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Blood-based Biomarkers for Amyotrophic Lateral Sclerosis

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Abstract: Early detection of amyotrophic lateral sclerosis (ALS) is critical for better therapeutic outcomes. The median time from symptom onset to diagnosis of ALS is 11 months, with a range of 6-21 months. Given that the median life expectancy is three years, it is important to shorten the diagnostic journey, initiate therapies promptly, and facilitate clinical research participation. Biomarkers may be the key to enhancing early diagnosis, tracking disease progression, and testing target engagement of promising therapeutics. Clinically valid biomarkers for ALS are currently lacking, and research has been ongoing to identify appropriate biomarkers. Ideal biomarkers should be minimally invasive, such as blood. In this chapter, we review our current understanding of blood-based biomarker research in ALS and discuss future directions.

Keywords: amyotrophic lateral sclerosis; biomarker; blood; mitochondria; TDP-43

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is mostly a sporadic disease that leads to progressive degeneration of the cortical, bulbar, and spinal motor neurons (1–3). The median age of onset of sporadic ALS is 55, with a male predominance (1.5:1) (2). Diagnosis is based on upper motor neuron signs (spasticity, increased tendon reflexes) and lower motor neuron dysfunction, which may be supported by electrophysiological findings (1). Weakness and atrophy begin either in the bulbar region or in the limb muscles in about a third of cases and spread to the contralateral limb. Respiration is usually affected late in the disease and up to 50% may have evidence of frontotemporal dementia (FTD). Patients with older onset age, bulbar dysfunction, greater clinical disability, and low respiratory function have the poorest prognosis (1, 2). The median life expectancy from symptom onset is approximately three years, with a five-year survival rate of 20–25% and a 20-year survival rate of 5% (2). Most cases are sporadic, but 10–15% are of autosomal dominant inheritance.

Biomarkers can serve as tools for early diagnosis, predictors of prognosis, indicators of target engagement or therapeutic response, and enablers of discovery of future therapeutics for ALS. Biomarker development efforts for ALS have been hampered by a number of issues including small sample size, methodological variation, and lack of standardized techniques. On average, time from symptom onset to clinical diagnosis spans 11 months and this time is critical for life-saving interventions and therapies (4). Biomarkers could hasten diagnosis to allow for earlier introduction of therapies. Prognostic biomarkers are critical due to the heterogeneous nature of ALS and could facilitate prediction of how a subgroup of ALS subjects might progress or respond to a therapy. The low prevalence of ALS is an important issue that negatively affects clinical trials and biomarker development (5–7). In general, recruitment to clinical trials in rare diseases like ALS is a challenge. In ALS, several factors reduce the likelihood of participation in clinical trials including delay or uncertainty in diagnosis, slow progression, respiratory compromise, short life expectancy, and in some cases, dislike of being assigned to the placebo group. Discovery of diagnostic, prognostic, and target-engagement biomarkers are essential for accelerating the research and development of ALS therapeutics. In this chapter, we provide an overview of our current understanding of blood-based biomarkers for ALS.

POTENTIAL BIOMARKERS FOR ALS

The body of knowledge on biomarkers of ALS is limited. Ideally, a biomarker for ALS should be easy to quantify, minimally invasive, specific, reliable with an uncomplicated measurement process, and reproducible across multiple laboratories (8). Figure 1 summarizes the main areas of biomarker research in ALS, all of which target pathological findings in the disease. These aim to measure neurodegeneration, neuroinflammation/systemic inflammation, oxidative stress, excitotoxicity, mitochondrial function, and protein aggregation/proteostasis. Tables 1 and 2 summarize the overall findings of blood-based measures (9–30).

