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Applying Proteomics to the Diagnosis and Treatment of ALS and Related Diseases

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Abstract

Protein-based biomarkers for amyotrophic lateral sclerosis (ALS) and other motor neuron diseases (MNDs) have many potential clinical utilities, including diagnostic, prognostic, and drug development indications. During the past decade a number of potential protein biomarkers have been proposed for MNDs. Further verification studies, followed by large validation and qualification studies, are required to advance these initial discoveries toward clinical use. Study of additional patient populations, including disease mimics, is required during the validation phase of biomarker development. Important regulatory issues are discussed that will affect the timing and strategy for biomarker assay development in ALS and other MNDs. The continued development of protein biomarkers for MNDs requires extensive collaboration between academic clinicians and scientists in conjunction with the biotechnology and pharmaceutical industries.

Keywords

proteomics; biomarkers; motor neuron disease

Motor neuron disease (MND) includes sporadic and familial amyotrophic lateral sclerosis (ALS), spinal muscular atrophy (SMA), hereditary spastic paraplegia (HSP), primary lateral sclerosis (PLS), and spinobulbar muscular atrophy (SBMA) or Kennedy disease. These heterogeneous syndromes typically manifest clinically by weakness, spastic paralysis, or both, reflecting a functional loss of upper and/or lower motor neurons. ALS includes dysfunction and loss of both upper and lower motor neurons, whereas SMA and SBMA result from the loss of lower motor neurons,¹ and PLS involves loss of upper motor neurons.² HSP is a complex group of inherited disorders that includes mutations of at least 20 genes and predominately involves upper motor neurons.³

While the genetic alterations that lead to familial forms of these disorders provide a diagnostic measure of the disease, each MND is typically diagnosed by clinical and electrodiagnostic features. Recent investigations have focused on the search for biomarkers that can distinguish MND from healthy or disease control subjects. The National Institutes of Health (NIH) defines

a biomarker as “a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.”⁴ The Food and Drug Administration (FDA) and NIH view development of biomarker and surrogate endpoints as an important component of drug development and personalized medicine. An FDA/Pharmaceutical Research and Manufacturers consortium on biomarkers has been established to assist in the development and validation of biomarkers.⁵ Biomarkers can include genomics, proteomic, or metabolomic-based molecules or biochemical signatures. To date, genomic-based biomarkers are more advanced with respect to translational medicine applications than proteomic or metabolomic-based biomarkers. Under the FDA Critical Path Initiative, a list of genomic biomarkers used in drug labeling for specific purposes has been published (http://www.fda.gov/cder/genomics/genomic_biomarkers_table.htm). While protein-based diagnostic assays, typically performed by enzyme-linked immunosorbent assay (ELISA), exist for many conditions, diagnostic tests for MND have yet to be achieved. This review will focus on protein-based biomarkers and their use in diagnostics and drug development for MND. Of note, sophisticated imaging modalities, such as magnetic resonance (MR) spectroscopy, diffusion tensor MR imaging (MRI), and electrodiagnostic techniques such as motor unit number estimation and transcranial magnetic stimulation may also be used to identify ALS biomarkers, but such techniques will not be discussed.^{6–8} In addition, MRI has also been used to demonstrate decreased thickness of the motor cortex in PLS patients.⁹

Comprehensive reviews of protein biomarker-based discovery efforts for ALS have recently been published.^{10–13} We will focus our discussion on the technologies and types of starting materials used in biomarker discovery efforts, the need for and utility of MND-specific biomarkers, and the regulatory process for translating biomarker discoveries to the clinic.

Proteomic Technologies

Proteomic biomarker discovery efforts require the ability to accurately measure and quantify proteins or protein fragments to identify specific individual or groups of proteins that can accurately differentiate MND from healthy controls and disease mimics. The type of technology platform used for proteomic discovery efforts will dictate the speed, resolution, and information flow of the results. There are gel-based techniques, antibody arrays, microsphere assays, and mass spectrometry-based proteomic methodologies that each have respective strengths and weaknesses. Gel-based approaches typically include standard SDS-polyacrylamide gel electrophoresis and 2-dimensional gel electrophoresis (2D-DIGE). Proteins are separated based on their molecular mass and/or charge. The 2D-DIGE permits quantification of different proteins using fluorescence labeling of proteins combined with imaging and software processing.¹⁴ Samples from control and MND groups can be analyzed on the same gel to increase the ability to find differentially expressed proteins between subject groups. A recent 2D-DIGE study comparing cerebrospinal fluid (CSF) from a small group of ALS and control subjects identified six proteins as candidate biomarkers for ALS.¹⁵ The main disadvantages are that 2D-DIGE is labor-intensive, and relatively few individual samples can be rapidly assayed using this technique.

