HHS Public Access

Author manuscript

Muscle Nerve. Author manuscript; available in PMC 2017 February 01.

Published in final edited form as:

Muscle Nerve. 2016 February ; 53(2): 169–182. doi:10.1002/mus.24979.

ALS Biomarkers for Therapy Development: State of the Field & Future Directions

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Abstract

Biomarkers have become the focus of intense research in the field of amyotrophic lateral sclerosis (ALS), with the hope that they might aid therapy development efforts. Notwithstanding the discovery of many candidate biomarkers, none have yet emerged as validated tools for drug development. In this review we present a nuanced view of biomarkers based on the perspective of the FDA; highlight the distinction between discovery and validation; describe existing and emerging resources; review leading biological fluid-based, electrophysiological and neuroimaging candidates relevant to therapy development efforts; discuss lessons learned from biomarker initiatives in related neurodegenerative diseases; and outline specific steps that we, as a field, might take in order to hasten the development and validation of biomarkers that will prove useful in enhancing efforts to develop effective treatments for ALS patients. Most important among these perhaps is the proposal to establish a federated ALS Biomarker Consortium (ABC) in which all interested and willing stakeholders may participate with equal opportunity to contribute to the broader mission of biomarker development and validation.

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Financial Conflicts of Interest

Andreas Jeromin is a paid employee and shareholder of Iron Horse Diagnostics, Inc. Seward Rutkove owns equity, receives consulting fees, and owns patent rights in Skulpt Inc.

Nazem Atassi receives consulting fees from Biogen.

James D. Berry has consulted with Biogen Idec and Neuraltus Pharmaceuticals and has received research support from Voyager Therapeutics, GSK, Cytokinetics, Brainstorm Cell Therapeutics, Novartis, ALS Therapy Development Institute, ALS Association, MDA, and NIH.

Keywords

prognostic; predictive; pharmacodynamic; disease progression; ALS; biomarkers

INTRODUCTION AND BACKGROUND

Interest in biomarkers relevant to amyotrophic lateral sclerosis (ALS) has grown steadily over the past decade. The current focus on biomarkers has been fueled, at least in part, by broad recognition of the value that they have added to therapeutic development efforts in disease areas such as multiple sclerosis, human immunodeficiency virus infection, cancer, and cardiovascular and cerebrovascular disease; and the sincere hope that biomarkers might be of similar value in the field of ALS. Notwithstanding the multitude of ongoing efforts, no biomarkers have yet emerged as validated tools clearly relevant to ALS therapy development. While the reasons are undoubtedly complex, conceptual clarity about the different types of biomarkers, the currently unmet needs they might fulfill, and the approach to development and validation of these 'fit for purpose' biomarkers is essential.

This white paper has emerged from several recent collaborative efforts to stimulate forward progress in biomarker development. These include a biomarker workshop co-sponsored by the ALS Association and ALS Therapy Development Institute (TDI) (Cambridge MA, May 19 2014), a biomarker symposium at the first annual ALS Research Group (ALSRG) meeting (Bloomington MN, September 2014), and the emergence of the Clinical Research in ALS and related disorders for Therapeutic Development (CReATe) Consortium, a National Institutes of Health (NIH)-supported Rare Diseases Clinical Research Consortium (RDCRC) that forms part of the NIH Rare Diseases Clinical Research Network (RDCRN) and which has, as a specific focus, the discovery and validation of biomarkers relevant to therapy development.

BIOMARKERS – THE FDA PERSPECTIVE AND BEYOND

The term 'biomarker' has been defined as a "characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathologic processes, or biological response to a therapeutic intervention" [1]. The concept of a 'biomarker', however, is not a unitary one. Instead, there are several different types of biomarkers, the desired characteristics of which may vary depending on the intended use or application. Moreover, methodological approaches to developing and validating a biomarker may very well differ depending on its intended use.

In their guidance document on the qualification of drug development tools, the United States Food and Drug Administration (FDA) recognizes 4 different types or applications of biomarkers: diagnostic, prognostic, predictive, and pharmacodynamic, acknowledging that some biomarkers may have more than 1 application depending on how the biomarker is used (Table 1) [2]. While the FDA guidance document does not specifically discuss the idea of disease progression biomarkers, they are centrally important to a neurodegenerative disease such as ALS, in which altering an otherwise inevitable decline is the goal of therapy. A biomarker of disease progression may be defined as a characteristic that is measurable over

time and which changes as disease advances. The ideal progression marker would be one that not only changes with disease progression, but also in response to effective therapy, and thus would serve as a pharmacodynamic marker as well. The level of urinary p75 neurotrophin receptor extracellular domain ($p75NTR^{ECD}$), for example, increases over time as disease progresses [3] and is therefore a promising progression biomarker. If an effective therapeutic also blunted the increase, stabilized, or even reduced urinary $p75NTR^{ECD}$ levels, then it might be considered a pharmacodynamic biomarker of drug effect. A critically important point is that we currently lack effective therapeutics for ALS that might be used to validate candidates as pharmacodynamic biomarkers.

Relevant to the discussion of biomarkers is the issue of surrogacy. From an FDA perspective, a "surrogate measure" can be defined as "…a laboratory measurement or physical sign that is used in therapeutic trials as a substitute for a clinically meaningful endpoint that is a direct measure of how a patient feels, functions, or survives, and is expected to predict the effect of the therapy" [4]. The difference between a surrogate measure and a biomarker is that the biomarker may be a "candidate" surrogate measure, whereas the surrogate measure is "used, and taken, as a measure of the effects of a specific treatment." A surrogate marker may be validated by showing that the treatment's effect on the surrogate reliably predicts the effect on the endpoint of clinical interest. The use of biomarkers as candidate surrogate measures is not a cause for regulatory concern in the early phases of drug development, since they may provide important insights in the drug development process. Controversy arises when a non-validated surrogate measure is proposed as the primary outcome measure in a clinical trial designed to provide evidence of effectiveness of a new treatment. We will not address this issue further, since biomarkers in the ALS arena are, at best, currently being developed as candidate surrogates rather than as validated surrogate markers. Within the context of phase II clinical trials in ALS, the hope for biomarkers is that they might be used as candidate surrogate markers if, for example, it could be shown that they were more sensitive to disease progression over a shorter interval than currently used clinical measures such as the revised ALS functional rating scale (ALSFRS-R).

