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# Biomarkers in Motor Neuron Disease: A State of the Art Review

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Motor neuron disease can be viewed as an umbrella term describing a heterogeneous group of conditions, all of which are relentlessly progressive and ultimately fatal. The average life expectancy is 2 years, but with a broad range of months to decades. Biomarker research deepens disease understanding through exploration of pathophysiological mechanisms which, in turn, highlights targets for novel therapies. It also allows differentiation of the disease population into sub-groups, which serves two general purposes: (a) provides clinicians with information to better guide their patients in terms of disease progression, and (b) guides clinical trial design so that an intervention may be shown to be effective if population variation is controlled for. Biomarkers also have the potential to provide monitoring during clinical trials to ensure target engagement. This review highlights biomarkers that have emerged from the fields of systemic measurements including biochemistry (blood, cerebrospinal fluid, and urine analysis); imaging and electrophysiology, and gives examples of how a combinatorial approach may yield the best results. We emphasize the importance of systematic sample collection and analysis, and the need to correlate biomarker findings with detailed phenotype and genotype data.

**Keywords:** biomarker, motor neuron disease (MND), ALS (Amyotrophic lateral sclerosis), neuroimaging, cerebrospinal fluid (CSF), electrophysiology, biofluid

## INTRODUCTION

Motor neuron disease (MND), or amyotrophic lateral sclerosis (ALS), is a neurodegenerative and ultimately fatal disease that causes progressive muscle weakness through loss of upper and lower motor neurons (UMN and LMN). Non-motor pathways are also affected and up to 50% of patients have detectable cognitive and behavioral changes (1). ALS can be classified as sporadic (sALS) or familial (fALS). Biomarkers in ALS have been the subject of intense research and discussion over the past 20 years. Sensitive and specific biomarkers have the potential to help clinicians and researchers better understand the disease, improve the design of clinical trials, develop novel therapeutics, and improve patient outcomes. A large body of research exists, although this has led to the provision of only a few validated biomarkers. In part, this reflects a wide variation in methodology, non-standardized analytical techniques, small sample sizes and paucity of longitudinal studies. To validate a biomarker there needs to be recognition of the limitations of the analytical technique by which it is being measured, the analysis must use a standardized operating procedure (SOP), and there must be test-retest reliability, ideally across different centers, to ensure replicability. This review aims to summarize progress in biomarker development in the domains of systemic measures

including respiratory function, biochemical analysis of biofluids, electrophysiology and imaging. **Table 1** summarizes the biomarkers discussed in the article.

## Diagnostic Biomarkers

ALS patients may initially present with subtle signs and symptoms and it has been shown that, on average, there is a 12-month period between symptom onset and neurological diagnosis (2). Current thinking is that there is pathological propagation of the disease through mechanisms such as axonal transmission of misfolded protein e.g., pTDP-43 [associated with diffusion tensor imaging (DTI)], abnormal RNA processing, “prion-like” spread, and cell-cell spread of dipeptide repeat proteins (3–8). It is hoped that by hastening the diagnosis future treatments will limit or halt progression, before patients are established on this progressive pathological course and before they suffer notable weakness and attrition of motor neuron numbers. A valid diagnostic biomarker will help guide the clinical diagnostic process at an early stage when signs are localized and subtle. This would allow for timely treatment and trial enrolment (**Figure 1**). Diagnosis for enrolment in trials is often based on the El-Escorial criteria which allows for a label of ALS-probable, lab-supported. Currently, this is based on evidence of active and chronic denervation on the electromyogram (EMG), together with the absence of other investigation findings that may suggest another pathological process. Further biomarkers may add to this laboratory support for more accurate enrolment and stratification. Ultimately this stratification may form the basis for a new classification system.

## Prognostic and Predictive Biomarkers

ALS is a heterogeneous condition with variability in site of onset, extra-motor involvement and rate of progression. Typical survival is 2–5 years, but life expectancy can range from several months to over 10 years. This heterogeneity is also seen when patients are investigated at a genetic level, and at post-mortem. It makes sense therefore to design clinical trials with this in mind: a subgroup of patients may be shown to benefit from a novel treatment when statistical analysis is not confounded by population heterogeneity. Additionally, if variability is decreased then sample-size can also be reduced, lowering the time and cost of clinical trials. A good prognostic biomarker will be useful in stratifying patients for better trial design by broadly distinguishing between disease subgroups. Predictive biomarkers are similar one-off measurements. However, they represent the chance of predicting a response to a particular treatment rather than the prognostic natural course of the disease.

## Pharmacodynamic and Disease Progression Biomarkers

Clinical trial endpoints typically involve measures such as survival and the revised ALS functional rating scale (ALSFRS-R), given that improvement in motor function and survival in a progressive disease are the ultimate outcomes being sought. Such outcomes may need to be monitored for several years before a conclusion can be drawn, which is an expensive process. Pharmacodynamic biomarkers reliably change in response to

treatment, and such markers would ensure that an experimental drug is having the desired effect on the pre-clinically identified therapeutic pathway. This could curtail ineffective therapeutic interventions at an early stage. Similarly, disease progression markers represent serial measures that change as the disease worsens, in the absence of treatment. This can provide another objective measure and time-saving approach to randomized control trial design.

## SYSTEMIC PROGNOSTIC BIOMARKERS

### Body Weight

An important facet of ALS management entails keeping weight records, prompt insertion of gastrostomy and prescription of nutritional supplements. Malnutrition (defined by a reduction in BMI or a >5% loss in pre-morbid weight) has a multifactorial adverse effect on life expectancy in ALS, in part due to neurotoxicity (9), and has been shown to give a 7.7-fold increased risk of death across a group of ALS patients at various time-points in the disease course (10). At time of diagnosis, 5% weight loss or more has been shown to be an independent adverse prognostic biomarker for survival (11). Therefore, patient stratification for trial entry, at any point in the disease course, should take into consideration the percentage of weight loss at baseline.

### Respiratory Function

Clinicians rely on patient-reported symptoms of respiratory insufficiency, such as orthopnea, early morning headache, interrupted sleep, daytime somnolence, reduced appetite, and results of respiratory function tests to assess the need for non-invasive ventilation (NIV), which has been shown to improve survival and quality of life in ALS patients (12). Several tests exist and they can be classified according to the time they take, how invasive they are, and whether they require patient volition. Tests such as vital capacity (VC), sniff nasal inspiratory pressure (SNIP), peak cough flow (PCF), maximal static inspiratory and expiratory mouth pressures (MIP and MEP) take a snapshot of respiratory function, but can be confounded by poor technique secondary to non-respiratory muscle weakness and cognitive dysfunction. Overnight sleep studies and transcutaneous carbon dioxide monitoring are passive tests. Tests involving phrenic nerve stimulation—phrenic nerve conduction studies (PNCS) and twitch trans-diaphragmatic pressure (Tw Pdi) are more invasive and complex as they require electrophysiology practitioners, but are objective and non-volitional.

Measures of VC, forced and slow, are widely used due to clinical availability and published validation (13, 14). In a recent study comparing tests as predictive for mortality or NIV usage, Polkey et al. concluded that, despite good sensitivity, decline in vital capacity only occurs 12 months before these endpoints. Furthermore, for prognostic time intervals beyond 3 months, the cut-off value for poor prognosis was >80% predicted, which is the clinically defined normal range, therefore making it an invalid biomarker for trial stratification. A better measure, they argue, would be Tw Pdi or SNIP (15). As Tw Pdi is considered more invasive and complex, SNIP therefore has better potential as a biomarker in clinical practice. This is supported by another study

**TABLE 1** | Summary of biomarkers across modalities.

Biomarker	Modality	Key findings	Salient characteristics and potential applications
<b>BIOMETRICS</b>			
Body weight		5–10% weight loss from baseline	Indicator of poor prognosis
Respiratory function	Sniff nasal inspiratory pressure (SNIP)	Reduction with disease progression or at presentation in respiratory onset disease	Non-invasive, effort-dependent Used clinically as a marker of respiratory function
	Forced/slow vital capacity (FVC/SVC)		Non-invasive, effort-dependent, limited in bulbar weakness Used clinically as a marker of respiratory function and as criteria for trial entry
	Phrenic nerve conduction study		More invasive and requires operator expertise but passive and objective
<b>BIOFLUID BIOMARKERS</b>			
Genetic mutation-linked proteins	CSF	C9orf72 poly(GP) present pre-clinically; stable over time	Pharmacodynamic potential for clinical trials
	Blood	SOD1 protein levels stable over time Level of SOD1 proteins in familial and sporadic disease poly(GP) repeats present in C9ORF72 disease	SOD1 used in current clinical trial Planned clinical trial specific to C9ORF72 mutations
		TDP-43 mislocalized but longitudinal readouts variable	Potential as markers for gene-specific disease
DNA methylation	Blood	Conflicting evidence in different cell types Global methylation shows promise	Potential, needs further investigation
Neurodegeneration	CSF	Neurofilament, increased levels of both NfL and pNfH, stable over time	Validated as diagnostic markers. Potential for prognostic and pharmacodynamic monitoring
	Blood	Steady increased NfL over time pNfH levels variable	Potential use of NfL as a diagnostic and prognostic marker
	Urine	p75ECD increased and increases over time	Potential, needs further investigation
Inflammation	CSF	Range of cytokines, chemokines, and immunological proteins up- and downregulated	Potential for diagnostic, prognostic, and disease progression; conflicting evidence currently
	Blood	T regulatory (Treg) cells altered Conflicting results across studies for cytokines, CRP, chitotriosidase	Tregs potential use as prognostic marker, targeted in current phase II trial Other targets need further investigation
Muscle denervation	Blood	Serum creatinine reduction Longitudinal changes in creatine kinase	Serum creatinine potential as prognostic marker Creatine kinase predicts slow vs. fast disease progression in panel in PRO-ACT database
miRNA	CSF	Differences in panels of miRNAs in patients Paucity of overlap across studies	Early potential for diagnostic, prognostic and pharmacodynamic; needs further investigation
	Blood	As per CSF	
Metabolism	CSF	Distinctive lipid profile identified through 1H-NMR and mass spectrometry Inconsistencies across studies	Potential for diagnostic and prognostic use Longitudinal studies needed
	Blood	Carbohydrate and lipid metabolism markers contradictory, but larger study promising Glutamate results contradictory in response to treatment Serum albumin reduction	Carbohydrate and lipid metabolism markers associated with disease risk in large 20-year study Glutamine and glutamate need further investigation Serum albumin predicts slow vs. fast disease progression in panel in PRO-ACT database
	Urine	Limited studies on F2-isoprostane (8-iso-PGF2 $\alpha$ ), Collagen type 4, and lucosylgalactosyl hydroxylysine (glu-gal Hyl)	Potential, needs further investigation
Oxidative stress	CSF	Raised levels of 4HNE, 3-nitrotyrosine NRF-2 pathway markers e.g., glutathione	Needs further investigation
	Blood	<sup>1</sup> Uric acid results contradictory, but larger study promising Ferritin, glutathione, 3-nitrotyrosine, 4HNE increase	Uric acid shows promise as prognostic in PRO-ACT database Other candidates need further investigation
	Urine	8-hydroxy-2'-deoxyguanosine (8-OhdG) increased and increases over time	Potential, needs further investigation

