



Pathophysiological Underpinnings of Extra-Motor Neurodegeneration in Amyotrophic Lateral Sclerosis: New Insights From Biomarker Studies

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Amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD) lie at opposing ends of a clinical, genetic, and neuropathological continuum. In the last decade, it has become clear that cognitive and behavioral changes in patients with ALS are more frequent than previously recognized. Significantly, these non-motor features can impact the diagnosis, prognosis, and management of ALS. Partially overlapping neuropathological staging systems have been proposed to describe the distribution of TAR DNA-binding protein 43 (TDP-43) aggregates outside the corticospinal tract. However, the relationship between TDP-43 inclusions and neurodegeneration is not absolute and other pathophysiological processes, such as neuroinflammation (with a prominent role of microglia), cortical hyperexcitability, and synaptic dysfunction also play a central role in ALS pathophysiology. In the last decade, imaging and biofluid biomarker studies have revealed important insights into the pathophysiological underpinnings of extra-motor neurodegeneration in the ALS-FTLD continuum. In this review, we first summarize the clinical and pathophysiological correlates of extra-motor neurodegeneration in ALS. Next, we discuss the diagnostic and prognostic value of biomarkers in ALS and their potential to characterize extra-motor neurodegeneration. Finally, we debate about how biomarkers could improve the diagnosis and classification of ALS. Emerging imaging biomarkers of extra-motor neurodegeneration that enable the monitoring of disease progression are particularly promising. In addition, a growing arsenal of biofluid biomarkers linked to neurodegeneration and neuroinflammation are improving the diagnostic accuracy and identification of patients with a faster progression rate. The development and validation of biomarkers that detect the pathological aggregates of TDP-43 *in vivo* are notably expected to further elucidate the pathophysiological underpinnings of extra-motor neurodegeneration in ALS. Novel biomarkers tracking the different aspects of ALS pathophysiology are paving the way to precision medicine

approaches in the ALS-FTLD continuum. These are essential steps to improve the diagnosis and staging of ALS and the design of clinical trials testing novel disease-modifying treatments.

Keywords: amyotrophic lateral sclerosis (ALS), neuroimage, cerebrospinal fluid (CSF), biomarker (BM), neuropathology, TDP-43 = TAR DNA-binding protein 43, frontotemporal lobar degeneration, frontotemporal dementia (FTD)

INTRODUCTION: USING BIOMARKERS TO REVEAL THE EXTRA-MOTOR UNDERPINNINGS OF AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic lateral sclerosis (ALS) is a progressive paralytic disorder defined by the neurodegeneration of motor neurons, while frontotemporal dementia (FTD) is the most common presentation of frontotemporal lobar degeneration (FTLD) and is characterized by progressive neurodegeneration of frontotemporal structures (1, 2). The diagnosis of ALS is based on the identification of motor signs and symptoms, while the diagnosis of FTD is based on cognitive, language, and behavioral features (3–5). However, recent evidence indicates that ALS can show varying degrees of cognitive and behavioral changes at diagnosis (6–9). In ALS, the presence of non-motor symptoms has been linked to frontotemporal neurodegeneration (10, 11), and current diagnostic criteria acknowledge the existence of a continuum of cognitive and behavioral changes between ALS without cognitive impairment (ALSno-cbi) and full-blown FTD (12). However, the exact mechanisms driving extra-motor neurodegeneration in ALS remain largely unknown. Filling this gap in our knowledge is essential to improve the diagnosis and management of patients with ALS and may also impact end-of-life legal decisions (13, 14).

From a clinical perspective, ALS and FTLD are the two heterogeneous diseases characterized by multiple clinical presentations and highly variable rates of functional decline (15, 16). The observed phenotypic and prognostic heterogeneity hampers the diagnosis and the prediction of disease progression at the single-subject level and the design of disease-modifying treatments (15). These problems underscore the importance of developing precise, objective, and reproducible biomarkers to improve the diagnosis, prognosis, and disease monitoring (17–19). A biomarker can be defined as objective, quantifiable characteristics of a biological process (either physiological or pathological) that can be objectively measured *in vivo* (20). Biomarkers have proven to be powerful tools to increase diagnostic certainty and predict disease progression in other neurodegenerative diseases (21). Biomarkers also have the potential to reveal the critical aspects of ALS pathophysiology and advance the field toward the implementation of precision medicine approaches (22).

Despite the cumulative evidence suggesting that ALS and FTLD lie on a clinical, pathological, and genetic continuum, the pathophysiological underpinnings of this continuum remain

largely unexplored (10). This review aims to (i) summarize the clinical and pathophysiological correlates of extra-motor neurodegeneration in ALS; (ii) review the diagnostic and prognostic value of biomarkers and their potential to characterize extra-motor neurodegeneration in ALS; and (iii) discuss how biomarkers could improve the diagnosis and classification of ALS.

EXTRA-MOTOR INVOLVEMENT IN ALS

Clinical Evidence for Extra-Motor Involvement in ALS

Although the first observation of the clinical overlap between ALS and FTD dates from the beginning of the twentieth century (1, 7–9, 23–26), the study of this clinical continuum has been boosted in the last decade using the emerging neuropathological, molecular, and genetic evidence connecting the two processes (10, 27–29).

Neuropsychological Evidence

The development of specific tools to assess the cognitive status of ALS patients with variable degrees of motor impairment has led to a major breakthrough for the study of the ALS-FTD continuum (26, 30, 31). When studied with specifically designed instruments, such as the Edinburgh ALS cognitive and behavioral screen (ECAS), the ALS cognitive behavioral screen, or the Arrows and Colors Cognitive Test, up to 20% of patients with ALS can also be diagnosed with FTD, and up to 50% of patients with ALS have a cognitive or behavioral impairment (ALS_{cbi}) (6, 10, 32–36). The frequency and characteristics of cognitive and behavioral impairment resemble those noted in FTD, and recent neuropathological studies have reported frontotemporal involvement consistent with the diagnosis of FTLD in more than 30% of patients with ALS (37). Among the most common cognitive symptoms developed in patients with ALS-FTD are executive dysfunction, language impairment, and social cognition, all of which are linked to the frontal and temporal lobes (6, 33). Some neuropsychological tools (i.e., *Sydney Language Battery*, *Test for Reception of Grammar*) have proven to be useful for characterizing the language performance in patients within the ALS-FTD clinical continuum. The presence of language impairment is almost universal in patients meeting the criteria for ALS-FTD. On the other hand, patients with ALS who do not meet the criteria for ALS_{ci} or ALS-FTD may show only subtle language deficits (38–46). From a behavioral perspective, frontal behaviors like apathy, stereotypic behavior, and disinhibition are the most common behavioral features in

patients with ALS (12, 47). However, behavioral changes in patients with ALS include subtle changes in social cognition. These changes can be adequately measured with specific tests such as the *Reading the mind in the eyes test* or the *faux-pas test* (41, 48). On the other hand, up to 15% of patients initially diagnosed with FTD may also develop motor neuron signs during follow-up (10, 49). It should be noted that the frequency of motor neuron signs in patients with FTD is probably underestimated because these signs are not always systematically investigated during follow-up in FTD cohorts.

