miRNAs as ALS biomarkers, highlighting the need for large validation studies to determine which miRNAs represent optimal biomarkers for ALS. It may also be necessary to combine miRNA signatures with other genetic risk factors to optimize biomarkers for subsets of patients with ALS.

Cu/Zn SOD1

SOD1 is an ubiquitously expressed antioxidant enzyme that is involved in the conversion of superoxide to oxygen and hydrogen peroxide, protecting cells from oxidative stress. Nearly 20% of fALS cases are caused by *SOD1* mutations and has been widely studied [121–123], though this represents a minor fraction of all patients with ALS. One study measured levels of CSF SOD1 levels between patients with ALS and neurologic disease controls and failed to find significant differences between the groups, though significantly higher levels of SOD1 were detected in the CSF of male patients with ALS, suggesting a potential gender stratification biomarker [124]. This study indicated that SOD1 CSF level is not a diagnostic biomarker for ALS. Prior animal studies have shown that silencing *SOD1* led to preservation of grip strength and a delay in the onset of motor function deficits [125, 126]. In a clinical trial for pyrimethamine, an antimalarial drug, SOD1 levels in the CSF decreased along with a trend towards declining Appel ALS scores, a measure of disease progression [127]. In addition, it was shown that while the baseline levels of SOD1 in the CSF varied between patients with ALS, the level remains relatively constant over time in individual patients [128]. These studies suggest that SOD1, while not a diagnostic biomarker, could function as a pharmacodynamic (PD) biomarker for SOD1 therapies that reduce SOD1 levels in the CNS. Recent studies have confirmed this hypothesis, as treatment with SOD1 antisense oligonucleotides greatly reduces SOD1 levels in the CSF in mice [128], thus providing strong support that measurements of SOD1 in the CSF will function as a pharmacodynamic biomarker for antisense treatments that are currently in clinical trials. Results from a phase I clinical trial of intrathecal delivery of SOD1 antisense oligonucleotides in patients with ALS with SOD1 mutations demonstrated safety for this treatment, though no substantial longitudinal changes in SOD1 CSF levels were detected [129]. Additional ALS clinical trials using SOD1 antisense oligonucleotides are currently in development and will likely include SOD1 measures as a PD biomarker of drug treatment and activity.

Biomarkers in Blood (Serum and Plasma)

While studies involving the CSF have identified many potential ALS biomarkers, mass transfer occurs between the blood and CSF at the blood–CSF barrier, predominantly in the subarachnoid space and the choroid plexus [130, 131], suggesting that the same biomarkers could be present in both fluid types. This was previously demonstrated where several studies have indicated significantly high correlations between blood (serum and plasma) and CSF levels of the same biomarker [34, 38, 44, 118]. However, other studies have also demonstrated that levels of biomarkers, that were discovered in blood, did not correlate with the levels of that same biomarker found in the CSF, suggesting that these 2 fluids are independently regulated [105, 117]. Blood-based biomarkers may also be generated by other organ systems affected during ALS, such as peripheral blood cells or degenerating muscle. Therefore, blood is an excellent biofluid for discovery and validation of biomarkers for ALS. Additionally, the lack of ethical implications and decreased difficulty of obtaining blood as compared with CSF makes blood a more viable option.

pNFH and NFL

With the success in validating neurofilament proteins as ALS biomarkers in the CSF, pNFH and NFL have also been examined as blood-based biomarkers in the serum and plasma. Higher levels of plasma pNFH were observed in patients with ALS as compared with healthy controls, but no difference was observed when compared to diseased controls [31]. Longitudinal studies have confirmed this finding and have also demonstrated stable levels over a 4-month study [132]. In a study consisting only of patients with ALS, high serum levels of pNFH was associated with rapid decline of ALSFRS-R scores at 4 months from baseline measurements but not 12 months [34]. However, higher plasma levels of pNFH were not correlated with decline in ALSFRS-R scores. In contrast to these findings, higher baseline levels of pNFH have been weakly correlated with the decline in ALSFRS-R scores [132]. Interestingly, increased levels of pNFH in plasma and serum was correlated with shorter survival time and bulbar onset [34]. Overall, these results highlight the utility of pNFH for prognosis and potential use in patient stratification.