(Continued)

**TABLE 1 |** Continued

Biomarker	Modality	Key findings	Salient characteristics and potential applications
<b>BIOMETRICS</b>			
Proteomic approach	CSF	Differential expression profiles identified e.g., cystatin C, chitinases, MCP-1, Subsequent failure of validation of individual markers	Potential as an unbiased investigation of novel markers but inconsistency across studies and validation of findings needed
<b>IMAGING BIOMARKERS</b>			
Central nervous system	Structural MRI	Focal atrophy	Employed in clinical practice to exclude mimics
Magnetic Resonance Imaging (MRI) and Magnetic Resonance Spectroscopy		Subcortical hyperintensities on T2 weighted, Proton Density weighted, and Fluid-Attenuated Inversion Recovery images	Cervical cord atrophy might have potential as a predictive and progression biomarker
		Cortical hypointensities on T2-weighted, T2*-weighted, and Susceptibility Weighted Images	The potential use of cortical hypointensities as a biomarker is currently being explored
	Diffusion tensor imaging	Fractional Anisotropy reduction Mean Diffusivity elevation	Potential use as a biomarker is under investigation
	Magnetization transfer imaging	Possible reduction in Magnetization Transfer Imaging ratios	Conflicting evidence
	Functional magnetic resonance imaging	Cortical reorganization	Useful primarily to explore pathogenesis; might provide evidence of target engagement in clinical trials
	Proton magnetic resonance spectroscopy	N-acetylaspartate reduction	N-acetylaspartate has been suggested as a diagnostic and disease progression biomarker and has been employed in a clinical trial
Peripheral nerve MRI	Diffusion tensor imaging	Fractional Anisotropy reduction	Potential use as a biomarker of disease progression
Muscle MRI and MRS	Anatomical imaging	Muscle volume reduction T2 hyperintensities	Potential use as a biomarker of disease progression
	Phosphorus magnetic resonance spectroscopy	Conflicting evidence	Technique's potential as a marker of energy dysmetabolism has not yet been fully explored
Positron emission tomography		Alterations in Fluoro-2-deoxy-2-D-glucose uptake Enhanced microglial activation Inhibitory inter-neuropathy Alterations of serotonergic neurotransmission Increased oxidative stress	Potential diagnostic biomarker and use in clinical trials to provide evidence of target engagement
<b>ELECTROPHYSIOLOGY BIOMARKERS</b>			
Motor unit number estimation	MUNE	Sensitive to disease progression Identifies pre-clinical LMN loss (MPS method)	Principally limited by operator-dependent variation in recording Newer methods (e.g., MScanFIT) expedite recording and overcome some technical limitations, but require dedicated software and evaluator training Potential for use diagnosis and follow-up Yet to be widely employed clinically
	MUNIX	Multicenter and multi-operator reliability and sensitivity demonstrated Positive influence of evaluator training Superior sensitivity to early disease change vs. conventional methods Identifies pre-clinical LMN loss	Relatively time-efficient and tolerable for patients Dependent upon patient cooperation as derived from muscle contraction Worldwide evaluation in clinical trials Commercially available
Neurophysiological index		Increased distal motor latency and F-wave frequency Decreased CMAP amplitude Sensitive to disease change in 4 weeks, greater rate of decline vs. ALSFRS-R, CMAP amplitude, and FVC	Utilizes standard neurophysiological measures Previously employed in clinical trials Potential to reduce required trial duration Further investigation required
Axonal excitability		Upregulation of persistent $\text{Na}^+$ conductances Reduction of slow and fast $\text{K}^+$ channel conductances Change with disease progression	Predictor for poor prognosis Specialist equipment Further investigation required

(Continued)

**TABLE 1 |** Continued

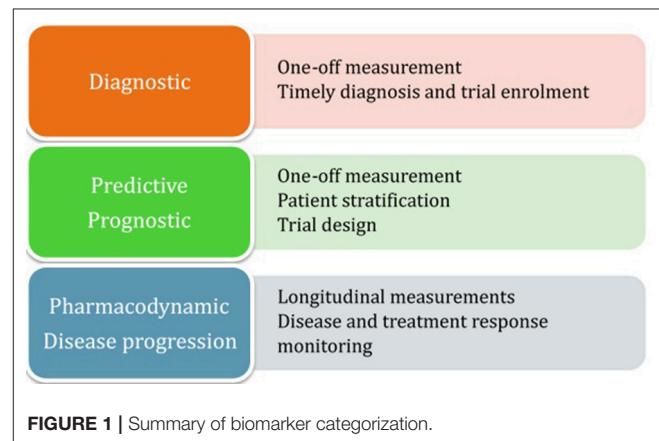
Biomarker	Modality	Key findings	Salient characteristics and potential applications
Electrical impedance myography		Multicentre demonstration of sensitivity to disease progression Applicable to bulbar musculature	Simple technique requiring limited patient cooperation or operator training Potential to reduce required sample size Further investigation into diagnostic utility and technique optimization required
Transcranial magnetic stimulation		Reduced short-interval intracortical inhibition, cortical silent-period duration, and resting motor threshold Increased intracortical facilitation and motor evoked potential Discriminates ALS from mimics	Specialist equipment/software Further multicenter investigation confirming diagnostic utility and evaluating longitudinal potential required

that investigated the ability of respiratory tests to predict the need for NIV over the following 3 month period and found significant reduction in SNIP values in patients going on to require NIV (16). Although 3 months is not long enough for a stratification tool, it strengthens SNIP as a predictive tool. Lending further support to SNIP as a prognostic biomarker, an Italian research team concluded that SNIP measurements at baseline represent an excellent predictor for mortality or tracheostomy within 1 year of follow-up (17).

Sniff nasal testing confers an additional benefit in that it does not rely on the patient being able to form a tight mouth seal around a device, therefore making it better in patients with bulbar weakness (18). It does not completely alleviate the problem however, as upper airway collapse and inability to completely close the mouth also affects SNIP readings to a degree (19). Jenkins et al. also raise concern about using volitional measures for this reason. They concluded from a large prospective study that PNCS to measure diaphragmatic compound muscle action potential (CMAP) has merit as a biomarker as it correlates well with ALSFRS-R, SNIP and FVC, and, after a period of practitioner familiarity, it is as reliable as normal nerve conduction studies and no more difficult to execute (20).

In addition to LMN weakness affecting the respiratory muscles, hypotonic, and weak upper airway muscles contribute to an obstructive picture, and there are central factors contributing to respiratory insufficiency with bulbar, motor, and extra-motor pathways involved. Dysfunctional breathing due to abnormalities in these pathways leads to overnight hypoxia and hypercapnia (21). Clinically, sleep studies are typically reserved for patients who are symptomatic or have fallen below a threshold on screening tests such as VC. They are more cumbersome for patients, and time and resource intensive, which reduces their utility as a biomarker. However, one longitudinal study demonstrated the prognostic value of assessing for obstructive sleep apnea, with mean survival being shorter in patients with a higher apnea/hypopnea index. Interestingly SNIP correlated with this measure (22).

Screening tests for respiratory insufficiency are sensitive tools and each modality has its advantages and disadvantages. As a balance in relation to ease of technique, serial measurements,

**FIGURE 1 |** Summary of biomarker categorization.

time, and expertise needed, and predictive power, SNIP stands out as a biomarker that could help in defining prognosis as well as the potential for sensitivity to change from therapeutic interventions. The exception is patients with severe bulbar or cognitive dysfunction and in those patients an electrophysiological modality could be of benefit.

## CEREBROSPINAL FLUID (CSF) BIOMARKERS

CSF is a useful biofluid for analysis due to the direct proximity with the brain and spinal cord. It is an ultrafiltrate of plasma [although there are CSF homeostatic mechanisms which, for example, maintain ion concentrations that are different to plasma concentrations (23)]. Thus, protein levels in the CSF are considerably less compared to plasma, making analysis likely to be representative of central nervous system (CNS) activity.

### Neurofilament Proteins

The most promising CSF biomarkers identified to date are neurofilament proteins, a cytoskeletal component of neurons that have been shown to accumulate following axonal damage and degeneration and can be measured in CSF (24, 25). Consisting of three subunits, the two of interest

are phosphorylated neurofilament heavy chain (pNfH) and neurofilament light chain (NfL). A substantial body of evidence supports neurofilament levels as a diagnostic element (26–30). Both subunits have been validated in one multi-center study as diagnostic biomarkers (29) and pNfH alone in another (31). These studies address the standardization needed by using carefully designed standard operating procedures (SOPs) for sample collection and processing, and checking consistency of neurofilament levels within patient samples and between centers.

Additional marker utility is still to be validated, although many studies provide supporting evidence. A longitudinal study comparing ALS patients with disease and healthy controls found higher NfL levels in ALS patients and higher levels were associated with worse prognosis (32). Similar results were found in a large cohort study but, when analyzing their longitudinal data, they found that only 67% of ALS patients had higher levels at subsequent time points, with some patients having decreasing values over time. This latter group had a higher baseline value suggesting that a plateau of CSF neurofilament levels is reached once the rate of neuronal death has peaked (33). Another study found that NfL (particularly blood-derived) was fairly stable over time, providing a potential pharmacodynamic monitoring tool, and provided further support for CSF NfL as a prognostic marker for patient stratification (34). The authors also found high correlation between serum and CSF NfL, useful as serial blood tests are easier to obtain than serial CSF samples. Furthermore, there is evidence that CSF NfL correlates to disease subtypes, with those with increased UMN burden (32) or more rapid rates of disease progression, independent of age, showing higher baseline levels (35).

Finally, a meta-analysis correlating pNfH with the ALSFRS-R and disease duration demonstrated a significant negative association (36). Validation efforts would therefore be useful for prognostic, disease progression and pharmacodynamic purposes.

## Tau

The tau protein stabilizes neuronal microtubules. Phosphorylated tangles, with tau as the major constituent, are seen in Alzheimer's disease, and ALS when associated with TDP-FTD. Raised total-tau has been reported in the CSF of ALS patients (37, 38), but no difference was found in another study (39) and there was failure to replicate this quantification in a multi-center, standardized collection analysis (31). Additionally, with no studies of tau showing correlation with disease severity or progression, neurofilament is currently the better marker of neuroaxonal degeneration.

