BIOMARKERS AND CLINICAL INDICATORS IN MOTOR NEURON DISEASE

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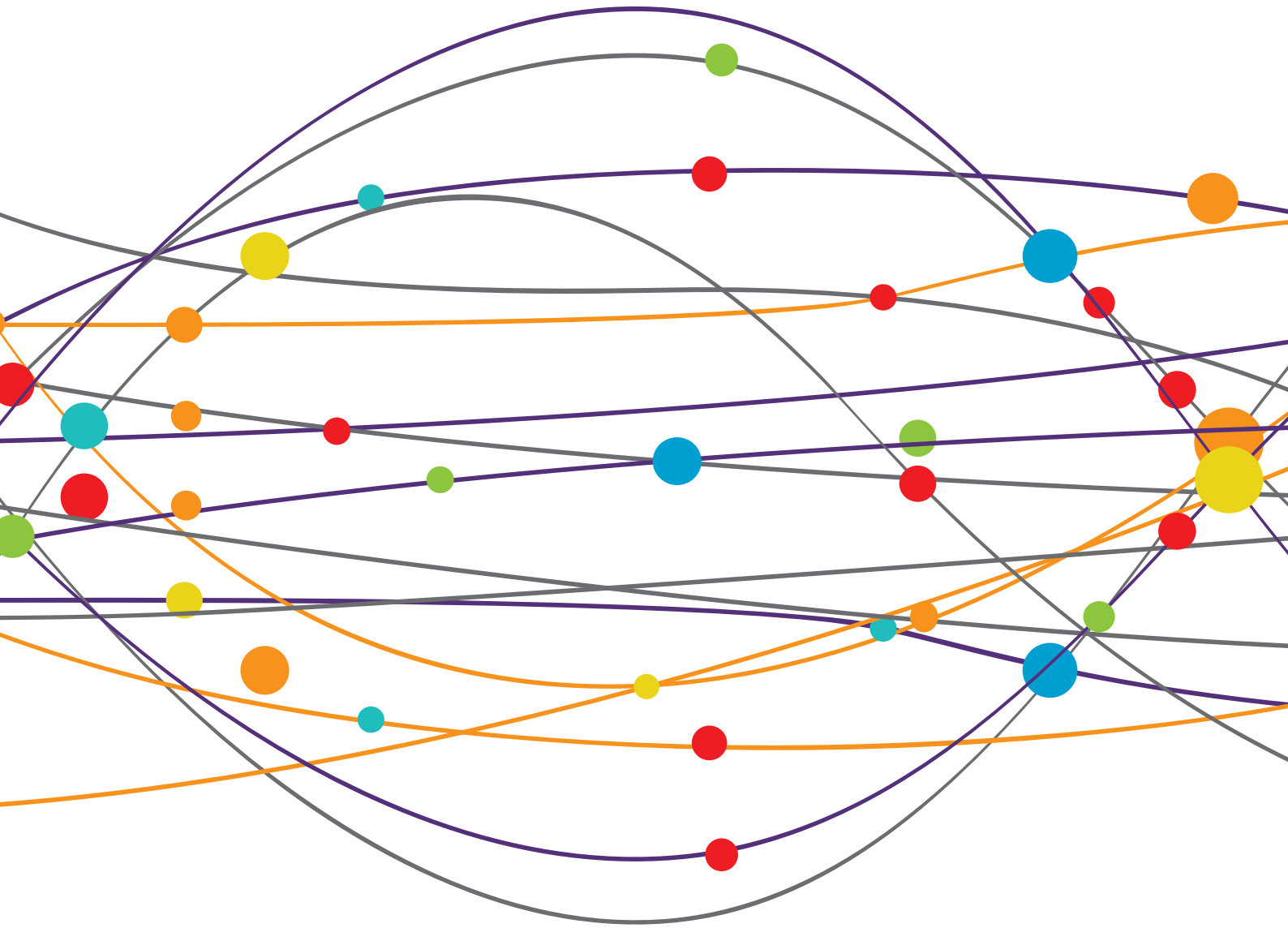

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BIOMARKERS AND CLINICAL INDICATORS IN MOTOR NEURON DISEASE

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Editorial: Biomarkers and Clinical Indicators in Motor Neuron Disease

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Editorial on the Research Topic

Biomarkers and Clinical Indicators in Motor Neuron Disease

Motor neuron diseases (MNDs) encompass a range of progressive neurodegenerative conditions with heterogeneous clinical presentations, disability profiles, prognosis, and age of onset. The umbrella term MND typically includes amyotrophic lateral sclerosis (ALS) (1), primary lateral sclerosis (PLS) (2, 3), progressive muscular atrophy (PMA), hereditary spastic paraplegia (HSP), spinal muscular atrophy (SMA) (4, 5), spinal and bulbar muscular atrophy (SBMA) (6), and rare conditions such as monomelic amyotrophy (MMA), juvenile muscular atrophy of distal upper extremity (JMADUE) (7), Mill's disease (8), ALS-FTD complex (9), and progressive bulbar palsy (PBP) (10). Despite the diversity of the clinical phenotypes, MNDs share a number of fundamental traits such as a long presymptomatic phase (11), insidious onset (12), considerable diagnostic challenges (13, 14), relatively low incidence (15, 16), extra-motor (Christidi et al.; Christidi et al.) and extra-neurological manifestations (6), relentless progression (17, 18), multidisciplinary care needs (19, 20), and lack of effective disease modifying therapies. These core similarities justify the discussion of various MNDs in a dedicated collection of articles and offer the opportunity to exchange research ideas between centres focusing on specific MNDs. There are other shared challenges across the MND spectrum, which are particularly relevant for therapy development, chief of which is the lack of validated biomarkers to serve as outcome measures in clinical trials. Pharmaceutical trials in MNDs mostly rely on functional rating scales and survival instead of objective, observer-independent markers which reflect on the underlying pathology of the condition (1, 21, 22).

INTRODUCTION

The dual relevance of biomarker development in MND lies in the characterization of dynamic pathological processes and its application to individualized patient care. From an academic perspective, biomarkers have the potential to elucidate the role of specific pathophysiological mechanisms, such as inflammation, cortical hyperexcitability, inhibitory dysfunction, cell to cell propagation and anatomical patterns of vulnerability. From a clinical standpoint, validated biomarkers have the potential to confirm an earlier diagnosis, thus enabling recruitment into clinical trials at an earlier stage. The key advantage of biomarkers however is their potential monitoring role in clinical trials; tracking disease progression *in vivo* and potentially detecting response to therapy. Biomarkers may also act as prognostic indicators which are indispensable both for patient stratification in clinical trials as well as individualized patient care.

The academic and clinical importance of biomarker development in MND is universally recognized by various scientific consortia and is regarded as a strategic funding priority by MND charities and funding agencies around the world. MNDA, ALSA, ARSLA, NISALS, NEALS, CALSNIC, RMN, JPND, IMNDA, SPF etc. are just some of the many organizations actively

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engaging in the development of multicenter data repositories, and establishing biobanking infrastructures for MND. Due to the limitations of single markers, it is generally accepted that a panel of biomarkers will most likely aid clinical management, guide care planning, and serve as monitoring markers in clinical trials. It is also increasingly clear that precision individualized therapies will be needed for specific phenotypes and genotypes instead of a "one-drug-for-all" approach.

The main themes of MND biomarker research include "wet biomarkers" which focus on disease-specific biofluid profiles and "dry biomarkers" such as electrophysiological and neuroimaging measures. Hypothesis-driven, targeted and high-throughput methods are both widely used in the so-called "omics" approaches: metabolomics, proteomics, lipidomics, and transcriptomics. One of the alluring aspects of international collaborations is that MND centers around the world have unique local expertise profiles which complement synergistically the skillset of other centers. Therefore, single ALS centers are in a position to provide authoritative reviews on specific aspects of biomarker research efforts. The editors of this collection are grateful for the expert contribution of 37 renowned research centers from around the globe. The 37 research papers included in this Research Topic discuss specific aspects of biomarker development in motor neuron diseases and embrace the diversity of MND phenotypes from SBMA to ALS-FTD. While the methodological focus of the papers differs depending the expertise profile of the authors, there is a cohesive theme among the papers to appraise biological, molecular, electrophysiological, and radiological markers which may potentially serve as pragmatic clinical indicators confirming the diagnosis, predicting the prognosis, detecting response to therapy or track longitudinal neurodegenerative changes. Beyond the practical relevance of ascertaining and quantifying pathological changes *in vivo*, biomarkers in MND also provide considerable academic insights such as the exploration of presymptomatic changes (23, 24), the description of genotype-associated signatures (25, 26), the delineation of natural disease trajectories (11, 27), the characterization of low-incidence phenotypes (2, 28, 29), confirmation of epigenetic and epidemiological factors (30, 31), and deciphering the pathological substrate of clinical symptoms [Finegan et al.; (32, 33)].

One of the commonest adult-onset motor neuron diseases is amyotrophic lateral sclerosis (ALS) which is an archetypical neurodegenerative condition with a presumed long presymptomatic phase (34), considerable delay between symptom onset and definite diagnosis (35), significant individual variations in disability profiles (Yunusova et al.), unrelenting motor decline (36), widespread non-motor symptoms (37, 38), and complex genetics (39, 40). In this collection of papers (https://www.frontiersin.org/research-topics/7659/biomarkers-and-clinical-indicators-in-motor-neuron-disease) wet and dry biomarkers are equally represented. "Wet" biomarkers typically refer to spinal fluid, serum or tissue-based indicators, whereas "dry" markers indicate non-invasive radiological, neuropsychological and or clinical indicators (41).