DISCOVERY VS. VALIDATION

Although there are no validated ALS biomarkers of relevance to therapy development, there is no shortage of promising candidates. These include biological fluid-based biomarkers [e.g. blood neurofilament light chain (NfL), phosphorylated neurofilament heavy chain (pNfH), CSF superoxide dismutase 1 (SOD1) levels, urinary p75 neurotrophin receptor extracellular domain ($p75NTR^{ECD}$)], neuroimaging techniques (e.g. magnetic resonance imaging, positron emission tomography), electrophysiological parameters [e.g. motor unit number estimation (MUNE), electrical impedance myography (EIM)], and motor assessments [e.g. accurate testing of limb isometric strength (ATLIS)]. This multitude of candidate biomarkers has emerged through intense efforts. While discovery is critically important and must continue, what is needed in parallel is a robust effort to test the utility of these putative biomarkers and to either move them "up" (i.e. validate for use in therapy development) or move them "out" (i.e. discard as not useful for advancing therapeutic development). Validation is a complex undertaking and may include harmonization and

standardization of both pre-analytic and analytic methods for multi-center implementation as well as independent replication; these may include longitudinal studies with attention to potential sources of bias such as incomparability of “baseline” data from patients enrolled at different points along the course of disease, a tendency to enrich for patients with slowly progressive disease (because those with more rapidly progressive disease have succumbed or accumulated sufficient disability to limit research participation), and the impact of loss to follow-up.

An important challenge is that both analytic and clinical validation studies may be perceived as less attractive by federal funding agencies, which may be more inclined to focus on discovery. Collective emphasis on the importance of validation studies, and perhaps seed funding from foundation partners, might provide the necessary impetus for federal agencies to also contribute to this essential undertaking. In the absence of such funding, the validation of these assays for commercial application in the clinical arena will require a hand-off from academic labs to commercial entities with the requisite motivation and resources. Such validation is not a trivial task. Based on guidelines issued by the FDA and the Clinical and Laboratory Standards Institute (CLSI), biomarker approval takes an average of 5 to 8 years with costs in excess of $10 million. While this process can be daunting when taken as a whole, the work, cost, and complexity of the biomarker discovery and validation process can be shared by multiple stakeholders, each uniquely suited to lead specific portions of the process. Successfully establishing appropriate partnerships that leverage strengths of each stakeholder in pre-competitive space increases the likelihood of success.

PRAGMATISM

Validation, however, is only part of the story. A technique may be ultimately proven to be valid, but for practical reasons it may be challenging to implement. These reasons could include general availability of the necessary equipment and expertise [e.g. a transcranial magnetic stimulation (TMS) device and expertise for threshold tracking], high cost, difficulty with application in a multicenter fashion, ease of access (e.g. urine is more readily collected than CSF), complexity of sample processing [e.g. peripheral blood mononuclear cells require more sophisticated lab processing than blood or urine), or patient preference. While such practical limitations could be overcome with sufficient effort or expense, this may be at the cost of other biomarkers that are easier to implement and are less cost-intensive. Biomarkers that require substantial patient time/discomfort to acquire, physician or evaluator time or expertise to perform, or more expensive or unusual equipment, or are limited in their applicability to only a subset of ALS patients, will have limited practicality in clinical trials. Some of these issues are discussed in more detail below.

EXISTING AND EMERGING RESOURCES

A growing number of resources are available to support biomarker discovery and validation. These include the biological repositories maintained by NINDS at the Coriell Institute, the Northeast ALS clinical trial consortium (NEALS), and the CReATe Rare Diseases Clinical Research Consortium (RDCRC) recently established under the auspices of the Office of Rare Diseases Research (ORDR) within the National Center for Advancing Translational

Science (NCATS). The Pooled Resources Open-access ALS Clinical Trials (PRO-ACT) initiative, which merges data from over 8,500 ALS patient records from multiple completed clinical trials, is an exciting new resource with potential to accelerate discovery in the field of ALS [5]. NeuroNEXT is a program at NINDS that aims to provide a robust, standardized, and accessible infrastructure to facilitate rapid development and implementation of biomarker informed phase II clinical trials in neurological diseases. The Neuroimaging Society in ALS is a multi-national consortium that has established a repository of magnetic resonances images at Jena University (Germany) and has developed an approach to the analysis of multi-center data that is typically collected through disparate imaging protocols at different sites [6].

PRE-ANALYTIC AND ANALYTIC ISSUES

As with any experimental procedure, biomarker studies are susceptible to error that may arise from a number of sources. Within the realm of biological fluid-based biomarkers, a distinction has traditionally been made between issues that arise in the pre-analytic (i.e. sample collection, processing and storage) and analytic (i.e. pertaining to the experimental assay itself) phases, although this conceptualization can also be used within the context of dry biomarker (e.g. neuroimaging, neurophysiological) studies.

Pre-Analytic Issues

Study Design—The design of every scientific experiment should be tailored to the specific scientific question that the study aims to address. This is certainly true of biomarker investigations in which the choice of study design (e.g. case control vs. cohort, cross-sectional vs. longitudinal sampling) and the definition of eligibility criteria (i.e. selection of cases and controls and whether the control group should include disease mimics) are critically important. For example, investigations of biomarkers with potential application to diagnosis should aim to differentiate patients with ALS from those with diseases that might cause diagnostic confusion at a stage in the diseases when the diagnosis of ALS is otherwise unclear; the diagnostic challenge is rarely how to differentiate between healthy controls and patients with established disease that is readily diagnosed clinically. The inappropriate use of a case-control design (rather than the more appropriate cohort design) for diagnostic studies tends to result in an inflated (and overly-optimistic) view of the sensitivity and specificity of the diagnostic test [7]. Similarly, studies of potential biomarkers of disease progression mandate a longitudinal design and will not require the inclusion of disease mimics, since the biological question is not whether the biomarker differentiates ALS from a mimic, but how the biomarker changes over time in someone already known to have ALS. As a field, we should also be cognizant of the limitations of study cohorts including: (a) the meaninglessness of aligning study participants at “baseline” (since patients are recruited at variable points along the trajectory/course of their own disease); (b) the tendency to enrich for patients with more slowly progressive disease (since those with more rapidly progressive disease are more likely to become physically disabled and to succumb to disease earlier); and (c) the risk of loss to follow-up in longitudinal studies in which those patients who accumulate greater physical disability may be more likely to drop out (e.g. loss to follow-up

in a longitudinal MRI study as respiratory muscle function declines and patients are no longer able to lay flat in the scanner) [8].

