HHS Public Access

Author manuscript

Brain Res. Author manuscript; available in PMC 2016 May 14.

Published in final edited form as:

Brain Res. 2015 May 14; 1607: 94–107. doi:10.1016/j.brainres.2014.10.031.

Use of biomarkers in ALS drug development and clinical trials

Nadine Bakkar, **Ashley Boehringer**, and **Robert Bowser***

Divisions of Neurology and Neurobiology, Barrow Neurological Institute, St. Joseph's Hospital and Medical Center, Phoenix, AZ, 85013 USA

Abstract

The past decade has seen a dramatic increase in the discovery of candidate biomarkers for ALS. These biomarkers typically can either differentiate ALS from control subjects or predict disease course (slow versus fast progression). At the same time, late-stage clinical trials for ALS have failed to generate improved drug treatments for ALS patients. Incorporation of biomarkers into the ALS drug development pipeline and the use of biologic and/or imaging biomarkers in early- and late-stage ALS clinical trials have been absent and only recently pursued in early-phase clinical trials. Further clinical research studies are needed to validate biomarkers for disease progression and develop biomarkers that can help determine that a drug has reached its target within the central nervous system. In this review we summarize recent progress in biomarkers across ALS model systems and patient population, and highlight continued research directions for biomarkers that stratify the patient population to enrich for patients that may best respond to a drug candidate, monitor disease progression and track drug responses in clinical trials. It is crucial that we further develop and validate ALS biomarkers and incorporate these biomarkers into the ALS drug development process.

Keywords

Biomarkers; ALS; clinical trial; pharmacodynamic biomarker; prognostic biomarker

1. Introduction

ALS drug development has traditionally used cell culture and transgenic animal model systems to identify and pre-clinically test compounds (McGoldrick et al., 2013). The most common transgenic animal models utilize overexpression of human SOD1 containing mutations that have been identified in familial ALS patients. While this animal model system has been extensively used for ALS preclinical drug development, none of the recent drugs that have shown efficacy in the animal model have proven to be beneficial in humans

*Corresponding Author: Robert Bowser, Ph.D., Division of Neurobiology, Barrow Neurological Institute, 350 W Thomas Rd, Phoenix, AZ 85013, USA. Phone: +1 602 406 8989. Robert.Bowser@Dignityhealth.org.

Conflict of Interest

RB is a founder of Iron Horse Diagnostics, Inc., a biotechnology company focused on diagnostic and prognostic biomarkers for ALS and other neurologic disorders.

with ALS. Part of the problem has been underpowered mouse studies that led to false positives and then translation to human trials of drugs that subsequently fail in appropriately powered mouse studies (Scott et al., 2008). Another problem is that model systems based on particular genetic mutations may best predict outcome in only the ALS patient population that harbor the particular gene mutation. New model systems or new approaches to investigate targets and pathways more relevant to the sporadic ALS population are needed to increase our success rate during ALS drug development. There is also a dire need for biologic and quantifiable substitutes for survival endpoints and clinical trial outcome measures to speed decisions regarding drug efficacy during clinical trials..

Biomarkers are laboratory objective measures that reflect various biological pathways and altered pathways during disease progression, and their benefit in both the drug development process and the clinic is multi-fold. Biomarkers will aid in a more rapid diagnosis of ALS, stratify the patient population to identify those that may best respond to the particular drug, provide prognostic indications regarding disease progression, demonstrate a drug is “hitting its target” within the periphery or central nervous system (CNS), or predict response to drug treatments. From a research and drug development perspective, biomarkers will identify new drug targets and aid pre-clinical drug development. In a disease that has inherent variability in both the sporadic and genetic forms of disease, patient stratification via biomarkers would greatly benefit clinical trial design and potentially reduce the number of patients required to power clinical trials.

Therefore, while the search for ALS biomarkers is quite challenging, the rewards will provide critical insights into the disease process and aid in our search for more effective treatments. There have been many efforts to uncover biomarkers using cell models, animal models and patient derived samples. The use of patient derived samples, while an obvious choice, is hindered by the inherent disease heterogeneity and how the samples were collected from the patients. The discovery and validation of biomarkers in patient derived samples has greatly benefited from a concerted effort in the field to standardize collection, processing and storage of samples that are used for biomarker studies. An example of this effort is the Northeast ALS Consortium (NEALS) biorepository that provides scientists with biologic samples collected using standard operating procedures (SOPs) and linked to clinical information for use in research studies. The past few years have seen great advancements in the discovery of biomarkers for ALS. We will focus this review on the identification and use of biomarkers to facilitate ALS drug development and more efficient design of clinical trials.

2. Pre-clinical Drug Development Pipeline

2.1. Biomarkers to Identify Drug Targets and Links to the Human Disease

The drug development pipeline relies heavily on the use of pre-clinical disease models such as transgenic animals, cell culture models, and more recently patient derived cell lines. While not as common in ALS drug development as in the drug development process for oncology (Hodgson et al., 2009; Simon and Roychowdhury, 2013), biomarkers are an ever more important component of the ALS drug development pathway to demonstrate drug effects and target engagement. They facilitate the discovery of novel targets and pathways

involved in ALS while at the same time providing a measurable commonality to determine the relevance of these targets in patient samples, both longitudinally (i.e. throughout the course of disease pathogenesis), and acutely. Therefore biomarkers provide a bridge between the pre-clinical disease models developed behind the bench and clinical measures and outcomes.

Both targeted and unbiased approaches have been used to identify biomarker candidates in model systems of ALS to explore as potential drug targets (Calvo et al., 2012). Others have tried to validate biomarkers identified in ALS patients within the transgenic mouse model systems (Kudo et al., 2010; Lilo et al., 2013). Recently these models have converged on a number of inflammatory pathways as therapeutic targets, and we will focus discussion on these findings. One needs to keep in mind however that these studies were performed in a transgenic model that may best model ALS patients that also harbor mutations within the same gene.

