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Biomarkers in amyotrophic lateral sclerosis: a review of new developments.

Nick Verber, Pamela J Shaw.

Sheffield Institute for Translational Neuroscience (SITraN)

University of Sheffield, United Kingdom.

Contact details for correspondence:

Professor Pamela J Shaw

Sheffield Institute for Translational Neuroscience,

University of Sheffield,

385 Glossop Road,

Sheffield S10 2HQ,

UK

Email: pamela.shaw@sheffield.ac.uk

Tel: +44 114 2222295 or 2222231

Abstract

Purpose of review: This review draws together the most recent findings in ALS biomarker research from biochemical, imaging and neurophysiology techniques.

Recent findings: The potential of circulating RNA is highlighted, including new retrieval techniques. With ongoing genetic clinical trials, the need for pharmacodynamic biomarkers is essential. There is a strong case for neurofilament proteins being validated in ALS; their biomarker profile is discussed. Oxidative stress and neuroinflammation studies offer insight into disease mechanisms and offer good biomarker potential. Recent metabolic studies include investigation of lipid profiles, creatinine and ferritin. The potential of chitinase proteins as pharmacodynamic and prognostic biomarkers is highlighted. The role of tau and amyloidβ is debated, as evidenced by the papers presented here. Proteomic approaches provide unbiased discoveries of novel biomarkers, together with confirmation of previous findings. The use of imaging techniques is outlined to demonstrate selective atrophy, volume loss, muscle and tract involvement. *In-vivo* imaging is discussed with reference to histone deacetylase, oxidative stress, neuroinflammation and metabolic changes. New applications of electrophysiology demonstrate objective muscle biomarkers and brain network perturbations.

Summary: The biomarker research field continues to provide insight into the disease. Multi-centre collaborations are needed to validate these promising recent findings.

Keywords: ALS; biomarker; imaging; electrophysiology

Introduction

Biomarkers are needed to identify therapeutic targets, stratify patients for clinical trial entry, for prompt diagnosis and subsequent trial access, as trial outcome measures, and to provide clinicians with objective prognostic and disease progression tools. This review highlights recent discoveries from biochemical, radiological and neurophysiological research.

Ribonucleic acid (RNA)

A substantial amount of recent work has explored circulating micro RNAs (miRNAs), circular RNAs (circRNAs) and messenger RNAs (mRNAs) (1). Extracellular vesicles (ECVs) are released as a means of intercellular communication. They cross the blood-brain barrier and so have garnered interest as a non-invasive source of central nervous system (CNS) biomarkers. Saucier et al. isolated plasma ECVs and used next-generation sequencing (NGS) to derive 27 miRNAs differentially expressed in ALS. MiR193a-5p was found to discriminate between high and low ALSFRS-R scores. Likely target pathways were identified and this included the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) antioxidant pathway (2). A second study enriched plasma ECVs for those that were neuronally-derived and identified 30 miRNAs using microarray. A comparable alteration was found in primary motor cortex (PMC) tissue. Gene Ontology (GO) analysis revealed the likely target genes are involved in synaptic vesicle and neurotransmitter secretion (3). Otake et al. analysed cerebrospinal fluid (CSF) exosomal mRNA using NGS, a first for this type of methodology. GO analysis demonstrated mechanisms that were enriched in ALS, namely the ubiquitin-proteasome pathway, oxidative stress response, and unfolded protein response (4). Leukocyte microarray revealed a circRNA profile and the best discriminators, with biological plausibility, were then validated with qPCR. Seven of these were discriminatory and two correlated with clinical measures (5).