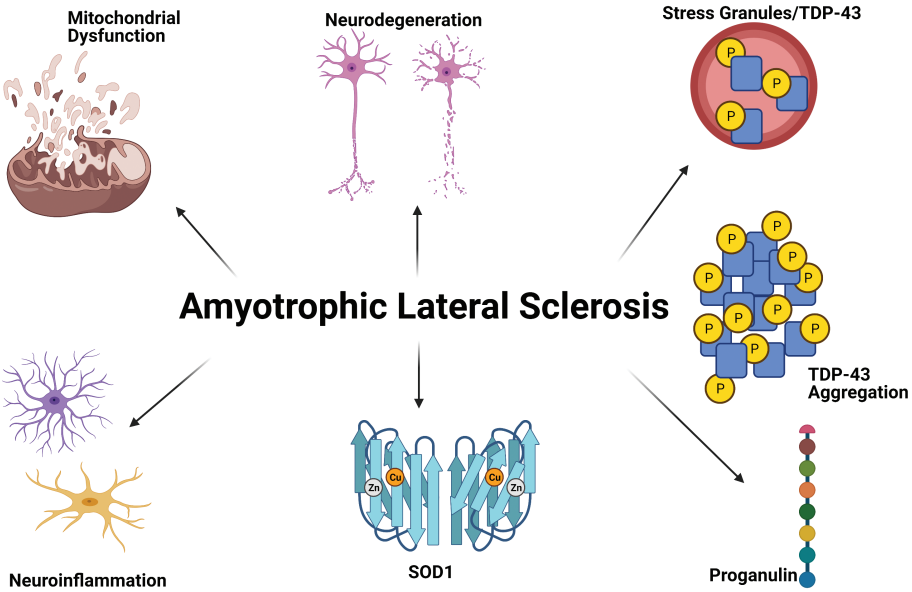

Figure 1. Biomarker Focus in ALS. Areas of biomarker development are focused on pathological findings in ALS. These include neuroinflammation/systemic inflammation, mitochondrial dysfunction, neurodegeneration, and protein aggregation/proteostasis. Created with BioRender.com

TABLE 1 Blood Based Biomarker Studies

Target	Source	Sensitivity/Specificity	n	Source
TDP-43	Plasma	NA	319	(9)
Exosome miRNA	Plasma	NA	40	(10)
Exosome proteomics	Plasma	NA	22	(11)
Proteomics	Plasma	58% and 90%	295	(12)
Glutamate Uptake	Platelet	NA	82	(13)
Mito-Respiration	Platelet	NA	15	(14)
Serotonin	Platelet	NA	114	(15)
NfL	Serum/Plasma	84–100% and 76–97%	248	(16)
NfH	Serum/Plasma	61–80%,72.1–83.7%	157–331	(16–18)
Cytokines	Serum/Plasma	NA	87–183	(19–21)
Ferritin	Serum/Plasma	NA	104–694	(22–24)
Creatine Kinase	Serum/Plasma	63.8% and 54.3%	216–834	(22, 25)
Non-coding RNA	Whole Blood	73.9–93.7%	88	(26)
Chromosomal Confirmation	Whole Blood	83.33–87.5%	58	(27)
Microarray analysis	Whole Blood	87%	1,116	(28)
Immune Cell Profiling	Whole Blood	NA	80	(29)
T-regs	Whole Blood	73.9–76.9%, 69.6–73.1%	217	(30)

TABLE 2 **Blood based biomarkers based on ALS categories (7)**

Familial ALS Biomarkers	Sporadic ALS Biomarkers
TDP-43	TDP-43
FUS	FUS
C9ORF72	Neurofilaments
	Extracellular RNA
	Stress granules
	Progranulin
	TNF-a and range of cytokines
	Metabolites (i.e., creatine kinase, platelet serotonin)
	Mitochondrial biomarkers (i.e., cytochrome oxidase, mitochondrial respiration rate, reduced Complex-II activity)

In this section, our current knowledge on biomarkers for both familial and sporadic ALS are discussed.