Transcriptome analysis performed on mice expressing $SOD1^{G93A}$ mutant protein showed enrichment of genes involved in T cell activation, macrophage activation and co-stimulatory regulation of the adaptive and innate immune systems in the spinal cord, skeletal muscle and peripheral nerves as well as up-regulation of proinflammatory chemokines (Lincecum et al., 2010). These results suggested that a monoclonal antibody targeted to CD-40L, a protein expressed on activated T-cells and necessary for induction of an immune response, may slow ALS progression in the animal model. Lincecum and colleagues found that treatment of $SOD1^{G93A}$mice with an antibody targeting CD-40L reduced neuroinflammation, delayed disease onset and extended survival by 9 days (Lincecum et al., 2010).

Similarly, an increase in expression of proinflammatory genes and microRNAs were seen in monocytes from the spleen of $SOD1^{G93A}$ mice prior to disease onset and these $Ly6C^{hi}$+ monocytes increasingly infiltrated the spinal cord of $SOD1^{G93A}$mice by the end stage of the disease (Butovsky et al., 2012). This increase was shown to be significant to the course of disease by treatment with an antibody against $Ly6C^{hi}$ that delayed onset of disease phenotype, prolonged survival by 16 days, and enhanced rotarod performance. Antibody treatment also decreased the number of $Ly6C^{hi}$ monocytes in the spinal cord and led to an increase in the number of motor neurons (Butovsky et al., 2012). Butovsky and colleagues also found a similar proinflammatory phenotype of CD14+/CD16- monocytes isolated from peripheral blood from ALS patients, suggesting that modulation of these monocytes may be an effective treatment. Furthermore, the use of peripheral monocyte measurements may be useful to identify patients that would benefit from a specific drug treatment that targets this class of monocytes.

Regulatory T cells have also been shown to play an important role in immune modulation and influence the rate of disease progression within the $SOD1^{G93A}$ mouse model of ALS and in ALS patients (Beers et al., 2011). Increased expression of CD4+ T-cells have been seen in mice (Chiu et al., 2008) as well as humans (Saresella et al., 2013) and decreases in the expression of regulatory T-cells have been seen during rapidly progressing disease in mice (Zhao et al., 2012) as well as in humans (Mantovani et al., 2009; Rentzos et al., 2012; Saresella et al., 2013). T-cells may instruct microglia to provide a neuroprotective

environment during the early stages of disease and maintain microglia/monocytes in a more neurotrophic phenotype (Chiu et al., 2008), suggesting that levels of these regulatory T cells may play an important role in modulating immune responses and homeostasis during ALS. The level of circulating myeloid (M2) cells has been shown to participate in regulating ALS progression in the $SOD1^{G93A}$ mouse model by modifying T cell functions (Vaknin et al., 2011). Therefore the combined and interacting functions of myeloid cells, T cells and monocytes/microglia likely influences the onset and progression of ALS in this mouse model of ALS.

A proinflammatory protein biomarker signature has also been observed in the CSF and plasma of ALS patients (Mitchell et al., 2009; Su et al., 2013). An increase in inflammatory gene expression and protein levels is linked to activation of microglia and astrocytes in the CNS with subsequent motor neuron degeneration, as shown by the secretion of neurotoxic proteins by activated astrocytes (Bi et al., 2013), the toxicity of astrocytes derived from iPS cells from ALS patients on motor neurons (Haidet-Phillips et al., 2011) and the presence of increased spinal cord GFAP and Iba-1 immunostaining in $SOD1^{G93A}$ mice (Evans et al., 2014). These peripheral and central proinflammatory biomarker signatures may be useful to monitor effects of drugs that target these pathways during clinical trials.

The innate immune system activates the complement pathway in response to pathogens, inflammatory states or infections and triggers the clearance of antibody complexes or dead cells. Complement activation on motor neurons and neuromuscular junctions was identified in the $SOD1^{G93A}$ mouse model of ALS prior to onset of symptoms and continued throughout the course of disease (Heurich et al., 2011; Lee et al., 2013; Woodruff et al., 2008). Increased levels of complement proteins have also been detected in ALS patients (Ganesalingam et al., 2011; Sta et al., 2011; Woodruff et al., 2008). Therefore the complement system represents another potential immune target for ALS based therapies that has been observed in both an animal model of ALS and in the patient population.

Finally, induced pluripotent stem cells (iPS) generated from ALS patients have been shown to model disease pathology observed in ALS patients (Dimos et al., 2008). TDP-43 aggregation can be seen in motor neurons derived from patient iPS cells and used to screen for drugs to modulate TDP-43 aggregation (Burkhardt et al., 2013; Egawa et al., 2012). Patient derived cells from patients with *C9ORF72* repeat expansion exhibit *C9ORF72* containing foci and repeat associated non-AUG (RAN) translation products that could be used as biomarkers during preclinical drug screens, with similar findings observed in ALS patients (Almeida et al., 2013; Su et al., 2014). These findings highlight the potential of patient derived stem cells and precision medicine approaches to identify new biomarkers and therapies for ALS.

2.2. Pharmacodynamic biomarkers

Increased levels of SOD1 have been found in the CSF of human patients and disease caused by mutant SOD1 is thought to be a toxic gain of function (Reaume et al., 1996; Winer et al., 2013). Antisense oligonucleotides against SOD1 have been used to lower SOD1 gene expression and protein levels, delaying disease onset in rat models (Miller et al., 2005; Ralph et al., 2005; Raoul et al., 2005). Lowering SOD1 levels in astrocytes derived from