Neuropsychological testing has proven to be helpful for the clinical characterization of patients with ALS, but neuropsychological evaluations are time-consuming and may not be well-tolerated by some patients. These limitations reinforce the need for the biomarkers that help us to trace the development of extra-motor manifestations (30, 35).

Clinical Evidence

As we will discuss in Section 2.3, the neuropathological correlates of cognitive and behavioral changes in ALS are still a matter of debate (6, 12, 33, 47, 49). Several studies have consistently noted that patients with ALS and having a bulbar onset of symptoms have an increased risk of cognitive and behavioral impairment (6, 32, 50). Distal upper-limb weakness, sometimes with no upper motor neuron signs, is also frequently observed in patients meeting the criteria for ALS-FTD (51). Other reports link the development of bulbar symptoms during the disease course with cognitive and behavioral symptoms (52, 53). Interestingly, other studies have also reported a higher presence of abnormal oculomotor findings, extrapyramidal signs, and autonomic dysfunction in patients with ALS and having prominent bulbar symptoms at disease onset (54).

Genetic Determinants of Extra-Motor Involvement

Major Genetic Findings Associated With Extra-Motor Impairment in ALS

Considerable progress has been made in unraveling the genetics of ALS and FTLT, and it is now clear that the genetics of these two neurodegenerative conditions overlap significantly (55). **Table 1** summarizes the main characteristics of genes associated with both ALS and FTD etiology. These genes shed some light on several pathophysiological processes, which are relevant for extra-motor involvement in ALS. For instance, the genes involved in the lysosomal function and autophagy process may have a prominent role in both sides of the spectrum and other neurodegenerative diseases (56).

The *C9orf72* hexanucleotide GGGGCC repeat intronic expansion is the most common genetic cause of both ALS and FTLT. Some noteworthy studies reported that patients with ALS and carrying *C9orf72* have a 4–5-fold higher risk of presenting with cognitive or behavioral changes than non-carriers (57). Physiologic *C9orf72* protein is normally localized in the nuclei, and it regulates membrane trafficking. On the other hand, the hexanucleotide repeat expansion is cytotoxic. While the precise molecular mechanisms underlying cytotoxicity are not fully understood, several mechanisms have been proposed, including

a loss of function through haploinsufficiency, RNA toxic gain-of-function through the sequestration and accumulation of toxic dipeptide repeat proteins (DRP) (10, 58–63). As noted in **Table 1**, other genes causing both ALS and FTLT are related to protein trafficking, cytoskeletal dynamics, protein degradation, and mitophagy (12, 64). Similarly, genes such as *TANK-binding kinase-1 (TBK1)* (65–69), Sequestosome-1 or p62 (*SQSTM1*) (70), Optineurine protein (*OPTN*), and Valosin-containing protein (*VCP*) that are associated with FTD, inclusion body myositis, motor neuron disease, and Paget's disease encode proteins related to protein degradation (71, 72). In addition, recent studies have suggested that the apolipoprotein E (*APOE*) gene may play an important role in the development of cognitive and behavioral impairment in ALS. The $\epsilon 4$ allele of the *APOE* gene is a major genetic factor in Alzheimer's disease and has also been shown to increase the risk of TAR DNA-binding protein 43 (TDP-43) proteinopathy and hippocampal sclerosis in a large community-based cohort. These results are in line with previous evidence suggesting that the $\epsilon 4$ allele of the *APOE* gene may also increase the risk of FTLT. Interestingly, recent evidence from well-characterized cohorts of ALS showed that the $\epsilon 2$ allele of the *APOE* gene (together with the presence of *C9orf72* repeat expansion) significantly increases the risk of FTD in participants with ALS (73–76). Recent studies on the evaluation of the impact of Huntingtin (HTT) pathologic expansions in the ALS-FTD spectrum suggest an ethiopathological link (77). Similarly, rare variants in the *GBA* gene encoding glucocerebrosidase (previously described as a potential risk factor of cognitive impairment in Parkinson's disease) are also overrepresented in patients with FTD-ALS and ALS_{cbi} compared to patients with ALS and having no cognitive impairment (78).

A recent transcriptome-based study of postmortem frontal cortex tissue from patients with ALS reported a coordinated upregulation of transcripts from a sub-population of microglia (termed disease-associated microglia) and overexpressed neuroinflammatory molecules, such as YKL 40 (also known as CHI3L1) and CHI3L2. Thus, microglial and inflammatory pathways are involved in the pathogenesis of extra-motor impairment in ALS (79).

In summary, genes associated with both ALS and FTD point toward the involvement of pathophysiological processes outside the motor regions that are relevant for extra-motor involvement in patients with ALS.

Genetic Findings Associated With Extra-Motor Sparing in ALS

It should be noted that the overlap in genes associated with ALS and FTLT is not complete. For example, superoxide dismutase 1 (*SOD1*), *FUS*, and *TARDBP* variants are most commonly associated with ALS and only rarely cause FTLT. Similarly, *GRN* is linked to the TDP-43 subtype of FTLT but not ALS (80). Interestingly, the reduced expression of *GRN* has been recently identified as a possible contributing factor in the development of different neurodegenerative diseases including ALS (81). In addition, experiments performed in animal models have identified the reduced expression of *GRN* as a sufficient factor to induce TDP-43 deposition (82). The gene encoding *SOD1* is

TABLE 1 | Genes in close association with both amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) etiologies.

Gene	Location	Pathway	MI
<i>C9orf72</i>	9p21.2	Nucleocytoplasmic transport/splicing	AD
<i>TBK1</i>	12q14.1	Autophagy/inflammation	AD
<i>TARDBP</i>	1p36.2	Nucleocytoplasmic transport/splicing	AD
<i>SQSTM1</i>	5q35.3	Autophagy	AD/Risk
<i>VCP</i>	9p13.3	Autophagy/mitochondrial function	AD
<i>FUS</i>	16p11.2	Nucleocytoplasmic transport/splicing	AD/AR
<i>UBQLN2</i>	Xp11	Autophagy/proteasome	XL
<i>CHCHD10</i>	22q11.2	Mitochondrial dysfunction/synaptic integrity	AD

MI, mode of inheritance; AAO, age at onset; DM, disease modifier; AD, autosomal dominant; AR, autosomal recessive; XL, X-linked.

rarely related to extra-motor impairment in ALS albeit that some mutations have been recently associated with an early cognitive impairment (83). *SOD1* is essential for antioxidant defense in the cytosol and mitochondria (84). Non native formations of *SOD1* have been detected in small granular *SOD1*-immunoreactive inclusions in the motor neurons of sporadic ALS patients without pathogenic *SOD1* variants and in patients carrying other ALS-associated genes (85, 86). These findings suggest that the misfolding of *SOD1* can be part of a joint downstream event in motor neuron neurodegeneration.