Another method to discover protein biomarkers utilizes antibody microarrays. This assay determines levels of protein expression with antibodies spotted onto a matrix or surface to capture specific proteins. Quantification is achieved by a fluorescent signal due to binding to the antibody attached to the array surface. The protein array technology is similar in concept to cDNA microarrays and has similar limitations with respect to signal intensity measurements and validation of the results. This technique can provide rapid assessment of many proteins, and the microarrays can be used to identify biochemical pathways altered in MND. This approach has been used to monitor protein expression in a model system for spinal muscular atrophy to discover alterations of particular RNA binding proteins and transcription factors.¹⁶

Single or multiplexed ELISA has been a mainstream tool for clinical diagnostic assays. A number of potential protein biomarkers for ALS and other MNDs have been identified and examined by ELISA.¹¹ A similar technology that combines color-coded or magnetic microspheres with flow cytometry was developed by Luminex Corp. for multiplex measurements of cytokines and other molecules. This approach can rapidly quantify levels of many proteins within a single sample. Recently, Mitchell et al.¹⁷ reported a cytokine profile in the CSF of ALS patients using this Luminex multiplex assay. A five-cytokine panel could predict ALS with 89% accuracy in the training set of 74 subjects. However, since altered cytokine expression occurs during inflammation, further verification and validation studies with additional disease mimics are required to determine the specificity of this CSF cytokine panel for ALS.

Mass spectrometry-based proteomics can be used to determine relative amounts of protein or protein fragments (peptides) in complex samples. A major problem with mass spectrometry experiments is the sensitivity of the instrument, which is negatively affected by the complexity of the sample. Given the large dynamic protein concentration range in serum, plasma, or tissue samples,¹⁸ enrichment for the proposed biomarker becomes a required step prior to mass spectrometry. Regardless of the type of enrichment, this sample preprocessing step introduces experimental variability and reduces sample throughput. One approach to help enhance sensitivity while reducing experimental preprocessing has been to use CSF, which has a greatly reduced protein complexity when compared to serum or plasma. Protein signatures for ALS have been discovered in CSF using mass spectrometry methodologies^{19–22,25}; however, large validation studies are required to confirm or refute these initial findings.

Regardless of the proteomic technology used to discover protein biomarkers for MND, this is only the first step in a long process to verify and further validate the candidate biomarker(s). Validation studies should include the use of alternative proteomic techniques to that used for the biomarker discovery process. An antibody-based assay, often ELISA, is typically generated and verified for continued assay development for regulatory testing purposes. This can be difficult and time-consuming if commercially available antibodies specific to the proposed biomarker are unavailable, or if the biomarker is a peptide or protein fragment that does not have specific commercially available antibodies. An alternative to antibody-based assays is the multiple reaction monitoring (MRM) mass spectrometry assay. This method uses a highly sensitive and accurate mass spectrometer to specifically target and quantify peptides of interest using an internal isotope-labeled peptide standard to obtain an absolute quantification of the peptide of interest.²³ Multiple peptides can be monitored within a single sample analysis, thus providing a multiplex approach to quantify biomarkers of interest. MRM is being used in biomarker validation studies, and this methodology has become more popular. It may become an accepted standard in future diagnostic tests.

Utility of ALS Biomarkers

As mentioned, the diagnosis of ALS is based on the presence and distribution of lower and upper motor neuron signs. Electrodiagnostic findings provide laboratory support for the lower motor neuron disturbance. Other disorders are excluded by appropriate imaging and laboratory studies, and a diagnosis may then be rendered using the El Escorial criteria.²⁴ Since a “definite” diagnosis is made in the minority at presentation, there may be heightened anxiety and the need to repeat clinical and electrodiagnostic examinations until findings evolve. This delay in diagnosis may also lead to a delay in treatment or in improper treatment. Identifying a diagnostic biomarker would simplify and hasten diagnosis.