C9ORF72 protein

The most common genetic abnormality in frontotemporal lobar degeneration (FTLD) and ALS is the expansion of GGGGCC $(G_4C_2)_n$ repeat in an intron of chromosome 9 open reading frame72, depicted as C9ORF72 (31, 32). GGGGCC repeat expansions are translated through a repeat associated non-ATG (RAN) mechanism that does not require the AUG start codon (33). This non-canonical type of protein translation takes place without frame shifting or RNA editing, resulting in production of dipeptide repeat (DPR) proteins. There are five known DPR proteins, Poly-GA, Poly-GP, Poly-GR, Poly-PA, and Poly-PR (34, 35). These DPR proteins display different profiles across neurodegenerative diseases and could be potential biomarkers. Poly-GA proteins are associated with inclusion bodies when TDP-43 aggregation is lacking (TDP-43-negative inclusions) (35). In the neurons of post-mortem brain, Poly-GA protein aggregates are surrounded by TDP-43 aggregates (36). Poly-GR and Poly-PR DPR proteins cause neurodegeneration in drosophila without TDP-43 aggregation (37). Some studies suggest Poly-GA aggregation can induce TDP-43 phosphorylation and aggregation (35). Thus, the precise role of DPR proteins in TDP-43 aggregation has not yet been resolved. The G_4C_2 repeats can be measured in blood (38) and could serve as a blood-based biomarker for ALS. For familial ALS cases, peripheral blood lymphocyte levels of mutated *SOD1* and mutated C9ORF72 were used to measure target engagement in a clinical trial. Although primary outcomes of clinical trials are focused on cerebrospinal fluid (CSF)-based biomarkers, blood cell profiles appear to be changing as well. For example, SOD1 levels were reduced in peripheral blood lymphocytes in a pyrimethamine clinical trial (39). Poly-GP repeats in C9ORF72-positive ALS cases are detected in peripheral blood mononuclear cells

(PBMCs) (39). The ability to detect these in blood is promising for target engagement in clinical trials with therapies aimed at restoring proteostasis.

Neurofilaments

Neurofilaments function to maintain axon structure and transport (40). Neurofilaments exist in three isoforms; high-molecular-weight subunit (180–200 kDa [NfH]), middle-molecular-weight subunit (130–170 kDa [NfM]), and low molecular-weight-subunit (60–70 kDa [NfL])—all are exclusively expressed in neurons (41). Neurofilaments are considered surrogate biomarkers of neuronal degeneration (42). Aberrant NfL accumulation is observed in both familial and sporadic ALS patients (43–47). CSF levels are considered better than blood levels for the diagnostic confirmation of ALS (48). NfL levels increase during early stages of ALS (18). Further studies show NfL increases as early as 12 months prior to symptom onset in ALS and could be a predictive biomarker (49). Single molecule array technology or SIMOA has enabled the quantification of NfL in serum and plasma at pictogram/mL sensitivity (50, 51). NfL is widely used as a biomarker of ALS. NfL levels in serum are higher in ALS subjects and correlate well with CSF measurements (52). Overall, NfL strongly correlates with survival, but levels are largely steady over time and show no correlation with functional diagnostic scores such as the El Escorial Criteria (7, 16, 53). Using the SIMOA assay, serum NfL may be not only a clinically validated prognostic biomarker for ALS but may also be a biomarker of treatment effect (54). Plasma neurofilament heavy subunit (pNfH) has shown variable results across studies (7, 53). One study showed elevated pNfH levels predict faster progression at 4 months while another study showed it was associated with higher mortality at 12 months (18). Other studies show pNfH levels are neither steady nor reliable longitudinally and are not correlated with disease progression. Overall, the rate of change in blood pNfH is not reliable to predict disease progression and its utility as a diagnostic marker remains to be realized (16–18).

TDP-43

Transactive response (TAR) DNA binding protein 43 (TDP-43) regulates gene transcription, mRNA splicing, stability, and translation (55). Mutations in *TDP-43* cause familial forms of ALS and TDP-43 aggregates are found in most ALS subjects on autopsy (56–58). TDP-43 and its post-translational modifications can be measured across numerous biofluid and could serve as a biomarker for ALS (59–65). Within the ALS field, CSF TDP-43 measurements are preferred over blood-based samples. However, lumbar punctures are invasive, and patients are less likely to agree to this procedure for CSF sampling. Mass spectrometry analysis of post-mortem brain tissue from ALS subjects revealed a number of TDP-43 post-translational modifications including hyperphosphorylation, acetylation, ubiquitination, deamidation, and oxidation (66). Hyperphosphorylation (67, 68) and lysine acetylation increase TDP-43 aggregation (69). Phosphorylation of TDP-43 between amino acids 220-414 is suspected to prevent TDP-43 degradation and increase its expression levels (70). Plasma TDP-43 is higher but is unchanged in serum (9). TDP-43 is mis-localized in cytoplasmic fractions of PBMCs while

overall expression of TDP-43 is not changed. TDP-43 levels in PBMCs correlate with disease burden over time (62, 71, 72). Longitudinal studies showed that TDP-43 plasma levels are highly variable over time, and between individuals (7). These variable findings could be a consequence of blood handling, hemolysis, and coagulation. Classification of TDP-43 expression and post-translation modifications in the blood of ALS subjects could be used as a biomarker for detection/diagnosis and therapeutic outcomes.