Biomarkers in genetic subtypes of ALS

The C9orf72 mutation causes an intronic hexanucleotide repeat expansion which is transcribed and undergoes non-ATG translation. The resulting dipeptide repeat proteins aggregate and are toxic to cells. The antisense oligonucleotide (ASO) trials in C9orf72-ALS (C9ALS) and SOD1-ALS are novel therapeutic approaches. To inform trial design, the study team prospectively recruited and analysed clinical and biological data from 116 C9ALS and 12 asymptomatic carriers. In agreement with previous findings, they found that CSF poly(GP) did not correlate with progression rate. It also negatively correlated with DNA expansion size (6). The value of poly(GP) remains as a pharmacodynamic marker, as levels seem to be stable over time and a reduction can be seen with ASO treatment in cell and mouse models (7).

Gertsman et al. demonstrated that a SOD1 peptide can reliably be detected in human CSF. Levels correlated with the mutation-specific stability of the full protein. They administered SOD1 ASO in rat models and observed a decrease in CSF levels (8)*. Given the challenges of measuring misfolded SOD1, this may prove a promising surrogate. SOD1 is present in plasma-derived ECVs, both in the exosome subset and the microvesicle subset (9). This is important as a potential mechanism of disease propagation and for consideration in ASO treatment design.

TDP-43 aggregates are found in nearly all ALS patients. Mislocalisation also occurs outside of the CNS, and platelet TDP-43 levels have been found to be higher in patients with ALS (10). Measuring CSF TDP-43 together with neurofilaments may improve the diagnostic utility of neurofilament levels (11).

Neurofilament proteins

Neurofilament levels have great potential to become a validated biomarker in ALS, as summarised in the review by Gagliardi et al. (12). There is utility in diagnosis (13-17), prognosis (14-16, 18-20) and pharmacodynamic monitoring (14, 18). Neurofilament light (NfL) levels tend to be strongly correlated in CSF and serum (13) allowing for a less-invasive blood sampling method. Using a molecular biomarker of disease progression in trials improves objectivity and it has been suggested that baseline serum NfL may facilitate a reduction in sample size (18). Finally, neurofilament exploration in asymptomatic gene carriers has consistently shown that there is unlikely to be a prolonged pre-symptomatic phase of neuronal injury in ALS (21).

Oxidative stress and neuroinflammation

Reactive oxygen and nitrogen species play important roles in the immune response and cell signalling. An excess of reactive species or a reduction in antioxidant capacity can cause DNA and RNA modification, lipid peroxidation, and oxidisation and nitration of amino acids. ALS-COSMOS, a multi-centre, longitudinal study, found fasting plasma uric acid (PUA) inversely correlated with disease duration (22). PUA has a direct role as an antioxidant and an indirect role in glutathione activity. They also found urinary 8-oxo-deoxyguanosine (8-oxodG) and 15-F_{2t}-isoprostane (IsoP) to modestly increase over time. Baseline 8-oxodG negatively correlated with ALSFRS-R. Baseline IsoP negatively correlated with ALSFRS-R at the last visit. Another large-scale study recently published the findings from their 20-year cohort study, in which they also measured PUA. They found no association with the development of ALS (23).

There has been recent interest in interleukin (IL)-6, which functions as a pro-inflammatory cytokine and anti-inflammatory myokine. Levels were found to be raised in paired ALS CSF and plasma (24), and raised and correlated with disease progression in astrocyte-derived exosomes (25). They have also been found not to be higher and inversely correlated with phrenic nerve compound muscle action potential (CMAP) (26). Researchers also demonstrated that CSF levels were only increased in patients with the IL-6 receptor C allele, suggesting a means of patient stratification in IL-6 receptor antagonist trials (24).

Combining quantification with other characterisation data, such as genotype, can reveal associations that are lost in the whole cohort, emphasising the need to stratify patients. Olesen et al. found that plasma TNF-α, IL-10 and TRAIL were negatively correlated with survival in patients without a C9orf72 or SOD1 mutation. Plasma IL-1β was associated with survival in C9ALS and CSF TRAIL was negatively associated in SOD1ALS (27).