Other desirable characteristics of a biomarker are as follows: (1) provide prognostic information as to rate of progression; (2) predict pattern of involvement, e.g., distribution of

upper and lower motor neuron dysfunction, presence of dementia, ventilatory dysfunction, etc.; (3) predict response to therapy by identifying an activated disease pathway; (4) correlate with response to therapy in serial assays; (5) provide additional insight into disease pathogenesis; and (6) act as a useful surrogate marker in clinical drug trials.

Biologic Samples Used for Biomarker Discovery Efforts

Sample Types

Protein biomarker discovery efforts for MNDs have utilized CSF, blood serum or plasma, urine, biopsied muscle tissue, or postmortem spinal cord tissue samples. Each starting material has its strengths and weaknesses with respect to use in clinical proteomic biomarker studies. The use of tissue samples may have the most clinical relevance, but such availability is limited and requires either a biopsy (muscle) or postmortem acquisition. The use of postmortem tissues for diagnostic biomarker discovery efforts is complicated by endstage disease effects and postmortem protein alterations that are not specific to MND. Therefore, diagnostic proteomic discovery efforts have focused on biofluid samples from patients and controls, typically using blood or CSF. Sera or plasma have been the focus of clinical proteomic biomarker studies in cancer and other diseases. The blood proteome is highly complex, with ≈ 12 log orders of magnitude concentration difference between the highest to lowest abundance proteins.¹⁸ Since most classical protein biomarkers reside in the lower abundance proteins, it is often necessary to prefractionate serum or plasma prior to initiation of the proteomic experiments. This step adds another level of experimental design complexity and potential for introducing artifacts into the final data analysis. CSF is in close anatomical contact with the brain and spinal cord interstitial fluid where biochemical alterations related to a chronic neurodegenerative disease are likely to be reflected and accumulate. However, CSF collection requires a more invasive procedure, and its availability is typically less than that of blood. Urine represents the most accessible biofluid with the most noninvasive type of collection for biomarker development. Urine harbors fewer proteins but higher concentrations of metabolites and peptides than blood. Unfortunately, urine would not be a good biofluid if the final assay requires detection of intact protein. Few studies have used urine in clinical proteomic biomarker studies for MND, although increased collagen metabolites and levels of markers for oxidation have been detected in the urine of ALS patients.²⁵

Table 1 lists protein biomarker discovery efforts in biofluids that have been reported to have diagnostic, disease duration and/or progression, or phenotypic utility for specific MNDs. Note that most efforts have focused on ALS, but other MNDs have been included primarily as disease controls. In 2008 the SMA Foundation initiated a pilot study to identify protein biomarkers in blood or urine for SMA that correlate with clinical measures of disease severity (ClinicalTrials.gov identifier NCT00756821).

To date, muscle has had a limited role in biomarker discovery. The presence of neurogenic atrophy is characteristic of motor neuron disease, but it is nonspecific. It is also present in other motor axon loss processes including radiculopathy and neuropathy. Baloh et al.²⁶ reported that the presence of mixed fiber types within an atrophic group of myofibers is more likely due to motor neuron disease, especially ALS, than in polyneuropathy. This study was relatively small and requires replication and analysis of more disease controls including other ALS mimics and MND subtypes.

Pradat et al.²⁷ assessed muscle biopsies for expression of Nogo-A, a protein involved in preventing outgrowth of neurites and regeneration in the central nervous system. They found that its expression in patients with a pure lower motor neuron syndrome predicts progression to ALS. This finding is controversial, as others report Nogo-A expression in any denervated

muscle fibers and Nogo-A expression in all neuropathies; therefore, this phenomenon is not specific to ALS.^{28,29}

It is conceivable that protein or genetic alterations that occur in ALS may be reflected in muscle, especially if the disturbances are systemic or present within the motor unit. Muscle biopsy is minimally to moderately invasive depending on the technique used. Genetic and some protein studies could be performed on percutaneous needle biopsy specimens that yield about 12–14 mg of tissue per harvest.³⁰ Further study of this tissue is warranted and is under consideration by the ALS Therapy Development Institute (ALS TDI), which has partnered with at least three academic institutions to further identify ALS genetic and protein biomarkers using blood and other tissues (<http://www.als.net/articles/articleDetail.asp?articleID=5334>).