In whole blood, levels of NFL were significantly higher in patients with ALS as compared with both healthy controls and patients who were asymptomatic but carried mutations in

C9orf72, *SOD1*, *TARDP*, and *FUS* [44]. Levels of NFL have also been shown to increase in serum and plasma of patients with ALS as compared with controls [38]. In this same study, no increases in plasma levels were observed over 15 months in fast, slow, and intermediately progressing ALS; however, a 4.6% increase per month was observed in the serum of patients with fast-progressing ALS. In contrast to these studies, measurements of NFL in the sera from patients with diseased MN could not be accurately quantified owing to significant variation between samples [40].

Additional studies using increased number of blood samples from patients with ALS and disease controls are needed to validate the above findings and to compare directly pNFH to NFL in the same samples. Ideally, these studies should also include CSF to determine the correlation between CSF and blood levels of pNFH and NFL. This type of head-to-head comparison will determine if one neurofilament protein is superior to the other, or if a particular neurofilament protein is best for identifying or monitoring a specific subset of the ALS population.

miRNAs

In addition to miRNAs in the CSF, investigations in the serum and plasma have also been performed (Table 2) [119, 120, 133]. Significant decreases in miRNA-1234-3p were only observed in sera from patients with sALS compared with healthy controls, but no significant difference was observed in sera from patients with fALS as compared with healthy controls [119]. This suggests that miRNA-1234-3p may allow for specific diagnosis of sALS. Plasma miRNA-4649-5p has been shown to increase in patients with sALS and was shown to be negatively correlated with disease duration [120]. miRNA-424 and miRNA-206 had significantly higher expression in ALS plasma compared with healthy controls [133]. Interestingly, levels of miRNA-424 correlated with the medical research council sum score, which is a measure of lower MN integrity, indicating its potential use in patient stratification. To the best of our knowledge, there have not been any studies that have used miRNA as a secondary marker for therapeutic development, and thus further studies are warranted.

Biomarkers of Inflammation

Similar to the results in CSF, increases in levels of inflammatory factors (IL-6 and IL-8) were also observed [134]. However, in contrast to levels in the CSF, decreases in immune factors (IL-5 and IL-2) and glutathione levels were also shown indicating decreased immune response with increased oxidative stress. In addition to inflammatory factors, levels of the anti-inflammatory factor transforming growth factor (TGF)-β1 were shown to increase in plasma from patients with ALS as compared with controls and was positively correlated with disease duration [135]. Similar observations were also observed in serum where TGF-β1 concentrations were higher in patients with ALS than in controls [136]. Given that one of the pathological mechanisms of ALS is inflammation, these findings seem counterintuitive to what would be expected as TGF-β1 is an anti-inflammatory mediator and thus should attenuate neuroinflammation. Nevertheless, these findings suggest that TGF-β could potentially modulate ALS progression and therefore further investigations will be required in order to determine the downstream effects and participation of this cytokine in the pathogenic process. Identification of prognostic inflammatory biomarkers has also been performed using plasma. Increases in creatine kinase, ferritin, TNF-α, IL-1β, IL-2, IL-8, IL-12p70, IL-4, IL-5, IL-10, and IL-13, and decreases in IFN-γ, have been observed [137]. Longitudinal analyses revealed that plasma IL-6 increased over 6 visits in those that had slow progressing ALS, those that are male, exhibited limb onset, less functional impairment, and also in patients with ALS treated with riluzole, further suggesting the utility of IL-6 in patient stratification. Median levels of TNF-α have also been shown to increase in the plasma of patients with ALS compared with healthy controls, and these elevated levels were observed over an 80-month period [138], implicating prognostic use of this inflammatory marker.

In addition to extracellular cytokines and chemokines, peripheral monocytes can enter the CNS and be detected in postmortem tissues from patients with ALS [139]. Regulatory T cells within the blood also enter and modulate inflammation within the CNS where reduced expression of FoxP3, a transcription factor required for regulatory T-cell function, correlated with the rate of disease progression and survival of patients [140]. Therefore, flow cytometric analysis of blood-borne T cells and subsequent measurement of FoxP3 levels may provide another prognostic indicator of disease progression.