Extracellular RNAs, exosomes and stress granules

Extracellular RNAs are found outside the cells in extracellular vesicles (EVs) such as exosomes, micro vesicles and apoptotic bodies, or RNA-binding proteins. Their association with lipids and proteins protect them from degradation and allows for their measurement. Extracellular RNAs are found in many forms, such as tRNA, mRNA, microRNA (miRNA), and circular RNA (circRNA) within EVs. tRNA fragments may be disease-specific and should be considered for biomarker development (73, 74). Next generation sequencing of neural enriched exosomes from plasma of ALS patients identified eight miRNAs that could discriminate ALS from healthy subjects (10). circRNA can be detected in extracellular fluid (75–78). The function of circRNA is largely unknown but regulation of gene expression is a likely function (79). High levels of extracellular circRNA in CSF suggest that the central nervous system (CNS) may secrete them (80–82). The potential of circRNA as a biomarker in ALS was recently reviewed (83).

Exosomes are 50-100 nm extracellular vesicles released from cells. In blood, exosomes are released by erythrocytes, platelets, endothelial cells, and lymphocytes (Table 3). Proteomic analysis of exosomes from ALS and Parkinson 's disease (PD) subjects was able to discriminate between these two diseases (11). Exosomes derived from blood, serum, or plasma show high contamination of blood proteins, which decreases the specificity of proteomic analysis (84).

Stress granules are cytoplasmic RNA complexes that form in response to environmental stress. Several ALS-associated proteins, such as FUS (85), TDP-43(86), Ataxin2 (87), and SOD1 variants (88) have been identified as integral components of stress granules. Currently, measurements of stress granules are limited to cell-based assays.

TABLE 3 Exosome Defining Markers

Exosome Donor Cell	Marker
Platelets	CD31, CD41, CD61, CD42b, GPIIb-IIIa
Endothelial cells	CD31, CD42B, CD51, CD105
Monocytes	CCR2, CD14, CD41a
Neutrophils	CD43, CD16
Lymphocytes	CD4, CD8
Erythrocytes	CD235a

Progranulin

Progranulin (PGRN) is a cysteine-rich secretory protein involved in cell proliferation, inflammation, and tumorigenesis (89). Brain progranulin is implicated in neuronal survival as well as pathogenesis of neurodegenerative diseases (90, 91). Progranulin levels can be measured in both CSF and serum of FTD, ALS, and Alzheimer's disease patients (92). Although no comprehensive study is available to compare progranulin levels in brain with CSF and serum values (92), blood levels are 35 times higher than CSF in ALS subjects with FTD (93). This suggests blood measures of progranulin could serve as a biomarker in ALS.

RNAseq and proteomics

Microarray analysis of blood cells has allowed for machine learning and identification of ALS subjects from the healthy (28) with an accuracy of 87%. Gene expression changes observed in ALS blood cells include increased neutrophil related genes with decreased erythroid lineage-specific genes. The expression of copper chaperone of superoxide dismutase (CCS) and other mitochondrial respiration-linked genes were significantly associated with survival in ALS subjects (28). Further, circulating non-coding RNAs have shown a 73.9-93.7% accuracy in discriminating the healthy from ALS populations (26). Proteomic analysis of ALS blood samples shows changes in proteins involved in the regulation of metabolism and mitochondrial function, particularly carbohydrate, creatine, and lipid metabolism (12). Nitric oxide and reactive oxygen species production are upregulated in macrophages of ALS patients (94). Protein expression of TDP-43, cyclophilin A, and ERp57 in PBMCs were found to associate with disease progression in ALS subjects. A multiprotein expression profile in PBMCs could discriminate ALS from healthy controls with 98% power, and discriminate ALS from other neurologic disease with 91% power. The multiprotein expression profile was further validated in the G93A *SOD1* ALS mouse model using both PBMCs and spinal cord tissue (62). Chromosomal conformation in blood samples can also discriminate between ALS and healthy subjects with a sensitivity of 83.33–87.5% and specificity of 75.0–76.92% (27).