Finally, neuropathological findings in sporadic patients with ALS and having *FUS* aggregates resemble those of patients with FTLN and having *FUS* deposition (87). However, mutations in the *FUS* gene are related to ALS and rarely cause FTLN (88). To date, the reasons behind the relative sparing of extra-motor regions in patients with ALS and having *FUS* mutations remain unclear.

Interestingly, some genes that are strongly associated with FTLN have scarcely been studied in the ALS-FTL spectrum, where their role remains unclear. This is the case for *MAPT* mutations, which cause the FTLN tau subtype, and *GRN* and *TREM2* mutations, which have been associated with the TDP-43 subtype and Alzheimer's disease (89–91).

Neuropathological Underpinnings of Extra-Motor Neurodegeneration

Neuropathological Changes in ALS

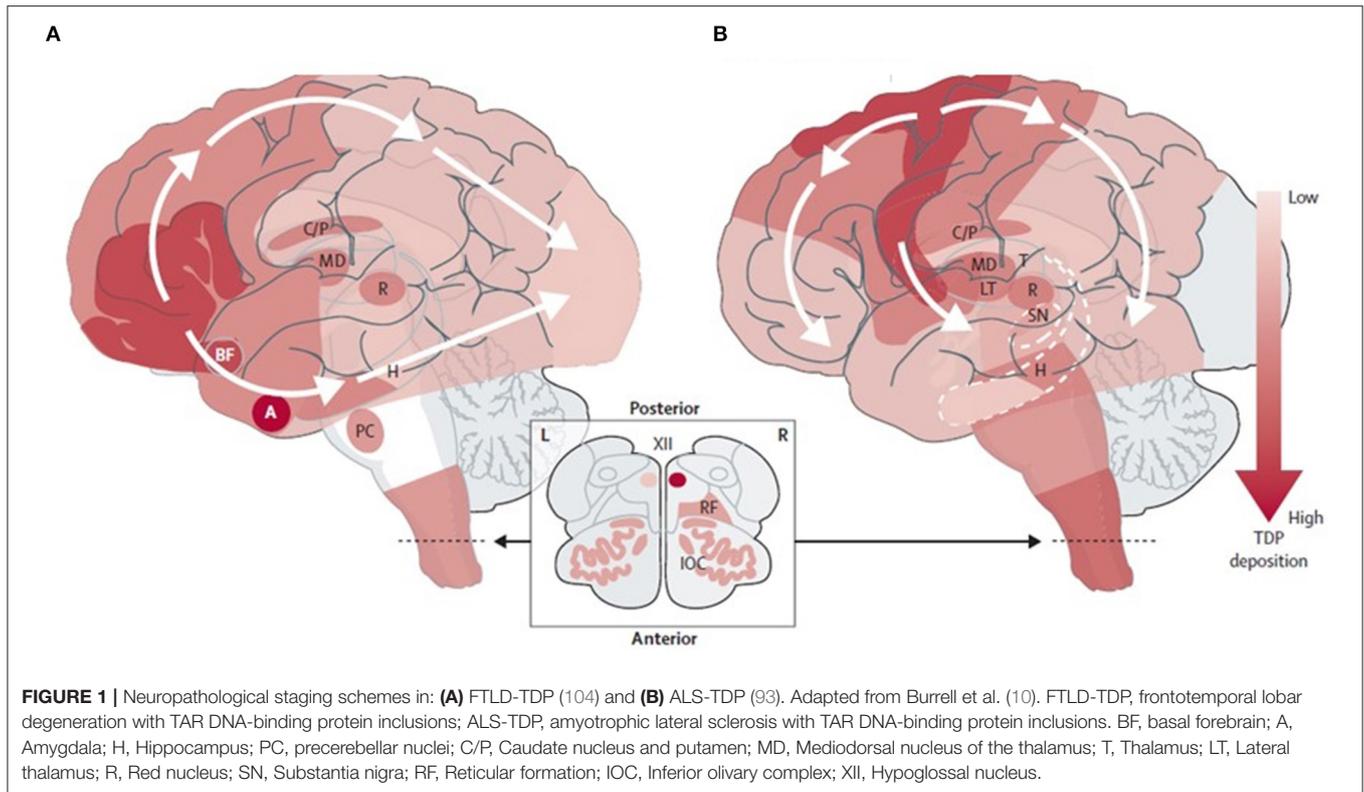
From a pathological perspective, TDP-43 aggregation is a frequent pathological feature of FTLN and the most typical neuropathological finding in patients with ALS (92–96). The pathological hallmarks of TDP-43 proteinopathies include mislocalization from the nucleus to the cytoplasm, deposition of ubiquitinated and hyperphosphorylated TDP-43 into inclusion bodies, protein truncation leading to the formation of toxic C-terminal TDP-43 fragments, and protein aggregation (97). ALS and FTLN with TDP-43 inclusions can be subdivided into different subtypes based on the anatomical distribution and morphology of abnormal TDP-43 aggregates. *Type A* is observed in patients with *GRN* mutations and is characterized by round “compact” intracytoplasmic TDP-43 inclusions, dystrophic short neurites, and occasional lenticular intranuclear inclusions, mainly in the upper cortical layers. *Type B* is the most frequent

subtype in patients with motor neuron diseases and shows many granular TDP-43 inclusions with a few dystrophic neurites in all the cortical layers. *Type C* shows long dystrophic neurites and a few intracytoplasmic inclusions. Finally, *type D* histopathology is associated with a mutation in *VCP*, which causes familial inclusion body myositis, Paget's disease of bone, FTLN with or without motor neuron disease involvement (98–100). Lee et al. reported a new histopathologic subtype for TDP-43 aggregates (*type E*) with specific TDP-43 aggregates, a uniform biochemical profile, and a rapid clinical course (101).

Misfolded TDP-43 propagation is believed to follow a “prion-like” mechanism, seeding of the native protein misfolding *in vitro* and synaptic transmission to the next neuron (93, 94, 102–105). This protein deposition is associated with neuronal and synapse loss and affects the different brain regions in ALS and FTLN, at least initially. In patients with ALS, the phosphorylated TDP-43 inclusions spread initially from motor neurons located in the motor cortex, spinal cord, and brainstem motor nuclei to other neocortical areas, cerebellum, and striatum (93). In contrast, in FTLN with TDP-43 inclusions, this spread begins in the amygdala and orbitofrontal cortex, spreading to the temporal and frontal non-motor cortex first and motor cortex later, and finally reaching the visual cortex (10, 93–95) (**Figure 1**). Most studies have shown moderate TDP-43 inclusions before synapse and neuronal loss in animal models (94); however, an intriguing minority of neurons may lack detectable nuclear TDP-43 despite the apparent absence of a cytoplasmic TDP-43 inclusion. These cells show neuronal atrophy comparable to inclusion-bearing neurons, suggesting that the loss of nuclear TDP-43 function promotes neurodegeneration even when TDP-43 aggregation is inconspicuous or absent (106).