TDP-43

Levels of TDP-43 have been assessed in plasma where increased levels were observed in patients with ALS compared with controls [141]. Longitudinal analyses showed consistent levels over time in most patients; however, 1 patient exhibited increased levels over time, potentially highlighting the heterogeneity of the disease. Another study has shown increased levels of phosphorylated TDP-43 in the plasma and CSF in patients carrying the *C9orf72* expansion [142]. In contrast to these findings, levels of TDP-43 in circulating lymphomonocytes were approximately the same in TDP-43 mutant-carrying cells, nonmutant cells, and controls [143]. However, given the limitations of the current TDP-43 immunoassays as described above, the potential biomarker utility of TDP-43 in blood remains uncertain.

Metabolic Biomarkers

Previous screening of amino-acid concentrations in plasma have shown increased levels of glutamate and lysine in patients with early-onset ALS, while leucine levels decreased [144]. Increased levels of plasma glutamate were positively correlated with disease duration [145], and was observed in patients who exhibited spinal onset [146]. Interestingly, while riluzole decreased disease progression, no effect on the levels of plasma glutamate or glycine was observed after 6 months of treatment [146]. This is in contrast to what has been shown in serum, where glutamate and total amino-acid concentrations were decreased after a 6-month treatment with riluzole [147]. The reasons for these discrepancies are not known. Nevertheless, these results demonstrate the utility of glutamate as a biomarker for ALS in serum in response to drug intervention.

Paganoni et al. also demonstrated that serum uric acid levels correlate with survival in men with ALS [148]. This study measured serum uric acid levels in a large number of ALS clinical trial participants, and by controlling patient body mass index, showed that higher baseline levels were associated with increased survival. The threshold cut-off value for improved survival was 4.8 mg/dl, which, interestingly, was a level detected in only a small fraction of women. Continued validation studies are required for this serum biomarker to determine if a separate, gender-specific cut-off level can be identified for women.

A global metabolomics study of plasma from over 250 subjects identified a 32-member panel of metabolites that differentiated ALS from healthy and disease controls [149]. Included in this biomarker panel were creatine, creatinine, urate, glutamine, and pyroglutamine, all identified in prior or subsequent metabolomics studies. While additional validation studies are needed, these studies suggest that specific metabolic alterations can be identified in patients with ALS that contribute to disease progression and survival.

Biomarkers in Urine

While CSF and blood-based screening has shown promise in identifying fluid-based biomarkers, the inherently invasive nature of obtaining these fluids, especially with CSF, presents difficulty. Additionally, ethical considerations limit the collection of CSF from healthy patients and, as a result, studies focusing on characterization of CSF from healthy individuals are limited [150, 151]. One way to circumvent this issue is through investigation of urine-based biomarkers. However, there are a limited amount of studies using urine from patients with ALS.

To the best of our knowledge, only neurotrophin receptor p75 (p75NTR) [152], glucosylgalactosyl hydroxylysine (Glu-Gal Hyl) [153], type IV collagen [154], and 8-hydroxyl-2'-deoxyguanosin (8OH2'dG) [155, 156] have been explored as potential biomarkers warranting further studies of urine for ALS biomarkers. Recently, the extracellular domain of p75NTR, which is a regulator of cell survival and death, was shown to exhibit increased levels in urine from ALS patients with limb or bulbar onset as compared with healthy controls [152]. Similar trends were also observed in $SOD1^{G93A}$ mice [152]. The p75NTR results using human urine also correlated with a decline in ALSFRS-R scores, such that patients with fast-progressing ALS exhibited higher levels of p75NTR. Degradation of collagen results in the excretion of the metabolites Glu-Gal Hyl and galactosyl hydroxylysine into urine, making these feasible biomarkers for ALS [153]. Glu-Gal Hyl exhibited decreased levels in ALS which continually decreased throughout the duration of the disease, while no change in galactosyl hydroxylysine levels were observed. These results correlate with earlier studies where total collagen levels also decreased in lateral corticospinal tract and anterior horn in the spinal cord of patients with ALS [157]. Decreases in type IV collagen levels in urine were also observed in patients with ALS as compared with diseased and healthy controls [154]. These results align with the established paradigm of ECM turnover during the progression of ALS. In addition to structural proteins, levels of 8OH2'dG, a marker of DNA damage due to oxidative stress, were elevated in patients with ALS as compared with healthy and diseased controls [155]. The change in urine levels of 8OH2'dG were negatively correlated with the rate of change of ALSFRS-R scores, suggesting that 8OH2'dG could serve as a potential prognostic marker, and also confirms that oxidative stress contributes to the ALS pathology. Similar observations were shown in another study using patients with sALS; however, no trend was observed between 8OH2'dG levels and ALSFRS-R scores [156]. Taken together, these results demonstrate the potential utility of urine for ALS biomarker discovery. Urine-based biomarkers may also provide further insight into the roles of ECM turnover and DNA damage related to ALS pathology.