Confounders—Factors that might confound the association between the biomarker and the clinically relevant phenotypic element (e.g. presence or absence of ALS for diagnostic studies, or some clinical measure of disease severity for disease progression biomarker studies) should be considered carefully. Examples of potential confounders might include age, gender, and medical comorbidities. Axiomatic is the need for carefully collected and detailed phenotypic data (including a standardized approach to collection of clinical data elements) to ensure that sufficient information is available to identify and control for potentially significant confounders.

Sources of Variability—It is essential to consider potential sources of variability (i.e. noise) that may impact the quantification of a biomarker. In biological samples, for example, diurnal fluctuations in biomarker levels, the potential effect of medications or of the fasting vs. non-fasting state, and the impact of differences in sample collection, processing and storage may all impact measurements. This underscores the critical importance of harmonized standard operating procedures that can be implemented across multiple centers and research laboratories. These have been largely developed, but they could be made more widely available and readily accessible.

Analytic Issues

Assay Methodology—Early discovery studies for protein-based biomarkers typically utilize “off-the-shelf” commercially available reagents. Immunoassays, for example, employ capture and detection antibodies coupled with different technologies for the detection of specific biomarkers in different biological fluids such as blood (plasma, serum), CSF, urine, and others. Understanding the performance of these antibodies in the biological fluid of interest is crucial, and concern has been raised over the lack of sufficient characterization of the analytical performance of these assays [9]. Variations in the so-called supply chain of these reagents (antibodies, calibrator) and changes in these reagents from lot to lot, often undisclosed by the manufacturer, have led to conflicting results. Assessment of the variability of the assays between different days, laboratories, and operators is another critical parameter for understanding how robust the assay is. One commonly employed approach to addressing the impact of such factors is the use of so-called proficiency or round-robin studies, in which identical clinical samples (also referred to as “technical replicates”) are sent to different laboratories around the world, with formal comparison of the results. The FDA (http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm368107.pdf) and CLSI (http://clsi.org/ldts/) have issued specific guidelines to address the necessary additional analytical and clinical validation of these assays, and thereby to advance these research-use-only assays toward use in the clinical context. The analytical validation of these assays includes documentation of the specificity of the assay(s), the sensitivity for detecting the specific biomarker in the biological fluid of interest, the precision and accuracy of the method, and robustness over different days and in different laboratories. The development of biomarker assays according to FDA guidance and

CLSI guidelines for use as “laboratory-developed tests” is time-consuming and requires a significant financial investment.

Neuroimaging—Standardization is as important for imaging acquisition protocols as it is for biological sample collections. Several acquisition parameters may add to the intra-subject variability, such as the magnet strength (1.5, 3.0, or 7.0 Tesla), model of scanner, version of pulse sequence, shimming, and coils. Subject movement during scanning also adds to the variability and can be addressed by pre- and post- acquisition techniques. After acquiring the imaging data, researchers have a choice of several imaging processing and analysis packages that also have different versions changing over time. Some of these packages are fully automated, and some rely on input and modifications done by hand by the research team. Finally, the processed imaging data can be taken out of these processing packages and further modified using other software such as MATLAB to prepare the data for final analyses. One can easily appreciate that changing any of these variables could produce different results even within the same study population. Standardizing data acquisition within the same study is feasible by using the same scanner/coil and pulse sequences in single site studies, and by using the same scanner/coil make, and testing phantoms and traveling heads before starting multi-center imaging studies. Robust data should survive the remaining variability and should be replicable. Other disease areas such as multiple sclerosis (MS) and Alzheimer disease (AD) that rely on neuroimaging for drug discovery can provide several examples of efforts to standardize imaging data acquisition, processing, and analysis.

LEADING CANDIDATES (Table 2)

Biological Fluid-Based Biomarkers

Phosphorylated neurofilament heavy and Neurofilament light chains—As neuron-specific structural components of motor axons, neurofilaments have drawn attention as potential biomarkers in neurological disorders [10]. The neurofilament subunit proteins, phosphorylated neurofilament heavy (pNfH) and neurofilament light (NfL), are readily detected by conventional antibody based immunoassays and are released into CSF and peripheral blood in a wide range of pathological states including ALS [10–18]. Published data include cross-sectional and longitudinal studies in blood and CSF.

Cross-sectional CSF studies show elevated pNfH compared to healthy and/or disease controls [12, 13, 15, 16, 19], but it has not been truly evaluated as a diagnostic biomarker in a population of patients with suspected (but unproven) ALS. Initial CSF pNfH levels, however, appear to predict faster disease progression and shorter survival [12, 13, 15]. While studies of plasma/serum pNfH similarly suggest that higher levels at initial evaluation predict more rapid future disease progression [10, 16, these studies differ with respect to whether plasma levels remain stable over time [11], or fluctuate [20]. While there are no longitudinal data on levels of pNfH in CSF, a single small study showed a moderate correlation between pNfH in CSF and plasma or serum (r = 0.47 and 0.51, respectively), and a strong correlation between plasma and serum pNfH (r = 0.97) [12]. More data are needed on longitudinal pNfH levels in CSF and peripheral blood, with consideration of specimen

handling and immunoassay methodology to evaluate technical factors that could potentially influence results of competing assays.