Inflammatory markers

Cytokine expression in blood is altered in ALS subjects but do not change over time. Tumor necrosis factor α (TNF-α) and downstream effector interleukins are increased in ALS subjects (19–21). Data from 25 independent studies examining serum and plasma levels of cytokines show that TNFα, IL-1β, IL-6, IL-8, TNF receptor 1, and vascular endothelial growth factor (VEGF) are elevated in ALS (7). Other inflammatory markers such as complement components, C reactive protein, and chitotriosidase have shown equivocal association with ALS (7). Immune cell profiling has shown that higher levels of lymphocytes, monocytes, and T cell subtypes are associated with longer survival times (29). $CD4^{+}CD25$High T-regs are lower in ALS patients (30, 95), and is a measure of ALS progression. Overall, inflammatory markers have not shown specificity for ALS diagnosis and no association with disease progression has been established yet.

Metabolites

Serum and plasma creatine kinase are elevated in ALS subjects and correlate with the revised ALS functional rating scale (ALSFRS-R) score and other functional outcomes in ALS (22, 25). Plasma and serum ferritin levels are higher in ALS subjects. In some studies, ferritin levels were associated with survival and in others it did not (22–24). Glutamate uptake is impaired in platelets and astrocytes derived from ALS subjects (13). Furthermore, platelet serotonin levels are reduced in ALS subjects and is associated with an increased risk of death (15).

Mitochondrial biomarkers

Mitochondrial dysfunction is observed across numerous tissues in ALS subjects. Spinal cord mitochondrial DNA shows higher levels of mutation, and reduced citrate synthase, complex I+III, II+III and IV activities (96). Induced pluripotent stem cells (iPSCs) derived from ALS patient fibroblasts show reduced mitochondrial function when differentiated into motor neurons. iPSC-derived ALS motor neurons had reduced ATP production and mitochondrial respiration and increased glycolytic flux (97). Muscle samples from ALS patients show a large number of cytochrome oxidase-negative fibers, and some of these patients had reduced enzyme activity (98, 99). Two separate studies of ALS muscles showed reduced mitochondrial respiration and changes in mitochondrial DNA (99, 100). Tissues outside of the spinal cord and muscle also show changes in mitochondrial function. Fibroblasts from ALS patients show reduced basal, uncoupled, and ATP-linked respiration (101). Hepatic mitochondria from ALS subjects show ultrastructural changes with enlarged mitochondria, inclusions, and disorganized structure (102). Lymphocytes from ALS subjects show increased calcium levels and reduced uncoupled respiration (103). These observations show that mitochondrial abnormalities are a systemic finding in ALS. While most mitochondrial respiration indices were reduced in ALS platelets, non-mitochondrial respiration and complex II activities were increased. Complex II activity reduction over three months correlated with decline in function on the ALSFRS-R scale (14). Two separate clinical trials, testing Rasagiline as a therapeutic for ALS, used lymphocyte apoptosis, mitochondrial superoxide, and mitochondrial membrane potential as secondary outcomes (65, 104). Based on abnormal lymphocyte mitochondrial membrane potentials (101), it would seem reasonable to pursue these as potential biomarkers. Blood cell respiration or enzyme V_{max} assays could be used to determine if a drug is engaging its target by altering mitochondrial function.