An alternative approach is to first identify biomarker candidates in a well-defined model system such as transgenic animal models of ALS. Recent work on biomarkers for cancer and cardiomyopathies has used this approach to identify potential biomarkers in a more homogeneous population (isogenic mouse strain) and then determine if these biomarkers can be translated to the human disease.^{31,32} One plasma protein elevated in a mouse model of dilated cardiomyopathy was also elevated in human patients with progressive dilated cardiomyopathy,³² indicating the potential use of such a strategy to identify disease biomarkers. A similar approach has been used to perform protein profiling in the mutant SOD1 transgenic mouse model of ALS to identify candidate biomarkers in this animal model of disease.^{33,34} For these studies transgenic and nontransgenic mice at different stages of disease were used to examine proteomic alterations early and late in disease. Continued work is required to determine if protein alterations identified in the transgenic mice are also observed in ALS patients. This approach requires that pathogenic mechanisms are shared between the human and animal model of disease.

Collection and Storage

As noted above, one critical preanalytical step is the collection and storage of samples for use in protein clinical proteomic biomarker studies. For biofluid samples, this includes the development of standard operating procedures for the collection, transport, and storage of the samples.³⁵ Failure to develop and adhere to standardized protocols will ultimately have significant consequences on the final data interpretations and conclusions.

Necessary Diagnostic Assay Validation Steps Required to Impact Patient Care

While the ultimate hope of MND biomarker investigations is to impact patient care, any biomarker must overcome a series of hurdles in order to obtain clinical practice utility. One important question to keep in mind during biomarker assay development is how the assay will be used in the clinic and how it compares to the current diagnostic standard. This is more problematic for MNDs that currently have no biochemical diagnostic test. For MND diagnostic tests, clinicians certainly desire a very low false-positive rate for a diagnostic biomarker given the implications of an ALS diagnosis. Therefore, a useful diagnostic biomarker assay must have high specificity, and clinicians would likely sacrifice some sensitivity for it. As more effective treatments become available, this balance could shift.

Given the prolific pace of protein biomarker discoveries, there is a need for regulatory oversight of analytic validation and qualification data to translate biomarkers to the clinic. Prognostic and predictive tests could identify patient populations that benefit from treatment, or equally important, those who would respond poorly to a specific treatment. As described below, the clinical utility of a protein biomarker-based diagnostic requires testing in prospective

randomized clinical trial designs and subsequent validation in follow-up clinical trials. This is quite similar to the traditional regulatory approval pathway for therapeutic agents.

Four phases of clinical biomarker assay development for diagnostics can be conceptualized (Fig. 1). These include the exploratory, discovery phase (I), the clinical assay development and verification phase (II), the retrospective validation phase (III), and the prospective screening and qualification phase (IV). The initial discovery phase typically uses retrospective samples to discover potential biomarkers. Often samples used for biomarker discovery experiments are collected at one site, although the use of samples obtained from multiple sites during this early phase may be beneficial for the next clinical assay development phase. This initial phase may also include a separate test group of samples to confirm the initial biomarker discovery results from the training set, but this is rare. The second phase further modifies the assay and verifies the protein so that it can be used in continued candidate biomarker validation studies. This phase may include optimizing antibodies used for protein detection, using another proteomic technology that increases sensitivity or specificity of the assay, or modifying the sample processing sets to enrich for the biomarker of interest. During phase II, one must establish assay performance parameters and generate assay reference standard calibrators, possibly a recombinant form of the biomarker or synthetic peptide recognized by antibodies used in the assay. The final assay plan should include standard calibrators, quality control (QC) samples, pooled sample controls from actual samples, and the true test samples. The candidate biomarker protein must be extensively verified with respect to tissue and cell type expression, physiologic functions, and potential biologic mechanisms related to the MND of interest. Thus, the verification stage will likely include multiple independent studies and may occur over multiple years. It is also imperative that standard operating procedures be described for the collection and storage of samples for use in all future studies. Assay development costs associated with verification studies can be extensive and therefore limit the total number of candidate biomarkers that will successfully complete this phase of development.