sporadic ALS patients reversed their toxicity when co-cultured with motor neurons (Haidet-Phillips et al., 2011). SOD1 antisense oligonucleotides delivered to pre-symptomatic SOD1 rats by intraventricular injection decreased mean levels of SOD1 mRNA and protein in frontotemporal cortex (Winer et al., 2013). Monitoring levels of SOD1 in the CSF is a promising pharmacodynamic (PD) biomarker as levels of SOD1 do not change significantly over time within individuals (Winer et al., 2013), so a change from baseline can be attributed to the action of the antisense oligonucleotide. Similarly, antisense oligonucleotides to acetylcholinesterase increased survival of the $SOD1^{G93A}$ mice and attenuated motor neuron loss, though only when provided at a pre-symptomatic state, which would be difficult to accomplish therapeutically in the sporadic ALS population (Marc et al., 2013). Recently, aggregated dipeptide RAN translation products have been localized to neuronal inclusions in the central nervous system of patients harboring the *C9ORF72* expansion, a phenotype that was recapitulated in vitro in iPS neurons (Ash et al., 2013; Mori et al., 2013). Antisense oligonucleotides to the *C9ORF72*transcript decreased its toxicity (Donnelly 2013 and Sareen 2013). In addition, small molecule binders of the GGGGCC repeat successfully inhibit RAN translation and foci formation in iPS neurons (Su et al., 2014). Interestingly, these RAN dipeptides were detected in the CSF of patients harboring the *C9ORF72*expansion, highlighting their usefulness as PD biomarkers to measure *C9ORF72* targeting therapeutic efficiency (Su et al., 2014). Last but not least, imaging has been proposed to create PD biomarkers for monitoring target engagement within the CNS for other disorders, such as pharmacodynamic A3 biomarkers for Alzheimer's disease (Blennow et al., 2014). Imaging related PD biomarkers represent a powerful approach that should be further exploited in future ALS clinical trials and drug development.

2.3. Biomarkers to monitor drug effects in model systems

Biomarkers should play an important role in monitoring drug effects within preclinical model systems and may include gene, protein or metabolic biomarkers that change in response to drug effect. Such biomarkers may or may not directly participate in disease pathogenesis or may be generated by cells not targeted by the drug. For example, neuroprotective drugs may reduce biomarkers of neuronal injury or death indirectly through drug actions on glial cells. One biomarker of neuronal injury is neurofilament light or heavy chain. Increased levels of neurofilament proteins can be detected in the CSF or blood of $SOD1^{G93A}$ mice prior to phenotypic symptom onset (Boylan et al., 2009). Plasma phosphorylated NFH (pNFH) levels have also been shown to correlate with decline in muscle force, motor unit loss, and motor neuron death in$SOD1^{G93A}$ mice (Lu et al., 2012). Treatment $SOD1^{G93A}$ mice with arimoclomol, a drug that increases expression of heat shock proteins, decreased plasma levels of pNFH and correlated with increased survival, suggesting that pNFH levels could be used as a biomarker of drug efficacy (Lu et al., 2012). Neurofilament positive aggregates were detected in iPS cells derived from patients harboring SOD1 A4V or D90A mutations that were not seen in control cells or in cells where the SOD1 mutation was corrected (Chen et al., 2014a). Therefore protein biomarkers may be useful biomarkers for drug screens or monitoring drug effects in many model systems.

Imaging based biomarkers represent another approach to uncover disease pathology and monitor drug effects in ALS model systems. Reliable imaging biomarkers are highly desirable for their noninvasive nature and can detect early disease related pathology. In $SOD1^{G93A}$ mice hyperintensity is present in T2-weighted MRI images prior to onset of disease phenotype (Marcuzzo et al., 2011). T2 weighted MRI also revealed early and progressive degeneration in the trigeminal, facial and hypoglossal brain stem (Evans et al., 2014) that was replicated in human SOD1 patients who showed cortical hyperexcitability prior to symptom onset (Vucic et al., 2008). Magnetic Resonance Spectroscopy (MRS) has been used to demonstrate decreases in N-acetylaspartate (NAA) in the motor cortex of $SOD1^{G93A}$ associated with the degree of motor dysfunction (Choi et al., 2009).

Electrophysiologic measures have gained ground as a way to monitor the muscle itself. They can be used to closely monitor and provide quantitative measures of muscle function. Electrical impedance myography (EIM) is a technique that uses the placement of high-frequency alternating electrical current producing electrodes on specific muscle groups and measuring the surface voltage patterns that are generated (Rutkove, 2009). EIM could monitor disease progression and rate of decline in rats expressing $SOD1^{G93A}$ (Wang et al., 2011). $SOD1^{G93A}$ mice exhibited a change in EIM from baseline at 8 weeks, detecting muscle changes much earlier than many other techniques (Li et al., 2013). Motor Unit Number Estimation (MUNE) has also been used to predict disease onset and progression in $SOD1^{G93A}$ mice (Shefner et al., 2006). Ngo et al used MUNE to show differences in the numbers of motor units in $SOD1^{G93A}$ mice at all tested time points, including before symptom onset, and further correlated MUNE results to the number of motor units as determined by histological analysis (Ngo et al., 2012).

3. Defining disease heterogeneity and progression

3.1. Biomarker based stratification of patients

The best current biomarkers to stratify the ALS patient population are genetic markers for familial causes of ALS, with the most common genetic factor for ALS being the *C9ORF72* repeat expansion (Majounie et al., 2012; Renton et al., 2011). Individual or panels of RNA or protein biomarkers have also been used to stratify patient populations (Puentes et al., 2014; von Neuhoff et al., 2012). An important use of patient stratification is to enrich for a particular mechanistic cause of disease or enrich patient populations for those that may best respond to a particular drug treatment. While reducing the potential number of patients that may be enrolled in a particular clinical trial, enrichment strategies may increase the chance of success for the trial. Given the failure of large phase III clinical trials for ALS, enrichment strategies using genetics or a combination of genetic and biochemical biomarkers will be a key component implemented in future ALS clinical trials.

3.2. Prognostic indicators of disease

Since ALS is a heterogeneous disease with multiple pathogenic mechanisms and variable site of disease onset and progression, it is not surprising that attempts to find prognostic biomarkers or biomarkers indicative of disease spread have spanned genomics, proteomics, metabolomics as well as neuroimaging, and focused on various biofluids and organ systems.

However to date there are no sensitive and validated quantitative measures of disease severity and progression. Instead, non-parametric clinical measures such as forced vital capacity and the ALSFRS-R score are typically used in clinical studies. In a large French study to identify clinical features present at the patient's first visit that predict survival, the authors found that older age, bulbar onset, shorter delay to first visit and poor motor function were expectedly the only predictors of shorter survival (Gordon et al., 2013). As we move forward, improved prognostic indicators are needed to assist in planning patient care, predicting disease course, and monitoring drug effects in clinical trials.