## TAR DNA-Binding protein (TDP-43)

Neuronal and glial inclusions of TDP-43 have been implicated in the pathogenesis of sALS and the linked fronto-temporal dementia (FTD) (40) but not SOD1-ALS (superoxide dismutase-1 mutation) (41). Subsequent studies have found elevated TDP-43 levels in the CSF of ALS patients as compared to healthy and neurological controls with neurodegenerative or neuroinflammatory disease (42–44), and higher in levels in ALS than in FTD (45). However, diagnostic accuracy was not demonstrated and a study by Feneberg et al. suggested that as

serum concentrations are 200 times higher than CSF levels, as a biomarker, serum TDP-43 may be more appropriate and with pharmacodynamic utility (46). There is little available evidence for use as a marker of disease progression or prognosis and longitudinal studies are needed.

## Proteomics

Another approach to identifying biomarkers is using liquid or gas chromatography (LC/GC) and mass spectrometry (MS) for proteomic analysis. An advantage is that it is an unbiased approach, yielding peaks for biochemical elements that may not have been previously recognized, and which may indicate a targetable, pathogenic pathway. Any protein identified must then be validated and the pathological pathway identified (47). A recent review outlined the problem with such approaches if they are not standardized: individual studies may find hundreds of proteins that differ between patients and controls, but there is only partial overlap between studies and attempts at replication have tended to fail (48). However, many proteins have been identified using these techniques and are currently undergoing further study.

Using LC-MS, Collins et al. demonstrated that the CSF proteome can be used to identify biomarkers and is relatively stable over time (49). In ALS, raised neurofilament, complement C3 and secretogranin I, and reduced cystatin C were amongst the top differentially expressed proteins identified. Additionally, using a machine learning approach they identified and used four classifier proteins—WD repeat-containing protein 63, amyloid-like protein 1, SPARC-like protein 1, and cell adhesion molecule 3—to differentiate between ALS, healthy controls and other neurological disease (83% sensitivity and 100% specificity).

Low levels of cystatin C in the CSF of ALS patients is well-recognized (50–52), although one study failed to find this difference (53). In a multi-center validation study, no difference between ALS patients and controls was seen (31) and there are conflicting data regarding the correlation with rate of disease progression (50, 51). The level of cystatin C has however been shown to correlate with survival time in limb-onset ALS (51) which lends further weight to the argument for careful clinical phenotyping and the need for longitudinal studies.

Other biomarkers analyzed in this six-center analysis (31) were monocyte chemoattractant protein-1, programulin, amyloid precursor protein and S100B. Of these, none demonstrated consistent change and some yielded conflicting results across the centers.

Chitotriosidase (CHIT1) was identified using a proteomic approach and levels were found to be significantly higher in ALS patients compared to controls (54, 55). A subsequent study using ELISA confirmed this and also found high expression in comparison to other neurodegenerative conditions, and that levels were correlated with progression rate and inversely correlated with disease duration (56). Immunohistochemistry (IHC) was then performed on post-mortem CNS tissue from ALS patients demonstrating CHIT-positive activated microglia and macrophages in the corticospinal tracts. The authors therefore tentatively concluded that CHIT may have a role as a diagnostic and prognostic marker. This is supported by a

recent LC-MS longitudinal study which demonstrated CHIT1 and other chitinases, CHI3L1 and CHI3L2, correlate with disease progression and indeed pNfH levels (57).

Levels of glutamate receptor 4 (GRIA4) expression in the CSF were found to be increased in ALS patients and to negatively correlate with disease severity, suggesting an early over-expression. This fits with glutamate excitotoxicity as a factor in neuronal damage and suggests that anti-glutamate therapy, like riluzole, may be more effective earlier in the disease course (55).

## Metabolomics

Like proteomics, an unbiased search can be done by performing LC/MS or proton-nuclear magnetic resonance (<sup>1</sup>H-NMR) on biofluids to identify metabolites that differ in quantity in ALS. One such <sup>1</sup>H-NMR study demonstrated lower CSF levels of acetate and increased levels of pyruvate and ascorbate (an antioxidant and linked with glutamate-mediated excitotoxicity) when comparing the ALS group with non-neurodegenerative disease controls. Subsequent modeling using the 17 identified metabolites achieved a discrimination rate between ALS and controls of 81.6% (58). A subsequent study from the same group increased the validity of CSF metabolomic <sup>1</sup>H-NMR spectroscopy as a means to discriminate, by testing their metabolite model on a validation cohort, achieving a sensitivity of 78.9% and specificity of 76.5% (59).

Another mass spectrometry approach investigated the CSF lipid profile of ALS patients (60). As discussed in the blood biomarker section, high lipid levels seem to confer survival benefit and, as the authors of this study explain, the brain composition is rich in lipids with many neuronal and systemic biological processes dependent on lipid homeostasis. They found that there was a distinct ALS lipidomic profile and, based on the baseline CSF analysis, they could provide a predictive model with 71% accuracy for disease progression thus providing a potential diagnostic and prognostic biomarker.

The review by Blasco et al. describes in more detail the large number of metabolites discovered and also the inconsistencies across the body of reported research (61). Longitudinal metabolomic studies with analysis of clinical data are scarcer, although one plasma analysis found that some metabolites did correlate with disease progression (62), and another demonstrated a distinctive plasma profile for patients with LMN disease, albeit only with a small sample size (63). There is promise and further work with pre-analytical and analytical SOPs is indicated.

## Oxidative Stress Biomarkers

Oxidative stress is associated with ALS pathogenesis (64–66), and has potential for novel therapies, as supported by the Japanese and American FDA approval of the free radical scavenger edaravone in recent years. In health, superoxide dismutase 1 has an antioxidant role in converting superoxide free radicals into oxygen and hydrogen peroxide. SOD1-mutations are implicated in a proportion of sporadic and fALS cases through toxic gain of function (67). Misfolded SOD1 can be measured in the CSF; it has been demonstrated that there is no significant difference between

SOD1 ALS patients and non-SOD1 patients and between all ALS patients and neurological controls (68, 69). The utility of measuring SOD1 protein levels in CSF is as a pharmacodynamic biomarker, as levels are stable in individual patients over time (69, 70) and antisense oligonucleotide (ASO) SOD1-lowering therapy is effective in rats (69). A phase I/II clinical trial is underway to determine whether ASO-therapy gives the same results in humans (NCT02623699). Furthermore, SOD1 ALS can be sub-classified based upon the specific mutation. This provides useful prognostic information for trial design: for example SOD1 A4V missense, the most common SOD1 disease-causing mutation in the United States, has a significantly worse prognosis compared to other mutations (71).

Other oxidative biomarkers that have been identified as raised in ALS patients are 8-oxodeoxyguanosine and 15-F(2t)-isoprostane in urine (72), 8-hydroxy-2'-deoxyguanosine (8OH<sup>2'</sup>dG) and 3-nitrotyrosine in CSF (73, 74), and 4-hydroxy-2,3-nonenal in serum and CSF (75). However, none are as yet validated for use in clinical trials.

The nuclear erythroid 2-related factor 2-antioxidant response element (Nrf2-ARE) is an important signaling pathway, shown to reduce oxidative stress and inflammation (76). By measuring markers of oxidative stress, it can be shown that novel therapeutics are having the desired preclinical and clinical effect on this pathway. For example, compound screening identified S[+]-apomorphine as an *in-vivo* inducer of Nrf2 in an ALS mouse model by measuring Nrf2 target genes, and as an attenuator of oxidative stress in patient fibroblasts (77). This therefore supports further exploration of Nrf2 activators, like S[+]-apomorphine, with measurable pharmacodynamic biomarkers.

Upregulated by Nrf2 activation, glutathione is another useful marker of oxidative stress, as it acts as a buffer for reactive oxygen species. Reduced serum levels have been shown when comparing ALS patients and controls (78). Measurable by *in-vivo* <sup>1</sup>H-MRS, this and other metabolites are discussed further in the imaging section.

As a more general measure of the oxidative system, one study showed that ALS patients had reduced antioxidant capacity with increased advanced oxidation protein products, although interestingly bulbar-onset patients had a protein composition similar to controls (79). Another study demonstrated a higher CSF oxidation-reduction potential (ORP) in ALS patients, and a negative correlation with ALSFRS-R in spinal-onset patients, leading the authors to conclude that it may be a marker of disease progression (80). However, their case-control groups were ALS and non-neurodegenerative neurological controls and a more varied control group encompassing all neurological disease may lend further weight to their preliminary findings.

## Biomarkers of Neuroinflammation

As well as measurable changes in antioxidants, immune and inflammatory mediators have a complex role in the pathophysiology of ALS. Whilst initial activation of microglia and astrocytes may be neuroprotective, a state of chronic activation tips the balance toward neurotoxicity, with up- and

down-regulation of a wide variety of humoral and cellular factors (81). Mitchell et al. performed a multiplex ELISA to identify potential biomarker candidates in the CSF of ALS patients. They reported that the 5 cytokines with the greatest difference between ALS and controls were IL-10, IL-6, GM-CSF, IL-2, and IL-15 and when combined, gave a differentiation accuracy of 89% (82). Other differentiating factors that have been identified are CHIT-1 and C3, as discussed earlier, IL-17, bFGF, VEGF, MIP-1 $\beta$ , MIP-1 $\alpha$ , MCP-1 $\beta$ , and IFN- $\gamma$  (83), and follistatin, IL-1 $\alpha$ , and kallikrein-5 (84).

Prediction of disease duration has also been proposed through multiplex analysis and immunoassays, with IL-9, IL-5, and IL-12 proving negative predictors and MIP-1 $\beta$  and G-CSF positive predictors (85). IFN- $\gamma$  has been shown to correlate with disease progression (83, 86), and bFGF, VEGF, and MIP-1 $\alpha$  have been shown to correlate with longer disease duration (83) further demonstrating the homeostatic attempt of the immune system. This immune profiling provides promise for sub-typing ALS patients and combining identification of pathophysiological factors with discovery of potential therapeutic targets.

## C9ORF72

The hexanucleotide repeat expansion associated with C9ORF72 disease causes accumulation of RNA foci and undergoes non-ATG (RAN) translation, forming C9RAN dipeptides (DPR). Toxicity is thought to be in part due to sequestration of RNA binding proteins (87). Like misfolded SOD1 protein, these DPRs are measurable in CSF (88). A cross-sectional study showed one of these, poly(GP), is detectable in the CSF of C9ORF72 ALS and FTD patients but not controls, and that levels are increased in patients pre-clinically (89). This concept was further explored longitudinally to show that DPR levels are stable over time, supporting their use as a pharmacodynamic biomarker (90). This latter study also demonstrated that poly(GP) levels are reduced with the use of ASOs in C9orf72 cell and mouse models. This provides promising proof-of-concept that a targeted approach to these RNA repeats can mitigate an important pathological process in this disease subtype; especially important for asymptomatic carriers. Indeed, a clinical trial is planned using anti-sense oligonucleotides to lower DPRs in human ALS patients with C9ORF72 mutations.