The third phase (III) involves large validation studies typically that use retrospective samples. A small confirmatory study of the final assay using additional samples from a single site may be performed prior to the large validation study. During this large validation study, it is necessary to include samples from multiple sites to determine the assay variability induced by sample collection and storage procedures. Proper power analysis must be performed to design a research protocol that will obtain statistically significant results concerning the sensitivity and specificity of the assay with large numbers of disease mimics. Many hundreds to thousands of total samples will be required to complete this phase of the biomarker assay development.³⁶ Appropriate disease mimics include polyradiculopathy, myeloradiculopathy, multifocal motor neuropathy, other motor neuropathies, focal axon-loss motor predominant mononeuropathies, the “spinal form” of multiple sclerosis, myelopathy, and possibly inclusion body myositis. Other MND subtypes, such as SMA, Kennedy's syndrome, and HSP, should also be included.

Whether it is important to include a broad range of ALS presentations and phenotypes is not certain, but it seems prudent to stratify the analyses based at least on features that vary among genetic forms of ALS. Although there is considerable overlap among sporadic ALS and genetic forms, especially ALS 1, 6, 7, and X,³⁷ there are noteworthy outliers. The best examples are in ALS 1, in which the A4V SOD1 mutation is associated with short survival of 12–18 months and limited upper motor neuron involvement,³⁷ while the H46R mutation is associated with an 18-year life expectancy.³⁸ There are also three juvenile onset forms, ALS 2, 4, and 5, that tend to progress slowly. Interestingly, ALS 2 (ALSIN) causes primary upper motor neuron or upper and lower motor neuron ALS. In contrast, dynactin mutations result in early adult onset, slowly progressive motor neuron disease with vocal cord paralysis as a primary manifestation.³⁷ The location of involvement at onset also appears to be a distinctive characteristic of a newly

discovered mutation in the fused in sarcoma/translated in liposarcoma (FUS/TLS) gene. In one kindred, affected patients presented with proximal upper extremity weakness without bulbar involvement³⁹; in another, cervical was twice as common as lumbar onset, and bulbar onset was uncommon.⁴⁰ There are also examples of unexplained phenotypic variability from the same mutation as seen in vesicle-associated membrane protein (synaptobrevin-associated protein) B (VAPB) mutations. They range from typical ALS to late-onset spinal muscular atrophy and slowly progressive ALS with tremor, all from the same mutations. Finally, there are inherited forms of ALS with frontotemporal dementia and parkinsonism.^{37,38,41}

At least partly based on the above variations, it would seem useful to stratify patients based on age of onset, rate of progression, type of motor neuron involvement (upper or lower, or both), associated features such as dementia and extrapyramidal signs, and site of initial involvement (bulbar vs. upper or lower limb in proximal or distal distributions).

However, any subgroup analysis will require sufficient numbers in each ALS subgroup to power the study and generate statistically significant results. The number of patients will determine how refined the stratifications may be and will limit the sensitivity and specificity capabilities of the testing conditions. Eventually, inherited as well as sporadic forms require subgroup analyses. The phenotypic variations that occur in the inherited forms could be due to environmental factors, and biomarker studies could provide insight into their origins.

Careful consideration of inclusion/exclusion criteria is necessary to properly address validation questions regarding the biomarker. Additional prospective validation studies are required when new standard operating procedures are incorporated into the biomarker assay development process. One must also remember that validation methods may differ for diagnostic (distinguish disease from controls) and prognostic (predict clinical outcome) MND biomarkers versus biomarkers for drug development (safety, efficacy, and exposure-effect relationships).⁴²