Multiple groups have presented cross-sectional data that show increased CSF neurofilament light chain (NfL) levels in patients with ALS compared to healthy and/or disease controls [14, 17, 18, 21–24, 25], but as with pNfH, NfL has not been truly evaluated as a diagnostic biomarker in a population of patients with suspected (but unproven) ALS. Higher CSF NfL levels at the time of initial evaluation do predict faster future disease progression and shorter survival [17, 18, 24, 25]. Based on a longitudinal study, NfL levels in both blood and CSF appear to remain stable over up to 15-months of follow-up [25], with a good correlation between CSF and serum concentrations (r = 0.70–0.78) [14, 25].

Based on these data, pNfH and NfL levels in CSF (and potentially in plasma) may have application as prognostic biomarkers in clinical trials by facilitating stratification of study participants into treatment arms on the basis of anticipated rates of disease progression. If pNfH and NfL in CSF are indeed relatively stable over time intervals relevant to clinical trial design, and assuming the elevation of these proteins in biofluids represents a consequence of neuronal damage, neurofilament levels may have a role as pharmacodynamic biomarkers in that effective treatment would be expected to reduce motor neuronal damage and the release of pNfH and NfL. This strategy was examined in a single arm, open-label study of menantine in ALS in which CSF pNfH levels (examined at baseline, 6 and 12 months in 19 patients) declined over the course of treatment but did not reach statistical significance [26]. While the foregoing suggests promise for pNfH and NfL in CSF as biomarkers in ALS clinical trials, no data are available regarding the half-life of pNfH or NfL in CSF or peripheral blood in humans in health or disease. This would be critical for understanding the time course of change to be expected from an effective therapy. In the absence of effective neuroprotective/neuroregenerative therapy in ALS, there is no way to investigate potential reductions in pNfH or NfL levels in CSF or peripheral blood in response to treatment as a function of neuronal preservation. Evaluation of pNfH and NfL as putative pharmacodynamic biomarkers as such requires further study and is an appropriate aim in ALS therapeutic trials in which CSF and/or blood biospecimens for assay of pNfH and NfL can be collected pre- and post-treatment.

Uric acid—Uric acid is the end product of purine metabolism in humans. It is a potent antioxidant in the blood and CSF and exists in relatively high concentrations [27]. Oxidative stress is thought to play a role in the pathogenesis of ALS, perhaps through formation of stress granules, or through protein damage conferred by reactive oxygen species [28, 29]. Uric acid might mitigate this oxidative damage through its antioxidant activity. Several studies including a meta-analysis, reporting lower serum uric acids in patients with ALS compared to controls [30–34] as well a possible association between lower uric acid levels and slower progression or improved survival [30, 31, 32], have garnered interest in uric acid as a potential biomarker. The data, however, do not support the use of uric acid as a potential diagnostic biomarker, since lower uric acid levels have also been reported in other neurodegenerative diseases including Parkinson disease[35] and Huntington disease [36], and differentiating ALS patients from healthy controls is not typically difficult. The clinically important diagnostic

question is how to differentiate patients with ALS from those who have a disease that might mimic ALS.

There are presently inconsistent data regarding the potential utility of uric acid as a potential prognostic biomarker, as some studies suggest an association between lower serum uric acid and a milder future disease course [30, 31, 32], but others have reported no such association [34, 37].

Independent of its utility as a biomarker, uric acid might represent a therapeutic target. An ALS study, building on a similar study in Parkinson disease [38], is now underway to examine the effect of inosine, a compound that can increase uric acid in the blood and CSF, in people with ALS (NCT02288091). In this trial, plasma uric acid will be used as a pharmacodynamic biomarker, since it is directly related to the mechanism of action of inosine.

p75 Neurotrophin Receptor Extracellular Domain—Neurotrophin receptor p75 (p75NTR) is 1 of 2 receptors for the neurotrophins, a family of growth factors that stimulate neuronal cells to survive and differentiate. In humans (and rodents), expression of p75NTR is high in motor neurons during the embryonic period, but declines after birth. p75NTR, however, is re-expressed following nerve injury, and experiments from the 1980s showed that the urine of adult rats contained increased amounts of the extracellular domain of p75NTR ($p75NTR^{ECD}$) following sciatic nerve injury [39]. Based on the idea that injured nerves and Schwann cells shed $p75NTR^{ECD}$ from cell membranes following up-regulation and binding of neurotrophins, it was hypothesized and subsequently demonstrated that $p75NTR^{ECD}$ is excreted into the urine of $SOD1^{G93A}$ mice and humans with ALS [3]. Subsequent experiments have provided evidence that urinary concentrations of $p75NTR^{ECD}$ are not only elevated in ALS patients compared to controls in cross-sectional studies, but also that urinary $p75NTR^{ECD}$ continues to increase as disease progresses[40]. Preliminary data also suggest that urinary $p75NTR^{ECD}$ does not increase pre-symptomatically in people at genetic risk for ALS, but rather that levels begin to rise around the time disease becomes clinically apparent [40]. These promising findings, if verified, suggest that urinary $p75NTR^{ECD}$ has potential as a pharmacodynamic biomarker worthy of further evaluation in the context of a therapeutic trial. These data also suggest that urinary $p75NTR^{ECD}$ may be a useful biological marker of phenoconversion from the pre-symptomatic to the symptomatic phase of disease.