## MicroRNAs (miRNAs)

Short, non-coding RNAs regulate gene expression by binding to mRNA, thereby reducing translation and promoting mRNA degradation. Specific miRNAs have been associated with neuronal cell identity, synaptic function and glial regulation, and neuroinflammation in ALS (91). Interestingly, miRNA biogenesis is linked to TDP-43 which, as described above, is a pathological hallmark of ALS. TDP-43 binding miRNAs are dysregulated in the CSF and serum of sALS patients (92). Several studies have demonstrated other specific miRNA changes in ALS CSF. For example, upregulation of miR-338-3p (93), and miR181a-5p and downregulation of miR21-5p and miR15b-5p (94). This latter study demonstrated a sensitivity of 90% and specificity of 87% when miRNA ratios were used to differentiate between ALS and healthy controls. Early potential for prognostic or

pharmacodynamic biomarker properties can be seen in a murine model which identified CSF miR-218 as correlating with motor-neuron loss and also responsiveness to therapy.

Due to discrepancy between methods and the specific miRNAs identified, further validation efforts are required; a recent study attempted to do this through optimizing RNA extraction and small RNA sequencing (91). Similarly, studying larger, longitudinal cohorts, will hopefully allow correlation of potential miRNA biomarkers with clinical phenotype.

As mentioned above, identification of SOD1 and C9ORF72 mutations is used for ASO trial enrolment, and the respective protein levels as pharmacodynamic biomarkers. In terms of prognosis, certain mutations have been found to infer a different disease course. As examples, C9ORF72 carriers have a higher incidence of fronto-temporal dementia, the specific A4V SOD1 mutation carries a poor prognosis (95), and certain UNC13A single nucleotide variants have been associated with shorter survival and others with longer survival (96). However, data are conflicting and the clinical significance of most mutations is unclear, lending support to larger phenotype-genotype studies. These should be systematic, including patients with seemingly sporadic disease, to accurately reflect the burden of genetic mutation in the population. Interested readers are directed to the Project Mine Project ([www.projectmine.com](http://www.projectmine.com)) and the recent review of Al Chalabi et al. on the topic (97).

## BLOOD BIOMARKERS

Blood based biomarkers are a useful medium between central and peripheral damage in ALS. While some markers show a correlation with CSF markers, as transfer occurs between CSF and blood, other candidate markers arise from peripheral effects of ALS such as muscle denervation.

## C9ORF72, SOD1, and TDP-43

As introduced above, downstream protein readouts linked to genetic mutations have been explored recently in response to current and planned clinical trials specific to SOD1 and C9ORF72 mutations. Although most studies have primary outcomes in CSF (89, 90, 98), SOD1 was reduced in leukocytes (99) but not erythrocytes (98, 99) in response to pyrimethamine treatment in SOD1 positive disease, and poly(GP) repeats were detected in peripheral blood mononuclear cell lysates in C9ORF72 positive disease, although levels were not compared to those in CSF (90).

In addition to mutation-specific disease, proteins linked to genetic mutations have been studied more broadly in sALS. For example, overall SOD1 levels are reported to be increased in leukocytes (100). The story for TDP-43 remains unclear; it is mislocalized to cytoplasmic fractions of circulating PBMCs in ALS cases (101), and although total TDP-43 level did not discriminate from controls in these cells (101, 102), increasing levels correlated with disease burden longitudinally (102). In plasma, total TDP-43 is increased in ALS, but longitudinal changes were variable between subjects (103) and in serum, TDP-43 levels were unchanged between disease states, with authors suggesting CSF TDP-43 is blood derived and not useful for ALS diagnosis (46).

## DNA Methylation

DNA methylation, as a readout of epigenetic influence, has gained interest in the last decade. Issues surround DNA methylation levels being influenced by variability between cell types and by immune factors, thus confounding methylation as a specific marker for disease phenotype (104). However, increased methylation of different components has been reported widely in ALS. Increased global DNA methylation been detected in ALS blood in some studies (105, 106), but not in a smaller study of two *SOD1* and two *TARDBP* carriers (107). In *C9ORF72* linked disease, *C9ORF72* itself (108, 109) or its promoter (110, 111) are hypermethylated, with *C9ORF72* hypermethylation showing correlation with G4C2 repeat size (109, 111) and promoter hypermethylation linked to reduced RNA foci and dipeptide repeat protein aggregates in the brain (112). Additionally, an increase in DNA methylation age was associated with disease duration in *C9ORF72* linked disease, with every 5-year increase in DNA methylation age correlating to age of onset 3.2 years earlier, and shorter disease length of 1.5 years. This finding fits with sporadic disease, where increased DNA methylation age was detected in four of five ALS-diagnosed monozygotic twins. In this study, although methylation patterns were most similar between twins, the changes in common across all with ALS implicated glutamate metabolism and the Golgi apparatus (113). Similarly in *SOD1*-linked disease, those with not-fully penetrant *SOD1* mutations showed increased DNA methylation in comparison to asymptomatic/pauci-symptomatic individuals, and levels showed a positive correlation with disease duration (106).

## Neurofilament Proteins

NfL levels in serum and CSF have been shown to be highly correlated (34). Blood NfL levels were shown to be significantly higher in ALS patients than healthy controls, and a high initial NfL level was a strong independent predictor of survival. However, levels remain steady over time (34) with high levels in early and later stage disease showing no correlation to El Escorial diagnostic categories (114). Hence, NfL appears to have utility as a diagnostic and prognostic marker, rather than a marker of disease progression.

pNfH has also been studied in blood, and correlates with CSF levels (115, 116). In a meta-analysis of two papers, the blood concentration of pNfH was non-significantly higher in ALS (36). One study showed an association between higher plasma pNfH concentrations and a faster disease progression, but this was only significant at 4 months of follow-up (117). Similarly, higher plasma and serum pNfH was associated with increased mortality over the 12 month follow-up period. The reliability of these results is limited by the small sample size and short follow-up period. A longitudinal study did not show a predictable trajectory of plasma NFH over time: levels increased, decreased, or remained steady as disease progressed (34). While a subgroup with fast progressing disease tended to start with higher pNfH levels which decreased over time, the rate of change could not be used to predict disease progression. Another study showed a tendency for pNfH levels to rise and then fall, but there was substantial variability between subjects (118).

## Inflammatory Markers

Various blood markers of immune activity have been studied. One study measured levels of multiple different immune cells and surface markers in order to generate immune phenotypes for familial and sporadic ALS patients (119). They found that ALS patients had increased immune activity, and could be grouped into two distinct immune profiles. Profile 1 patients were reasonably similar to healthy volunteers, but Profile 2 patients had elevated levels of total leukocytes and mononuclear cells, as well as CD3+, CD4+, CD8+, CD4+CD28+, CD3+CD56+ T-cells, and CD8+CD45RA+ naïve T cells. Profile 2 was associated with younger age, familial ALS and significantly increased survival (a median of 344 weeks, vs. 184 weeks for Profile 1). Within profiles, different leukocyte phenotypes were found to influence survival; for example, Profile 1 patients with higher levels of PD-1+ CD4 T cells survived longer, whereas Profile 2 patients with more CD3+CD56+ T cells survived longer, but neither association held true in the other group. It is unclear whether the altered immune profile in ALS is related to the pathophysiology of the disease or a response to disease activity. There was no longitudinal sampling in this study, so it is unclear how the profiles may change over time, but this study shows they are likely useful for prognosis. Another study found that levels of leukocytes, monocytes and NK cells were increased in ALS patients, and that they increased over time. An increase in total leukocytes and neutrophils, and a decrease in CD4 T cells, were correlated with a decrease in ALSFRS-R (120).

T-regulatory cells (Treg) represent a promising biomarker candidate and a possible therapeutic target. These cells suppress various components of the immune response, including cytokine production and T lymphocyte proliferation. One study found that levels of CD4+CD25High Tregs were reduced in patients with ALS, and that the number of Tregs was inversely correlated with rate of disease progression (121). Another study found that the Tregs from ALS patients had reduced ability to suppress activity of T responder lymphocytes, and that Treg dysfunction was correlated with the rate of disease progression (122). These results support the use of Tregs as a prognostic biomarker. In the latter study, disease burden as measured by the Appel ALS score (AALS) at the time of venepuncture was correlated with Treg dysfunction, which implies a decrease in function over time. However, a longitudinal study is needed to confirm this.

Blood levels of cytokines have been studied widely, including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (123–125) interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, and IL-13 which were reported to be increased, while interferon- $\gamma$  (IFN- $\gamma$ ) was decreased in ALS patients in cross-sectional studies. However, cytokine levels did not change over the course of disease (123). In serum, IL-1 $\beta$  (78), IL-6 (78, 83, 126) IL-8 (60, 78), and IFN- $\gamma$  (83, 86, 127) are also reported to be increased, whereas serum IL-5 levels are decreased. Serum IL-2 and IL-10 results have been less conclusive (78, 83). A recent meta-analysis (128) combining serum and plasma measurements from 25 studies found TNF- $\alpha$ , TNF-receptor 1, IL-6, IL-1 $\beta$ , IL-8, and VEGF were significantly elevated in ALS, but of note is that results for IL-1 $\beta$ , IL-6,

and VEGF may have been skewed by one study. Products of complement activation are also increased in ALS patient blood samples; specifically C3b-alpha-chain in serum (129), and C5a (130, 131) and C5b-9 (131) in plasma, along with a wide range of complement factors in another plasma study (130).

Other inflammatory markers have shown varying results, such as C reactive protein (CRP), which showed no differences in plasma (132) or whole blood (60) at baseline. In serum, CRP was increased in ALS and did not associate with ALS risk or survival in one study (133), but correlated with ALSFRS-R and survival in another (134). Similarly, chitotriosidase, expressed by active tissue macrophages, was increased in dried blood spots of ALS patients compared to healthy individuals, and was higher in those with rapidly progressing disease (135). However, Steinacker et al. (56) found no change in chitotriosidase serum levels in ALS compared to controls in the same study in which CSF levels correlated with disease progression and severity.

## MUSCLE DENERVATION BIOMARKERS

Lower serum creatinine in ALS has been reported, and although some studies have found levels differing by onset site (136) or gender (137) the majority link levels to prognosis (136–140). A recent analysis of trial data from over 1,200 people with ALS found strong longitudinal correlations between serum creatinine and ALSFRS-R score, muscle strength, and overall mortality, indicating that using serum creatinine in trials over 18 months in length would allow a reduction in sample size by 21.5% (141). Lending further support to this pathway as a useful biomarker of muscle denervation, serum creatine kinase (CK) is increased in plasma (132), and serum (140, 142) and correlates with survival in some studies (140, 142). This discrepancy may be attributed to differing rates of disease progression. Modeling of the PRO-ACT database showed those with slow disease progression had stable or slowly declining creatine kinase, whereas people with rapidly declining disease had quickly declining levels. Indeed, along with decreases in weight, alkaline phosphatase, and albumin, creatine kinase decline was able to predict slow vs. fast disease progression (143).