Biomarkers in Saliva

Similar to studies involving urine-based biomarkers, there are relatively few studies that have investigated potential biomarkers for ALS in saliva, as shown in Table 3. These studies have focused on markers that are synthesized by the endocrine system, suggesting its potential involvement in the pathogenesis of ALS. However, in one study chromogranin A levels did not correlate with many measures from the ALS assessment questionnaire, such as physical mobility, activities of daily living, eating and drinking, and communication. Conversely, cortisol levels did correlate with accepted

Table 3 Endocrine amyotrophic lateral sclerosis (ALS) biomarkers in saliva

Protein	Function	Levels in patients with ALS *vs* controls	Correlations (±)	Reference
Chromogranin A	Endocrine stress marker	Increased (controls = moderate ALS, vascular dementia, and healthy)	+ with emotional functioning	[158]
Cortisol	Steroid hormone	Decreased at 30 min postawakening	+ with ALSFRS and MMT measures—with depressive status measures	[159]

ALSFRS = ALS Functional Rating Scale; MMT = manual muscle testing

measures of ALS progression. Therefore, much further in-depth studies using accepted measures of ALS progression such as the ALSFRS-R scores, manual muscle testing, and forced vital capacity are required in order to validate this biomarker, as well as others derived from saliva and their relation to ALS pathogenesis. Nevertheless, given the limited amount of information using this easily obtained biofluid, future studies could focus upon identifying additional biomarkers and increased sample sizes, as well as verification in clinical trials.

Clinical Applications

Many of the studies discussed previously have demonstrated the diagnostic and prognostic potential for these biomarkers for ALS. In follow-up studies, applying these biomarkers in clinically relevant situations is used for further validation, as summarized in Table 4.

A few studies have already utilized inflammatory mediators as biomarkers during clinical trials [160–162, 164]. Given that inflammation is an enriched process in patients with ALS as compared with controls [47], treatments have been studied that are aimed at reducing inflammatory marker mRNA and protein abundances. Nineteen patients with moderately progressing ALS and lower MN disease, treated with a combination of anakinra and riluzole, were tolerant to the administered doses. However, no effect on disease progression was observed [160]. These patients also demonstrated a slight, yet insignificant, decrease in serum IL-6 and TNF-α over the trial period. However, fibrinogen levels were significantly decreased in both fluids, while levels of C-reactive protein increased in serum, indicating that this drug combination may target specific inflammatory pathways. Patients with ALS have also been treated with thalidomide but did not show any improvement in ALSFRS-R rate of decline nor did they exhibit any changes in serum TNF-α level over 9 months of treatment [164]. Tocilizumab (Actemra®) is an antibody-based therapeutic that inhibits IL-6 and soluble IL-6 signaling, and has been shown to decrease mRNA expression and protein secretion of inflammatory mediators in peripheral blood mononuclear cells from patients with sALS alone and in co-culture with activated macrophages after 24 h of treatment [162]. Direct infusion of Actemra into patients has been shown to decrease mRNA expression of many inflammatory cytokines in the serum of patients with sALS with high basal inflammation both acutely and longitudinally, and this correlated with attenuated decrease in ALSFRS-R scores [161]. Further studies utilizing Actemra in phase II clinical trials is