Neuropathological Correlates of Cognitive and Behavioral Changes in ALS

Pathological aggregates of TDP-43 in extra-motor cortical and subcortical areas are believed to play an essential role in cognitive impairment in ALS. The burden of pathological TDP-43 pathology in extra-motor regions is higher in patients with ALS_{cbi} and ALS-FTLN than those with ALS_{no-cbi} (39, 107, 108). Also, synapse loss has been identified using high-resolution imaging of postmortem brain tissue samples in extra-motor cortical areas, such as the prefrontal cortex of sporadic



patients with ALS (109). Notably, the degree of synapse loss correlated with the severity of the patients' cognitive impairment independent of cortical atrophy, supporting the idea that synapse loss precedes neuronal loss (109, 110). Finally, Tau pathology and hippocampal sclerosis may also play a secondary role in the presence of cognitive impairment in patients with ALS (37, 111, 112).

Neuroinflammation

Neuroinflammation in ALS and FTD is primarily characterized by the activation of innate immune sensing pathways in microglia and astrocytes resident to the central nervous system but also with the involvement of peripheral-derived inflammatory cells, such as T-lymphocytes, mast cells, monocyte-derived macrophages, and dendritic cells (113–116). As part of the innate immune system, some components of the innate complement system have been identified in postmortem studies and their eventual role in disease progression (117, 118). Also, microglial density and microglial activation measured using immunostaining for glial fibrillary acidic protein (GFAP) and CD6, show an increase in patients with ALS and FTD and correlate well with synaptic, axonal, and neuronal loss and faster motor and extra-motor disease progression (119–121). There is also a higher presence of reactive astrocytes, which are believed to have neurotoxic properties and have been described involving the gray matter of motor and non-motor cortex in patients with ALS and also in affected cortical areas in FTD (122–124). In addition, oligodendrocytes pathology mediated by phosphorylated

TDP-43 deposition in postmortem tissue is a common finding in multiple cortical and subcortical areas of patients with ALS, but its significance remains uncertain (125, 126). Taken together, these findings highlight the importance of concurring neuroinflammatory processes in the pathophysiology of both ALS and FTD.

Other Neuropathological Hallmarks of ALS: C9orf72, FUS-FET

As aforementioned (refer to *Genetic determinants of extra-motor features*), patients carrying C9orf72 mutation have been associated with more cognitive impairment and more extensive TDP-43 deposition following predominantly a Type B or a Type A + B pattern (93, 127). A p62 and ubiquitin negative intracytoplasmic and intranuclear inclusions corresponding to dipeptide repeat are found in extra-motor areas, such as the frontal cortex, hippocampus, and cerebellum (12, 62, 110, 128). Interestingly, some reports have also identified C9orf72 patients with ALS and FTD presenting with cognitive impairment but with minimally TDP-43 inclusions underscoring the influence of other physiopathological mechanisms such as dipeptide repeat accumulation and RNA foci in the appearance of cognitive impairment (63, 108).

Conversely, in the case of FUS protein deposition neuropathology, it is believed that the pathological processes underlying the aggregate formation and cell death between FTD and ALS differ. While ALS with FUS mutations seems to be more restricted to the dysfunction of FUS protein, a more complex dysregulation that includes the deposition of all FET proteins

(TAF15 and EWS) and Transportin-1 (Trn1) is thought to be involved in the subtypes of FTLT with FUS pathology (88).

BIOMARKERS

Biomarkers can be used for four primary purposes: (i) to guide clinical diagnosis by identifying the key pathophysiological changes of a particular disease (so-called diagnostic markers), (ii) to estimate the risk or speed of progression of a particular disease (prognostic markers), (iii) to monitor the progression or response to therapy (theragnostic markers) (129), and (iv) to characterize the relevant aspects of disease pathophysiology (i.e., inflammation). Section Fluid Biomarkers will describe the currently available biomarkers for the study of extra-motor neurodegeneration in the ALS-FTLD continuum and their diagnostic and prognostic value.

Fluid Biomarkers

The cerebrospinal fluid (CSF) represents an invaluable sample for studying neurodegenerative diseases due to its close relationship with brain parenchyma (130). However, the study of CSF biomarkers may be limited because of their invasive nature. Recently, blood biomarkers have shown promise as minimally invasive clinical tools in neurodegenerative diseases (129, 131). Compared to CSF, the accessibility and cost-effectiveness of blood samples make them more suitable for first-line clinical use and facilitate clinical trial recruitment and monitoring.

Neurodegeneration Biomarkers

Neurofilaments are the principal constituents of the neuro-axonal cytoskeleton and play an essential part in axonal transport and the synapse (132). Neurofilament light chain (NfL) is the most abundant and soluble neurofilament subunit, and increased levels in CSF and blood reflect axonal damage in the CNS. The CSF and plasma levels of NfL are highly correlated and have been found to increase in a wide variety of diseases (133, 134). Both CSF and plasma levels of NfL are increased in patients with ALS and have proven helpful to differentiate between patients with ALS and other ALS mimics (135–137). Importantly, multimodal biomarker studies have shown that the CSF and plasma levels of NfL correlate well with frontotemporal cortical thickness and white matter microstructure in patients within the ALS-FTD continuum (138–140). The CSF and blood levels of NfL also correlate with measures of disease severity, disease progression rate, and the prediction of longitudinal clinical deterioration and survival (21, 137, 138, 141–148). The CSF NfL levels seem to increase over time but further longitudinal data are needed (149, 150). Moreover, the combination of plasma NfL with other measurements of neuroinflammation in plasma may improve disease progression at the single-subject level (151–153). Because biofluid levels of NfL are strongly correlated with age, recent multicenter studies have determined highly specific age-adjusted cut-offs (154). Overall, both CSF and plasma levels of NfL may be helpful to support the diagnosis in clinically relevant scenarios, disease staging, and the prediction of disease progression. In addition to NfL, neurofilament heavy chain (NfH) has also been studied in patients with ALS and can discriminate between ALS,

ALS mimics, and healthy controls with similar accuracy to NfL (155). Of note, a recent study showed that the difference in the abundance of NfH between ALS and FTD was markedly stronger for NfH than that of NfL (155). On the other hand, studies have reported a better correlation between the serum and CSF for NfL compared to NfH, albeit that NfL has been more widely investigated (156). Overall, CSF NfL and NfH as well as serum NfL are equally suited for the differential diagnosis of ALS, whereas serum NfH performs less well (155). Other serum biomarkers, such as peptides linked with eating behavior and metabolism, for instance, leptin and neuropeptide-Y, have been recently studied in the ALS-FTD continuum and show a promise as neurodegeneration biomarkers, but further investigations are needed to clarify their role in the pathophysiology of ALS and FTD (157).

Patients within the ALS-FTD clinical continuum also show increased levels of total tau (t-tau) in CSF but the diagnostic and prognostic value of this biomarker is lower than NfL (145, 158). Some studies have reported that low levels of both phosphorylated tau at threonine 181 (p-tau) and the p-tau:t-tau ratio in CSF may differentiate TDP-43 and tau subtypes of FTLT (159–162), but these findings seem to be driven by the higher levels of t-tau in patients with ALS (162–164) and not by lower levels of p-tau in FTLT-TDP (165).