WET BIOMARKERS

Two papers are dedicated to the academic and biomarker role of micro RNAs; Joilin et al. discuss the diagnostic and prognostic utility of specific microRNAs and Rob Layfield's group propose the targeted study of four miRNAs; hsa-miR-124-3p, hsa-miR-127-3p, hsa-let-7a-5p, and hsa-miR-9-5p as particularly promising biomarkers (Foggin et al.). Tan and Guillemin discuss the potential biomarker role of kynurenine pathway metabolites in ALS, as these are involved in inflammation, excitotoxicity, oxidative stress, immune responses, and energy dysregulation. Chen et al. base their study on the inflammatory hypothesis of ALS etiology and not only demonstrate increased IL-6 levels in astrocyte-derived exosomes in ALS patients but identify associations with rate of progression. Dr. Duguez's group meticulously reviews the literature and suggest a multi-tissue biomarker panel encompassing markers of motor neuron integrity (pNFH and NF-L, cystatin C, Transthyretin), inflammation (MCP-1, miR451), muscle integrity (miR-338-3p, miR-206) and metabolism (homocysteine, glutamate, cholesterol). They argue that biomarker panels should reflect the multi-system, multi-tissue nature of ALS-pathophysiology (Vijayakumar et al.). As ALS is increasingly recognized as a metabolic disorder (42), De Aguilar provides an eloquent overview of metabolic markers with a particular focus on proposed lipid biomarkers. Kirk et al. elaborate on the metabolic spectrum of ALS from cellular to multi-organ systemic involvement. Dr. Blasco's team discusses advances in metabolomics and advocates for a pharmacometabolomic approach to evaluate individual response to therapy, to develop personalized treatments for ALS (Lanznaster et al.). Poesen and Van Damme review the diagnostic, monitoring and prognostic role of neurofilaments in ALS.

CLINICAL INDICATORS AND THERAPEUTIC STRATEGIES

Zhang et al. draw the reader's attention to comorbid extra-neurological manifestations in ALS, such as autoimmune syndromes. Lule et al. contribute an authoritative review of the key determinants of quality of life in ALS, and underline the lack of a direct link between physical disability and quality of life. Professors Lule and Ludolph also emphasize the key ethical principles of supportive care in ALS which are centered on patient autonomy, dignity, beneficence and caregiver support (20, 43–47). Li Hi Shing et al. highlight the complex symptomatology of post-polio syndrome and discuss the etiology of under-researched symptoms such as fatigue. Finegan et al. perform a comprehensive review of the pathophysiology of pseudobulbar affect (pathological crying and laughing) which is one of the most prevalent and distressing symptoms of PLS and ALS, yet it remains surprisingly understudied (32, 33, 48). Chipika et al. undertake a systematic analysis of the most promising markers to track pathological progression *in vivo*, which may detect response to therapy in future clinical trials of

ALS. Their primary perspective is the assessment of the pragmatic utility [Grollemund et al.; (14, 35, 49–51)] of emerging markers in pharmaceutical trials (Chipika et al.). Christidi et al. reviews the evidence for memory deficits in MND with a painstaking analysis of the available clinical (52), radiological (1) and post mortem literature (53). Professor Mioshi's group eloquently reviews the impact of cognitive and behavioral deficits in ALS on patients and caregivers drawing attention to an important aspect of ALS care which is relatively understudied (54, 55). The novelty of their analysis lies in the identification of viable non-pharmaceutical strategies to improve patient and caregiver well-being (Caga et al.). Grollemund et al. perform an in-depth analysis of the ever expanding literature of machine-learning in MND, and discuss the advantages and drawbacks of specific mathematical models. Professor de Carvalho's group gives an authoritative overview of respiratory markers and diaphragmatic neurophysiology in ALS (de Carvalho et al.). Professor Yunosova's group appraises the most commonly utilized clinical tools for assessing and monitoring bulbar dysfunction in ALS and advocate for the development and validation of novel assessment protocols (Yunosova et al.). Professor Kabashi's group gives an elegant overview of neuromuscular junction involvement in ALS and examines the evidence from animal models to clinical observations (Campanari et al.). Dr. Floeter's group discuss genotype-specific biomarker panels and presymptomatic alterations. They review candidate imaging, electrophysiology, and biofluid markers in patients with *C9orf72* hexanucleotide expansions (Floeter et al.). Christidi et al. discuss the clinical (38, 56–58), imaging (31, 36, 59, 60), and pathological correlates of cognitive and behavioral dysfunction in ALS giving specific screening and assessment recommendations. They describe which domains are most likely to be affected and review the impact of neuropsychological deficits on patients and their caregivers (Christidi et al.). Querin et al. evaluate monitoring strategies in spinal and bulbar muscle atrophy (SBMA) and discuss the spectrum of motor, extra-motor, and extra-neurological manifestations in detail. They give specific recommendations to screen for endocrine, cardiac and respiratory involvement (Querin et al.).

From a therapeutic viewpoint, Gouel et al. discuss the role of neurotrophic growth factors (NTF) in neuroprotection and neurorestoration. Professor Bogdahn's group give a real-life example of using biomarkers in a therapeutic trial of Granulocyte-colony stimulating factor (G-CSF). They evaluate the biomarker potential of serum cytokines in ALS and perform a meticulous analysis of MDC, TNF-beta, IL-7, IL-16, and Tie-2 levels in relation to clinical outcomes (Johannesen et al.).

DRY BIOMARKERS

Electrophysiology is one of the most widely utilized clinical and research tools in motor neuron diseases [Proudfoot et al.; (5, 61, 62)]. Professor Kiernan's group provides an expert review of electrophysiological markers of upper and lower motor neuron degeneration and discuss the clinical value of specific neurophysiological indices (Huynh et al.). Wang et al. present an elegant electrophysiology study, investigating the neurophysiological substrate of the split-hand phenomenon. Imaging is another promising dry biomarker of ALS-associated degenerative change. In recent years imaging in ALS confirmed extensive extra-motor pathology in cerebellar (63, 64), extra-pyramidal (65, 66), subcortical (26, 67), hippocampal [Christidi et al.; (68, 69)], hypothalamic (42), brainstem [Yunusova et al.; (70)], and frontotemporal involvement [Christidi et al.; (71)]. Imaging in ALS also helped to decipher the pathological underpinnings of specific symptoms, such as pseudobulbar affect [Finegan et al.; (32, 33, 48)], compensatory changes (72), executive dysfunction (73), extrapyramidal manifestations (65), metabolic dysfunction (42), memory deficits (59, 74). Imaging in ALS has also been instrumental to link disability profiles to pathological TDP-43 burden patterns (36, 53, 75–78) and track progressive pathological changes [Chipika et al.; (11, 79)]. In this collection of papers, Fortanier et al. demonstrate how structural imaging data may be used to characterize alterations in connectivity patterns. Rajagopalan and Pioro elegantly demonstrate how clinically well-defined ALS sub-populations have distinctive neuroimaging signatures. Instead of the most commonly used quantitative techniques, such as diffusion tensor imaging (80, 81) they demonstrate the utility of alternative imaging cues on T2-wighted, Flair and proton density imaging (Rajagopalan and Pioro). Kalra a pioneer of MR spectroscopy, gives an eloquent overview of the achievements, practical utility and future applications of metabolic imaging in ALS. Professor Filippi and Dr. Agosta's research group contributed an expert review of diffusion imaging in ALS, discussing methodological advances, the contribution of network analyses, and the potential of DTI to track progressive pathological changes (Basaia et al.). Their observations also highlight the paradigm shift from the analysis of focal diffusivity changes (80, 81) to the assessment of network integrity (41, 82). Muller and Kassubek review the utility of diffusion tensor imaging in ALS with respect to detecting pathological TDP-43 burden *in vivo*. They describe how *in vivo* measurements may relate to pathological stages and provide an expert overview of the most frequently utilized analysis methods (Muller and Kassubek). The majority of imaging studies in motor neuron disease focus on cerebral pathology (72, 83–85), despite the pathognomonic involvement of the spinal cord in ALS [El Mendili et al. (86)], SBMA (6, 10), SMA (4, 5), PLS (2), juvenile muscular atrophy of distal upper extremity (7, 28, 87) and PPS (29). In this Research Topic, Professor Pradat's group gives a methodological update on advances in spinal imaging and outline future research directions (El Mendili et al.). Chew and Atassi discuss how PET radioligands unveil specific pathophysiological mechanisms such as neuroinflammation, metabolic changes, neuronal dysfunction, and oxidative stress and how PET may be utilized both in natural history studies and pharmaceutical trials. Professor Turner's group reviews the advances in functional imaging discussing the contribution of functional MRI, MEG and EEG studies to ALS research (Proudfoot et al.). Dr. Grosskreutz's group discusses the benefits of data sharing and gives an expert overview of the methodological and logistical challenges of data harmonization, hosting large data repositories, generating consortium bylaws and data protection policies (Steinbach et al.). Barritt et al. summarize some of the most exciting new imaging

methods in MND and discuss emerging methods such as Neurite Orientation Dispersion and Density Imaging (NODDI) (88), and quantitative Magnetization Transfer Imaging (qMTi) and data analysis approaches such as Event-Based Modeling (EBM). A shared aspiration of both "wet" and "dry" biomarker studies is the transition from describing group-level observations to the precision categorization and tracking of individual patients (53, 76, 83, 84, 89–91).