Table 4 List of biomarkers used in preclinical and clinical applications

Drug or therapy	Mechanism of action	Biomarker(s) used for evaluation	Biofluid	Reference(s)
Anakinra + riluzole	IL-1 receptor antagonist	IL-6, TNF-α, of fibrinogen levels, C-reactive protein	Serum	[160]
Tocilizumab (Actemra®)	Inhibits IL-6 and soluble IL-6	IL-1β, IL-6, IL-10, GM-CSF, and TNF-α, and evaluation of mRNA levels of many inflammatory cytokines	PBMC	[161, 162]
Arimoclomol	Inducer of heat shock response	pNFH	Plasma	[163]
Thalidomide	Immune suppression	TNF-α	Serum	[164]
Memantine + Riluzole	NMDA receptor antagonist	Tau and pNFH	CSF	[165]
Antisense oligonucleotide against SOD1	Short DNA sequence that binds mRNA and triggers degradation	SOD1	CSF	[128]
Pyrimethamine	Antimalarial drug	SOD1	CSF and leukocytes	[127]
Celecoxib	NSAID that inhibits COX-2	Prostaglandin E2	CSF	[166]
Dexpramipexole	Improves mitochondrial efficiency	Creatinine	Plasma	[88]

IL = interleukin; TNF = tumor necrosis factor; GM-CSF = granulocyte macrophage colony-stimulating factor; PBMC = peripheral blood mononuclear cells; pNFH = phosphorylated neurofilament heavy chain; NMDA = N-methyl-D-asparate; CSF = cerebrospinal fluid; SOD = superoxide dismutase; NSAID = nonsteroidal anti-inflammatory drug; COX = cyclooxygenase

already planned and will need to be performed in order to validate fully its effects [167].

Prior studies identified up to a 10-fold increase in the prostaglandin E2 levels in the CSF of patients with ALS as compared with healthy control patients [51, 52]. Prostaglandins, especially prostaglandin E2, are generated by cycolooxygenase (COX) activation and stimulate the release of glutamate by astrocytes. Given the large difference in the levels of this inflammatory mediator in the CSF of patients with ALS, it was evaluated as a secondary endpoint in a clinical trial for celecoxib, a nonsteroidal anti-inflammatory drug that inhibits COX-2 [166]. However, similar prostaglandin E2 levels were observed in both the celecoxib-treated and nontreated groups, though no changes in clinical parameters of disease or survival were observed by drug treatment, and levels were not elevated in the ALS patients. These results suggest that prostaglandin E2 is not a biomarker for ALS.

Early-phase clinical trials have also been performed using pNFH as biomarker of axonal injury and neuronal degeneration. Neurodegeneration and axonal injury releases neurofilament proteins into the CSF and, ultimately, the blood. Therefore, this biomarker has been considered for neuroprotective-based therapies. Preclinical studies utilizing $SOD1^{G93A}$ mice demonstrated the use of pNFH as a biomarker to test the effects of arimoclomol [163]. Plasma levels of pNFH were observed to decrease in these mice over approximately 60 days of treatment. Prognostic utility of pNFH and Tau has been used in a pilot study aimed at assessing the safety and tolerability of a combination of memantine and riluzole [165]. Patients who completed the treatment showed a decrease in the rate of disease progression along with a trend toward decreases in CSF levels of both Tau and pNFH. Further studies utilizing increased samples sizes are needed to validate these results. Nevertheless, this demonstrates the application of pNFH as a biomarker for therapeutic interventions.

Clinical trials have also demonstrated the use of SOD1 as a biomarker for ALS [127, 128]. SOD1 mutations are common among many patients with fALS and are a contributing factor to increased oxidative stress. As a result, therapies have been developed and tested that are aimed at decreasing levels of SOD1. Winer et al. measured SOD1 levels in the frontotemporal cortex and in the CSF as a biomarker for SOD1 antisense oligonucleotide therapy in rats and demonstrated a decrease in SOD1 levels, in both cases, as compared with untreated controls [128]. Interestingly, in humans, SOD1 levels in the CSF did not correlate with disease severity or progression, but these levels did remain relatively stable with repeat measurements, which suggests its potential utility as a PD biomarker for antisense-based therapies. In contrast, the antimalarial drug, pyrimethamine, has also been shown to decrease levels of SOD1 in leukocytes and the CSF; however, this was only shown in 2 patients [127]. While these trials demonstrate SOD1 as a potential prognostic biomarker, further studies with increased samples sizes are still needed.