Synapse Degeneration Markers

The amyloid precursor protein (APP) is a type I single-pass transmembrane protein with a large extracellular domain and a short cytoplasmic tail that undergoes very complex proteolytic processing, yielding biologically active fragments (166). These include the full-length A β 1–42 and other N-terminal and C-terminal truncated forms of A β , sAPP α , sAPP β , etc., that can be detected in human CSF (167–169). Numerous studies suggest that reduced neuronal-synaptic activity may also lead to less A β production (170, 171) and that A β may also have an important role in synaptic function (172). Recent studies on autopsy-confirmed cases have reported reduced CSF concentrations of sAPP β in FTLT in the absence of comorbid Alzheimer's pathology (173). Interestingly, CSF concentrations of sAPP β are decreased in ALS and FTD compared to healthy controls and correlate with frontotemporal cortical thickness and cognitive impairment suggesting that sAPP β levels may reflect the neuronal loss in frontotemporal areas (140, 174). Unfortunately, plasma concentrations of sAPP β poorly correlate with CSF concentrations (175), potentially due to a confounding effect of abundant non-CNS-derived sAPP β in the blood. On the other hand, CSF concentrations of neurogranin, the most studied synaptic marker to date, were not elevated in patients with ALS (176). In another study, label-free mass spectrometry-based proteomic analyses of CSF from patients with ALS revealed a concomitant decrease in proteins from biological pathways related to synapse organization and inflammation-related proteins (177). Recently, other synaptic markers have been identified by unbiased proteomics-based approaches. Still, more studies are needed to clarify the role of these new biomarkers and their potential use for the diagnosis, staging, and prognosis of patients with suspected ALS (178, 179).

Biomarkers Linked to Neuroinflammation and Glial Activation

A variety of systemic inflammatory responses (pro- and anti-inflammatory cytokine profiles, altered immune cell populations) have been consistently reported in patients with ALS and FTD (113, 180). These responses do not seem to be a mere passive phenomenon and may contribute to the neurodegeneration/neuroprotection balance (181, 182). For instance, increased CSF and serum levels of some monocyte-derived cytokines linked with the inflammatory response such as TNF- α , IL-1 β , IL-17, and TGF- β reveal a microglial and white blood cell participation in the ALS-FTD pathologic continuum (113, 182, 183). In addition, increased CSF levels compared to controls of Matrix Metalloproteinase-10 and decreased CSF levels of some chemokines and growth factors (such as IL-8, IL-12B, TGF- α among others) have been recently reported in a multicentric study (184). Other surrogate markers of acute-phase systemic inflammation have been reported in ALS, including lipopolysaccharide-binding protein, C reactive protein, and soluble CD14 (181, 185).

In addition to a systemic inflammatory response, several biomarkers in the blood and CSF have been found to be consistently altered in the ALS-FTD continuum (186). Some of these biomarkers may be useful to predict disease progression and survival (140, 187). Increased CSF levels of YKL-40 (also known as Chitinase like-3 protein 1) in ALS were first observed in a small study (188), a finding that has been later replicated in larger studies involving participants along the ALS-FTD continuum (140). Recent proteomic studies confirmed that three macrophage-derived chitinases (YKL-40, chitotriosidase, and chitinase like-3 protein 2) were increased in ALS and predicted a faster disease course but only YKL-40 levels increased over time in those with low initial levels (187). These results support a crucial role for microglial activity in ALS (152), offering novel target engagement and pharmacodynamic biomarkers for neuroinflammation-focused ALS therapy. However, YKL-40 is not specific to the central nervous system, and their levels in the CSF and blood are poorly correlated, thus limiting its use as a non-invasive biomarker. Conversely, GFAP is a brain-specific protein and an established marker of astrogliosis. This protein can also be accurately measured in the blood with new ultrasensitive technology (189). Interestingly, different neuroinflammatory profiles between ALS and FTD have been recently reported, with increased CSF levels of the chitinase chitotriosidase 1 in ALS and high levels of GFAP in FTD (190).

The microglial transmembrane receptor (TREM2) represents another important molecule linked to microglial activity. One study reported that the *TREM2* p.R47H genetic variant may represent a risk factor for sporadic ALS (191), but this finding has not been replicated in subsequent studies (192, 193). In addition, the soluble part of the microglial transmembrane receptor (sTREM2) is measurable in the blood and CSF, but the information regarding its role within the ALS-FTD continuum is scarce (194). Finally, immune innate complement factors involved in the inflammatory process have been studied in the CSF of patients with ALS finding an upregulation of C3 in CSF

and increased levels of C3 cleavage products in the serum of patients with ALS (195, 196).

Biomarkers Linked to Abnormal TDP-43 Aggregation

Cytoplasmic aggregation of ubiquitinated, phosphorylated, and truncated TDP-43 is a unifying pathologic observation across the neuropathological continuum of ALS and FTL. However, standard immunoassays of TDP-43 have not proven to be useful to identify patients with pathological TDP-43 aggregation, and it is not yet clear whether TDP-43 would be expected to increase in the CSF due to the degeneration of nerve cells and the release of intracellular proteins or whether it would be expected to be low in the CSF due to aggregate formation (197–199). This is because most currently available techniques for quantifying TDP-43 use commercially available antibodies, which only detect the full-length form of TDP-43 rather than the disease-specific forms of TDP-43 (200, 201). However, several recent advances hold promise to the detection of abnormal TDP-43 aggregation *in vivo*. A recent study used immuno-IR sensors to study the secondary structure of all TDP-43 isoforms in the CSF (202). In addition, a recent study showed that real-time quaking-induced conversion reaction (RT-QuIC) antemortem prion detection was a robust technique for prion amplification of TDP-43 (203).

Some promising biomarkers like truncated Stathmin-2 (STMN2) are linked to TDP-43 function and deposition. The expression of STMN2 is increased in the frontal cortex of patients with the TDP subtype of FTL, which makes it a good candidate biomarker related to TDP pathology (204). Finally, in *C9orf72* carriers, high CSF levels of DRPs can be detected even in pre-symptomatic stages, suggesting a role of DRPs early in ALS-FTD pathogenesis in early stages (145, 205). Although not currently relevant as clinical fluid biomarkers, DRPs may become more widely used in detecting the *C9orf72* expansion prior to genetic screening or in prodromal stages, which would make a valuable addition to the biomarker arsenal in the future clinical trials.