CONCLUSIONS

The ensemble of these articles showcases the determination, drive and momentum in motor neuron disease research worldwide. We are particularly proud that renowned research groups from Australia, France, China, Greece, United Kingdom, Ireland, United States, Canada, Germany, Belgium and Italy shared their unique perspective, methodological expertise and their vision for future research directions. The diversity of research strategies and the unrelenting enthusiasm of the various research groups give cause for optimism for the development of precision biomarkers, and ultimately, a cure for MND.

AUTHOR CONTRIBUTIONS

Both authors contributed equally to the drafting of this editorial.

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MINI REVIEW
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The Clinical and Radiological Spectrum of Hippocampal Pathology in Amyotrophic Lateral Sclerosis

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Hippocampal pathology in Amyotrophic Lateral Sclerosis (ALS) remains surprisingly under recognized despite compelling evidence from neuropsychology, neuroimaging and neuropathology studies. Hippocampal dysfunction contributes significantly to the clinical heterogeneity of ALS and requires structure-specific cognitive and neuroimaging tools for accurate *in vivo* evaluation. Recent imaging studies have generated unprecedented insights into the presymptomatic and longitudinal processes affecting this structure and have contributed to the characterisation of both focal and network-level changes. Emerging neuropsychology data suggest that memory deficits in ALS may be independent from executive dysfunction. In the era of precision medicine, where the development of individualized care strategies and patient stratification for clinical trials are key priorities, the comprehensive review of hippocampal dysfunction in ALS is particularly timely.

Keywords: hippocampus, amyotrophic lateral sclerosis, neuropathology, neuroimaging, cognition

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is relentlessly progressive neurodegenerative condition with considerable clinical heterogeneity (1). One of the key clinical dimensions of disease heterogeneity in ALS is the varying severity and profile of cognitive impairment. The quality of life implications of cognitive impairment in ALS and its impact on caregiver burden (2), compliance with assistive devices (3) and survival (4) are now universally recognized. The discovery of hexanucleotide expansions in *C9orf72* in 2011 (5) has given fresh momentum to neuropsychology research in ALS by confirming shared etiological factors between frontotemporal dementia (FTD) and ALS. The momentous conceptual advances in the neuropsychology of ALS have taken place in a remarkably short period of time, from sporadic observations, through the development of diagnostic criteria (6), to robust family aggregation (7) and genetic studies, to the development of disease-specific screening instruments (8, 9). The current consensus criteria (6) distinguish ALS with cognitive impairment; ALS with behavioral impairment; ALS with cognitive and behavioral impairment; ALS-FTD; ALS-dementia (non-FTD, i.e., Alzheimer dementia (AD), vascular dementia,

mixed dementia). One of the most exciting aspects of ALS neuropsychology studies is their localization potential to specific anatomical circuits and that their observations are widely corroborated by neuropathology (10–12) and neuroimaging studies (13). Memory deficits in ALS have traditionally been regarded as atypical and considered suggestive of coexisting AD-type pathology. The recognition that memory deficits are part of the spectrum of ALS-associated cognitive impairment is relatively recent.

MEMORY IMPAIRMENT IN ALS

Early neuropsychology studies of ALS have predominantly examined frontal lobe-mediated neuropsychological domains, and highlighted executive dysfunction, impaired phonemic fluency, poor set shifting, reduced cognitive flexibility, impaired response inhibition, planning deficits, problem-solving difficulties, selective attention, and impaired social cognition (14). More recently, the spectrum of memory impairment has been specifically evaluated, including encoding and retrieval functions (primary memory system) (15, 16) and storage/consolidation domains (secondary memory system) (17). Furthermore, population-based studies identified cognitive phenotypes without executive impairment (18, 19). The description of episodic memory deficits without coexisting executive dysfunction in ALS drew attention to temporal lobe network dysfunction which has been elegantly corroborated by a series of neuropathology and neuroimaging studies (20).

ANATOMICAL OVERVIEW

The hippocampus (**Figure 1A**) is a bilaminar structure and consists of the cornu ammonis (CA) and the dentate gyrus (DG). Based on its cytoarchitecture and projections, the CA is further divided into four histological subfields, named CA1-CA4 by Lorente de No in his seminal paper (21). The dentate gyrus is a narrow, dorsally concave structure which envelops CA4. The cornu ammonis, the dentate gyrus, and the subiculum together form the "hippocampal formation" (**Figure 1B**). The subiculum is divided into the following segments: the prosubiculum, the subiculum proper, the presubiculum, and the parasubiculum.

Each segment of the hippocampal formation receives afferentation from its neighboring regions but these connections are not all bidirectional (22). For example, the "trisynaptic circuit" (23) is a unidirectional network, which arises from layer II of the entorhinal cortex, its axons perforate the subiculum, and form the "perforant pathway" (PP). Duvernoy (24) coined the term "polysynaptic pathway" for the intrinsic hippocampal circuitry, which refers to a circuit of at least four synapses that connect the entorhinal cortex, the dentate gyrus, the CA subfields, and the subiculum. A direct intrahippocampal pathway has also been identified, which originates from layer III of the entorhinal cortex and projects directly to the CA1 but not through the PP (25). The perforant pathway (**Figure 1C**) arises from layer II-III neurons of the lateral and medial entorhinal cortex (26), which is also the origin of the polysynaptic pathway (27). The PP perforates the subiculum to reach the dentate gyrus and the hippocampus proper, but minor projections also originate from the presubiculum and parasubiculum (28). The majority of the PP fibers reach the stratum moleculare of the dentate gyrus through the vestigial hippocampal sulcus (24). The PP contributes to the "Papez circuit" (26, 29, 30) which is relayed through the following structures; entorhinal cortex → dentate gyrus → hippocampus → hypothalamus → thalamus → cingulate cortex→ presubiculum → entorhinal cortex. In addition to the intrinsic hippocampal circuitry, there are numerous extrinsic hippocampal projections to subcortical and cortical regions (31). The main input to the hippocampus enters via the entorhinal area (31).

INSIGHTS FROM NEUROPATHOLOGY

Neuropathological changes have been consistently reported in the hippocampus in ALS (**Table 1**). Early reports highlighted ubiquitin-positive intraneuronal inclusions (32–35) in medial temporal structures, neuronal loss in the medial cortex of the temporal tip (36, 69) and focal depletion of pyramidal neurons in the pes hippocampi in both patients with and without dementia (33, 36, 69). A specific focus of interest in histopathological studies is the PP zone, which has been comprehensively studied in most neurodegenerative conditions, especially in AD. While AD is characterized by the extraneuronal deposits of the amyloid β-protein (Aβ) and the intraneuronal tauopathy (70), ALS is primarily associated with TAR DNA-binding protein 43 (TDP-43) deposits (71). ALS patients with and without dementia (37, 38) show neuropathological changes in the dentate gyrus and the outer lamina of the molecular layer where the PP terminals are distributed (26, 30, 72). In ALS, the molecular layer of the dentate gyrus is primarily affected, a pattern which is distinctly different from AD. The inner molecular layer, which is innervated by the CA4 (73), is the least affected layer in ALS (38). The intermediate layer, which receives projections from layer II of the medial entorhinal cortex, is affected (38), but the outer layer, is the most affected region in ALS (38). Despite considerable mesial temporal lobe involvement in both ALS and AD, the distribution of disease-specific inclusions is strikingly different. Neurofibrillary tangles in AD are mostly found in the entorhinal cortex and are positive for tau, whereas the main proteinopathy of ALS is TDP-43 and mostly affects the transentorhinal cortex (38).

It is now widely recognized that phosphorylated TDP-43 (pTDP-43) aggregates are the hallmark pathology of sporadic

Abbreviations: ALS, amyotrophic lateral sclerosis; ALSFRS-r, revised ALS functional rating scale revised; ALSnci, ALS with no cognitive impairment; aMCI, amnestic mild cognitive impairment; C9orf72 HRT, chromosome 9 open reading frame 72 hexanucleotide repeats; CA, cornu ammonis; DG, dentate gyrus; DTI, diffusion tensor imaging; DWI, diffusion weighted imaging; ECAS, Edinburgh cognitive and behavioral ALS screen; FTD, frontotemporal dementia; GM, gray matter; HARDI, high angular resolution diffusion imaging; HC, healthy control; MND, motor neuron disease; NeuroC, neurological controls; NODDI, neurite orientation dispersion and density imaging; PP, perforant pathway; PtwoCI, patients without cognitive impairment; PtwCI, patients with cognitive impairment; PtwoD, patients without dementia; PtwD, patients with dementia; TBSS, tract-based spatial statistics; VBM, voxel-based morphometry; WM, white matter; MRS, magnetic resonance spectroscopy.