Current Status and Future Directions

With respect to the studies reviewed here, many promising biomarkers have demonstrated diagnostic and prognostic utility for ALS. However, some of these biomarkers may not be sensitive and/or specific enough to segregate patients with ALS from neurologic disease or healthy controls. Table 5 lists results from the top 5 specific biomarkers with published data on sensitivity and specificity for ALS.

The vast majority of the biomarkers listed have been studied in the CSF, highlighting this biofluid in the discovery of biomarkers related to ALS. All of the biomarkers listed in Table 5 have been studied with adequate sample sizes and have demonstrated both high sensitivities and specificities for ALS. To date, the most advanced biofluid-based biomarkers for ALS are pNFH and NFL. These proteins appear as soon as symptoms are present, are inherently stable proteins in the blood and CSF, and correlate with the rates of disease progression and survival. Additionally, these proteins are highly abundant and changes in levels over time due to neurodegeneration are easily detected by immunoassays. Multicenter studies have also validated pNFH and NFL, something not yet accomplished for any other biofluid-based ALS biomarker. Additionally, neurofilament-based immunoassays are being moved into the clinic by Iron Horse Diagnostics, Inc. as an aid for earlier diagnosis, for prognostic indicators of ALS, and to monitor drug effects in clinical trials that attempt to reduce neurodegeneration. While studies on p75NTR are somewhat limited, its discovery in urine makes it a promising fluid-based biomarker for ALS for continued investigation. Further studies are needed to confirm its sensitivity and specificity for ALS in larger numbers of patients with ALS and disease controls.

One of the more interesting approaches to biomarker discovery is using combinations or ratios of biomarkers from different pathogenic pathways, which has been shown to increase the sensitivity and/or specificity of potential ALS biomarkers [31, 39, 116, 168]. For example, as mentioned previously, studies from our group have shown that a ratio of pNFH/C3, which combines data representing both axonal degeneration and inflammatory processes, maintains the sensitivity and slightly increases the specificity for distinguishing ALS from controls compared with pNFH alone (see Table 5) [31]. Using vector machine algorithms, combinations of IL-10, IL-6, granulocyte macrophage colony-stimulating factor, IL-2, and IL-15 not only exhibited adequate sensitivities and specificities, but have also demonstrated 89% accuracy when using these 5 biomarkers to distinguish patients with ALS

Table 5 Top 5 fluid-based biomarkers that have been used to distinguish patients with amyotrophic lateral sclerosis (ALS) from diseased and nondiseased controls

Biomarker	Biofluid	Patient information	Sensitivity (%)	Specificity (%)	Reference
pNFH	CSF	40 ALS, 40 non-ALS DC	97.3	83.8	[49]
pNFH	CSF	253 MND, 85 DC	83.0	80.0	[40]
pNFH	CSF	71 ALS, 52 DC, 40 HC	87.7	93.7	[31]
pNFH/C3	CSF	71 ALS, 52 DC, 40 HC	87.7	94.6	[31]
NFL	CSF	254 MND, 85 DC	77.0	88.0	[40]
NFL	CSF	64 ALS, 36 HC	97.0	95.0	[38]
NFL	Serum	64 ALS, 36 HC	89.0	75.0	[38]
NFL	Plasma	103 ALS, 42 HC	90.0	71.0	[38]
Combination of IL-10, IL-6, GM-CSF, IL-2, and IL-15	CSF	41 ALS, 33 DC	87.5	91.2	[53]
p75NTR	Urine	28 ALS, 12 HC	93.0	100.0	[152]
p75NTR	Urine	28 ALS, 19 DC	93.0	79.0	[152]

pNFH = phosphorylated neurofilament heavy chain; CSF = cerebrospinal fluid; DC = diseased controls; MND = patients with motor neuron disease; HC = healthy controls; NFL = neurofilament light chain; IL = interleukin; GM-CSF = granulocyte macrophage colony-stimulating factor; p75NTR = neurotrophin receptor p75