3.2.1. Blood and CSF biomarkers—As noted above, neurofilament proteins are major structural components of axons; their breakdown results from axonal injury, releasing their subunit proteins into the CSF and ultimately the blood. Neurofilament light and heavy chain proteins have been investigated as potential diagnostic and prognostic biomarkers of ALS. Several groups have reported increases in the levels of pNFH in the CSF of ALS patients compared to healthy and neurologic disease controls (Ganesalingam et al., 2011; Tortelli et al., 2012). Levels of pNFH in CSF and blood were found to correlate with rate of disease progression and survival in ALS (Boylan et al., 2013; Ganesalingam et al., 2013; Lehnert et al., 2014). Higher pNFH levels in plasma, serum and CSF correlated to a faster ALSFRS-R rate of decline and shorter survival.

Another cytoskeletal protein, tau, can also be measured in CSF and blood and has been investigated as a prognostic biomarker. However conflicting results are seen in the literature. While Sussmuth and colleagues reported higher total levels of tau in ALS and upper motor neuron diseases compared to controls using an ELISA-based approach (Sussmuth et al., 2010), others reported similar tau levels in control and ALS patients(Jimenez-Jimenez et al., 2005; Lehnert et al., 2014). More recently, measurements of phosphoTau/Tau ratio from CSF showed a significant decrease in ALS compared to healthy controls or patients with 4-repeat tauopathy (Grossman et al., 2014). In addition, the authors correlated this ratio with clinical measures of disease such as ALSFRS-R scores and white matter reduction neuroimaging, highlighting the potential of this phosphoTau/Tau ratio, though no links were found to patient survival or disease progression. Therefore tau does not appear to be a promising prognostic biomarker for ALS.

Since the *TARDBP* gene is mutated in some familial patients and TDP-43 is a core component of cytoplasmic inclusions in ALS and FTLD, many laboratories have pursued the use of TDP-43 as a biomarker in various biofluids. Antibody based approaches to quantify levels of the RNA binding protein in CSF using ELISA showed increased levels in ALS patients, with lower levels associated with reduced survival (Noto et al 2011). TDP-43 levels were similarly significantly increased in plasma of patients with ALS and positively correlated with age (Verstrate et al 2012). Free TDP-43 in CSF was subsequently found to originate mainly from blood (Feneberg et al 2014). However as TDP-43 is a self-aggregating protein, antibodies to the protein may fail to recognize a significant amount of protein within a biofluid contained in protein aggregates. Continued efforts to optimize assays for detecting TDP-43 and other self aggregating proteins such as SOD1 and FUS are necessary to determine the utility of these proteins as biomarkers within patient derived biofluid samples.

Various biomarkers of inflammation are deregulated in the blood of ALS patients, reflecting the activation and recruitment of microglia and other immune cells (Robelin and Gonzalez De Aguilar, 2014). High levels of high mobility group box 1 (HMGB1) autoantibody (Hwang et al., 2013), increased granzyme B (Ilzecka, 2011), higher CSF IL-8 levels (Mitchell et al., 2009) and wide-range C-reactive protein (wrCRP) (Keizman et al., 2009) correlated with disease severity as measured by ALSFRS-R. In addition, increased levels of blood MCP-1, TNF-3 and GM-CSF correlated with disease duration (Kuhle et al., 2009; Mitchell et al., 2010). Two glial-derived proteins, sCD14 (a soluble monocyte receptor involved in inflammation in neurodegenerative diseases) and S100B (an astrocyte-derived neurotrophic protein) show decreased levels in CSF of patients with ALS (Sussmuth et al., 2003; Sussmuth et al., 2010). Lower S100B and higher sCD14 levels were independently associated with better patient survival. Interestingly, when the quotient of these two proteins was calculated and correlated to patient survival, a sensitivity of 75% and a specificity of 91% were achieved (Sussmuth et al., 2010). Whether the quotient of sCD14/S100B in CSF is a valid prognostic indicator of survival remains to be confirmed in larger patient cohorts. However inflammatory based prognostic biomarkers would be useful in the many current and upcoming clinical trials using drugs that modulate the inflammatory system.

Connor and colleagues identified a combination of blood and CSF based inflammatory biomarkers that were prognostic indicators for fast versus slow disease progression (Su et al., 2013). High levels of plasma IP-10, IL-5, L-ferritin, CSF MCP-1 and particular ratio of CSF to plasma IFN-3 predicted longer patient survival, whereas higher CSF levels of IL-8 predicted shorter lifespan. These findings must be verified in much larger set of samples in a separate research study.

Serum albumin has also been associated with the state of inflammation. While decreased in ALS compared to healthy controls (Ghasemzadeh et al., 2008), serum albumin levels were shown to significantly correlate with patient's clinical status, with lower levels associated with reduced ALSFRS-R scores and survival (Chio et al., 2014). Reduced serum albumin also correlated with increased total leukocytes, neutrophils and monocytes in the blood, further suggesting that decreased serum albumin were a result of inflammation. Longitudinal studies on the variation of albumin blood levels should help determine the prognostic utility of this biomarker candidate.

Many studies have reported decreased CSF levels of the cysteine protease inhibitor cystatin C using ELISA or various mass spectrometry methods (SELDI-TOF, MALDI-TOF). Our group determined that CSF cystatin C levels correlated with patient survival, with the best correlation being for patients with limb-onset disease, thus making it a candidate prognostic biomarker of survival (Wilson et al., 2010). One should note that a recent quality control evaluation of cystatin C across multiple European institutes failed to demonstrate differences in CSF cystatin C levels between ALS and control groups though did not evaluate correlations to patient survival, highlighting the need for further verification studies of cystatin C as a prognostic biomarker for ALS (Lehnert et al., 2014).

A new addition to CSF protein biomarkers is the poly (GP) repeat-associated non-ATG translation (RAN) proteins. Su et al investigated RAN protein levels in CSF from 14 patients

harboring the *C9ORF72* repeat expansion, 25 ALS patients with no *C9ORF72* mutation and 3 healthy controls (Su et al., 2014). RAN dipeptides were only detected in patients with the repeat expansion, thus providing a potentially very valuable biomarker for stratifying patients that harbor the *C9ORF72* expansion. Further studies are needed to validate this finding an independent and larger number of subjects, and to assess whether varying levels of the RAN dipeptide are associated with differences in disease severity and/or progression.