Another method of investigating the inflammatory milieu is by assessing T-cell subtypes. One such study found pro-inflammatory T-helper (Th)17 cells and Th1 cells upregulated in blood, and the anti-inflammatory Th2 and regulatory T cells (Treg) were downregulated. Concordantly, levels of pro-inflammatory IL-1β, IL-6 and IFN-γ were elevated, and anti-inflammatory IL-10 was reduced. Th1 and Th17 also correlated with disease severity (28). This lends further biological plausibility to the ongoing clinical trials investigating low-dose IL-2 (NCT03039673) and Tecfidera (ACTRN12618000534280) as treatments to ameliorate the pro-inflammatory cascade and increase Treg levels.

Metabolism

Substantial weight loss can occur before the onset of weakness and is a predictor of poor survival in ALS. It has therefore been hypothesised that metabolic regulation plays an important role in the disease. Ingre et al. measured the lipid profile of patients at diagnosis. Survival analysis demonstrated a beneficial prognostic effect in the range of 6-12 months for total cholesterol, LDL-cholesterol, apolipoprotein B, increased LDL-cholesterol/HDL-cholesterol and increased apolipoprotein-B/apolipoprotein-AI. It is the first time that

apolipoprotein-B and apolipoprotein-AI levels have been explored (29). Loss of fat-free mass has been shown to correlate with a reduction in LDL-cholesterol (and an increase in ferritin). These markers could be used as surrogates for nutritional status (30).

A meta-analysis suggested plasma creatinine (PCr) as a promising biomarker (31). ALS-COSMOS measured this in fasting samples and found baseline levels predicted survival and correlated with ALSFRS-R at both baseline and last visit. The authors suggest that plasma and urinary creatinine reflect both a state of muscle health as well as a wider set of metabolic processes (22).

Iron metabolism may have a pivotal role in ALS, both in conjunction with, and independently of oxidative stress (32). A longitudinal study measured plasma ferritin, transferrin and hepcidin, from the large dataset generated from the MITOTARGET trial (NCT00868166). Ferritin was a significant predictor of progression. Other significant biomarkers were NfL, 4-hydroxynonenal, and 8-oxodG (20)*. Serum ferritin was also raised in a Chinese ALS population and higher levels were associated with poorer survival (33).

Chitinase

Non-neuronal cells have been implicated in ALS pathophysiology, and activated microglia and astrocytes cause chitinase expression. Thompson et al. demonstrated that chitotriosidase-1 (CHIT1) and chitinase-3-like protein 2 (CHI3L2/YKL39) could discriminate from mimics, but their diagnostic performance was weaker than neurofilament. CHIT1 and CHI3L2 correlated with rate of progression, and CHIT1 correlated with survival when incorporated into a multivariate model (34). Gille et al. found CHIT1 and chitinase-3-like protein 1 (CHI3L1/YKL40) to poorly discriminate and weakly correlate with disease progression; CHI3L1 was independently associated with survival. (35) A longitudinal study showed CHIT1 and CHI3L1 to be associated with progression rate and, consistent with previous findings, levels were constant over time (36). These proteins are promising prognostic and pharmacodynamic biomarkers.

Tau and amyloidβ

There have been previous explorations of total tau (tTau), phosphorylated tau (pTau) and amyloidβ (Aβ) levels in ALS, albeit with contradictory results. Lanznaster et al. measured CSF tTau, pTau and Aβ1-42 in a larger cohort than previous studies and demonstrated a role for these analytes as diagnostic and prognostic biomarkers (37). Whilst this does provide pathophysiological information, there is overlap between tau and neurofilament, with an increase in both seen with neuronal degeneration. Another study to look at tTau included discovery and validation cohorts, but with smaller participant numbers. CSF tTau was higher in patients, but only when both sets of cohort data were combined. By measuring NfL in the same samples, the authors found it to be the superior biomarker (11). Finally, a proteomic study found no difference in CSF levels between patients and controls (21).