SOD1 Levels—Several studies have described changes in the expression of SOD1 in tissue and biological fluids, using ELISA for total SOD1 and/or misfolded SOD1. These latter assays reportedly employed conformation-specific antibodies to misfolded SOD1. So far, these results have been conflicting. For example Zetterstrom *et al* [41] reported no differences for misfolded SOD1 in familial (with SOD1 mutations) vs. sporadic forms of ALS in CSF. Winer et al [42] did not observe any differences in CSF for total SOD1 in ALS vs. neurological controls, but since CSF SOD1 levels were elevated in ALS patients vs. controls and remained stable over time, these authors suggested SOD1 as a potential pharmacodynamic biomarker. There are several strategies in place to improve the performance of the species-specific antibodies either for therapeutic or diagnostic

applications, and rigorous analytical validation in different biological fluids will be required to advance the detection of specific misfolded species of SOD1.

c9ORF72 Dipeptide Repeat Proteins—A hexanucleotide repeat expansion in C9orf72 [r(GGGGCC)exp] reported in late 2011 is the most common genetic cause of ALS, frontotemporal dementia (FTD), and combined ALS/FTD phenotypes (c9FTD/ALS). It accounts for approximately 40% of familial ALS (fALS) and approximately 6% of sporadic ALS (sALS) cases, or about 10% of cases overall. [43–45] This high prevalence of the C9orf72 repeat expansion in ALS patients, combined with recent studies demonstrating the potential for mutation-specific therapeutic strategies, have motivated efforts to identify biomarkers to support drug development for c9FTD/ALS. [46–48] A current focus in c9FTD/ALS biomarker development builds on the discovery that RNA species bidirectionally transcribed from the C9orf72 repeat expansion undergo unconventional translation to produce dipeptide repeat proteins. [49–51] This repeat-associated non-ATG translation (RAN translation) of sense and anti-sense RNA containing r(GGGGCC)exp in each of 3 possible reading frames gives rise to 6 dipeptide repeat protein species, Gly-Pro, Gly-Arg, and Gly-Ala from the sense sequence and Pro-Arg, Pro-Gly, and Pro-Ala from the anti-sense sequence. An increasing body of data support the concept that c9RAN proteins contribute directly to disease pathogenesis in c9FTD/ALS. [52–56]. Development of clinical assays for c9RAN proteins has emerged as a key strategy in particular for development of clinically useful biomarkers for c9FTD/ALS.

Neuronal nuclear and cytoplasmic inclusions widely present in post-mortem brain and spinal cord of c9FTD/ALS patients are immunoreactive with antibodies to c9RAN protein species. [49, 57, 58]. In standard ELISA and electro-chemiluminescent immunoassays these reagents also detect c9RAN proteins in CSF of clinically affected c9FTD/ALS patients [48]. Current challenges include a lack of availability of suitable specific immunoreagents for each of the individual c9RAN protein species and limited data on expression levels and pathological relevance of the various c9RAN proteins in c9FTD/ALS. Data on longitudinal levels of c9RAN proteins in relation to clinical features, particularly the emergence of clinical deficits in early-stage disease, will be essential to any future application of c9RAN proteins as biomarkers in clinical trials.

Electrophysiological Biomarkers

Nerve conduction studies and needle electromyography remain the main approaches for confirming a diagnosis of ALS. Electrophysiological methods might also be used to track clinical disease progression and the effect of therapy.

Compound motor action potential (CMAP)—The CMAP, obtained with supramaximal stimulation of a nerve while recording over a specific muscle of interest, represents the near-simultaneous depolarization of all muscle fibers underlying the electrode over the muscle [59]. A reduction in CMAP amplitude generally corresponds to motor axon loss and is commonly observed in ALS at the time of diagnosis, yet, somewhat surprisingly, its potential use as a marker of ALS progression has not been pursued. ALS studies have instead focused on related measures, including the neurophysiological index (NI) or motor

unit number estimate (MUNE), each of which is discussed below in more detail. However, since calculation of both NI and MUNE rely on generation of a CMAP, there is actually considerable CMAP data embedded in these studies. Indeed, median and ulnar CMAPs show substantial decline over time in most ALS patients, as demonstrated by 2 recent MUNE-focused studies [60, 61] and 1 of the NI [62].

Like most biomarkers of disease progression and drug effect, the challenge to using the CMAP is its repeatability in subjects, which is sensitively dependent on a number of factors, including electrode positioning, limb and hand positioning, electrode size, and limb temperature. In fact, studies of CMAP reliability have shown mixed results [63, 64]. However, by carefully trying to maximize the CMAP value and being consistent about electrode placement and limb position, it may be possible to more effectively use it as a primary marker of disease progression. Additional studies focused specifically on the CMAP as a biomarker should be pursued.

The Neurophysiological Index (NI)—The neurophysiological index (NI) is defined as NI = (ulnar CMAP amplitude/distal motor latency) X F-wave persistence and was introduced by Swash and de Carvalho in 2004.[65] By dividing the CMAP by the distal motor latency and then multiplying by the F-wave persistence (the former increasing and the latter decreasing in progressive disease), the premise was that the NI should be a sensitive biomarker of disease progression. Importantly, these data are easy to gather, since they are obtained during standard electrophysiological evaluation and only require a few more stimuli (to fully evaluate for F-wave persistence) beyond a typical nerve conduction study. While there has been relatively limited follow-up of this concept, a recent study has shown that the NI is very sensitive to disease progression over a several month period. This appears mainly to be due to changes in CMAP amplitude rather than F-wave persistence or distal motor latency [62]. In addition, there has been no longer-term study (e.g., over the typical clinical trial length of 6 months or 1 year), and its test-retest repeatability is unknown. Nevertheless, further study of this easily obtained value is clearly warranted.

Motor unit number estimation and motor unit number index—MUNE is perhaps the most theoretically compelling electrophysiologic biomarker for evaluating ALS progression and drug efficacy. Put simply, MUNE attempts to estimate the number of motor neurons innervating a muscle or muscle group [66]. This is accomplished by first obtaining a standard CMAP, usually from the abductor pollicis brevis or abductor digiti quinti muscle in the hand. Then, a variety of techniques are used to identify the average size of the single motor unit potentials (SMUPs) that contribute to that CMAP. The first described and most conceptually straightforward approach is via incremental stimulation [66]. In this MUNE version, stimulus intensity over the nerve is gradually increased, and the size of the steps in amplitude with successive elevations in stimulation is measured. The average size of the steps is then calculated, and that size divided into the CMAP to obtain the MUNE. A second conceptually straightforward approach is the multipoint stimulation technique in which the nerve is stimulated at different points along its length at very low levels with an effort to obtain a series of unique low-threshold SMUPs, and the amplitude of each is measured [67]. The average size of 10 of these SMUPs is then divided into the CMAP to obtain the MUNE.