Metabolic profiling of plasma and CSF from ALS patients has been used to identify metabolomic biomarkers of ALS (Blasco et al., 2013; Kumar et al., 2010). However these studies have focused on differentiating ALS from control subjects and other neurologic diseases for diagnostic potential (Lawton et al., 2014), with less emphasis on identifying prognostic markers of disease. Interestingly, one of these metabolic biomarkers, urate, correlated with ALSFRS-R scores and higher uric acid levels were independently shown to predict longer survival in males (Paganoni et al., 2012). Further studies are needed to identify prognostic metabolic biomarkers for ALS.

3.2.2. Muscle Biomarkers—As a neuromuscular disease, it is reasonable to suggest that peripheral blood or skeletal muscle tissues can be a valuable source of ALS biomarkers to monitor muscle loss and disease progression. The neurite outgrowth inhibitor Nogo-A is expressed in human skeletal muscle (Dupuis et al., 2002), and several studies have shown that its increased expression correlates with disease severity and degree of muscle fiber atrophy (Dupuis et al., 2002; Jokic et al., 2005; Pradat et al., 2007). Later studies however challenged the specificity of Nogo-A increases to ALS, associating them instead with denervation and peripheral neuropathies (Askanas et al., 2007; Tagerud et al., 2007). A first in-human trial with a monoclonal antibody against Nogo-A (Ozanezumab) was performed earlier this year to assess safety as well as muscle targeting of the treatment (Meininger et al., 2014). A major limitation of muscle biomarkers, though easily accessible, remains the invasiveness of performing muscle biopsies.

3.2.3. Genetic biomarkers—The number of genetic alterations and risk factors linked to ALS continues to increase and currently numbers 36 genes (Leblond et al., 2014; Renton et al., 2014). A recent genome wide analysis of heritability of ALS estimates the total heritability of the disease to be at 21% (95% CI), significantly higher than previously reported, indicating that many risk loci are yet to be identified(Keller et al., 2014). Identification of genetic alterations will assist in enrolling patients for targeted therapies related to specific genetic defects, such as antisense oligonucleotide based therapies for *SOD1* or *C9ORF72* based ALS. Pre-symptomatic subjects that harbor genetic alterations will also be critical to identify biomarkers that correlate to symptom onset or predict onset of disease.

In addition, specific genes have been proposed as prognostic indicators of disease. Expression levels of EPHA4**,** a receptor in the ephrin axonal repellent system, inversely correlated with disease onset and survival in patients(Van Hoecke et al., 2012). In addition, two loss-of-function mutations in *EPHA4*, R571Q and R514X were associated with longer survival (Van Hoecke et al., 2012).

The hexanucleotide repeat (GGGGCC) expansion within the first intron of *C9ORF72* is by far the most frequent genetic cause of ALS, FTLD and ALS/FTLD (Majounie et al., 2012; Renton et al., 2011). ALS patients harboring this *C9ORF72*repeat expansion represent a recognizable phenotype characterized by a lower age of onset, presence of cognitive and behavioral impairment, specific neuroimaging changes, a strong family history of neurodegeneration, and reduced survival. Specifically, an age-matched univariate analysis showed shorter survival (20 months vs 26 months) in patients with the repeat expansion (Byrne et al., 2012). This study was however limited by the number of patients with the repeat expansion.

In a recent study of a Spanish cohort of sALS patients, the *CX3CR1* V249I genetic variant associated with a reduced survival time by a median of 25 months as well as faster rate of disease progression (Lopez-Lopez et al., 2014). This variant was previously shown to alter the activity of the Fractalkine receptor protein and suggests that function of this receptor modulates ALS disease progression.

The *SLC11A2* gene encodes the divalent metal transport 1 (DMT1) protein mediating iron transport in endosomes, and an intronic polymorphism (rs407135) was associated with a 17 months shorter duration of ALS in patients with lower limb onset (Blasco et al., 2011). Such preference of the *SLC11A2* polymorphism for lower motor neurons could stem from a previously described iron accumulation in spinal neurons (Kasarskis et al., 1995), although the reason for this phenomenon is unknown. There was no association between the *SLC11A2* gene and sporadic ALS.

A common variant of the *UNC13A* gene, which encodes a protein that regulates the release of neurotransmitters such as glutamate at neuromuscular synapses, is not only associated with susceptibility but also with shorter survival of ALS patients (Chio et al., 2013; Diekstra et al., 2012). Data collected from multiple studies in Europe showed a reduction in survival ranging from 5–10 months, resulting from a minor allele of *UNC13A*.

Another promising candidate genetic prognostic indicator is the *ZNF512B* gene. A single nucleotide polymorphism (SNP) in this gene was found to be a prognostic factor influencing survival, independent of age and site (bulbar/spinal) of symptom onset or riluzole use (Tetsuka et al., 2013a). ALS patients with the risk allele of this SNP had a markedly shorter survival compared with ALS patients without the risk allele, by 72 months.

A number of putative genetic risk loci for ALS were identified in genome-wide association studies (GWAS), including *FGGY* and *DPP6* (Dunckley et al., 2007; van Es et al., 2008; van Es et al., 2009), but their involvement has yet to be replicated in other GWAS studies. This suggests these genes are not likely linked to ALS and prior GWAS studies were likely under powered. A variant in the *KIFAP3* (kinesin-associated protein 3) gene was initially found to be associated with increased survival in sporadic ALS, conferring up to 14-month survival advantage in a multi-center study (Landers et al., 2009). However subsequent independent studies (Traynor et al., 2010), including a recent larger multi-center survival analysis (van Doormaal et al., 2014), failed to show a beneficial advantage of this genotype.