from neurologically diseased controls [53]. In a subsequent study from the same group, mathematical multivariate modeling was employed [20]. These models included a variety of inflammatory cytokines, growth factors, and proteins involved in iron metabolism, as an accurate measure of predicting disease duration in both CSF and plasma with coefficient of determination (R^2) values of 0.617 and 0.769, respectively, indicating that a combination of factors can be used for prognosis. Combining the 2 models into 1 comprehensive model that includes both CSF and plasma factors improved the R^2 value to 0.962. Another study identified a negative correlation between CSF and serum NFL levels with fractional anisotropy measurements [39], demonstrating the potential use of both protein levels with neuroimaging measurements. Ratios of miRNA181a-5p/miRNA15b-5p and miRNA181a-5p/miRNA-21-5p showed a considerable improvement in specificity with a slight decrease in sensitivity compared with each individual miRNA [116]. However, studies with increased sample sizes would be beneficial and are needed to verify these results. Taken together, these studies highlight the need for routinely measuring sensitivity and specificity for potential ALS biomarkers and it would be beneficial if appropriate disease controls are used in the study. To date, few studies have attempted to combine biomarkers across multiple biofluids (blood, CSF, urine), and this represents another area for future investigation. Studies should not only combine biofluid-based biomarkers, but also genetic risk factors and/or imaging-based biomarkers to optimize patient stratification or patient selection for specific disease-modifying therapies.

While many other biomarkers discussed here have shown initial promise, they must be further validated by multiple independent laboratories or via collaborative multicenter studies. In addition, assays used to measure these biomarkers must be optimized and validated in order to move toward clinical utility. Biomarkers should also be incorporated into all stages of ALS drug development, from preclinical studies to early-stage and, ultimately, late-stage clinical trials. Biomarkers should be identified that permit demonstration of drug–target engagement and downstream pathway effects. In addition, the inclusion of disease progression biomarkers will also help demonstrate therapeutic efficacy. These biomarkers should also augment the current clinical measures used in ALS clinical trials. While a few small ALS clinical trials have incorporated biofluid-based biomarkers to monitor drug effects, it should be noted that these studies have shown limited, and even in some cases no, drug efficacy, which, consequently, does not permit proper evaluation of these biomarkers for monitoring drug action. Use of SOD1 or DPR measurements in the CSF as PD biomarkers for clinical trials of SOD1 antisense or anti-*C9orf72*-directed therapies may have more immediate impact.

One area receiving greater attention is determination of the change in biomarker levels over time within individual patients (longitudinal studies). Some examples of these approaches, albeit from targeted analyses, have been performed [38, 105, 169], and have been discussed throughout this review. These studies require large sample sizes consisting of patients with ALS, neurologic disease controls, and healthy controls, in order to assess how particular biomarkers change over time. New longitudinal studies aimed at discovering changes in biomarker levels using unbiased methodologies will identify new biomarkers that track specific patient subpopulations during disease progression, as well as new therapeutic targets.

Finally, one other biomarker discovery method is a comprehensive screening of metabolites in each of these biofluids

[170]. Many untargeted metabolite studies have been previously performed in CSF [171, 172], plasma [149, 173], and serum [174], but metabolite identification is usually confined to searches using in-house databases, which biases the results. Further untargeted studies could yield deeper insights into metabolic dysregulation associated with ALS. One of the challenges of an untargeted metabolomic study is the lack of a comprehensive metabolite database, which limits the use of automated algorithms [175]. The most comprehensive human metabolomics databases to date is the human metabolome database [176]. For those metabolites that are not in the database, researchers rely on *de novo* sequencing, which may not be feasible for many in-depth untargeted metabolomics analyses. Further development of these databases would provide another avenue for biomarker discovery in biofluids for all diseases.

While ALS biomarker discovery efforts have been quite successful in the last decade, continued efforts are necessary to appropriately validate candidate biomarkers and the assays used to measure them. Incorporation of biomarkers in all stages of ALS drug development will vastly improve our ability to test drugs properly in clinical trials and, ultimately, find therapeutic treatments for ALS. With increased efforts, the hope is that a biomarker will not only aid in making faster Go/No-Go decisions in clinical trials, but also act as surrogate markers for primary endpoints in late-stage clinical trials. For now, the field of ALS biomarkers remains an active area of investigation with much promise as biomarkers are reaching clinical utility.

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