Other methods incorporate various versions of these basic approaches, some incorporating the use of F-waves or needle recording of the action potential [61, 68]. Another somewhat different approach, termed the motor unit number index (MUNIX), has been developed. After obtaining the CMAP, voluntary contraction of the muscle is made at various intensity levels, and the surface interference pattern is measured and divided into the CMAP value to provide an index of motor units rather than a true MUNE value [69].

There can be little question that MUNE is conceptually appealing; however, it is challenging to perform, since it requires considerable training and much real-time decision-making. Nonetheless, with practice and patience, it does become fairly straightforward. Studies have suggested good repeatability across a group of individuals [70], but test-retest variation for individual data can be high, with variation of up to 20% [71]. MUNIX, in contrast, is simpler to perform than MUNE since it does not require as much real-time decision-making, but its reliability is less well studied. The one major advantage of MUNE or MUNIX over CMAP is that each is theoretically capable of capturing disease progression very early in the disease course at a time when CMAP size remains relatively stable due to ongoing reinnervation [72]. Unfortunately, this potential benefit is offset by the fact that repeatability of MUNE and MUNIX is usually lowest early in the disease and improves only as the disease progresses toward end-stage when there are fewer, larger SMUPs. Nonetheless, MUNE, and to a lesser extent MUNIX, have been used in several clinical studies and remain a focus of investigation in ALS and other motor neuron disorders, including spinal muscular atrophy [68].

All electrophysiologic biomarkers based on standard techniques, including CMAP, NI, and MUNE are limited by the fact that they can only be applied to nerves that can be effectively stimulated and muscles that can easily be measured. Accordingly, most methods have been used only on the median and ulnar nerves, although peroneal or tibial motor conduction studies could also be used. Superimposed compression neuropathies, common in ALS, also could impact the accuracy of these measures to at least some extent.

Electrical impedance myography (EIM)—In EIM, a set of 4 strip electrodes is placed in parallel lines over a muscle or muscle group of interest; a small high-frequency electrical current is applied between the 2 outer electrodes, and the consequent surface voltages are measured between the inner 2 [73]. The measured surface voltages reflect the conductive and capacitive properties of the underlying tissue. The method is not truly electrophysiological, since it is not producing excitation of muscle fibers, but rather provides electromorphological data. Studies have shown that EIM has strong sensitivity to disease progression as well as high reliability [74–76]. Additional SOD1 mouse and rat studies have also supported this concept [77, 78]. The technique is easy to perform and requires minimal training. Unlike standard electrophysiological measures, it can be applied to most superficial muscles including proximal, truncal, and even bulbar muscles. A recent study has also shown that it correlates with strength testing in ALS patients [79]. A simple way of interpreting EIM is to think of it as being analogous to the CMAP, since both provide a measure of muscle integrity and health. But unlike CMAP, EIM does not require nerve stimulation and thus can be performed on any superficial muscle. Although EIM appears to have promise, a single laboratory has been primarily responsible for its development and

application. Thus, additional research incorporating the technology into clinical trials is needed to ascertain its long-term value in serving as a *bona fide* ALS biomarker of disease progression.

Peripheral nerve excitability testing—Excitability testing of peripheral nerves, including measurement of threshold electrotonus, the recovery cycle, and the strength-duration time constant, has revealed alterations of excitability of motor neurons in ALS [80, 81]. One study also confirmed that higher levels of excitability predicted shorter survival [82]. However, unlike some of the measures described above, the literature using excitability testing in ALS is relatively small, and test-retest reproducibility in diseased populations has not been performed. Thus, like EIM, excitability metrics will require longer-term study to determine their potential value in ALS therapeutic trials.

Transcranial magnetic stimulation (TMS) measures—In the early 1990s, several studies showed that cortical excitability, as measured by TMS, was altered in ALS [83–85]. Further research has shown that a variety of specific TMS measures appear to be consistently disturbed in ALS, including motor threshold (generally reduced early in the disease in keeping with increased excitability), motor evoked potential (generally also increased early in the disease), central motor conduction time (generally prolonged in the disease), and cortical silent period (reduced in the disease) [86]. Like EIM and excitability testing, TMS is in the relatively early stages of study for application in ALS therapeutic trials. And although some studies have been performed using TMS to track progression, the results have been inconsistent [87, 88].

Neuroimaging Biomarkers

Over the course of the ALS disease process, the central nervous system undergoes gross structural changes as neurons degenerate, and cell-level changes that reflect both pathophysiologic and compensatory processes, including neuroinflammation. In theory, all of these changes are amenable to study with neuroimaging techniques. Because these changes as a group are, in essence, the disease itself, they represent a potentially rich source of biomarkers for diagnosis, progression, and response to therapy.

Voxel-based morphometry (VBM)—VBM is an automated magnetic resonance imaging (MRI) technique that allows assessment of atrophy across large groups of subjects. This technique typically uses T1-weighted volumetric MRI scans and performs statistical tests across all the voxels in the image to identify volume differences between or within groups. VBM analysis is widely used, since this technique relies on standard MRI acquisitions and is fully automated [89].

Several cross-sectional and few longitudinal ALS imaging studies have implemented VBM analysis [90–96]. Cross-sectional comparisons of VBM between ALS patients and healthy volunteers revealed grey matter (GM) atrophy in the precentral gyrus (PCG) and frontal regions in the patients [94]. The GM atrophy correlates with the revised ALS functional rating scale (ALSFRS-R) subscores, verbal fluency, and the estimated rate of functional decline [94]. Longitudinal VBM studies have shown GM atrophy in the PCG, frontotemporal regions,

and basal ganglia [94, 97]. Baseline GM atrophy does not always correlate with ALS functional rating scale (ALSFRS-R) or upper motor neuron signs [75, 76]. In 1 study, the rate of ALSFRS-R decline correlated negatively with the GM volume in the left PCG [94].