Image-Based Biomarkers

Macrostructural and Microstructural Changes in MRI

Routine structural MRI has a limited utility in ALS as signal intensity, and gross volume changes in T1- and T2-weighted images are not typically observed in most ALS cases. On the contrary, quantitative analysis of MRI can improve the detection of neurodegeneration-related abnormalities by measuring small changes in cerebral structure. The *in vivo* quantification of motor and extra-motor cortical cerebral changes with MRI analyses is of great value in monitoring disease progression in the ALS-FTD continuum (Figure 2) (206–208). In sporadic ALS cases, recent meta-analyses of neuroimaging studies report consistent gray matter loss in the precentral gyrus, inferior frontal gyrus, cingulate/paracingulate gyrus, and rolandic operculum (209, 210). This cortical thinning in extra-motor cortical areas correlates with cognitive and behavioral impairment in the neuropsychological test (211, 212). On the other hand, *C9orf72* cases and a subgroup of sporadic cases without the *C9orf72* expansion are characterized by widespread cortical changes characterized by cortical thinning involving bilateral

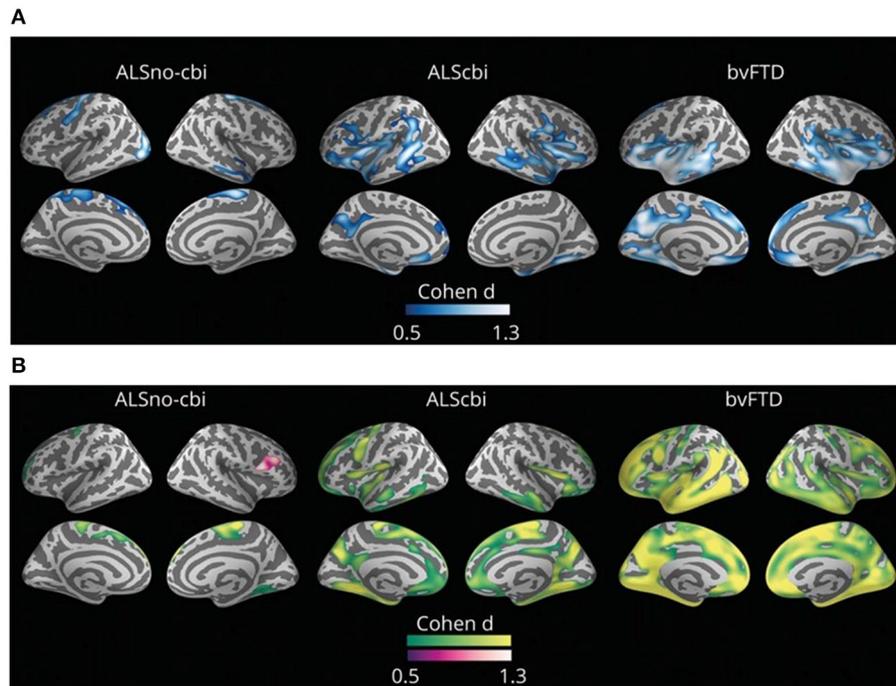


FIGURE 2 | Group comparisons between those with amyotrophic lateral sclerosis (ALS) without cognitive or behavioral impairment (ALSno-cbi; left column), those with ALS with cognitive or behavioral impairment (ALScbi; middle column), and those with the behavioral variant of frontotemporal dementia (bvFTD; right column) compared to controls for **(A)** cortical thickness and **(B)** cortical mean diffusivity. Regions in blue represent thinner cortex; regions in green represent higher cortical mean diffusivity; and regions in purple represent lower cortical mean diffusivity. All analyses were adjusted for age, sex, education, and MRI equipment. Only clusters that survived family-wise error correction $p < 0.05$ are shown. ALSno-cbi, Amyotrophic Lateral Sclerosis without cognitive and behavioral impairment; ALScbi, Amyotrophic Lateral Sclerosis with cognitive and behavioral impairment; bvFTD, Behavioral variant of frontotemporal dementia.

pars opercularis, fusiform, lingual and parietal cortex, and also smaller volumes in the right hippocampus and bilateral thalamus (213, 214).

Interestingly, Dadar et al. were able to replicate the neuropathological stages of TDP-43 *in vivo* with a novel neuroimaging technique (215). However, some neuroimaging methods, which quantify brain atrophy (i.e., gray matter loss or cortical thickness), show limited sensitivity to the earliest cortical changes related to cortical dysfunction (158, 216). Diffusion tensor imaging (DTI) is a diffusion weighted imaging sequence that measures the diffusion of water molecules in the different directions of space (217). The main advantage of DTI is that it is able to detect subtle changes at the microstructural level reflecting the breakdown of biological barriers and neuroinflammation (218, 219). Thus, DTI studies allow the study of microstructural changes of the cerebral cortex (i.e., cortical mean diffusivity) and the white matter (i.e., microstructural changes in different white matter tracts). Several studies on neurodegenerative diseases, including FTD, have shown that DTI can reveal pathology that is not detected with cortical thickness measurements (216, 219–223). A recent cross-sectional study showed that ALScbi displayed cortical mean diffusivity changes in extra-motor regions in the absence of cortical thinning and showed a better correlation with cognition than with cortical thickness. These results suggest that cortical mean diffusivity may be more sensitive than cortical thickness to detect extra-motor cortical

changes in ALS, but more studies are needed to characterize the longitudinal changes of cortical microstructure in ALS (158). On the other hand, longitudinal studies using DTI to study the white matter microstructure in ALS have shown the usefulness of this approach to detect extra-motor changes and monitor disease progression (222, 224–226).

Functional Magnetic Resonance

fMRI based on the measurements of the fluctuation in blood flow and blood oxygen level as a consequence of neuronal activity helps us to trace the underlying networks between the areas that coactivate simultaneously. Assessing the resting-state brain connectivity in patients with ALS has revealed either decreased or increased connectivity in premotor, sensorimotor, and basal ganglia (227, 228). Advanced network-based neuroimaging techniques based on connectomics and a whole-brain connectivity analysis have shown different patterns of motor and extra-motor involvement between different MND phenotypes (229).

Magnetic Resonance Spectroscopy

MRS provides a means of measuring cerebral metabolites relevant to neurodegeneration *in vivo* [for a review, Kalra et al. (230), Ernst et al. (231)]. In ALS, neurochemical changes reflecting neuronal loss or dysfunction (i.e., decreased N-acetylaspartate) are mostly significant in the motor cortex and

corticospinal tracts. Other neurochemical changes observed include increased myo-inositol, a putative marker of gliosis. Some studies also reported cerebral metabolite changes in extra-motor regions. Previous studies performed in asymptomatic genetic carriers of mutations associated with FTL using MRS-derived markers exhibit an encouraging discriminatory ability to identify patients from healthy controls; however, more data are needed to determine their ability to assist with the diagnosis in early stages and in distinguishing from disease mimics (232–239).