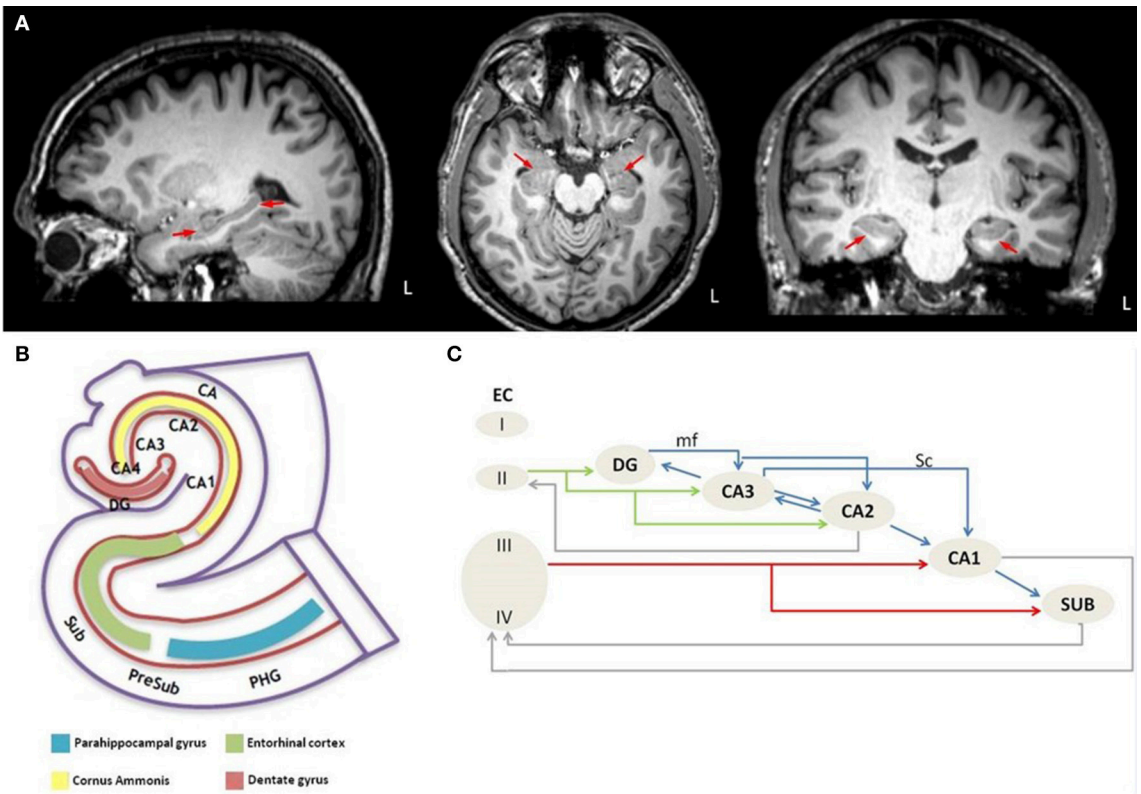

FIGURE 1 | Anatomical depiction of hippocampus on sagittal, axial and coronal plane of high resolution T1 image from a healthy control subject **(A)** and schematic representation of **(B)** the anatomy of the hippocampus-entorhinal cortex-parahippocampal gyrus system and **(C)** the intrahippocampal connections. L, left hemisphere; PHG, parahippocampal gyrus; PreSub, Presubiculum; Sub, Subiculum; CA, Cornu Ammonis; CA1-CA4, Cornu Ammonis subfields; DG, Dentate gyrus; EC, Entorhinal cortex; I-IV, Layer I-IV; mf, mossy fibers; Sc, Schaffer collaterals.

ALS (39, 74, 75). Based on post mortem observations, a sequential staging system of pTDP-43 pathology has been proposed, using stage-defining involvement of specific cortical and subcortical regions (12). According to this four-stage model of disease propagation, the PP is predominantly affected in stage IV. A three-stage model has also been suggested for PP degeneration (38) where stage I is the "inclusion stage" defined by TDP-43-positive cytoplasmatic inclusions appearing in the granular cells of the dentate gyrus, stage II is the "early perforant stage" where gliosis and neuronal loss of the transentorhinal cortex are observed, and stage III is the "advanced perforant stage" defined by the degeneration of the molecular layer of the dentate gyrus and neuronal loss in the transentorhinal cortex (38). The chronological stages of hippocampal pathology are closely linked to its structural anatomy, confirming that disease propagation occurs along connectivity patterns (76). The TDP-43 stages of ALS are in line with the notion that gray matter (GM) regions become sequentially involved via the WM pathways that connect them (77–79).

THE CONTRIBUTION OF NEUROIMAGING

Neuroimaging studies have already contributed meaningful structural, metabolic and functional insights in ALS (80, 81) and recent technological advances in imaging techniques offer unprecedented opportunities to characterize hippocampal changes *in vivo*. Following sporadic reports of hippocampal degeneration (82–85) in whole-brain exploratory studies, recent studies have specifically focused on the evaluation of this structure (43) (**Table 1**). Emerging imaging methods not only highlight hippocampal volume reductions, but have the potential to characterize specific sub-regions (78), shape changes (42), density alterations (20), progressive longitudinal changes (43), altered connectivity profiles, and functional changes (40, 46, 47).

Structural Neuroimaging

Computational neuroimaging techniques have consistently captured hippocampal GM changes which was initially thought to be more significant in ALS patients carrying the *C9orf72* hexanucleotide repeats (40), but later studies showed similarly extensive hippocampal degeneration in *C9orf72* negative ALS-FTD patients (78). Interestingly, unilateral hippocampal changes were not only captured in patients with cognitive impairment (42), but also in cognitively intact cohorts (41). Shape and density analyses of the hippocampus in ALS highlighted phenotype-specific patterns of hippocampal degeneration (42). A longitudinal study of hippocampus, which included a small (∼6%) number of *C9orf72* positive patients, identified baseline

TABLE 1 | Research studies with hippocampal-related neuropathological, neuroimaging, or neuropsychological findings in ALS included in the present review.

References	Authors (Date)	Sample size	Diagnostic criteria	Genetic Status	Dementia	Cognitive status
NEUROPATHOLOGICAL STUDIES						
(32)	Wightman et al., 1992	33 ALS	N/A	N/A	Included	19 PtwoCI; 14 PtwCI-D*
(33)	Okamoto et al., 1991	27 ALS/50 HC	N/A	N/A	1 PtwD	N/A (1PtwD)*
(34)	Okamoto et al., 1992	10 MND	N/A	N/A	10 PtwD	Dementia*
(35)	Okamoto et al., 1996	2 ALS	N/A	N/A	N/A	Mental changes*
(36)	Nakano et al., 1993	54 ALS/35 non ALS	N/A	N/A	10 PtwD	44 PtwoD; 10 PtwD
(37)	Takeda et al., 2007	12 ALS	N/A	N/A	12 PtwD	Demented
(38)	Takeda et al., 2009	14 ALS	N/A	N/A	9 PtwD	9 PtwD* (6 PtwMI)
(39)	Brettschneider et al., 2012	102 ALS	El Escorial-R	N/A	12 PtwD	88 PtwoD; 12 PtwD; 2 unknown
(12)	Brettschneider et al., 2013	76 ALS	El Escorial-R	11 C9orf72(+)	5 PtwD	71 PtwoD*; 5 PtwD*
NEUROIMAGING STUDIES: STRUCTURAL GM						
(40)	Bede et al., 2013	39 ALS/44 HC	El Escorial	9 C9orf72(+)	N/A	Cognitive exam; Unspecified cognitive groups
(41)	Abdulla et al., 2014	58 ALS/29 HC	El Escorial-R	3 C9orf72(+)	N/A	Cognitive exam; Unspecified cognitive groups
(42)	Machts et al., 2015	67 ALS/ 39 HC	El Escorial-R	C9orf72(-)	7 PtwD	Cognitive exam; 42 PtwoCI; 18 PtwCI; 7 PtwFTD
(43)	Westeneng et al., 2015	112 ALS/60 HC	El Escorial-R	7 C9orf72(+)	N/A	N/A
(44)	Sage et al., 2007	28 ALS/26 HC	El Escorial	N/A	PtwoD	No behavioral or cognitive changes; Unspecified cognitive exam
(45)	Sage et al., 2009	28 ALS/26 HC	El Escorial	N/A	PtwoD	No behavioral or cognitive changes; Unspecified cognitive exam
NEUROIMAGING STUDIES: STRUCTURAL WM						
(46)	Barbagallo et al., 2014	24 ALS/22 HC	El Escorial-R	N/A	N/A	13 Pt cognitively examined; Unspecified cognitive groups
(47)	Thivard et al., 2007	15 ALS/25 HC	El Escorial-R	N/A	PtwoD	N/A
(48)	Prell et al., 2013	17 ALS/17 HC	El Escorial-R	N/A	PtwoD	No significant frontal or cognitive dysfunction; Unspecified cognitive exam
(49)	Keil et al., 2012	24 ALS/24 HC	El Escorial-R	N/A	PtwoD	No cognitive exam
(50)	Kassubek et al., 2014	111 ALS/74 HC	El Escorial-R	N/A	N/A	N/A
(51)	Christidi et al., 2017	42 ALS/25 HC	El Escorial-R	N/A	PtwoD	Cognitive exam; Memory impairment based on normative data; Unspecified cognitive groups
(52)	Steinbach et al., 2015	16 ALS/16HC	El Escorial-R	N/A	16 PtwD	Cognitive exam; Cognitive categories based on Phukan criteria[#]
NEUROIMAGING STUDIES: TASK fMRI						
(53)	Stoppel et al., 2014	14 ALS/14 HC	El Escorial-R	N/A	PtwoD	Cognitive exam; Memory impairment based on normative data; Cognitive categories based on Phukan criteria[#]
NEUROIMAGING STUDIES: RESTING-STATE fMRI						
(54)	Agosta et al., 2011	26 ALS/15 HC	El Escorial-R	N/A	PtwoD	N/A
(55)	Zhu et al., 2015	22 ALS/22 HC	El Escorial-R	N/A	PtwoD	Cognitive exam; Unspecified cognitive groups
(56)	Heimrath et al., 2014	9 ALS/11 HC	El Escorial-R	N/A	PtwoD	Cognitive exam; Unspecified cognitive groups
(57)	Loewe et al., 2017	64 ALS/38 HC	El Escorial-R	N/A	PtwoD	Cognitive exam; Specified cognitive groups[#]