Surface-based morphometry (SBM) is a similar analytic technique that measures cortical thickness rather than cortical volume. SBM has been used in several ALS studies [98, 99]. One of the large cross-sectional SBM studies in ALS showed cortical thinning by approximately 0.1 mm in the PCG compared to healthy controls, which is consistent with GM atrophy in the PCG seen in VBM studies. Cortical thinning in the PCG correlates with ALSFRS-R subscores. Longitudinal SBM analyses have shown increased cortical thinning in the temporal regions (0.1 mm/year) but not in the PCG [99]. The longitudinal changes in PCG cortical thinning did not correlate with changes in ALSFRS-R [99].

In summary, structural imaging analysis techniques (VMB and SBM) are widely used, automated, and easy to implement. They consistently show atrophy in the PCG in people with ALS compared to healthy volunteers. Progressive atrophy in the PCG may or may not be detected depending on the technique used. Any potential for VBM to serve as a disease progression biomarker, therefore, would require larger longitudinal studies to define the sensitivity of this approach for detecting change over time compared to more established clinical metrics such as the ALSFRS-R.

Diffusion Tensor Imaging (DTI)—Diffusion tensor imaging (DTI) is an advanced MRI technique that measures the isotropic and anisotropic diffusion of water molecules in the brain, which is represented by several DTI metrics, such as fractional anisotropy (FA), mean diffusivity (MD), and axial diffusivity (AD) [100]. The difference in diffusivity of water molecules within the white matter tracts compared to free water allows generation of maps of white matter tracts.

Cross-sectional DTI studies in ALS show widespread white matter changes in ALS subjects compared to healthy volunteers [101–105], and these changes correlate with upper motor neuron signs, ALSFRS-R scores, verbal fluency, and the estimated rate of functional decline between the reported date of symptom onset and the date of scanning [94]. Longitudinal DTI studies in ALS show more focal pathology, involving the corticospinal tract, corpus callosum, and posterior limb of the internal capsule in some studies [94, 103] and other studies have shown more diffuse changes, including the cerebellum and the temporal and parietal lobes [102]. The longitudinal DTI changes in the PCG and the corticospinal tract were correlated with disease duration and ALSFRS-R scores [94, 102].

Thus, DTI is an automated MRI analysis technique that can track white matter changes in the CST as the disease progresses. This suggests that it could be considered as a potential biomarker of disease progression. It should be noted, however, that the longitudinal changes in DTI parameters are small and may not translate to more efficiency in trial designs compared with the currently available clinical measures such as ALSFRS-R. DTI might, however, have an added benefit if combined with other imaging and clinical measures. Larger longitudinal studies are needed to replicate the above findings and to test the sensitivity of DTI for quantifying changes over time compared to ALSFRS-R.

FDG-PEG—Fluorodeoxyglucose (FDG)-positron emission tomography (PET) is a widely used and relatively automated imaging technique that can estimate energy consumption or metabolism in the brain. Several small cross-sectional FDG-PET studies have been conducted in the past 25 years and reveal a consistent reduction in glucose uptake in the PCG in people with ALS compared to healthy volunteers [106–108]. Recent large cross-sectional FDG-PET studies revealed hypometabolism in the PCG and frontal regions in people with ALS compared to healthy volunteers [108, 109]. In addition, severe hypometabolism in the frontotemporal regions was an independent predictor of shorter survival in ALS patients [109]. FDG-PET, therefore, could be a potential prognostic biomarker in ALS. In the absence of longitudinal FDG-PET studies, it is impossible to estimate its value as a disease progression biomarker.

TSPO-PET—The translocator protein (TSPO), formerly known as the peripheral benzodiazepine receptor (PBR), is highly expressed in activated microglia and astrocytes and serves as marker of neuroinflammation [110, 111]. Older-generation TSPO radioligands such as [^{11}C]-(R)-PK11195 suffered from high levels of non-specific binding and poor signal-to-background ratio compared to second-generation ligands such as [^{11}C]-PBR28, which has an 80- fold higher specific binding [112].

The first application of TSPO PET imaging in patients with ALS was conducted with the radioligand [^{11}C]-(R)-PK11195 and showed increased binding in the motor cortex, pons, dorsolateral prefrontal cortex, and thalamus in a group of ALS patients [113]. Increased TSPO expression, assessed using the radioligand [^{18}F]-DPA-714, was subsequently reported in primary motor, supplementary motor, and temporal cortex of patients with ALS, thereby providing additional support for a role for inflammatory processes in the disease [114]. Finally, Zurcher *et al* used [^{11}C]-PBR28 to evaluate binding in people with ALS compared to age- and binding affinity-matching healthy volunteers. PBR28 binding was increased in the PCG in the ALS group, and the distribution of PBR28 binding correlated with the site of onset (bulbar vs. limb). In addition, the degree of binding in the PCG correlated negatively with the functional status and positively with upper motor neuron signs [115].

Based on these findings, TSPO PET imaging is a promising molecular imaging modality that could serve as a pharmacodynamic biomarker for ALS therapies that target neuroinflammation. In the absence of longitudinal studies, however, it is impossible to estimate its value as a disease progression or prognostic biomarker.

Combinatorial Approaches to Biomarkers

ALS is a complex disease, likely involving multiple pathogenic processes, including neuronal dysfunction, spread of misfolded proteins, and neuroinflammation, at different stages of the disease. As such, there are likely to be multiple changes in blood, CSF, electrophysiology, and neuroimaging that could be monitored simultaneously, potentially providing a more in-depth picture of the disease over time. For instance, the combination of, say, plasma uric acid and diffusion tensor imaging might be a more powerful predictor of rate of decline than either alone. Further, it is likely that some changes may be “leading indicators,” presaging new stages in the disease process, while others are “lagging

indicators," confirming the completion of transition to a new stage. It is possible that combining biomarkers of different types may provide a more dynamic understanding of disease progression and response to therapy than any single measure alone. Any use of such a combination will, of course, require first validating the individual biomarkers, and then further exploring the significance of the pair. The value of a robust and widely accessible biorepository is precisely to allow researchers to explore such questions without the delay involved in collecting new samples to test each new hypothesis.