## microRNA (miRNA)

Whole blood (93, 144), serum (145–149), and plasma (150, 151) sourced microRNAs have been studied as possible biomarkers, due to their role in regulating gene expression. In whole blood, six downregulated miRNAs and one upregulated miRNA were identified (144) and a later study confirmed upregulation of miR-338-3p in leukocytes and serum (as well as in CSF and spinal cord) (93). A plasma based study (151) found increased levels of hsa-miR-4649-5p and decreased levels of hsa-miR-4299 in ALS patients vs. controls, but found no significant trend over time. Similarly, a second plasma study identified steady upregulation of two different miRNAs longitudinally (150), one of which, miR-206, is also increased in serum (147).

Serum miR-206 was also increased in a study which reported an increase in miR-143-3p and decrease in miR-374b-5p compared to controls (148). Additionally, this longitudinal study

reported that miR-206 levels remained steady, while miR-143-3p levels increased and miR-374b-5p levels decreased over time, and that riluzole had no effect on miRNA levels. Further studies identified different panels of miRNAs differentially expressed in ALS serum compared to controls (146, 149) and also to neurological disease controls (149), noting longitudinal changes in separate sets of miRNAs (149) and higher variability across sporadic disease (146) compared to familial cases. Interestingly, one study identified 30 downregulated miRNAs in ALS, 22 of which were also downregulated in presymptomatic ALS mutation carriers, with some showing a greater degree of downregulation after disease onset (145). MicroRNAs seem to have promise as biomarkers, but there is a lack of overlap in microRNAs identified across different study groups, and to date, little longitudinal evidence reported.

## METABOLIC BIOMARKERS

Markers of carbohydrate and lipid metabolism have been studied extensively, with contradictory results [reviewed in (152)] although dysregulation of these processes is clear. A large 20-year study in Sweden showed lower levels of serum glucose and higher levels of low-density lipoprotein cholesterol (LDL-C), apolipoprotein B (apoB), and apoB/apoA-I ratio during the 20 years before diagnosis, and increasing levels of LDL-C, high-density lipoprotein cholesterol (HDL-C), apoB and apoA-I in the 10 years before diagnosis, in 623 ALS patients. As such an increased risk of ALS was observed with increasing serum LDL-C, apoB, and apoB/apoA-I ratio, and high LDL-C/HDL-C and high apoB/apoA-I ratios, whereas high serum glucose was associated with lower ALS incidence (152).

A decrease in glutamine (153), and an increase in its metabolite glutamate (154), the principal excitatory neurotransmitter in the CNS have been identified in ALS plasma, with increased glutamate levels seen in males, those with spinal onset, and correlated with longer disease duration (155). Interestingly, Riluzole treatment had no effect on plasma glutamate (156) but decreased serum glutamate in another study (157) suggesting usefulness of this measure in response to therapies in serum.

A large 2014 study of 638 ALS patients showed the utility of serum albumin at diagnosis as a biomarker of survival, with levels decreased in ALS, better survival seen with increasing levels, and that albumin levels correlated with markers of inflammatory state (137). A more recent study of 42 ALS patients and 18 healthy controls also showed a decrease in plasma derived serum albumin in ALS regardless of cognitive impairment, but could not detect disease severity or survival time using albumin at one time-point alone (130). Most convincingly, longitudinal modeling of ALS from the PRO-ACT database (143) showed that albumin decline, was one of four factors able to predict disease progression rate.

## Proteomics

While many groups have performed mass spectrometry analyses in blood (102, 154, 158–161), there is not often an overlap in the specific proteins identified and those identified require validation. However, pathways known to be dysregulated in

ALS are implicated. For example, the largest study of 172 ALS patients and 50 healthy controls (154) identified a panel of 32 differentially expressed proteins, showing dysregulation of carbohydrate and lipid metabolism, mitochondrial function, and creatinine. A recent study in 42 ALS patients and 18 healthy controls showed downregulation of lipid/cholesterol, and coagulation pathways, inhibition of NO and ROS production in macrophages, and increases in acute phase response and the complement system (130).

## Oxidative Stress Biomarkers

An increase in ferritin, suggesting iron misregulation which promotes oxidative stress, is present in plasma (132) and serum of ALS patients (85, 136, 162), with higher levels associated with poorer survival in some studies (123, 136, 162), but not all (85).

While excess uric acid is harmful, it is also a powerful antioxidant and so could be useful to combat the oxidative stress seen in ALS. In cross sectional studies, serum levels are decreased in comparison to healthy controls (163–166). Higher serum uric acid levels correlated with a moderately decreased risk of the future development of ALS (167), but its link to increased survival is less clear, showing positive results in one study (164), only for men (168), or not at all (165). However, a recent study of the PRO-ACT database including 1,736 ALS cases showed an 11% reduction in risk of death for every 1 mg/dl increase in serum uric acid (169). Uric acid levels have also shown promise in plasma, identifying ALS from neurological disease mimics with high sensitivity as part of a 32 metabolic panel biomarker set although levels were no different between groups alone (154).

## URINE BIOMARKERS

The search for urinary biomarkers in ALS include small cross-sectional studies, often with contradictory results, such as the usefulness of urinary trace elements (170–172). Those showing promise include the oxidative stress marker 8-hydroxy deoxyguanosine (8-OHdG) a product of nuclear and mitochondrial DNA oxidation which was increased in ALS in cross-sectional studies (72, 74) and increased longitudinally over 9 months in ALS patients (2.9 ng/mg creatinine/year) but not in disease controls (74). F2-isoprostane (8-iso-PGF2 $\alpha$ ) is also increased in ALS patient urine (72), but the existence of an inflammation-induced pathway for F2-isoprostane generation in addition to lipid peroxidation (173) needs to be considered when interpreting results.

Collagen type 4 (174) and collagen metabolite glucosylgalactosyl hydroxylysine (glu-gal Hyl) (175) levels were decreased in people with ALS as compared to neurological disease controls and healthy individuals, levels were lower in people with longer duration of ALS symptoms in cross-sectional analysis, and correlated with decreased levels in skin (collagen type 4), but did not correlate with muscle power rating (174, 175).

More recently, an increase in the extracellular domain of neurotrophin receptor p75 (p75ECD) was reported in ALS patient urine (176–178), which increases longitudinally as disease progresses (2.3 ng/mg creatinine/year), and provides prognostic potential advantages over clinical parameters of disease onset

and change in ALSFRS-R alone (178). These findings suggest that urinary p75ECD has potential for use as a prognostic and pharmacodynamic biomarker.

## IMAGING BIOMARKERS

### Magnetic Resonance Imaging (MRI)

Magnetic resonance imaging (MRI) is an attractive candidate as a biomarker tool as it is non-invasive, relatively inexpensive, and does not involve ionizing radiation. The multi-modal nature of MR lends itself to the study of various anatomical and pathological changes and processes *in vivo* (179). There is a large body of published work in the context of ALS, predominantly focused on the brain, with fewer studies relating to the spinal cord, muscle, and peripheral nerve.

## CENTRAL NERVOUS SYSTEM

### Conventional Anatomical Magnetic Resonance Imaging (MRI)

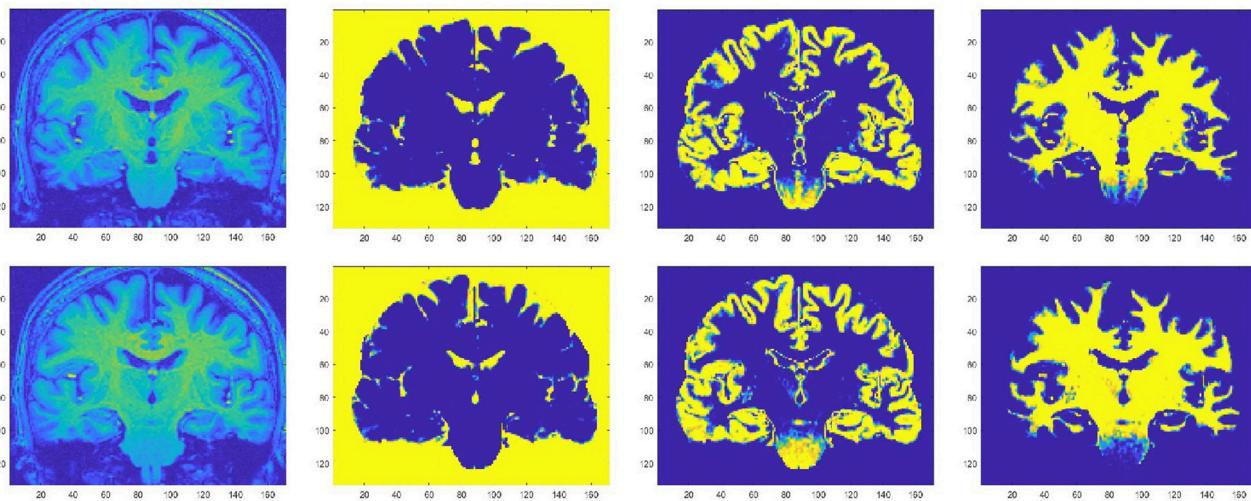
Focal cortical atrophy has been demonstrated in the precentral gyrus (180–182) (Figure 2), as well as in other motor and non-motor areas, including frontal (181), parietal (184), temporal (185), limbic (186, 187), thalamic (188), bulbar (189), and spinal regions (190). Precentral atrophy predominates in regions of the motor homunculus that correspond to areas most affected by disease (191), whilst frontal cortical atrophy is especially pronounced in patients with associated cognitive dysfunction (192) or fronto-temporal dementia (193). White matter atrophy has been demonstrated in the corticospinal tract (194), front-temporal (192), cerebellar, callosal, and occipital regions (195), but, overall, global atrophy tends to be mild (185).

Atrophy is thought to be a surrogate of neuroaxonal loss (196, 197) and MRI studies have supported the concept that neurodegeneration in ALS is not confined to motor regions. However, volumetric analysis in isolation is not sufficiently sensitive at individual level and, at present, the role of conventional structural MRI in clinical practice is mainly for the exclusion of ALS mimics as part of routine diagnostic workup (198).

Longitudinal studies assessing primary motor cortex (191, 199), subcortical regions (186), and cervical spinal cord (189, 190) have demonstrated worsening atrophy over time, and that the rate of volume loss is greater in rapidly progressive patients, compared to slow progressors (199). Reduction of cervical spinal cord surface area has been shown to correlate with clinical measures of disability, for example ALSFRS-R scores (189), and cervical spinal cord volume decrease over 3 months was predictive of respiratory dysfunction in the subsequent year in one study (190). Cervical atrophy therefore may have potential as a predictive and progression biomarker.