(Continued)

TABLE 1 | Continued

References	Authors (Date)	Sample size	Diagnostic criteria	Genetic Status	Dementia	Cognitive status
NEUROPSYCHOLOGICAL STUDIES						
(58)	Abrahams et al., 1997	12 ALS/25 HC	N/A	N/A	N/A	Cognitive exam; Unspecified cognitive groups
(59)	Chari et al., 1996	50 MND/27 HC/23 NeuroC	El Escorial	N/A	PtwoD	Cognitive exam; Unspecified cognitive groups
(60)	Frank et al., 1997	74 ALS/56 HC	N/A	N/A	N/A	Cognitive exam; Unspecified cognitive groups
(61)	Hanagasi et al., 2002	20 ALS/13 HC	El Escorial	N/A	PtwoD	Cognitive exam; Unspecified cognitive groups
(62)	Iwasaki et al., 1990	18 ALS/15 HC	N/A	N/A	PtwoD	Cognitive exam; Unspecified cognitive groups
(63)	Ludolph et al., 1992	17 ALS/12 HC	N/A	N/A	PtwoD	Cognitive exam; Unspecified cognitive groups
(64)	Massman et al., 1996	146 ALS	El Escorial	N/A	N/A	Cognitive exam; Cognitive impairment based on normative data; Unspecified cognitive groups
(65)	Mantovan et al., 2003	20 ALS/20 HC	El Escorial	N/A	PtwoD	Cognitive exam; Unspecified cognitive groups
(66)	Christidi et al., 2012	22 ALS/22 HC	El Escorial-R	N/A	PtwoD	Cognitive exam; Unspecified cognitive groups
(67)	Machts et al., 2014	40 ALS/39 aMCI/40 HC	El Escorial-R	N/A	3 PtwD	Cognitive exam; Unspecified cognitive groups
(68)	Burke et al., 2017	203 ALS/117 HC	El Escorial-R	C9orf72(–)	30 PtwD	Cognitive exam; 117 PtwoCI; 56 PtwCI; 30 PtwD

*ALS, amyotrophic lateral sclerosis; HC, healthy control; MND, motor neuron disease; N/A, non-available; PtwoCI, patients without cognitive impairment; PtwCI-D, patients with cognitive impairment-dementia; PtwD, patients with dementia; PtwoD, patients without dementia; PtwMI, patients with memory impairment; PtwCI, patients with cognitive impairment; PtwFTD, patients with frontotemporal dementia; *unspecified cognitive status; #no comparison between cognitive groups; El Escorial-R, El Escorial revised criteria; C9orf72(+), C9orf72 positive status; GM, gray matter; WM, white matter.*

changes in the left presubiculum, and progressive CA2/3, CA4 and the left presubiculum involvement at follow-up (43).

While diffusion-weighted imaging (DWI) is primarily used to study white matter (WM) structures, there is increasing evidence that it may provide useful information on aspects of GM integrity (86). Evaluation of diffusion tensor imaging (DTI) metrics have consistently shown low fractional anisotropy (44, 49) and increased mean diffusivity in both hippocampal (44–47) and parahippocampal regions (48).

DTI has been initially used to characterize medial temporal lobe WM regions and later to assess limbic circuit integrity (i.e., fornix; uncinate fasciculus) (87–89). One of the most unique applications of hippocampal DTI in ALS however is the ability to reconstruct and evaluate of the PP. (50, 51). Based on *in vivo* assessments, these studies have not only confirmed previous neuropathological observations but also revealed structure-specific clinical correlations (51). The use of DWI-based PP imaging (90) has contributed to our understanding of impaired memory processing in a range of conditions from mild cognitive impairment, through AD, to traumatic brain injury (91–94). PP imaging is therefore a relatively well-established approach which has only recently been applied to ALS. A longitudinal tractography study of ALS (52) found increased connectivity between the visual cortex and medial temporal lobe regions which increased further at 3-month follow-up. Increased connectivity over time in ALS is not an isolated finding (95) and is often interpreted as a compensatory mechanism.

Functional Neuroimaging

There are relatively few paradigm-based functional magnetic resonance imaging (fMRI) studies specifically evaluating hippocampal function, but a longitudinal fMRI study identified increased novelty-evoked hippocampal activity over time (53). Resting-state studies have consistently captured increased connectivity between the left sensorimotor cortex and contralateral cortical regions including the parahippocampal gyrus (54). Additionally, increased low-frequency amplitudes have been observed in the right parahippocampal cortex (55). Increased functional connectivity was also identified between parahippocampal components of the default-mode network (56). In a relatively large sample of ALS patients with only minor cognitive changes, (57) decreased functional connectivity was identified between temporal lobe structures, including hippocampal and parahippocampal regions. This was thought to represent early metabolic disturbances before cell-loss occurs but highlight the fact that increased and decreased connectivity is both reported in fMRI studies of ALS.

INSIGHTS FROM NEUROPSYCHOLOGY

Contrary to the consensus around executive dysfunction in ALS (96–99), there are strikingly inconsistent reports about the incidence of memory impairment in ALS (**Table 1**). Intact memory function, mild dysfunction, executive function mediated memory impairment, and moderate memory deficits have all been reported (58–65, 97). The primary substrate of amnestic deficits is still under investigation. Most studies agree that the primary deficit is in encoding-retrieval (65) which is often linked to frontal dysfunction, attention, and other executive-based processes (65–68). However, recognition deficits and memory consolidation difficulties are likely to be just as important (66). Compelling evidence also exist for pure episodic memory dysfunction based on impaired picture recall, word list-learning, pair associations, and story-recall. These observations would suggest that memory impairment in non-demented ALS patients cannot be exclusively attributed to executive dysfunction (100–102).

In a combined neuroimaging-neuropsychology study, abnormal immediate and delayed recall scores were identified in 23% of non-demented ALS patients (102). While the ALS cohort of this study did not exhibit reduced hippocampal volumes in comparison to healthy controls, their memory performance correlated with hippocampal volumes. These findings are echoed by other studies which rely on volumetric analyses and verbal list-learning test and report significant correlations between the hippocampal volumes and verbal memory indices such as total learning, delayed recall, and recognition (41).

While direct clinico-radiological correlations are often regarded as contentious (103), a positive association has been reported between verbal memory indices and hippocampal volumes in several ALS subgroups, including ALSci and ALS-FTD (42). DTI studies have consistently revealed correlations between memory performance and memory-associated WM tracts such as the fornix (88), the uncinate fasciculus (87, 88), and the hippocampal PP (51). Emerging reports of similar episodic memory performance in ALS and amnestic mild cognitive impairment patients (67) corroborates neuropathological findings of comparable PP changes (37, 38).

Testing Recommendations

Traditionally, the assessment of episodic memory includes tests for immediate and delayed recall, and performance evaluated from a learning, retention and recognition perspective. More recently, distinct memory processes are specifically assessed, such as encoding, consolidation, and retrieval. (104–106) List-learning tests (e.g., California Verbal Learning Test; Rey Auditory Verbal Learning Test; Hopkins Verbal Learning Test etc.) are particularly useful to assess hippocampus-mediated verbal memory dysfunction in ALS. These tests enable the clinician to evaluate immediate recall, delayed recall, and recognition and can be readily interpreted in terms of encoding, consolidation, and retrieval performance (66). Story-recall tests, such as the Wechsler-Memory Scale, are also sensitive to detect episodic memory impairment and ideally, both list-learning and story-recall should be performed to comprehensively evaluate episodic memory in ALS. The accurate assessment of visual episodic memory is often confounded by motor disability in in ALS or by coexisting executive dysfunction which may affect the organization and encoding of complex figures (e.g., Rey-Osterreith Complex Figure Test). The limitations of short, non-ALS, cognitive screening tools such as MMSE; ACE; MoCA are widely recognized in the ALS research community, as these tests have been developed for other neurodegenerative conditions. The administration of ALS specific screening tools (ECAS, ALS-CBS) should be followed by specialist neuropsychological evaluation if memory impairment is identified or reported by the patient or caregiver.

DISCUSSION

The synthesis of insights from neuropathology, neuroimaging and neuropsychology enables the systematic discussion of structural and functional aspects of hippocampal degeneration in ALS and helps to integrate focal pathology into a network perspective.

While hippocampal pathology used to be primarily evaluated in ALS patients with comorbid dementia (34, 37, 38), recent studies have increasingly focused on non-demented patient cohorts (12, 32, 69, 71). With the increased recognition of neuropsychological deficits beyond executive dysfunction, imaging studies of ALS have gradually started to evaluate mesial temporal lobe structures and memory domains have now been incorporated in ALS-specific cognitive screening tools (8). The targeted evaluation of memory function and reliance on more sophisticated indices of episodic memory (65–68) not only help to characterize the heterogeneity of cognitive profiles but also confirm that pure episodic memory dysfunction is not uncommon in ALS and can be detected in the absence of FTD.