CONCLUSION

ALS is a rare disease. We estimate the ALS population in the US to be about 17,000 people (13,000–24,000) based on a US population of 329,450,000 (105). This is one of the main reasons affecting biomarker development for ALS. The exact mechanisms underlying motor neurodegeneration and muscle impairment in ALS are unknown. Current hypotheses include neuroinflammation, mitochondrial dysfunction, oxidative stress, excitotoxicity, and protein aggregation (1, 106–112). Lack of understanding of how these mechanisms interact at different stages of the

disease is another issue limiting the progress of biomarker development and subsequent drug development for ALS. The lack of validated biomarkers for ALS has directly affected drug development. There are three FDA approved therapies for ALS: riluzole and edaravone for modulating the course of the disease, and dextromethorphan/quinidine for symptomatic treatment of sialorrhea. The effect of riluzole is modest, extending the lifespan by 2–3 months (113–115). Edaravone appears to slow progression and preserve function in ALS patients (115–117). Like riluzole, edaravone (Radicava) can have some side effects but its intravenous route of administration can be an obstacle at times. Nuedexta targets pseudobulbar symptoms and has no known effect on life span (118, 119). Current clinical trials for ALS are listed on https://clinicaltrials.gov/ [accessed on 17 June 2021]. There are 448 ongoing studies in Unites States, and most of these would benefit from a host of exploratory and confirmatory biomarkers.

Blood-based biomarkers are considered non-invasive and have the potential to be cost-effective. Disagreements exist regarding the utility of blood measures as surrogate for reflecting the status of motor neurons in the spinal cord or muscle. However, as shown in Figure 2, neurodegeneration and reactive gliosis contribute to blood brain barrier (BBB) breakdown. This BBB breakdown can lead to leakage of CNS exosomes/EVs and other molecules into the blood stream. Further studies are required to assess the correlation between blood measures and spinal cord/muscle tissue disease status. Validated biomarker application in people with ALS

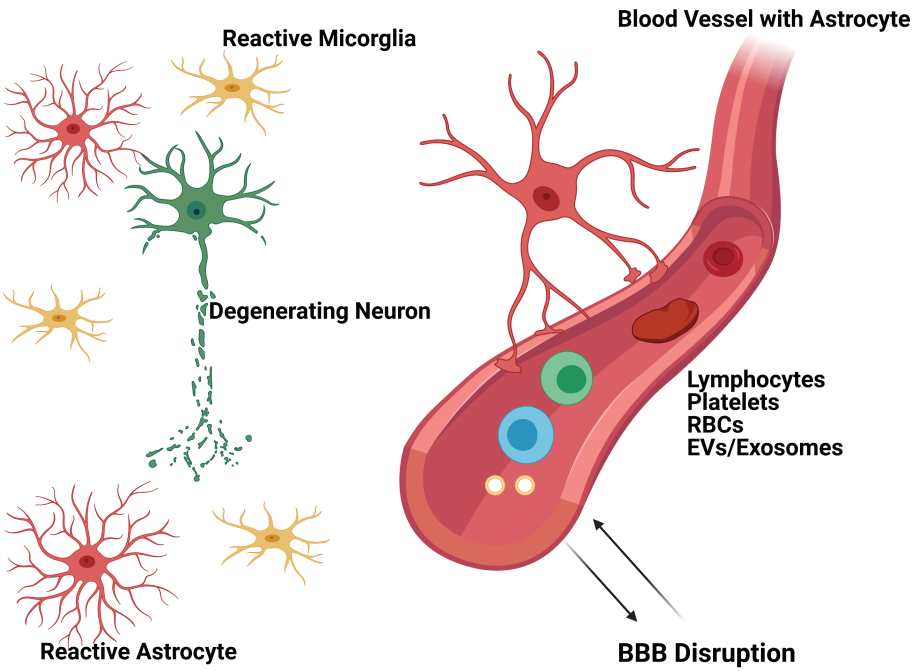

Figure 2. Blood Brain Barrier Breakdown and Circulating Biomarkers. Neurodegeneration and reactive gliosis can lead to blood brain barrier (BBB) disruption (and vice versa). This BBB disruption could allow for CNS derived circulating biomarkers to be measured. Created with BioRender.com

would derive numerous benefits. In addition to shortening the diagnostic journey, disease biomarkers may generate some cost-savings and enhance enrollment in clinical trials. Timely diagnosis will also reduce the time to starting currently available therapies. Biomarkers have the potential to provide valuable information about disease trajectory and critically important early insight into the effectiveness of experimental therapeutics. There is a great unmet need for cost-effective, reliable, accurate, non-invasive and reproducible biomarkers for ALS.

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