PET: 18F-Fluorodeoxyglucose Radiotracer

PET represents a powerful imaging biomarker for the study of neurodegenerative dementias (240, 241). An increasing number of 18F-labeled tracers are not available for use at the clinical site without the requirement of an on-site cyclotron, thus turning brain PET scans into a widely applicable tool. Brain hypometabolism can be detected with PET and the 18F-fluorodeoxyglucose radiotracer (18F-FDG-PET). Of note, 18F-FDG-PET imaging has some advantages over MRI as it can be performed in patients needing mechanical ventilation. Cerebral metabolism reflects the local intensity of brain glutamatergic synaptic and astrocyte activity, and cerebral hypometabolism is considered an indirect marker of neurodegeneration (240, 242). In ALS, previous studies investigating 18F-FDG-PET revealed a marked frontal and prefrontal relative hypometabolism in ALS_{cbi} when compared to patients with ALS_{no-cbi} (243, 244). Unexpectedly, previous studies also reported hypermetabolism in the midbrain, temporal poles, and hippocampus (245). It has been hypothesized that hypermetabolism in patients with ALS may be related to microglial activity in these areas but more multimodal biomarker studies are needed to understand the pathophysiological underpinnings of cortical and subcortical metabolism in ALS (206, 245, 246). Overall, previous studies support the view that 18F-FDG-PET could be a useful tool to assess the spread of brain pathology *in vivo*.

PET to Detect Neuroinflammation

Multiple imaging studies have reported *in vivo* microglial activation in motor and extra-motor regions in both ALS and FTD using different radiotracers with affinity to the translocator protein or TSPO (which is expressed in the mitochondria of activated microglia) (247–250). Nevertheless, some caveats of those radiotracers might influence their usefulness; the most used radiotracer 11C-PK11195, which labels peripheral benzodiazepine binding sites, has a low signal-to-background ratio and binding of the subsequent generation of radiotracers (11C-PBR28, 18F-DPA714, and 18F-FEPPA) is influenced by the TSPO polymorphism (247, 250–252). Thus, more work is needed for the validation of radiotracers that can detect and monitor microglial activation *in vivo*.

PET to Detect TDP-43 Pathology

The development of a radiotracer able to bind phosphorylated TDP-43 and allow the *in vivo* visualization of pathological TDP inclusions would be a major breakthrough in the field. Unfortunately, such a biomarker is not available at this moment (251).

OTHER BIOMARKERS

Neurophysiological Biomarkers

Neurophysiological studies have shown that hyperexcitability of the frontotemporal cortex is an early feature of ALS that may precede motor neuron degeneration and can be assessed using transcranial magnetic stimulation with single or multiple pulses (253). Transcranial magnetic stimulation with multiple pulses evaluating the short-interval intracortical inhibition is one of the most robust tests developed for assessing cortical hyperexcitability and has shown good sensitivity and specificity for ALS diagnosis and a good correlation with other biomarkers of peripheral neurodegeneration (254, 255). Recent studies have demonstrated a more prominent cortical hyperexcitability in patients with ALS presenting with cognitive symptoms than in cognitively unimpaired patients with ALS (256). Hyperexcitability in the sensory cortex may induce increased amplitudes that can be detected early in ALS and correlate with a shorter disease survival (257). Some groups have tried to assess cortical connectivity and the interaction between the motor and non-motor network using other techniques, such as electroencephalography and fMRI, but the clinical utility of these techniques is still limited (258, 259).

Genetic Biomarkers

MicroRNAs are short (about 22 nucleotides in length) non-coding RNA molecules that play a relevant role as endogenous regulators of gene expression (260). Emerging studies have shown that circulating microRNAs can serve as potential biomarkers in ALS. However, there are still many problems to be solved before microRNAs could be used in clinical practice [for a detailed review please refer to Wang and Zhang (261)].

DISCUSSION: ADVANCING ALS CLASSIFICATION WITH BIOMARKERS

Phenotypic classification of ALS relies on clinical observations such as site and region of onset and predominance of upper or lower motor neuron signs, while the diagnosis of FTD is based on clinical criteria (2, 24). Although ALS and FTD share partially overlapping patterns of neurodegeneration, the generation of clinically defined subtypes is not straightforward due to the observed clinical and prognostic heterogeneity. The definition of precise and reproducible classification systems for both ALS and FTD is urgently needed to guide present and future treatments, provide an indication of prognosis and enable the analysis in clinical trials of homogenous groups for a more personalized approach to therapy (24, 262). These refined classification systems would ideally consider the etiology (i.e., proteinopathy responsible for the patient's symptoms), other clinical, or biological features for robust disease staging at the single-subject level (i.e., scales measuring a motor or cognitive function, or frontotemporal atrophy burden on neuroimage) and finally the quantification of other pathophysiological processes that are relevant for prognosis (i.e., microglial activation at diagnosis). In the following section, we will discuss how biomarkers (alone or in combination with multimodal biomarkers studies) could

be integrated into multidimensional classification systems to further improve the diagnosis and prognosis of these complex neurodegenerative diseases.

Etiology: Early Identification of TDP-43 Proteinopathies

There is no definitive diagnostic test for ALS or due to abnormal TDP-43 aggregation (1). Because clinical-pathological correlations are imperfect, biomarkers, which can detect abnormal TDP-43 aggregates *in vivo*, are needed to provide an early etiological diagnosis (i.e., ALS caused due to abnormal TDP-43 vs. FUS or tau aggregates in FTD). Recently developed RT-QuIC assays for TDP-43 are promising (203). Concomitant TDP-43 pathology can also be seen in a significant number of patients with Alzheimer's disease neuropathological changes, as well as in a smaller proportion of other neurodegenerative diseases (263–268). Thus, an understanding of how abnormal TDP-43 aggregates impact brain function may also be valuable to understanding its role in other neurodegenerative dementias. Of note, pathological TDP-43 aggregates are a key neuropathological finding in the recently described neuropathological entity named “Limbic predominant Age-Related TDP-43 pathology encephalopathy” or simply “LATE” (269). This new neuropathological construct may account for up to 22% of the attributable risk of developing dementia of the Alzheimer's type and has shown to impact the clinical presentation and prognosis in patients with Alzheimer's disease neuropathological changes. However, it is unclear whether “LATE” is the underlying pathology in cases with the FTLTDP-43 subtype (263, 270).

A recent study on neuropathologically confirmed FTLTDP-43 suggests the existence of disparate patterns of gray and white matter involvement in FTLTDP-43 subtypes, with more prominent involvement of white matter in tauopathies than in TDP-43 proteinopathies (271). We envision that the combination of cortical mean diffusivity with additional measures of subcortical white matter microstructure and the use of fluid-based biomarkers of neurodegeneration and synaptic loss (i.e., NFL, sAPP β) could reveal important insights into the etiology of neurodegeneration by identifying specific neurodegenerative signatures *in vivo*. This approach may also improve clinical-pathological correlations in some challenging clinical scenarios. For example, it may help identify patients presenting with a non-fluent agrammatic variant of primary progressive aphasia with underlying TDP-43 pathology and possibly benefit from future protein-specific treatments (272).