Proteomics

Proteomic approaches allow for an unbiased exploration of biosamples. This has the potential to implicate novel proteins and pathways for further validation, as was the case for CHIT1 discovery. For a discussion on the advances and limitations, readers are directed to the review by Hedl and colleagues (38).

Hayashi et al. analysed CSF-derived exosomes and found 14 proteins differentially expressed in ALS, with novel INHAT repressor (NIR) being the most upregulated. Immunohistochemical analysis of anterior horn cells showed a corresponding reduction in NIR, a protein involved in nucleolar stress (39). Oeckl et al. report 4 novel proteins that were upregulated in CSF of patients: ubiquitin C-terminal hydrolase-L1 (UCHL1);

microtubule-associated protein 2; capping actin protein, gelsolin-like (CAPG); and glycoprotein non-metastatic melanoma protein B (GPNMB). CAPG and GPNMB were also increased in spinal cord tissue. Transcriptional pathways were most affected (21). An additional study also found CSF UCHL1 and GPNMB to be raised associated with a poorer outcome (40). Leoni et al. used two complementary workflows: peripheral blood mononuclear cells and brain-tissue enhanced plasma. As well as confirming known biomarkers, they proposed myosin-9, fructose-bisphosphate aldolase and plectin as new candidates, as well as highlighting several perturbed pathways. Their approach is to be commended for attempting to overcome the low concentration of biomarkers that is commonly a limiting factor in CNS exploration (41)*.

Imaging

Magnetic resonance imaging (MRI) is integral to neurological biomarker exploration and its utility is extended by combining sequences, new protocol development, machine learning and by pairing with positron emission tomography (PET) and proton spectroscopy.

Klickovic et al. demonstrated lower limb hyperintensities on MRI assessment of skeletal muscle. This is caused by oedema, which itself is secondary to denervation. Intuitively, imaging-derived functional muscle scores correlated with the ALSFRS-R leg domain, suggesting value as a progression marker (42). van der Burgh et al. combined sequences of the cervical cord to compute cross-sectional areas and concluded that atrophy is a sensitive diagnostic and prognostic tool (43). Combining volumetrics with relatively new protocols – diffusion kurtosis and quantitative susceptibility mapping (QSM) – provides a marker with early diagnostic potential and insight into brain microstructure (44). Bede et al. combined structural data with a segmentation algorithm to better categorise brainstem atrophy. They found marked loss of volume of the medulla and moderate pontine loss in both primary lateral sclerosis (PLS) and ALS (45).

Recognising upper and lower motor neuron (UMN/LMN) involvement is important for diagnosis and prognostication. Diffusion tensor imaging (DTI) has been used to demonstrate corticospinal tract (CST) involvement and correlation with clinical measures (46). Sako et al. used deterministic tractography, an extension of DTI, to show CST track number decreases and correlation with severity (47). Contarino et al. used QSM to specifically assess the PMCs in relation to UMN function. Increased susceptibility skewness showed promise and their protocol was automated to reduce potential bias (48). Ultrasound-determined measures of the median nerve and abductor pollicis brevis have been suggested as evidence of LMN involvement (49). Ultrasound has been shown to have interrater and intrarater reliability when measuring longitudinal muscle loss and represents a potential marker of progression (50). When combined with MRI, tongue ultrasound predicted bulbar progression and, interestingly, overall motor function (51).

PET advances include radioligand development and creative use of well-established protocols. De Vocht et al. used [18F]FDG-PET/MRI to demonstrate changes in glucose metabolism in pre-symptomatic C9-carriers across various brain regions. Furthermore, only a small proportion of subjects had elevated neurofilament levels, suggesting this may be a sensitive measure of disease onset (52)*. Longitudinal studies would be useful to establish the prognostic and pharmacodynamic value. Histone deacetylase (HDAC) is linked with epigenetic processes, both of which have been shown to be dysregulated in ALS. Using a novel HDAC-radioligand, Dios et al. were unable to validate these findings *in vivo* (53). To determine *in vivo* oxidative stress, Ikawa measured ^{62}Cu–ATSM mitochondrial uptake, and found it localised to both PMCs and correlated with disease severity (54). Neuroinflammation can be measured through expression levels of translocator protein (TSPO) on activated glia. Van Weehaeghe and colleagues analysed uptake data from two TSPO radioligands and preferential uptake was seen in both PMCs. They concluded that data from both tracers could be pooled; an important step for increasing the power of multi-centre trials (55)*.