3.3. Biomarkers of disease spread

Mechanistically one of the more poorly understood facets of ALS is the progressive nature of the disease and the spread of the disease through the body. While some have proposed that disease spread occurs in an orderly process with a focal initiation and contiguous spread (Ravits and La Spada, 2009), others have suggested a “multiple hit” hypothesis and propagation (Sekiguchi et al., 2013). Regardless of single or multiple “hits” for disease initiation, spread may occur via local connectivity. Recent studies suggest that contents of exosomes released from affected cells may help initiate the spread of disease to neighboring cells. Cultured cells were shown to transfer aggregated SOD1 from one cell to another via direct synaptic connection or exosome release (Sekiguchi et al., 2013). Mice expressing $SOD1^{G93A}$ show increased exosome release in spite of overall decreases in their secretary pathways. These released exosomes contained large amounts of SOD1 (Basso et al., 2013), though exosomes also contain many other proteins, mRNAs and microRNAs (Rani et al., 2011). Further studies are necessary to confirm the role of exosomes or interstitial release of proteins or microRNAs as a means of disease spread in ALS and may provide both novel insights into disease pathogenesis/spread and new therapeutic targets.

Muscle atrophy is a characteristic disease feature of ALS, and typically correlates with progressive weakness and worsened prognosis. Therefore careful muscle measures may be a useful biomarker to monitor disease spread. Muscle ultrasound (MUS) is a painless and relatively simple technique that can be used to visualize structural muscle changes. Affected muscles have decreased thickness and appear whiter (reflected by increased echo intensity) (Pillen et al., 2008). Several groups have used MUS to quantitate ALS muscle loss in an attempt to correlate results to survival, with some focusing on biceps and wrist flexors, and others examining quadriceps and tibialis anterior as well (Arts et al., 2011; Lee et al., 2010). Results showed decreased muscle thickness over disease course, and increased echo intensity strongly correlated with ALSFRS-R scores. Although promising as a biomarker of disease spread, the heterogeneity of ALS itself (various sites of onset, unilateral muscle weakness etc.) warrants further assay standardization and examination of larger patient cohorts before it can be included for ALS prognostics.

MUNE is another method to assess the intact motor units that innervate a muscle by monitoring its maximum response action potentials and estimating the number of individual units that innervate it. Several studies have suggested that increased changes in MUNE strongly predicted rates of progression and survival and enabled stratification of patients according to speed of disease progression (Ahn et al., 2010; Armon and Brandstater, 1999; Shefner et al., 2011). Reproducibility and sensitivity of the assay thus suggest that MUNE can be used not only as a biomarker of disease spread, but also as a secondary outcome measure in clinical trials.

Another physiological measure of muscle loss is EIM (Rutkove, 2009). Similar to the results obtained in $SOD1^{G93A}$ mice discussed above, EIM measurements decline over time in patients with ALS (Rutkove et al., 2012). In addition, EIM measurements moderately correlated with ALSFRS-R upper and lower extremity subscores, but not total ALSFRS-R scores. Single muscle but not averaged whole body EIM strongly correlated with MUNE

(Rutkove et al., 2014). Continued studies examining the role of EIM to monitor disease progression in clinical trials are warranted.

On a systemic level, serum levels of creatinine, a product of creatine phosphate and a key molecule for energy production in muscle, is usually associated with whole muscle mass. It is transported from muscles to the kidneys via circulation and is currently considered a useful blood parameter to reflect motor dysfunction in various diseases including spinal and bulbar muscular atrophy, Duchenne muscular dystrophy and ALS (Hashizume et al., 2012; Ikeda et al., 2012). Serum creatinine levels are significantly downregulated in ALS (Chen et al., 2014b; Chio et al., 2014; Ikeda et al., 2012), and inversely correlate with ALSFRS-R scores and forced vital capacity, rendering this factor a useful blood biomarker of disease spread and severity. However, because the levels of creatinine are dependent on renal function, its accuracy has been questioned. In order to eliminate the effect of renal function on serum creatinine, Tetsuka et al (2013) proposed the use of the ratio of creatinine to cystatin C. Cystatin C levels are independent of muscle volume, but depends on renal function similarly to creatinine, and the authors believe that by using the ratio, they eliminate the effect of renal function on serum creatinine(Tetsuka et al., 2013b). Tetsuka et al showed a linear decrease in the creatinine to cystatin C ratio with increased ALS severity, and a stronger correlation of the ratio with the ALSFRS-R scores than the serum creatinine levels alone. Their results suggest that the creatinine/cystatin C ratio is a reliable surrogate marker of residual muscle mass. Recently, Bozik et al. proposed that blood creatinine levels may be a biomarker to monitor effects of dexpramipexole, based on a post-hoc analysis of the large phase III clinical trial (EMPOWER) of dexpramipexole in ALS patients (Bozik et al., 2014).

4. Biomarker use in clinical trials

The incorporation of biomarkers into ALS clinical trials has been slow when compared to the use of biomarkers within clinical trials for other disorders such as oncology(Simon and Roychowdhury, 2013). This has likely been due to the lack of biomarker incorporation into the early drug development process, though that is changing within our field. One advancement during the past 5 years has been the standardized collection of blood, CSF and clinical information in ALS clinical trials and clinical research studies to aid in the retrospective discovery of biomarkers that then can be further pursued in prospective studies. The Northeast ALS Consortium biorepository (http://www.alsconsortium.org/neals_samples.php) is a centralized repository of patient derived samples matched to a wealth of clinical information that is to available to investigators for biomarker related studies.

Biomarkers should be used in all ALS clinical trials to enrich for a patient sub-population that may best respond to a particular therapy, measure target engagement, monitor downstream effects of a drug treatment or act as a surrogate marker of drug efficacy. This will include genetic and/or biochemical biomarkers to enroll a particular patient genotype/phenotype within the trial and other biomarkers and clinical parameters to monitor treatment effects. One can also use biomarkers to retroactively analyze samples collected during past clinical trials to identify patient sub-populations that may have responded to the drug

therapy but could not otherwise be detected using clinical parameters of the whole subject population. There are a few examples of using biomarkers in ALS clinical trials for these purposes. A small phase II clinical trial of memantine examined CSF levels of pNFH and tau as markers of neuronal injury (Levine et al., 2010). This open-label trial treated patients for one year, with CSF collected at baseline, 6-months and 12-months. Patient inclusion criteria included elevated baseline CSF levels of tau and pNFH. ALSFRS-R was used as a clinical measure of disease progression. Patients treated with memantine exhibited a reduction in the rate of decline as measured by ALSFRS-R when compared to the slope using a 3-month lead-in period. Patients that exhibited the greatest reduction in the rate of decline also exhibited the greatest reduction in CSF tau and pNFH levels, suggesting that memantine provided some neuroprotection (Levine et al., 2010). A larger clinical trial of memantine is planned to further examine the potential benefit of memantine in ALS patients with elevated CSF levels of cytoskeletal proteins.