### Signal Changes

High signal may be seen in motor areas on T2-weighted, proton density, or fluid-attenuated inversion recovery (FLAIR) images (199–201), especially in the subcortical precentral white matter and in the posterior limb of the internal capsule. T2 signal change



**FIGURE 2** | Motor cortical atrophy in a patient with ALS, more pronounced on left side (which correlated with the pattern of weakness clinically). Sequence: 3T, T1w IR, TR 8.4 ms, TE 3.9 ms, TI 1000, FOV 240 mm, Acq voxel 1 × 1 × 1 mm Recon matrix 0.94 × 0.94 × 1 mm. Segmentation algorithm according to Chuang et al. (183).

can reflect a number of different underlying mechanisms, for example, oedema, inflammation, demyelination, or, in ALS, most likely neuroaxonal loss or gliosis, either alone or in combination (202, 203), and is neither sensitive nor specific in ALS. T2 signal change in the corticospinal tracts does not appear to correlate well with clinical measures (200, 203).

Cortical hypointensities assessed both qualitatively and quantitatively, on T2-weighted (204), T2\*-weighted (205), and susceptibility-weighted images (206) are thought to reflect reactive ferritin-laden microglia accumulating in the deep layers of the precentral gyrus (206, 207). Ferritin contains iron which is paramagnetic and alters T2\* relaxation, leading to hypointensities on T2-weighted, T2\*-weighted, and susceptibility-weighted images, a feature that increases with static magnetic field strength. Although these findings were not replicated by another study (208), and such changes appear rather non-specific since they have also been shown in healthy individuals, T2-weighted hypointensities do correlate with UMN signs in ALS patients (207, 209, 210) and can appear early in the disease process (190).

## Diffusion Tensor Imaging (DTI)

Diffusion tensor imaging (DTI) exploits differences in local directionality of water diffusion to assess tissue architecture and is especially suited to the study of white matter tracts. Fractional anisotropy (FA) is a derived measure which can represent tract integrity. In ALS, FA reduction in the corticospinal tracts and corpus callosum is a consistent finding (211–214) which correlates with clinical measures of disease progression (190, 211, 215–217). Associated elevations in mean diffusivity (MD), a scalar measure representing total diffusion within a voxel, have been reported in a number of these studies (211, 218). Low FA has also been demonstrated in the cervical spinal cord (219, 220) and in extra-motor regions (217, 221, 222). Longitudinal reductions

in FA over time have been shown in both motor and extra-motor areas (223, 224).

DTI has demonstrated widespread white matter tract damage supporting the concept of ALS as a multi-system disorder. Diagnostic sensitivity and specificity of 68 and 73%, respectively, has been reported (225). Recent work has applied DTI to create *in vivo* disease staging models, to probe hypotheses of pathophysiological spread in ALS (5, 226).

## Combination of Structural MRI and DTI

Machine learning algorithms combining both volumetric gray matter and DTI measures have been reported to discriminate ALS patients from healthy controls with 86% sensitivity, 67% specificity, and 78% accuracy (227), and ALS patients from ALS-mimics with 92% sensitivity, 75% specificity, and 87% accuracy (228).

## Magnetization Transfer Imaging (MTI)

Magnetization can undergo transfer between bound water, macromolecular groups and free MR-observable water. This interaction can be used to provide the tissue contrast exploited in Magnetization Transfer Imaging (MTI), often interpreted as a measure of myelin integrity or neuroaxonal damage. Reduced MTI ratios have been reported in the corticospinal tracts and extra-motor gray matter of patients with ALS compared to controls (229–231) although these findings were not replicated in one report (199).

## Functional Magnetic Resonance Imaging (fMRI)

Blood oxygen level-dependent (BOLD) functional MRI (fMRI) can detect regions of neuronal and synaptic activation in response to experimental stimuli. A localized vascular response to energy use and demand causes “active” regions to receive an increased oxygenated blood supply, and the MR signal

is differentially attenuated according to blood oxygenation level. Aspects of brain physiology can therefore be assessed, based on an assumption of neurovascular coupling. Cortical reorganization has been demonstrated in patients with ALS, with increased activation of contralateral and ipsilateral motor areas including sensorimotor cortex, supplementary motor areas, basal ganglia and cerebellum during motor tasks (232–235). Contralateral over-activation correlates with disease progression (236). Reduced activation has been observed in dorsolateral prefrontal cortex (235) and in other studies which investigated tongue movements in patients with bulbar dysfunction (237, 238). Longitudinal studies have demonstrated that increased sensorimotor cortical activation (perhaps attributable to loss of intracortical inhibition) is followed by decreased activation later (probably as motor neurons degenerate) (238). Contrasting results were obtained following motor imagery experiments. Increased activity was seen in patients compared with controls in one study (239), but reduced activity in another (240). In addition to an external stimuli-driven BOLD response, resting state abnormalities have been demonstrated (241). Patients have been shown to demonstrate abnormalities in cerebral regions associated with executive functions (242), and emotional (243, 244), sensory (245), and language (246) processing.

## Magnetic Resonance Spectroscopy

Magnetic resonance spectroscopy (MRS) is a promising advanced MR technique which provides insights into tissue neurobiology through direct measurement of metabolites (Figure 3). Proton spectroscopy of the brain ( $^1\text{H}$ -MRS) enables detection of the neuronal molecule N-acetylaspartate (NAA), the glial marker myoinositol (mI), choline-containing compounds (Cho), amino acids and neurotransmitters such as glutamate, glutamine, and gamma-aminobutyric acid (Glu, Gln, and GABA), and creatine, phosphocreatine, and glutathione (Cr, PCr, and GSH) which are compounds related to cellular bioenergetic and oxidative status. Brain  $^1\text{H}$ -MRS studies have demonstrated a widespread reduction in NAA correlating with UMN burden (247–249) in regions spanned by the pyramidal tract (247, 248, 250–265) and in other cortical and subcortical areas (266). NAA has been proposed as an objective indicator of UMN dysfunction and as a potential diagnostic biomarker: sensitivity and specificity of the NAA/Cho ratio have been reported to be 100% and 85% (267), and to be superior to anatomical MRI (268, 269), DTI (267), and transcranial magnetic stimulation (270). The combination of  $^1\text{H}$ -MRS and DTI to diagnose ALS yields a high positive likelihood ratio (6.20) and low negative likelihood ratio (0.08), with potentially useful sensitivity and specificity of 90 and 85%, respectively (271). Although publications assessing longitudinal NAA changes have reached inconsistent conclusions (272–274), NAA concentration has also been used as a marker of treatment response in a number of clinical trials (275–281).

Total creatine (Cr and PCr) appears unchanged (248, 259, 282), but studies measuring Glu, mI, Cho, GABA, and GSH have produced conflicting results and, at present, it is unclear whether the concentration of these molecules is altered in ALS (255, 258, 259, 263, 264, 282).

As highlighted above, published findings from studies that utilize  $^1\text{H}$ -MRS have reported conflicting results at times. In addition to differences between study groups, MR system manufacturer, and spectroscopic analysis methodology, the basic acquisition technique can vary (e.g., echo-generation type, localization method, TR, TE), which may partially explain the lack of consensus. As with standard MRI, the relative contributions from different spectral resonances can be weighted by intrinsic factors such as proton density, T1-, and T2-relaxation rates for each of the metabolites. For  $^1\text{H}$ -MRS, to further our understanding and provide indications of pathophysiology, disease stage and potential therapeutic response, well-characterized and appropriately standardized  $^1\text{H}$ -MRS acquisition methodology is warranted.

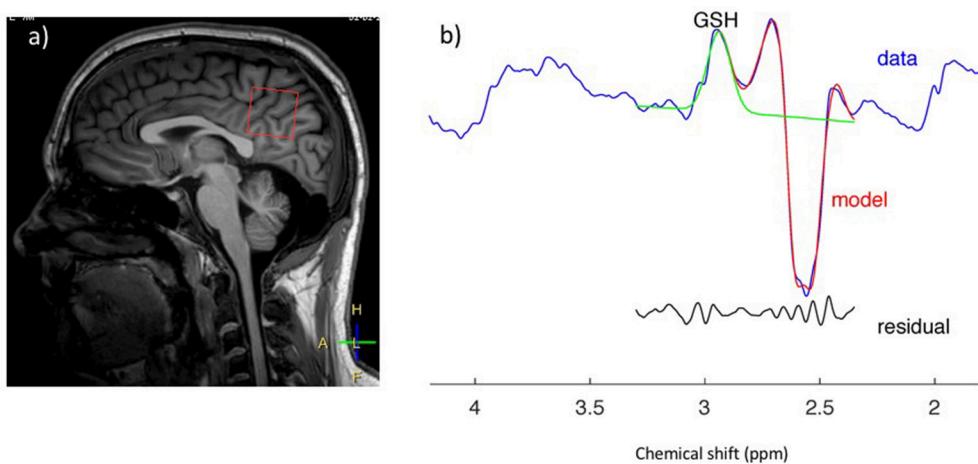
## PERIPHERAL NERVE IMAGING

In ALS, secondary effects on peripheral nerve are the least studied anatomical location with MRI, but the technique shows potential and has been investigated (283, 284). In a recent longitudinal study, the FA of tibial and peroneal nerve was shown to decrease with disease progression and to correlate with ALSFRS-R, showing potential as a biomarker of disease progression (285).

## MUSCLE IMAGING AND SPECTROSCOPY

Anterior horn cell denervation in ALS leads to secondary signal change and atrophy in muscles and nerves which can be assessed with MRI and potentially employed as a marker of disease progression. An early study showed reductions in the volume of the tongue in up to two-thirds of ALS patients (284). Tibialis anterior volume reduction and increased T2-relaxation times were observed in a longitudinal study of 11 patients (286) and correlated with clinical (maximal voluntary isometric contraction, MVIC) and electrophysiological (CMAP) measures. Limb muscle signal changes have been demonstrated in cross-sectional studies using qualitative observer assessment scales (283, 287). A more recent longitudinal whole-body muscle MRI assessment demonstrated semi-quantitative T2 changes in multiple body regions in ALS patients compared with controls, as well as associations with clinical power and MUNIX, and longitudinal increases signal changes in the tibialis anterior muscle over 4 months (288) (Figure 4).

Metabolites related to cellular bioenergetics, such as adenosine triphosphate (ATP), PCr, and inorganic phosphate (Pi), as well as intracellular pH, have been measured in muscle using phosphorus-31 spectroscopy ( $^{31}\text{P}$ -MRS); some studies have also employed dynamic protocols to assess PCr and pH variations during muscle contraction. PCr recovery (a parameter that correlates with mitochondrial oxidative capacity) was found to be prolonged in patients in one study (289) but was reported unchanged in another (290). Additionally, there appears to be a decreased drop in PCr upon muscular contraction in ALS patients, likely due to lack of available motor units to recruit (291), although other hypotheses, such as impaired central



**FIGURE 3 |** GSH spectrum (B) from medial parietal cortex (A) (MEGA-PRESS sequence, HERMES spectral editing). (B) Green line showing spectral edited GSH peak.

activation or even existence of ALS related primary muscular changes, have also been proposed (292, 293). The potential of  $^{31}\text{P}$ -MRS being a putative marker of energy dysmetabolism and disease progression has not yet been fully explored.