Despite the momentous advances in characterizing hippocampal degeneration in ALS, considerable shortcomings and inconsistencies can be identified. The commonest problem is sample size limitations followed by the inclusion of poorly characterized patients. The comprehensive neuropsychological assessment of patients is paramount and administering screening tests alone is not sufficient. Reliance on non-ALS specific batteries, such as Addenbrooke's Cognitive Examination, Mini-Mental State Examination, Montreal Cognitive Assessment, is not sufficient to characterize ALS-associated cognitive change. A common shortcoming of ALS neuropsychology papers is overlooking the confounding effect of medications which affect cognitive performance. Anticholinergics commonly used for sialorrhea, tricyclic antidepressants, opiates, benzodiazepines are all widely used in ALS and have a significant impact on attention, registration, and recall. Other disease-specific confounding factors such as hypoxia, hypercapnia, physical discomfort, fatigue, apathy, low mood, depression also need careful consideration. Despite established consensus criteria (6) different batteries are used in different centers to test memory. There is a paucity of reports where caregivers or family members are interviewed about the sort of memory impairment they may have observed. A few targeted questions if the patient

gets lost in familiar places, misplaces items, forgets names, or dates etc. may be worth asking from the caregivers. Given the strikingly quick progression rates observed in ALS compared to other neurodegenerative conditions, resource allocation, care planning, assessment of capacity may be important at an early stage of the disease. ALS patients have to make a number of important financial, personal, and end-of-life decisions which may or may not be affected by memory impairment.

The practice of excluding patients with dementia in neuroimaging studies (44, 47, 49, 55) to evaluate clinically homogenous samples may also be counterintuitive. More recent imaging papers include comprehensive cognitive testing (55–57) which aids the interpretation of extra-motor changes (107). The lack of cognitive profiling of the healthy controls in many neuroimaging studies also precludes robust statistics as only the patient group is then used for correlative analyses. Often, reference normative neuropsychology data are used for the interpretation of patient's memory performance, data which is independent from the given study and originate from volunteers who have not been scanned as part of the given study. The patients' neuroimaging data on the other hand are contrasted to scans of controls who had no detailed neuropsychological evaluation. This unfortunately is a common study design, which essentially uses a different imaging and neuropsychology control group. Another common shortcoming of ALS neuroimaging studies is the lack of adjustment for education, which may impact on both structural and functional imaging data (80). A binary, comparative study design of patients versus controls and the contrasting of two clinically or genetically defined cohorts is not entirely satisfactory either. The inclusion of mimic cohorts, or a "disease-control" group with an alternative neurodegenerative condition such as MCI, AD, or Parkinson disease would also be desirable. The selection bias of relatively well patients who are able to lie flat in the scanner and able to make the journey to a radiology department is seldom acknowledged. It is conceivable that progressive hippocampal changes occur as the disease progresses, but these patients are no longer able to partake in imaging studies. Clinical trial designs are not only hampered by late recruitment of clinically heterogeneous cohorts, but they overwhelmingly rely on motor, respiratory, nutritional markers (108–110). Patient stratification based on cognitive performance prior to inclusion and monitoring performance during the trial seems essential, especially given the survival implications of cognitive impairment (3, 4, 111).

Despite initial enthusiasm that hexanucleotide repeats account for most of the ALS-FTD cohort (112, 113), it has quickly become apparent that *C9orf72* hexanucleotide repeats only explain a minority of ALS-FTD cases (114). Emerging studies confirm that a subgroup of *C9orf72* negative patients may show neuroanatomical alterations similar to the ones observed in patients carrying the hexanucleotide expansion. Furthermore, temporal lobe changes have been captured in asymptomatic hexanucleotide carriers, who also exhibited subcortical gray matter degeneration prior to symptom onset (115).

Existing multimodal studies which combine neuroimaging and neurocognitive measures either support a close association between anatomical changes and memory performance or highlight a relative dissociation between the two methods. This inconsistency is epitomized by reports of absent neuroimaging changes in patients with established memory deficits and the detection of significant hippocampal changes in patients with mild memory impairment (41, 42, 102). Based on the shortcomings of existing hippocampal studies in ALS, future studies should include large sample sizes, disease-controls, longitudinal designs, paradigm-based fMRI, comprehensive neuropsychological profiling, "disease-controls," anatomical corrections for education, and genetic screening for mutations implicated in ALS, FTD, and AD. Furthermore, reliance on high directional diffusion models such as neurite orientation dispersion and density imaging (NODDI), high angular resolution diffusion imaging (HARDI), or Q-ball imaging may be desirable to characterize early WM alterations in parahippocampal regions. Finally, combined imaging and post-mortem studies may provide a validation of the *in vivo* findings.

In conclusion, hippocampal pathology is a clinically and academically relevant field of ALS research which has gained unprecedented momentum in recent years and is likely to contribute important further insights in the coming years.

AUTHOR CONTRIBUTIONS

The paper was drafted by FC, EK, and PB and has been reviewed for intellectual content by GV, PF, MR, NK, and IE.

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MINI REVIEW
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MRI-Based Mapping of Cerebral Propagation in Amyotrophic Lateral Sclerosis

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Neuropathological studies revealed the propagation of amyotrophic lateral sclerosis (ALS) in a sequence of four separate disease-related regional patterns. Diffusion tensor imaging (DTI)-based analysis was established for the individual mapping of sequential disease spreading in ALS as the *in vivo* transfer to neuroimaging. The aim of this review is to summarize cross-sectional and longitudinal results of these technical approaches in ALS as an *in vivo* tool to image ALS propagation stages. This concept was also applied to restricted phenotypes of ALS, e.g., lower motor neuron disease (LMND) or primary lateral sclerosis (PLS). In summary, the regional disease patterns in the course of ALS have been successfully mapped by DTI *in vivo* both cross-sectionally and longitudinally so that this technique might have the potential as a read-out in clinical trials.

Keywords: amyotrophic lateral sclerosis, motor neuron disease, diffusion tensor imaging, fractional anisotropy, magnetic resonance imaging

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INTRODUCTION

The potential of neuroimaging as a technical biological marker for cerebral microstructural alterations in neurodegenerative diseases like motor neuron disorders (MND) is under investigation (Turner et al., 2011, 2012). This review was designed to summarize diffusion tensor imaging (DTI)-based approaches for mapping the established propagation patterns in the brain in amyotrophic lateral sclerosis (ALS) and its variants (restricted phenotypes Ludolph et al., 2015). Classification of MND is a challenge of growing importance given that the therapeutic portfolio for ALS might expand in the future, as reflected in the efforts to revise the diagnostic criteria (Ludolph et al., 2015). With respect to the clinical presentation of ALS, the current revision of the El Escorial criteria addressed a validated staging system, and it was held that the development of non-invasive investigations including MRI will assist (Ludolph et al., 2015). For the staging concept, post-mortem studies of the brain pathology of ALS based on phosphorylated 43 kDa TAR DNA-binding protein (pTDP-43) revealed a possible dissemination in a regional sequence of four disease-related patterns (Braak et al., 2013; Brettschneider et al., 2013; Jucker and Walker, 2013), with the sequential protein pathology spreading initially from the motor neocortex toward the spinal cord and brainstem, followed by spreading to frontal, parietal and, ultimately, anteromedial temporal lobes (Ludolph and Brettschneider, 2015). This corticoefferent spreading model has been transferred *in vivo* to MRI-based concepts by *in silico* models (Schmidt et al., 2016), microstructural data (Kassubek et al., 2014, 2018b), and functional connectivity analysis (Schulthess et al., 2016). Specifically, DTI can be used to detect pathology within the corresponding neuronal white matter (WM) tracts and to obtain *in vivo* staging at an individual patient level by fiber-tract of interest (TOI)-based DTI mapping, i.e., a hypothesis-driven approach that revealed sequential

involvement of the corresponding WM tracts in cross-sectional data (Kassubek et al., 2014) and longitudinal data (Kassubek et al., 2018b). To assess the axonal damage and myelin degradation, the statistical analysis of DTI metrics can be performed by various approaches: (1) unbiased voxelwise comparison by whole brain-based spatial statistics (WBSS) (Müller et al., 2012) or tractwise comparison by tract-based spatial statistics (TBSS) (Smith et al., 2006), or (2) hypothesis-guided tract-based quantification by analyzing DTI metrics in tract systems by probabilistic tools (Sarica et al., 2017), or TOI-based tractwise fractional anisotropy statistics (TFAS) (Müller et al., 2007b). In this review, results of DTI-based cross-sectional and longitudinal analyses in ALS were summarized including applications to clinical variants, i.e., lower motor neuron disease (LMND) and primary lateral sclerosis (PLS).