A similar trial design was used to test a combination therapy of pioglitazone and tretinoin in ALS patients. Combination therapy was performed for 6-months but failed to demonstrate any changes in the rate of ALSFRS-R decline nor any change in the levels of CSF biomarkers during drug treatment (Levine et al., 2012).

SOD1 antisense oligonucleotides are a promising treatment for patients that harbor mutations in the SOD1 gene (Miller et al., 2013). The presence of SOD1 mutations stratifies the patient population and is an inclusion criterion for drug treatment. Measurements of SOD1 mRNA or protein within the CSF were used as biomarkers of drug treatment and reductions in both SOD1 mRNA and protein were observed in response to treatment with SOD1 antisense oligonucleotides but not control oligonucleotides (Miller et al., 2013). Similarly targeting SOD1 (albeit less specifically) is a pilot trial with the antimalarial drug pyrimethamine that had been shown in human cells to reduce SOD1 protein levels (Lange et al., 2013). In a small cohort of ALS patients harboring SOD1 mutations, pyrimethamine decreased leukocyte SOD1 levels, a change that was sustained throughout the remainder of the study.

Antisense oligonucleotide based therapies as well as small molecule inhibitors of the GGGGCC G-quadruplex structure may also be beneficial for patients harboring the *C9ORF72* repeat expansion, and many laboratories and drug companies are pursuing this therapeutic approach (Donnelly et al., 2013; Fernandes et al., 2013; Riboldi et al., 2014; Su et al., 2014). Imaging based biomarkers may be developed to demonstrate the therapy reaches and engages the target, and biomarkers of downstream pharmacologic effects such as a reduction of RNA foci or reduction of repeat expansion RAN translation products in CSF may be useful biomarkers to monitor drug effects in patients.

Muscle or skin biopsies represent another method to examine biomarkers during a clinical trial. One study used a nasal biopsy to measure PD changes induced by a therapy that targets astrocytes (Sattler et al., 2011). The authors measured astrocyte expression of EAAT2 as a biomarker of thiamphenicol effects in both an animal model and healthy volunteers to demonstrate the potential for nasal epithelium biopsies to monitor drug effects in astrocytes.

A number of recent and upcoming clinical trials that target neuroinflammatory pathways are using biomarkers as secondary outcome measures. NP001 (Neuraltus Pharmaceuticals) is a small molecule regulator of inflammatory macrophage activity and peripheral inflammatory biomarkers CD16 and HLA-DR were used as a secondary endpoint in their phase I clinical trial (Miller et al., 2014). Tocilizumab/Actemra is a humanized monoclonal antibody to the IL-6 receptor and has been used in early phase clinical trials of ALS to reduce an inflammatory signature in patients (Fiala et al., 2013; Mizwicki et al., 2012). A larger phase II clinical trial of Actemra in ALS is planned for 2014 and uses a peripheral inflammatory signature as an inclusion criterium and to monitor drug effects. A current phase II trial of Gilenya in ALS, a drug FDA-approved for some forms of multiple sclerosis that reduces neuroinflammation, will measure effects of drug treatment on circulating lymphocyte populations as a secondary outcome measure.

5. Future directions

There remains a critical need to identify and verify biomarkers common between the various model systems and the human disease, as well as longitudinal studies to explore how biomarkers change within individual patients during disease progression. With respect to model systems, continued studies using patient derived cells and iPS cells will help identify mechanisms of ALS and further the translation of findings between *in vitro* models, animal models and the human disease. However iPS model systems currently rely on complex differentiation protocols that generate heterogeneity within the cultures and challenges to reproducible results. Introduction of methods that induce more rapid and consistent cell differentiation will produce a reliable model system that can be used across multiple laboratories to identify and screen new drug compounds.

We remain at an early stage in combining data from the various –omics technologies (genomics, proteomics, metabolomics, transcriptomics, etc.) and linking this data to patient clinical information to optimize our search for disease specific biomarkers. Investigators must be willing to share data generated across the technology platforms using the same patient derived samples, and hopefully a large ALS data “warehouse” will be created that links all data generated using patient derived samples to clinical information and imaging results. This data warehouse must have open access for analysis by all members of the field. We also must discover and validate biomarkers using longitudinal samples to best identify prognostic and predictive biomarkers for ALS. Our hope is that combining clinical information, genetic information, imaging and longitudinal –omics datasets, we will identify biomarker panels that best stratify the patient population and also provide a means by which one can best optimize drug development and clinical trial design.

Biomarkers must be incorporated into the early drug development process and used in clinical trials to demonstrate target engagement and downstream drug effects (Figure 1). Biomarkers common between ALS patients and model systems can aid in the selection of pathways and targets for drug development that may successfully translate to the patient population. It is well accepted that ALS is a heterogeneous disease and this heterogeneity adds considerable challenges to the ultimate success of large clinical trials. Stratification of the patient population using biomarkers will aid in enrolling patients that may be respond to

a particular treatment and create a more homogeneous patient population in which to perform the clinical trial. PD biomarkers (biologic and/or imaging) will demonstrate the drug candidate is hitting its target, and prognostic biomarkers will assist in demonstrating drug efficacy and shorten the length of clinical trials. Such strategies will increase our chance of success in large clinical trials and provide improved treatment options for ALS patients.

Acknowledgments

This work was supported by National Institutes of Health/National Institutes of Neurological Disorders and Stroke grants NS061867 and NS068179 to RB.