LESSONS FROM OTHER BIOMARKER INITIATIVES

The ALS community stands to benefit from experience gained through large-scale collaborative biomarker initiatives such as the Alzheimer Disease Neuroimaging Initiative (ADNI) [116], the Parkinson Progression Markers Initiative (PPMI), and the Parkinson Disease Biomarkers Program (PDBP). Large-scale initiatives such as these have revealed the importance of standardization of biomarker measures at the pre-analytical and analytical levels, both for diagnostic purposes and in support of therapeutic development. They have also developed procedures and processes for data and biospecimen sharing. De-identified data collected by these consortia are made broadly available through some form of a web-based, open-access portal. Access to biological specimens is generally more laborious, variably requiring a formal application, some form of scientific review (considering the significance of the proposed project, the expertise of the investigator, the proposed methodology, and the availability of relevant support), and appropriate acknowledgement of the consortium in publications.

CONCLUSION – NEXT STEPS

The most pressing need in the field is for biomarkers that will be most relevant to therapy development. Discovery and early development (including analytic validation) of such biomarkers will appropriately utilize samples housed within established repositories. However, the development and clinical validation of biological fluid-based pharmacodynamic and disease progression biomarkers will require prospective cohorts in which large numbers of patients are systematically and longitudinally studied using harmonized approaches to clinical phenotyping as well as standardized protocols for biological specimen collection, processing, and storage. Standardized protocols are similarly essential for multi-center implementation of 'dry' biomarker protocols (e.g. neuroimaging). Of necessity, such studies will need to be multi-center in nature, in part to achieve the required sample size and in part to help ensure broad 'buy-in' from as many relevant stakeholders as possible. Multi-center clinical trials through consortia such as the Northeast ALS (NEALS) and Western ALS (WALS) are ideal opportunities for add-on biomarker studies. Similarly, multi-center collaborative projects such as CReATe are ideally suited to help accomplish this goal.

This is an opportune time for the establishment an ALS Biomarker Consortium (ABC) that includes all relevant stakeholders. If it is to succeed, the structure and governance of this consortium must recognize and accommodate the complexity of the ALS research landscape, including the number and diversity of stakeholders who may often have

competing interests. Individual academic investigators must find ways to work collaboratively and synergistically while simultaneously sustaining support for the activities of their individual research groups and consortia. Similarly, small biotech and large pharmaceutical companies must be willing to support collaborative efforts in pre-competitive space. All parties must (and indeed do) recognize that all efforts are ultimately directed towards, and expended in, serving the needs of our ALS patient population. In light of these considerations, we would venture that a centralized model in which all efforts are coordinated by a single group will likely fracture the field and fail. Instead, we propose a federated model, in which all interested and willing stakeholders may participate with equal opportunity to contribute to the broader mission of biomarker development and validation. Such an organized structure would attract industry partners and funding opportunities from NINDS for biomarker discovery and validation. Building on experiences from the Michael J. Fox Foundation and NINDS, The ALS Association is now prepared to invest significant funds to establish a *bona fide* ALS Biomarker Consortium and seeks immediate and dedicated involvement from all stakeholders. An initial call for participants will be forthcoming.

Acknowledgments

We are grateful to Richard Robinson for his careful review and edits to this manuscript. This work was supported in part by the CReATe Consortium (U54-NS-092091), which is part of the Rare Diseases Clinical Research Network (RDCRN), an initiative of the Office of Rare Diseases Research (ORDR), NCATS. CReATe is funded through collaboration between NCATS and NINDS.

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Table 1

Biomarker Types Recognized by the FDA

Biomarker Type	Description [2]	Examples relevant to ALS
Diagnostic	Disease characteristics that categorize people by the presence or absence of a specific physiological or pathophysiological state or disease	• EMG for demonstrating the presence and distribution of subclinical lower motor neuron pathology
Prognostic	Baseline characteristics that categorizes patients by degree of risk for disease occurrence or progression of a specific aspect of disease (i.e. inform the natural history of the disorder in a particular patient in the absence of a therapeutic intervention)	• Mutations in ALS susceptibility genes categorize individuals as being at risk for developing ALS. • Some specific mutations, such as the A4V mutation in the SOD1 gene, predict an aggressive form of disease and portend a very poor prognosis for survival.
Predictive	Baseline characteristics that categorize patients by their likelihood of response to a particular treatment relative to no treatment. Such biomarkers may be used as an enrichment strategy to identify a subpopulation likely to respond to treatment intervention in a particular way	• The presence of mutations in the SOD1 gene or a hexanucleotide repeat expansion in the C9ORF72 gene might be used to select for a clinical trial those patients most likely to benefit from SOD1 and C9ORF72 antisense oligonucleotides.
Pharmacodynamic	Markers that show that a biological response has occurred in a patient who has received a therapeutic intervention	• Biological measurements that are abnormal (e.g. elevated) but stable over time in the absence of therapy (e.g. neurofilament light chain) as well as biomarkers of disease progression • May also be drug - rather than disease- specific, indicating that a drug has, for example, engaged its intended target and exerted the intended biological effect.

Table 2

Most Promising Biomarkers for ALS Therapy Development

Biomarker	Potential Utility		
	Prognostic	Progression	Pharmacodynamic
CSF pNfH	X[1]		
CSF NfL	X[1]		X[2]
Urinary p75	X[1]	X[1]	X[2]
CSF SOD1			X[3]
CSF C9RANT			X[4]
CMAP		X[5]	
MUNE		X[5, 6, 7]	
MUNIX		X[5, 7]	X[2]
EIM		X[5, 1]	X[2]

[1] Ready for further validation in a multi-center study

[2] Ready for evaluation in a suitable clinical trial

[3] Only in patients with SOD1 mutations

[4] Only in patients with C9ORF72 mutations

[5] Sensitive to electrode placement

[6] Conceptually appealing, but technically challenging.

[7] Lowest reproducibility early in disease