Proton magnetic resonance spectroscopy (^{1}H-MRS) combines metabolic measurements with MRI. For a summary of recent papers and implicated brain regions and metabolites see Table 1.

Brain region	Metabolite	Comment	Reference
PMC	Glutamate/GABA	No change in ALS	(56)
	GABA/Cr	No change in ALS	(56)
	Glutamate/Cr	↓ in ALS	(56)
	NAA/Cr	↓ in ALS	(56)
	tNAA/Cr	↓ in ALS Correlates with foot tapping rate	(57)
	tNAA/Cho	↓ in ALS Correlates with foot tapping rate Associated with progression rate	(57)
	tNAA/Ino	↓ in ALS Correlates with foot tapping rate Correlates with finger tapping rate Correlates with upper limb function Reduction at baseline associated with fast progression	(57) (58)
	Glutathione/Cr	↑ in ALS	(56)
	NAA	Correlates with hand strength and disease severity	(56)
Prefrontal cortex	tNAA/Cr	Associated with verbal and semantic fluency, and digit span forwards and backwards	(57)
Pons	Glutamate and glutamine	Inversely correlated with bulbar function	(58)

Table 1: Metabolic dysregulation as determined by ^{1}H-MRS studies.

PMC = primary motor cortex; GABA = Gamma aminobutyric acid; Cr = creatinine; NAA = N-acetylaspartate; tNAA = total N-acetylaspartate; Cho = choline; Ino = myo-inositol

Electrophysiology

Advances in electroencephalography (EEG) have improved the spatial resolution and signal-to-noise ratio. Using 128-channel EEG and multiple localisation methods, McMackin and colleagues demonstrated mismatch negativity at various neuroanatomical regions; this correlated with impaired attention switching (59). The same team also used spectral EEG and MRI to show neural connectivity changes associated with motor and non-motor function (60).

Jenkins et al. combined motor unit number index (MUNIX), a measure of denervation, with MRI and clinical scores to demonstrate effective multi-modal, longitudinal muscle profiling (61). Bashford et al. developed an automated, surface-electromyogram to detect fasciculations. This has potential for longitudinal use but will need validating in larger cohorts and with clinical correlates (62). They also used this non-invasive tool to characterise daytime fasciculation activity, something that would be unpleasant for patients with needle-electromyogram (63). Another non-invasive technique is the motor unit size index (MUSIX), a surrogate for reinnervation derived from CMAP and MUNIX. Researchers tested MUSIX in a single-subject round-robin and a multi-centre assessment of healthy volunteers to confirm its reliability. This gives confidence to develop this within ALS cohorts (64). Finally, Alix et al. provide further evidence of electrical impedance myography as an objective, non-invasive and passive measure of bulbar function. By applying electrodes to both planes of the tongue they argue it better captures atrophy in ALS patients (65)*.

Conclusions

The proof-of-principle explorations give fascinating insight into ALS pathophysiology. There are excellent examples of the value of large, prospective studies with standard operating procedures. Further validation is required as there are often inter-study discrepancies. A concerted effort is now needed to move some of these promising biomarkers into clinical trials and incorporate them into the clinic to facilitate optimal clinical management.

Key points:

1. The development of current technologies and the advent of new ones allow researchers to gain insight into ALS.

2. There is the potential to create biomarker panels from across different disciplines, which pave the way for personalised medicine both in the clinic and for more effective clinical trials.

3. Continued efforts are needed to recruit and carefully phenotype patients to allow for further biomarker discovery.

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