DTI DATA ANALYSIS TECHNIQUES

The post-processing and statistical analysis of WBSS and TFAS was performed by use of the analysis software tensor imaging and fiber tracking (TIFT) (Müller et al., 2007a). In order to assess the axonal damage and myelin degradation, DTI metrics effects at the group level are reported by voxelwise WBSS comparison (Müller et al., 2012) and tract-based quantification by TOI-based TFAS (Müller et al., 2007b). Standard pre-processing procedures contain quality control of the DTI data including elimination of corrupted DTI volumes (Müller et al., 2011), motion correction of individual DTI data sets, in case of longitudinal data an alignment of baseline data and follow-up data by a halfway rigid-brain co-registration (Menke et al., 2014), normalization to the Montreal Neurological Institute (MNI) stereotaxic standard space (i.e., non-linear and iterative normalization to a study specific template – Müller et al., 2012), and, in case of DTI data from different scanners, a 3-D inter-protocol correction which can be applied ex post facto (Rosskopf et al., 2015). The covariate age should be regressed out due to an age dependency of FA values (Lim et al., 2015). In case of longitudinal analyses, the FA differences between the baseline and follow-ups were normalized to an identical time interval representing comparable disease durations for all patients before group level comparison as previously described in detail (Kassubek et al., 2018b), in order to control for variable follow-up intervals. Post-processing and statistical analysis was performed by a differentiated analysis, i.e., unbiased WBSS (Müller et al., 2012) that statistically compares voxelwise FA values of two subject groups and hypothesis-based tractwise quantification by analyzing FA values along tract systems (TFAS – Müller et al., 2007b).

Fiber tracts were reconstructed from an averaged DTI data set of MNI transformed controls' data (Müller et al., 2007b) by a seed-to-target approach (Kassubek et al., 2014, 2018b); here, for a given pathway, the corresponding TOI is defined by all tracts that originate in a defined seed ROI and end in a target ROI. For quantification of the directionality of the underlying tract structures, the TFAS technique (Müller et al., 2007b) was applied. The four-stage corticoefferent sequential axonal spread of pTDP-43 has been transferred *in vivo* by a hypothesis-driven TOI-based analysis that revealed sequential involvement of the corresponding WM tracts in cross-sectional data (Kassubek et al., 2014) and longitudinal data (Kassubek et al., 2018b). Staging categorization for a given patient at the individual level is possible using an FA-based categorization scheme with sequential involvement of the specific tract structures (Kassubek et al., 2014, 2018b).

IN VIVO TRANSFER OF THE STAGING CONCEPT

The TOI-Based Staging Approach

The hypothesis-guided TOI-based staging approach was suggested to image the neuropathologically proposed sequential progression of ALS in the respective cerebral tract systems, i.e., the CST (as a correlate of ALS-stage 1), the corticorubral and corticopontine tracts (ALS stage 2), the corticostriatal pathway (ALS stage 3), and the proximal portion of the perforant path (ALS stage 4) (Kassubek et al., 2014). The statistical analyses of TOIs showed differences between ALS patients and healthy controls for all tract systems; the significance level of the cross-sectional comparison at the group level in the corresponding fiber tracts was lower, the higher ALS-stage was (Kassubek et al., 2014). After a cross-sectional study with 111 ALS patients and 74 healthy controls with MRI data from 1.5T as well as at 3.0T scanners, a follow-up (mono-centre) study confirmed the results in 382 ALS patients and 149 healthy controls (Kassubek et al., 2018b). In a subsample of 67 ALS patients and 31 healthy controls who obtained at least one follow-up scan after a median of 6 months, longitudinal FA changes showed significant alterations in ALS patients compared with healthy controls in all ALS-related tracts as well as for the grand average of all tract systems (Kassubek et al., 2018b).

By applying the *in vivo* categorization cascade at the individual level (Kassubek et al., 2014), staging categorization for the baseline scans of 387 ALS patients revealed that 72% of the ALS patients were categorized into ALS stages with a homogeneous distribution over the stages. The longitudinal follow-up study with 67 patients with ALS demonstrated that 27% of the longitudinally scanned ALS patients showed an increase in ALS stage after about 4 months, while the other ALS patients remained stable or had already been classified as ALS stage 4 (Kassubek et al., 2018b).

The Unbiased Approach Confirms Results at the Cross-Sectional and Longitudinal Group Level

A multicentre study of eight contributing centers with 253 ALS patients and 189 healthy controls (Müller et al., 2016) confirmed the most significant alterations to be localized in the CST (corresponding to stage 1) and found additional significant WM tract changes in the frontal lobe, the brainstem, and hippocampal regions (corresponding to stages 2–4). The localization of these DTI-based *in vivo* results were in accordance with the definition

of the post-mortem neuropathological stages (Brettschneider et al., 2013; Braak et al., 2017).

In a longitudinal study with 67 ALS patients and 31 healthy controls and an average inter-scan interval of 6 months (Kassubek et al., 2018b), longitudinal significant FA alterations were also observed in the CST, the frontal lobe, the brainstem, and in hippocampal regions, that way imaging longitudinal alterations of FA during disease progression.

Hypothetical Longitudinal FA Dependence in ALS Patients

The cross-sectional and longitudinal FA alterations in ALS patients for unbiased WBSS and hypothesis-guided TFAS suggested a hypothetical FA alteration model for the mean FA values in ALS staging-related tract systems (**Figure 1**). After a certain time interval after disease onset in the ALS patients, FA alterations at the group level appear first in the CST; these FA alterations increase during the disease course, and FA alterations in the corticopontine and corticorubral tract as well as in the corticostriatal pathway can be observed. Finally, FA alterations in the proximal portion of the perforant path contribute to the FA alteration pattern. This hypothetical course is based upon the assumption of almost linear FA alterations. However, there is no proof yet which mathematical model (linear or polynomial) could be assumed for the FA decrease. A solution to this challenge could be the analysis of high-frequency DTI scanning (monthly or even bi-weekly) in a group of about 10 ALS patients during the course of the disease.

A study with 65 DTI scans from ALS patients and healthy controls with several follow-up measurements (Baldaranov et al., 2017) showed an FA decrease in the CST that correlated with the revised ALS functional rating scale (ALS-FRS-R – Cedarbaum et al., 1999). In other studies, both the clinical severity as assessed by the slope of the ALS-FRS-R and the disease duration significantly correlated with the resulting staging scheme (Kassubek et al., 2014, 2018b). Furthermore, the results were recently supplemented by neuropsychological data: 139 patients with ALS were tested with the Edinburgh Cognitive and Behavioral ALS screen (ECAS), in addition to DTI brain measures of pathological spread. Executive function, memory and disinhibited behavior were selected for cognitive staging criteria, since these cognitive functions are attributed to cerebral areas analogous to the pattern of MRI markers of TDP-43 pathology, showing that cognitive impairment follows specific patterns in ALS and, in analogy to DTI-based staging, these patterns are useful to set up a cognitive staging (Lulé et al., 2018).

APPLICATION OF THE *IN VIVO* STAGING APPROACH TO PHENOTYPIC VARIANTS OF ALS

Lower Motor Neuron Disease and Primary Lateral Sclerosis

The current revision of the El Escorial criteria for ALS addressed restricted phenotypes in the sense of clinical variants (Ludolph et al., 2015). Adult LMND without clinically overt upper motor neuron (UMN) pathology accounts for about 10% of all cases of MND types and is also traditionally named progressive muscular atrophy (PMA) (Norris et al., 1993; Traynor et al., 2000). On the other hand, PLS is considered a MND which almost exclusively affects UMN (Wais et al., 2017).

In a monocentric study of 37 LMND patients vs. 53 healthy controls, WM microstructure showed characteristic alteration patterns in patients with LMND (clinically differentiated in fast and slow progressors according to van den Berg-Vos et al., 2003), especially along the CST with regional FA reductions in the motor system; the TOI-based tract-specific analysis in fast progressing LMND showed significant FA reductions in ALS-related tracts beyond the CST when compared to slow progressors or healthy controls (Rosenbohm et al., 2016). These results were confirmed by a bicentric study of 65 LMND patients compared to 92 matched healthy controls and 101 matched ALS patients with a "classical" phenotype: the tract-specific analysis demonstrated significant alterations in ALS-related tract systems for fast progressing LMND patients vs. slow progressors and healthy controls (Müller et al., 2018a).

There is also a longstanding debate if PLS could be classified as a disease entity separate from ALS or as a slowly progressing ALS variant with UMN predominance (Singer et al., 2007). In the revision of the El Escorial criteria, PLS is described as a restricted phenotype that evolves into ALS in the majority of patients (Ludolph et al., 2015). *In vivo*, the analysis of WM integrity by regional FA reductions in 50 PLS patients vs. 50 controls showed the alterations along the CST and additionally in frontal and prefrontal brain areas in PLS and ALS patients (Müller et al., 2018b). The ALS-staging-related tract-specific analysis demonstrated identical alterations of ALS-related tract systems for PLS and ALS when compared with controls and showed no differences for the comparison between ALS and PLS (Müller et al., 2018b).

APPLICATION OF THE *IN VIVO* STAGING APPROACH TO BEHAVIORAL VARIANT OF FRONTOTEMPORAL DEMENTIA

The characteristic longitudinal distribution pattern of the underlying pTDP-43 pathology in the behavioral variant of frontotemporal dementia (bvFTD) across specific brain regions was demonstrated (Brettschneider et al., 2014). The *in vivo* staging approach was transferred to bvFTD (without MND) and showed an alteration pattern in the involved major WM tracts (Kassubek et al., 2018a): the TOIs of bvFTD-pattern 1 (uncinate fascicle), 2 (corticostriatal pathway) and 4 (optic radiation) demonstrated significant differences for bvFTD patients vs. controls, whereas the TOI representing the CST (bvFTD-pattern 3) showed no differences for bvFTD vs. controls. Aspects of the heterogeneous neuropathology of bvFTD which is based upon pTDP-43 only in about 50% of the cases are an issue of discussion (Kassubek et al., 2018a).