## POSITRON EMISSION TOMOGRAPHY

Positron emission tomography (PET) is another imaging modality that has been employed primarily to investigate ALS pathophysiology, but has shown some potential as a diagnostic biomarker. Relatively fewer PET studies have been conducted in ALS, possibly because this modality, albeit non-invasive, involves exposure to ionizing radiation, and because radiotracer development is a complex process that requires a cyclotron and a specialized multidisciplinary team.

[ $^{18}\text{F}$ ]Fluoro-2-deoxy-2-D-glucose (FDG) PET measures cellular glucose uptake and can assess metabolic activity of brain regions. In ALS, decreased FDG uptake, a probable corollary of neurodegeneration, has been reported in the motor, premotor, and prefrontal cortices as well as in the basal ganglia (294, 295). Notably, the severity of hypometabolism in the front-temporal cortex was associated with cognitive decline and was predictive of shorter survival (296, 297). Interestingly, increased FDG uptake has also been reported in midbrain, pons, hippocampus, superior temporal gyrus, and cerebellum (295, 298). This could perhaps reflect neuronal hyperexcitability, adaptive cellular changes within metabolically active pathways, and/or astrocytic proliferation (295). These findings further corroborate the hypothesis that ALS-related dysmetabolism does not pertain exclusively to motor areas. In addition, midbrain hypermetabolism appears to be relatively specific to ALS and could potentially be valuable in the diagnostic workup of ALS patients (295, 297). Data on altered glucose uptake in the amygdala, parietal, and occipital cortices is more equivocal: lack of consensus could be due to differences either in study protocols or control groups (299).

Neuroinflammation is considered a potentially important contributor to the pathophysiological cascade in ALS and there have been ongoing efforts to develop immune-modifying therapeutics. In this context, assessment of *in vivo* microglial activation by PET could potentially be employed in clinical trials to provide evidence of target engagement and, possibly, to be used as a biomarker of disease response. Microglial activation can be investigated using radiotracers targeting the 18 kDa translocator protein (TSPO), also known as the peripheral-type benzodiazepine receptor, such as [ $^{11}\text{C}$ ]-( $\text{R}$ )-PK11195 (a first generation tracer which is relatively non-specific and has a low signal to background ratio), [ $^{18}\text{F}$ ]-DPA-714, and [ $^{11}\text{C}$ ]-PBR28 (second generation, more specific tracers). TSPO is thought to be expressed specifically by activated microglia and astrocytes. These studies have shown enhanced microglial activation in primary and premotor cortices, prefrontal and temporal cortices, thalamus, and brainstem (300–303). Findings correlated with UMN burden and ALSFRS-R score and were associated with concomitant alterations of the glial marker mI and with DTI and spectroscopic measures of tissue damage (300, 302, 303).

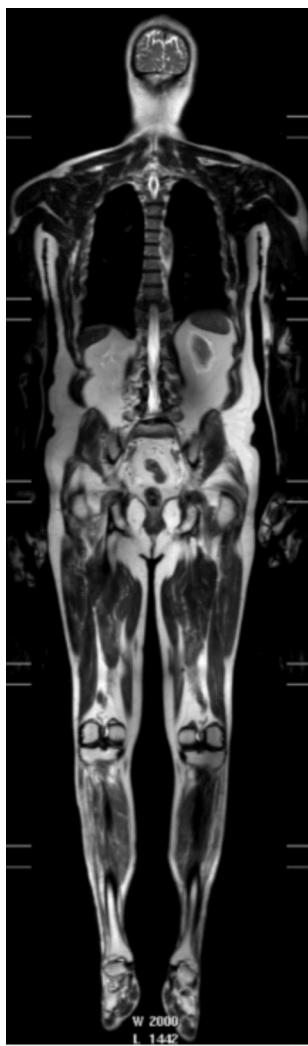
Other work has provided further insights into ALS pathogenesis by showing evidence of inhibitory interneuronopathy (employing the GABA-A ligand, [ $^{11}\text{C}$ ]flumazenil) (304, 305), alteration of serotonergic neurotransmission [using the radiotracer [ $^{11}\text{C}$ ]-WAY100635] (306), and increased oxidative stress [by [ $^{62}\text{Cu}$ ]-ATSM] (307).

In summary, whilst MR and PET studies have made important contributions toward elucidating disease mechanisms *in vivo* in patients with ALS, a fully validated biomarker sensitive and specific to disease change at individual level remains elusive. This represents an important area of need in the field (308).

## ELECTROPHYSIOLOGY BIOMARKERS

### Motor Unit Number Estimation (MUNE)

First developed in the 1970s, motor unit number estimation (MUNE) aims to provide a reproducible, quantitative measure



**FIGURE 4 |** T2-weighted whole body image acquired in a patient: 3T, single shot TSE, TR 1107 ms, TE 80 ms, FOV 37 × 55 cm, voxel size 1.25 × 1.5 × 5 mm recon 0.78 × 0.78 × 5—used with permission from Jenkins et al. (288).

of the number of functional motor units (309). Numerous MUNE methods have emerged predominantly based on the same underlying principle. First, a summated value for the total motor unit population within a nerve, the maximum compound muscle action potential (CMAP) amplitude, is obtained. This is then divided by a value representing the average single motor unit in that nerve, thus providing an estimate of motor unit number (309, 310).

MUNE calculations differ in the approach taken to measuring a typical single motor unit (311). For example, the original incremental method utilized the concept of different axons having differing excitation thresholds, with step-wise increases in stimulus intensity used to recruit additional discrete motor units (309). However, subsequent work determined that repeated presentation of the same stimulus may activate different motor axons with similar stimulation thresholds, thus resulting in

CMAP changes not representative of single motor unit size, a phenomenon termed alternation (310). The multiple point stimulation (MPS) method (and later adaptations) attempted to circumvent this through stimulation at distinct points along the nerve in an attempt to sample different motor axons (312). Further developments included a multipoint incremental MUNE, combining incremental and MPS methods. This technique had a number of practical advantages over other methods in that it is simple, relatively rapid to perform (~5 min per muscle), well-tolerated (as multiple supramaximal stimuli are not performed), and does not require specialized equipment (313). Statistical approaches to the *post-hoc* analysis of data have also been proposed (314, 315).

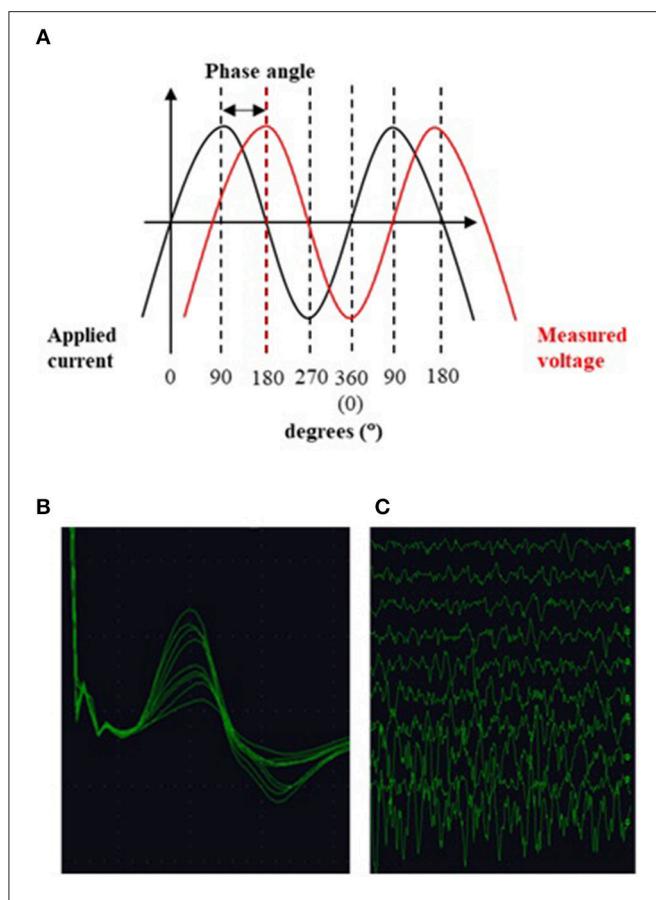
Incremental, MPS and multipoint incremental MUNE methods have been reported as reliable and sensitive tools, correlating with and outperforming other functional clinical measures in demonstrating disease progression (249, 316). MPS has additionally been observed to identify preclinical LMN loss (249). Despite promising findings in familial (317) and sporadic cohorts (318), Poisson statistical methods were unable to account for the increased motor unit variability found in patients with ALS (318). Similarly, despite promising initial results (315), dissemination and validation of Bayesian statistical methods, which allow for sources of variability and uncertainty, has been limited by the technically intensive nature of the process (310, 319).

High-density MUNE utilizes a large number of electrode channels to resolve alternation, whilst also enabling the measurement of proximal and distal muscles, a feature not offered by most MUNE techniques (320). The requirement for specific equipment and software has precluded its widespread use thus far (319).

Recently, a novel MUNE method, MScanFIT MUNE (MScan), has been proposed, using detailed stimulus-response curves, or “CMAP scans,” which provide information on all motor units contributing to the CMAP, unlike other MUNE methods (321). Preliminary findings appear promising, demonstrating superior reproducibility, detection of motor unit loss, and disease progression compared to other MUNE methods (322).

Motor unit number index (MUNIX) applies a mathematical model based on the CMAP and surface EMG interference pattern at different voluntary activation levels (Figures 5B,C) (323). It overcomes a number of MUNE limitations by enabling fast (<5 min/muscle), easy to perform measurements of any proximal or distal muscle from which a supramaximal CMAP can be elicited (324).

The sensitivity and reliability of MUNIX as a marker of disease progression in ALS was highlighted in a 15-month longitudinal multicenter study (325), with further work demonstrating a significant correlation between MUNIX and various MUNE techniques (27, 311, 326, 327). The importance of optimizing maximum CMAP amplitude during MUNIX recording has been emphasized (324). MUNIX measurement reliability has been shown to improve when employing a qualification process including face-to-face teaching and training, with ongoing support for evaluators (328).