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Highlights

- Biomarkers in common between ALS patients and animal model systems
- Need to incorporate biomarkers in ALS drug development
- Pharmacodynamic biomarkers for ALS
- Prognostic biomarkers for ALS
- Use of biomarkers in ALS clinical trials

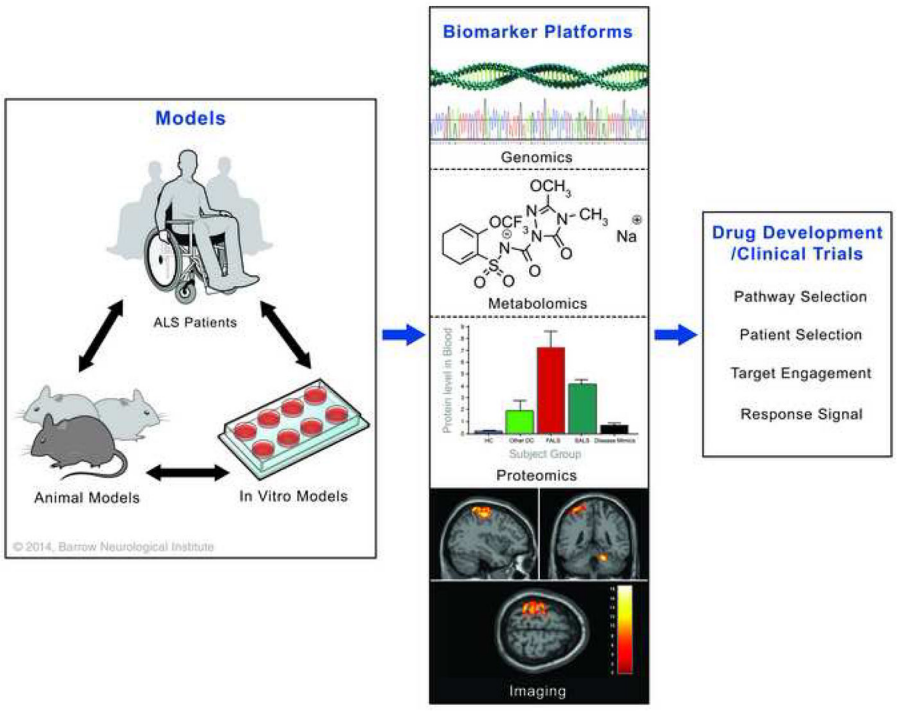

Figure 1.

Use of biomarkers in ALS drug development and clinical trials. Biomarker discovery and validation efforts incorporate various model systems and patient population, using –omics based technologies and imaging. These biomarkers are used to identify pathways to target for drug development, selection of patients for clinical trials, target engagement of the drug and determining efficacy within the patients.

Table 1

Biomarkers in Common between Preclinical Models and ALS Patients

Biomarker	Pre-clinical	Patient Population
Proinflammatory monocyte phenotype, microglia or cytokine expression	(Butovsky et al., 2012; Chiu et al., 2013; Hensley et al., 2002; Lincecum et al., 2010)	(Butovsky et al., 2012; Lincecum et al., 2010; Mitchell et al., 2009; Su et al., 2013)
Increased expression of CD4+ T cells	(Chiu et al., 2008)	(Saresella et al., 2013) (Mantovani et al., 2009)
Decreased expression of Tregs	(Zhao et al., 2012) (during rapidly progressing stage)	(Mantovani et al., 2009; Rentzos et al., 2012; Saresella et al., 2013)
Complement activation	(Heurich et al., 2011; Lee et al., 2013; Woodruff et al., 2008)	(Ganesalingam et al., 2011; Sta et al., 2011; Woodruff et al., 2008)
SOD1 in the CSF	(Miller et al., 2005; Ralph et al., 2005; Raoul et al., 2005)	(Winer et al., 2013)
Increased neurofilament in blood/CSF	(Boylan et al., 2009; Chen et al., 2014a; Lu et al., 2012)	(Boylan et al., 2013; Ganesalingam et al., 2011; Ganesalingam et al., 2013; Lehnert et al., 2014; Tortelli et al., 2012)
T2 weighted MRI structural changes	(Evans et al., 2014; Marcuzzo et al., 2011)	(Vucic et al., 2008)
Alterations in EIM	(Li et al., 2013; Wang et al., 2011)	(Rutkove et al., 2012; Rutkove et al., 2014)
Alterations in MUNE	(Ngo et al., 2012; Shefner et al., 2006)	(Ahn et al., 2010; Armon and Brandstater, 1999; Shefner et al., 2011)
RAN dipeptide proteins	(Su et al., 2014)	(Su et al., 2014; Zu et al., 2013)

Table 2

Prognostic Markers of Disease

Biomarker	Prognosis	Reference
Blood and CSF		
Low serotonin	57% increased risk of death	(Dupuis et al., 2010)
High sCD14	Increased survival time	(Sussmuth et al., 2010)
Low S100B	Increased survival time	(Sussmuth et al., 2010)
Low cystatin C	Shorter survival time	(Wilson et al., 2010)
High pNF-H	Increased risk of death/correlate with ALSFRS-R decline	(Boylan et al., 2013; Ganesalingam et al., 2013; Lehnert et al., 2014)
Low phosphoTau/Tau	Correlate with lower ALSFRS-R and white matter reduction	(Grossman et al., 2014)
High HMGB1 autoantibody	Correlate with lower ALSFRS-R scores	(Hwang et al., 2013)
High granzyme B	Correlate with lower ALSFRS-R scores	(Ilzecka, 2011)
High wrCRP	Correlate with lower ALSFRS-R scores	(Keizman et al., 2009)
High urate	39% reduction in risk of death per 1mg/dl increase	(Paganoni et al., 2012)
Peptides/amino acid/phosphate	Correlate with ALSFRS-R scores	(Lawton et al., 2014)
Low serum albumin	Correlate with ALSFRS-R scores	(Chio et al., 2014)
Muscle		
High Nogo-A	Correlate with lower ALSFRS-R score	(Dupuis et al., 2002; Jokic et al., 2005; Pradat et al., 2007)
Genetic biomarkers		
Mutations in EPHA4	Longer survival (14month increase)	(Van Hoecke et al., 2012)
Variant of CX3CR1	Reduced survival (25.49month shorter)	(Lopez-Lopez et al., 2014)
SLC11A2	Reduced survival (17month shorter)	(Blasco et al., 2011)
UNC13A	Reduced survival (5–10month shorter)	(Chio et al., 2013; Diekstra et al., 2012)
C9orf72	Reduced survival (6month shorter)	(Byrne et al., 2012)
SNP in ZNF512B	Reduced survival (72month shorter)	(Tetsuka et al., 2013a)