Disease Staging: Looking Beyond Corticospinal Tract Neurodegeneration

Imaging studies have consistently reported that extra-motor neurodegeneration in ALS is related to cognitive impairment and that the frequency of cognitive and behavioral impairment seems to increase with disease progression (33). Prefrontal changes detected with MRI *in vivo* predict the progression rate, and recent studies suggest that the topography of extra-motor atrophy in patients with ALS

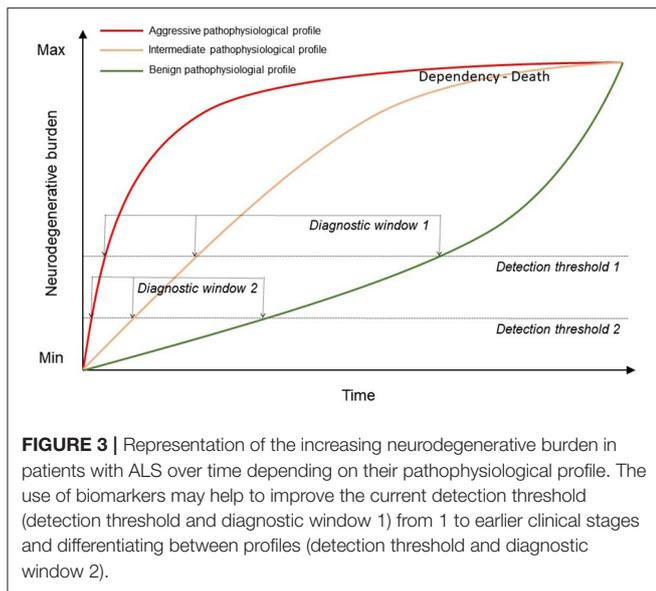
parallels the neuropathological stages of TDP-43 (215). However, neuroimaging studies investigating the structural correlates of cognitive and behavioral impairment have failed to define a consistent topography of extra-motor involvement (210, 212, 273). One limitation of previous studies is the application of neuroimaging techniques that may not capture the earliest cortical changes driving cognitive and behavioral impairment in ALS such as gray matter density or cortical thickness (274). On the contrary, other DTI-based MRI biomarkers have been shown to be sensitive to the earliest neurodegeneration-related changes and may be of great value to monitor disease progression (158, 207). More studies are needed to determine the potential utility of DTI-based imaging techniques for quantifying disease burden within first and second motor neurons and extra-motor neurodegeneration. This information could allow the refinement of current classification systems and advance the field toward a multidimensional approach for the classification of neurodegenerative diseases (275).

The Relationship Between Astroglial Activity and Disease Progression in the ALS-FTD Continuum

As discussed previously, several biofluid biomarkers have proven to be useful to predict disease progression in patients with ALS. Due to the role of microglial reactivity in the neurodegenerative process, YKL-40 might be useful for the prognostic stratification in the ALS-FTD spectrum (187). The combination of multiple biomarkers, such as CSF cytokines and innate complement system fragments in a single study may provide important clues to understand the role of neuroinflammation in ALS and FTD pathophysiology. However, one important limitation of biofluid biomarkers is that they do not provide information regarding the topography of neurodegeneration (190). A recent study combined structural MRI with the use of a PET ligand to measure microglial activation and showed that the areas of ligand binding were correlated with motor signs, cortical thinning, reduced fractional anisotropy and increased diffusivity (248). Interestingly, this study did not identify a change in ligand binding in subjects undergoing repeat imaging over 6 months despite clinical progression, suggesting that glial activation is present early and does not change at least in terms of TSPO imaging. This study illustrated the potential of multimodal biomarker studies to understand the role of neuroinflammation in ALS. More studies and better tools are needed to clearly define how neuronal-glia signaling occurs at the different stages of the disease, and where potential therapeutic interventions can be made to modulate inflammation to slow disease progression (113).

Beyond TDP-43: Identifying Fast and Slow Progressors

Although TDP-43 is the major neuropathological hallmark of the disease in most ALS cases, other pathophysiological



processes play an important role. Clinical-pathological studies investigating the relationship between pathological aggregates of TDP-43 and cognitive impairment have revealed conflicting results (33, 108). However, a recent study reported that pre-mortem cognitive performance was better correlated with postmortem synaptic density than with postmortem cortical thickness or neuronal loss (109). In addition, cognitive and behavioral changes were better correlated with cortical microstructure than with cortical thickness in a recent neuroimaging study involving patients from the ALS-FTD clinical continuum (158).

Of note, early neurodegenerative changes and synapse loss can be captured with novel radiotracers capturing synaptic density but to date, these novel imaging methods have not been assessed in ALS (276, 277). Taken together, these findings suggest that synaptic loss and other neurodegeneration-related changes may antedate cortical atrophy and pathological aggregates of TDP-43 but additional studies are needed to clarify the relationship between non-motor symptoms, synaptic density, cortical microstructure, and cerebral metabolism. In addition, the exact significance of cortical mean diffusivity changes in ALS is unknown. Future multimodal biomarker studies combining ^{18}F -FDG-PET, cortical mean diffusivity, and measures of microglial activity and neuroinflammation will be helpful to understand the pathophysiology of the earliest microstructural changes in ALS.

Figure 3 illustrates the potential use of biomarkers to advance the diagnosis of ALS in earlier clinical stages when future targeted therapies may be more effective. The information provided by biomarkers at diagnosis could also be considered for the prediction of disease progression and the selection of candidates for clinical trials. This information may be crucial for the candidate selection for future trials targeting a specific aspect of ALS pathophysiology (i.e., anti-inflammatory drugs). On the

other hand, the development of novel imaging biomarkers more sensitive than conventional imaging biomarkers may increase our sensitivity for the detection of FTLT-related neurodegenerative changes by lowering the detection threshold. This, in turn, may allow at diagnosis the earliest clinical stages. Finally, pathophysiological biomarkers measuring the key aspects of the neurodegenerative process (i.e., neuroinflammation or FTLT subtype) could play an important role in future multimodal classification schemes, similar to the recently proposed biomarker-based classification systems for other neurodegenerative diseases (278).

CONCLUSIONS

The fact that some patients diagnosed with ALS develop cognitive and behavioral changes while others remain cognitively intact is intriguing. This observation may provide important clues to the specific vulnerability of the frontotemporal cortex in neurodegenerative dementias. As we have shown in this review, biomarkers have the potential to improve our understanding of the pathophysiological underpinnings of extra-motor neurodegeneration in ALS. Emerging imaging biomarkers have shown a significant potential to improve the diagnosis and staging of patients within the ALS-FTD clinical continuum. In addition, several biofluid biomarkers have the potential to increase the diagnostic certainty of underlying ALS or FTLT, predict disease progression at the single-subject level, and characterize the pathophysiological underpinnings of extra-motor neurodegeneration in ALS. Future studies for determining the benefit of current and future biomarkers to advance ALS classification and monitoring are the essential steps for improving the design of clinical trials testing novel disease-modifying treatments.

AUTHOR CONTRIBUTIONS

II-G and DR-L designed and drafted the manuscript. OD-I, EC-V, JT-S, NL, RB, OB, VM, DA, JF, AL, and RR-G provided critical feedback and helped review and edit the manuscript. All authors contributed to the article and approved the submitted version.

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