**FIGURE 5 | (A)** EIM: alternating current is applied to the muscle and the ensuing voltage measured. The phase angle is a metric of tissue impedance and describes, in degrees, the angle of asynchrony between the two sinusoidal waveforms. **(B)** Multiple compound muscle action potentials recorded during the incremental motor unit number estimation technique. Each change in amplitude is thought to represent the addition of a new motor unit. **(C)** Surface interference patterns obtained during the motor unit number index technique. After recording a maximal compound muscle action potential the subject performs isometric contraction of the muscle of interest with increasing force. Parameters from these recordings are then used together with data from the CMAP to compute the index.

Despite these promising results, some authors have suggested that values depend too heavily on CMAP amplitude to provide useful estimates of motor unit numbers (326). However, MUNIX has been shown to exhibit superior sensitivity to early change when compared to ALSFRS-R, manual muscle testing, and CMAP amplitude (329). Furthermore, a capacity to detect pre-symptomatic LMN loss has also been reported (330).

Multi-muscle global MUNIX scores have been investigated as a measure of multi-segment involvement (331), allowing more broad evaluation of motor unit loss and insight into the pattern of disease spread (330, 331). Such instruments have shown increased sensitivity to progression when compared to single-muscle MUNIX (331) and ALSFRS-R (325), reducing the time required to detect therapeutic change (325). This approach may, however, over-represent individual segments (331) and negative

results have also been reported (332). Nonetheless, MUNIX offers interesting insights into disease progression and is undergoing worldwide evaluation in clinical trials.

## Neurophysiological Index

The neurophysiological index (NI) has been proposed as a quantitative measure of peripheral disease burden in ALS patients. It collectively expresses changes observed during disease progression using standard neurophysiological measures: increases in distal motor latency and F-wave frequency, and a decrease in CMAP amplitude (333, 334). Previous studies report NI to be a reliable measure (335), differentiating fALS and sALS cohorts from healthy controls (336, 337). As a surrogate measure of disease progression, NI has displayed decline at a greater rate (41.9% at 6 months) than ALSFRS-R (18.4%), FVC (15.4%), and CMAP amplitude (25.5%) (316), with sensitivity to change in as little as 4 weeks (329). While further work is needed, the NI has been implemented in clinical trials and has been proposed as a method to expedite completion of future phase II trials (334).

## Axonal Excitability

Axonal excitability measurement techniques allow non-invasive, *in vivo* assessment of the biophysical properties of peripheral axons (338). Employing threshold tracking methods allows sensitivity to changes in the membrane potential caused by activation of ion channels and electrogenic ion pumps (339). Indices used in threshold-tracking axonal excitability testing have provided information of pathological significance in ALS (338). Upregulation of persistent  $\text{Na}^+$  conductances and reduction of slow and fast  $\text{K}^+$  channel conductances have been demonstrated, with the net result being motor axonal hyperexcitability (340).

Axonal ion channel dysfunction has been observed in sALS and fALS cohorts, and supported by mouse models (341–343). Such membrane hyperexcitability is postulated to promote the generation of fasciculations and muscle cramps (341), with intra-axonal  $\text{Ca}^{2+}$  accumulation due to persistent  $\text{Na}^+$  influx implicated in the neurodegenerative process (338, 344). In keeping with this, changes in axonal excitability have been reported to correlate with more standard measures of motor axon degeneration, such as CMAP amplitude (345). A persistent  $\text{Na}^+$  conductance has been observed to be a predictor for shorter survival time and rapid inter-regional spread (346, 347). Changes in the pattern of abnormal membrane properties with disease progression have also been reported (341). Availability of the specialist hardware/software may limit uptake; however further study of longitudinal utility and test-retest reproducibility is warranted.

## Electrical Impedance Myography (EIM)

Electrical impedance myography (EIM) provides a non-invasive, painless and quantitative method for the evaluation of muscle (Figure 5A). Low-intensity, high-frequency alternating electrical current is applied via surface electrodes to a muscle (or muscle group) of interest and the resulting surface voltages measured. The fundamental basis of EIM is that these recorded surface voltages reflect the conductive and capacitive properties of the underlying tissue, with disease-related changes in muscle

morphology, such as muscle fiber atrophy, resulting in altered impedance values (348).

EIM is easy to perform, allows study of proximal and distal muscles, and requires limited subject cooperation and evaluator training (349, 350). It has been shown to be a highly reproducible tool, correlating with established electrophysiologic and functional measures of disease severity (351, 352). Multicenter data have reported sensitivity of EIM to disease progression, demonstrating its potential to expedite phase II clinical trials by reducing the sample size required to detect a treatment effect by more than 50% compared to the ALSFRS-R (353). Evidence for the utility of EIM in the diagnosis of ALS is preliminary, with further study required into its ability to distinguish ALS from other neuromuscular diseases (354).

More recently, EIM has been applied to the evaluation of bulbar dysfunction in ALS, an area of particular importance given the prognostic implications and lack of objective, quantifiable bedside measures of bulbar status (355, 356). Initial investigation has indicated tongue EIM to be a reliable technique, significantly correlating with tongue endurance and the ALSFRS-R bulbar subscore, and distinguishing healthy and diseased muscle (357, 358). Despite the encouraging results emerging principally from a single laboratory, EIM remains in need of development and optimization (355). Further interdisciplinary investigation would allow greater appreciation of the utility of EIM as an objective clinical measure.

## Transcranial Magnetic Stimulation (TMS)

The diagnosis of ALS relies on identification of a combination of UMN and LMN features (359). Conventional electrophysiological techniques objectively assess LMN function. Evaluation of UMN involvement, however, remains solely based on clinical examination (360). Pioneered by Barker and colleagues (361), transcranial magnetic stimulation (TMS) is a non-invasive neurophysiological technique that assesses UMN functional integrity (360, 362). Differences in a number of TMS parameters, signifying a change in cortical excitability, have been identified as an early and specific feature in patients with both sporadic (337) and familial ALS (336). Such abnormalities, including reductions in short-interval intracortical inhibition and cortical silent period duration, and increases in intracortical facilitation and motor evoked potential amplitude (362), precede evidence of LMN dysfunction (363, 364), correlate with measures of peripheral disease burden (337), and relate to the pattern of disease spread (365). These findings provide pathological insight and lend support to the dying-forward hypothesis of ALS as a primary disease of the cortical motor neuron (360).

Recently developed, threshold tracking TMS (TTTMS) (337) has produced important results, including facilitating reliable differentiation of ALS from mimic disorders (366), an improvement in diagnostic sensitivity when compared to the Awaji-Shima criteria, and a reduced time to diagnosis (364). To date, this technique has been largely pioneered by a single group; if reproduced in other centers, the case for incorporation of TTTMS as an objective tool for assessing in future ALS diagnostic criteria would be strong. Evidence supporting the use of TMS as a biomarker assessing longitudinal change is, however, more

preliminary and has employed traditional TMS techniques, with conflicting conclusions reached in the ability to monitor disease progression (367, 368), in addition to limited application in ALS therapeutic trials (369). This area remains an exciting field for the ALS community to develop over the coming years.

## CONCLUSIONS

The breadth of the research outlined above is an indication of the efforts being undertaken to better understand the pathophysiology of ALS and to discover and validate biomarkers. Common themes occur in each described modality.

Biomarker exploration is dependent on replication. Using biofluid samples as an example, by using agreed SOPs for sample collection and for analysis, more robust conclusions can be drawn. In this way results from multiple centers can be pooled, providing sufficient statistical power to label a biomarker as useful or not. Once a potential biomarker is identified, it can be validated using round-robin or “reverse” round-robin methodology (31). If not successful then a consensus approach should be established to shift focus onto other promising markers. A similar approach in imaging has been established: The Neuroimaging Society in ALS (NiSALS) is a collaboration of neuroimaging scientists to discuss imaging methodologies in the disease as well as providing a solution to the challenge of analyzing MRI data from different sites and protocols (308). Successful validation from meticulous research methodology unfortunately then has the additional hurdle of becoming valid in clinical practice, wherein there is new heterogeneity, with reliance on healthcare professionals and hospital laboratories to collect and process samples in a comparable way.

Despite excellent attempts in each field, single useful biomarkers of ALS are as of yet out of reach. Combining biomarkers within a modality is a useful way to improve their utility, although this increases the risk of false positives, and the more biomarkers that are used the higher the sample number needed to confirm significance (370). Additionally, combining markers across modalities is a logical approach to maximize the strengths and sensitivities of each method. With the vast amount of data that this yields, particularly with the use of “-omic” approaches, machine learning techniques may yield the best combinations to maximize sensitivity and specificity. To this end, collaboration with bioinformaticians is essential.

Collaborative efforts like the Pooled Resource, Open access ALS clinical trials (PRO-ACT) database, provide researchers with a large body of well-categorized, longitudinal, patient data sets. This is especially useful in a relatively rare disease like ALS. It can be used to increase the statistical power during analysis of single biomarkers and for machine learning models. Prize4Life, a non-profit organization, asked for models that best predicted survival based on the PRO-ACT data. Algorithms and machine learning approaches were submitted and shown to improve prediction as compared to clinician assessments, and that these methods could reduce the cost of trials through a reduction in sample size. Additionally, this approach identified features previously unrecognized in their contribution to prediction such as creatine

kinase, pulse and blood pressure (371). Other research groups continue to use the PRO-ACT data and have developed models of disease progression (372) and survival (373), and have clarified, for example, the predictive utility of urate as a biomarker (169). Whilst this exercise is undoubtedly useful, the importance of standardized collection and analysis methods remains.

The majority of studies explore diagnostic biomarkers, and many exist contrasting patients with healthy controls. However, if a patient with typical ALS is seen by a neurologist, particularly a neuromuscular specialist, then there is rarely a diagnostic dilemma. Ideally, comparisons should be made between ALS and those patients with disease mimics e.g., multifocal motor neuropathy with conduction block or monomelic amyotrophy. Moreover, as explained above, the survival and disability heterogeneity in ALS is large and to this end longitudinal studies assessing how the disease changes over time, measured through surrogate biomarkers, will provide improved information to better sub-classify patients and their prognosis and ensure trial success.

Future biomarker studies should aim to encapsulate all phenotype data as well as genetic and biological information to help stratification. The above point is well-explained by Benatar et al. (374) and furthermore they outline general points for researchers to be aware of in ALS longitudinal studies. During longitudinal follow-up, studies may enrich with slow-progressors, implying that conclusions that are drawn are not necessarily applicable for the whole population. Secondly, attempting to define disease onset is difficult, given that disease is likely to be active before presentation to healthcare; “baseline”

comparisons are therefore not valid. However, most ALS progresses linearly and as such there is value in measuring fixed interval time points from the “recruitment baseline.”

## AUTHOR CONTRIBUTIONS

PS and NV conceived the concept and structure of the review. NV wrote the sections on introduction, cerebrospinal fluid and conclusion. SS and SM wrote the section on blood and SS wrote the section on urine. HM wrote the section on electrophysiology with oversight from JA. MS wrote the section on imaging with oversight from TJ and IW. All authors reviewed the final manuscript and offered critical feedback.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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