The final phase (IV) will apply the diagnostic biomarker assay in a prospective manner across multiple sites to monitor the predictive value of the test when compared to the final clinical diagnosis for each subject. This is the phase in which the ability of the biomarker assay to impact patient care is answered and actively qualified as a biomarker-based test for the clinical indication.⁴³ Long-term outcome measures are required to determine the final sensitivity and specificity of the assay. Substantial costs and time will be required to complete a biomarker validation study. This phase may also include incorporation of the biomarker assay into clinical trials to evaluate its use as a surrogate marker of drug efficacy. To determine the biomarker's utility as a surrogate marker, the natural history of the biomarker has to be assessed in controls via serial analyses. Surrogate markers to monitor disease progression are not equivalent to diagnostic biomarkers; therefore, surrogate markers of disease progression may not have diagnostic utility. When incorporated into drug development-based clinical trials, biomarkers should be an exploratory substudy within the clinical trial and not a component of the regulatory submission for the drug. However, the results may provide critical validation data such that the biomarker could be used as a useful endpoint in future registration studies for the therapeutic agent. Again, biomarkers of drug efficacy may not have diagnostic utility and should be selected based on the proposed mechanism of action of the drug candidate.

Thus far, protein biomarker studies for MND remain predominately in the discovery phase of the development process. One common problem with the published biomarker studies is that they are generally underpowered, use limited numbers of MND patients, and typically lack the appropriate disease controls. Very few have used greater than 100 total subjects during biomarker discovery experiments.^{11,17,27,44} These studies are all retrospective in nature, using subjects with clinically defined MND and control subjects. A few words of caution are also in order regarding the methods used for data analysis when discovering and validating

biomarkers. In general, multivariate statistics and machine-learning algorithms are prone to experimental overfitting and generation of false-positive biomarkers.⁴⁵ Multivariate analysis may perform well on the original sample set (i.e., the training set) but often fail when applied to an independent test set. Therefore, careful biomarker validation studies must be formulated, especially for panels of candidate biomarkers.

While most acknowledge that standard operating procedures for the collection and storage of clinical samples are required for clinical proteomic biomarker discovery and validation efforts,⁴⁶ few individual studies have carefully defined such standard operating procedures.^{35,47} Additional standard operating procedures must be developed for any sample processing step necessary to enrich the biomarker prior to analysis as well as the methods for data capture, normalization, and statistical analysis. The Human Proteome Organization (HUPO) has developed an HUPO-Proteomics Standard Initiative and recently published guidelines for standardizing the collection, analysis, and reporting of proteomics data.⁴⁸ Careful definition of protocols and procedures early in the biomarker development process will not only reduce the chance of generating false-positive biomarkers in the discovery phase, but it will also enhance all validation studies and will ultimately be required for regulatory approval.

The task of taking biomarker discovery results and generating a validated and accepted clinical assay requires extensive experimentation that must be carefully formulated and documented. There are two general paths to get a diagnostic assay to the clinic in the United States.⁴⁹ The first is FDA approval of the diagnostic test, whereas the second is via the Center for Medicare & Medicaid Services (CMS) that regulates all laboratory testing through the Clinical Laboratory Improvement Amendments (CLIA). The FDA is charged with regulating all in vitro diagnostic devices (IVDs) to ensure safety and effectiveness as defined in the Code of Federal Regulations. Safety and effectiveness refers to the consequences of reliance on the IVD to make clinically significant diagnostic or clinical care decisions. Unless the candidate biomarker is already established to detect disease or monitor therapeutic efficacy, all biomarkers will require extensive clinical studies to support a claim of effectiveness and safety before receiving FDA approval. Meetings with the FDA via a preinvestigational device exemption (pre-IDE) are required prior to the initiation of trials designed to evaluate a new IVD. In addition to this expensive clinical validation process, thorough documentation and validation of all reagents and equipment used in the IVD is required by the FDA. Therefore, considerable time and collaborative efforts will be required to generate an FDA-approved diagnostic test for an MND.

A less cumbersome path for translation from biomarker discovery to the clinic is the CLIA approved in-house or “home brew” test assay for “research only” purposes. The laboratory developed test (LDT) is generated within the CLIA-approved clinical laboratory using analyte-specific reagents (ASR) that typically are antibodies manufactured elsewhere and used in the LDT. The FDA exempted most ASRs from 510(k) clearance requirements and does not regulate the in-house laboratory tests that are generated from these ASRs. It is critical to adhere to FDA guidelines describing ASRs so that the final assay conforms to all federal regulations (CLIA, 42 U.S.C. 263a 62 FR 62252). CMS oversight with state regulatory agencies ensures that biomarker assays generated with ASRs are accurate, safe, and available for use while their potential clinical utility is being examined before submission to the FDA for approval.

Regardless of the pathway used to translate the biomarker discovery efforts to the clinic, large validation studies are required to properly test the diagnostic accuracy of the proposed assay. As noted above, no proper validation studies have been performed to date for MND diagnostic assays. By contrast, clinical trials for MND therapies typically include hundreds to thousands of subjects, and the results are often replicated in separate studies prior to approval of the therapy. Ideally, diagnostic biomarker assays should also be validated in analogous prospective, well-controlled clinical studies of diverse patients across multiple institutions.

Standard operating procedures for sample collection, processing, storage, and shipping must be incorporated in these clinical studies.

Next Steps for MND Diagnostic Assays to Become a Reality

The complexity and heterogeneity of MNDs will require strong collaborative efforts between basic scientists and clinicians to discover and develop protein-based diagnostic assays with clinical utility. Large multiinstitutional teams participating in collaborative groups are required to develop and advance the standard operating procedures for patient sample collection and analytical tools necessary for biomarker discovery and validation studies. Proactive efforts within the MND community are necessary to initiate sample collections and share resources in order to successfully translate proteomic biomarker discoveries to the clinic. Within the ALS community, we have initiated such efforts by establishing a consortium of academic institutions that collect and store patient samples under identical conditions. A repository of plasma and CSF from ALS, other neurodegenerative diseases, and healthy control subjects has been initiated for use in biomarker discovery and validation experiments

(<http://www.alsconsortium.org/news.html>). A committee of scientists and clinicians will review requests for samples from the repository to ensure these valuable resources are used most effectively toward biomarker discovery efforts and ultimately more successful treatments for ALS. In addition, biomarker assays must be incorporated into MND clinical trials to obtain critical validation data and to evaluate their role as markers of disease progression and/or ability to monitor drug efficacy. However, as noted above, this biomarker validation substudy is not included within the regulatory submission of the drug but used internally to establish the utility of the biomarker. Such studies will require the additional collaboration with biotechnology and pharmaceutical companies that typically manage and fund MND clinical trials.

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Abbreviations

2D-DIGE	2-dimensional gel electrophoresis
ALS	amyotrophic lateral sclerosis
ASR	analyte-specific reagents
CLIA	Clinical Laboratory Improvement Amendments
CSF	cerebrospinal fluid
ELISA	enzyme-linked immunosorbent assays
FDA	Food and Drug Administration
HSP	hereditary spastic paraplegia
HUPO	Human Proteome Organization
IVD	in vitro diagnostic device
LDT	laboratory developed test
MND	motor neuron disease
PLS	primary lateral sclerosis
SBMA	spinobulbar muscular atrophy
SMA	spinal muscular atrophy

FIGURE 1.

Biomarker assay development phases from discovery to validation and qualification. The flow diagram depicts the process of biomarker discovery to regulatory approval as a diagnostic or surrogate biomarker. Few biomarkers discovered will ultimately pass through this process for regulatory approval. SOP, standard operating procedure.

Table 1

Selected studies of MND protein biomarkers.

Biofluid/tissue	Protein(s)	Reported utility	Reference
Blood serum or plasma	MMP-9	Diagnosis	50
	Angiogenin	Diagnosis	51
	Creatine kinase	Disease progression	52
	ApoE	Disease progression	53
	SMN	Correlation to SMN gene copy number	44
	Fibrinogen, C-reactive protein	Disease state and progression	54
CSF	Cystatin C, modified transthyretin, 7B2	Diagnosis	20
	Cystatin C, VGF, 6.7 kDa mass peak	Diagnosis	21
	5 cytokine panel: IL-10, IL-6, GM-CSF, IL-2, IL-15	Diagnosis	17
	VGF	Disease progression	55
	Tau and S100beta	Disease progression	52
	IGF-1, insulin, growth hormone	Diagnosis	56
	NF-L	Diagnosis and Disease duration	57
	NF-H	Diagnosis and Disease duration	58

MMP, matrix metalloprotease; ApoE, Apolipoprotein E; SMN, survival of motor neuron; CSF, cerebrospinal fluid; VGF, neurosecretory protein VGF; IGF-1, insulin-like growth factor-1; NF-L, neurofilament light chain; NF-H, neurofilament heavy chain.