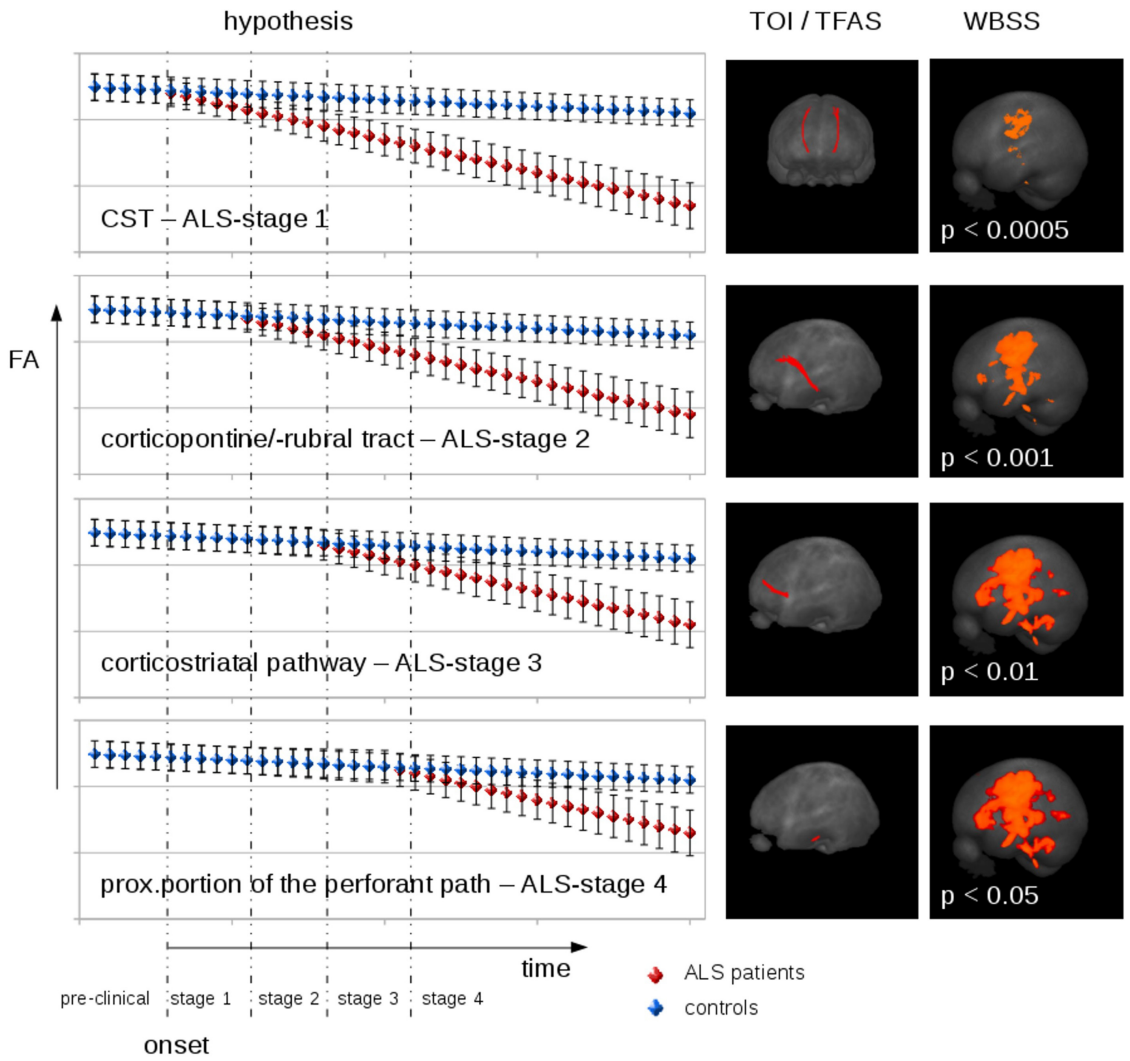

FIGURE 1 | Hypothetical FA development/decrease model for the mean FA values in ALS staging related tract systems. Left panel: At baseline, mean FA was supposed to be identical in patients and controls (with individual error bars). After a certain time interval after disease onset, FA alterations appear first in the CST (related to ALS stage 1). During the disease course, these FA alterations manifest, and FA alterations in frontal and prefrontal areas as well as in the brain stem are observed (corticopontine and corticorubral tract as well as corticostriatal pathway, related to ALS stages 2 and 3, respectively). With higher disease duration, FA alterations in the CST further decrease and alterations in hippocampal areas (proximal portion of the perforant path, related to ALS stage 4) contributed to the FA alteration pattern. Central panel: Projectional views of fiber tracts used for tractwise fractional anisotropy statistics (TFAS) for each of the four stages. Right panel: Projectional views of the corresponding whole brain-based spatial statistics (WBSS).

DISCUSSION

In this review, the approach to use DTI metrics in the assessment of axonal damage and myelin degradation in ALS is specifically addressed. An unbiased voxelwise comparison by WBSS (Müller et al., 2012) is an approach to assess microstructural alterations with an imaging resolution in the order of millimeters. WBSS directly compares DTI metrics of subjects at the group level after stereotaxic normalization for the whole brain without any prior restriction to specific brain areas. On the other hand, a tractwise comparison by TOI-based TFAS (Müller et al., 2007b) addresses DTI-based alterations along specific tract structures both at the group level and at the individual level; the hypothesis-guided TOI approach provides a higher statistical accuracy compared to voxelwise analysis since the whole tract structure is taken into account. An alternative approach to assess ALS-related microstructural alterations is TBSS (Smith et al., 2006; Agosta et al., 2010) that aims at analyzing changes in WM across individuals, that way relying on the precise changes in WM across individuals. TBSS is a probabilistic method that generates multiple solutions to reflect the variability or uncertainty of the estimated fiber orientation restricting the statistical comparisons to the centers of WM tracts after non-linear registration (using FA measurements to realign subjects and extract the centers of WM tracts).

In vivo Imaging of TDP-43 Pathology in ALS and Its Variants

Post-mortem studies demonstrated a concept for patterns of TDP-43 pathology in ALS with a sequential progression of pTDP-43 aggregates (Braak et al., 2013), the task remained to investigate if *in vivo* neuroimaging measures might be identified that were consistent with these patterns of pTDP-43 progression (Kassubek et al., 2018a). The TOI-based staging approach (Kassubek et al., 2014) was able to map *in vivo* the proposed neuropathological progression of ALS cross-sectionally as well as longitudinally, that way supporting DTI as a candidate technical marker to image ALS stages *in vivo* (Kassubek et al., 2018b). The microstructural alterations were supplemented by alterations in functional brain organization: specific intrinsic functional connectivity networks revealed significantly increased functional connectivity for the motor network (as the correlate of the neuropathological stage 1), the brainstem network (neuropathological stage 2), the ventral attention network (neuropathological stage 3), and the default mode/hippocampal network (neuropathological stage 4) in a cross-sectional as well as in a longitudinal study design (Schulthess et al., 2016). Increased functional connectivity is strongly indicative for abnormal brain functioning. First, patterns of increased functional connectivity in ALS that result from abnormally strong functional coupling within a specific functional brain network have been attributed to a gradual loss of the inhibitory influence (Douaud et al., 2011). Second, the patterns of increased functional connectivity also present as a network expansion (Schulthess et al., 2016) which is a commonly observed phenomenon in neurodegenerative diseases (Gorges et al., 2015). A straightforward interpretation of adaptive changes is that additional brain areas become functionally integrated, i.e., additional functionally segregated resources are recruited for compensating the ongoing cell loss in within-network modules in order to maintain "normal" performance (Hillary and Grafman, 2017). The application of the *in vivo* techniques to specific MND phenotypes (ALS variants) demonstrates central nervous system involvement of the corticofugal tracts in fast progressive LMND, in support of the hypothesis that LMND is an ALS variant (Müller et al., 2018a). Furthermore, the clinical approach to the phenotype of PLS as an ALS variant was confirmed, in accordance with the latest revision of the El Escorial criteria (Agosta et al., 2015a; Ludolph et al., 2015), in favor of the conclusion that these patients can be treated like ALS and also may be included into clinical trials of ALS (Müller et al., 2018a).

Hypothesis Guided Tract-Based Analysis

The DTI-based TOI approach is a microstructural correlate of the progressive pathological process; this analysis technique identifies defined anatomical tract systems that represent the proposed progression patterns based upon histopathology (Braak et al., 2013) and are not *per se* determined by a data-driven analysis (Kassubek et al., 2018a). The approach of analyzing a "propagation pattern" is longitudinal in nature. Thus, the analysis according to the progression concept – which has been developed on the basis of cross-sectional post-mortem data – targets the identification of patterns that can be consistently found in a diverse group of neurodegenerative disorders, each of which entails the aggregation of abnormal protein inclusions in characteristic locations (Jucker and Walker, 2013). The longitudinal access of categorizing patients with ALS could be by longitudinal DTI scans followed by confirmation by post-mortem pathology analyses, i.e., the combination of the *in vivo* staging with post-mortem classification in the same subjects. However, the availability of such data is limited. The role of other neuroimaging modalities including molecular imaging has to be evaluated in future studies.

Limitations

A limitation of the staging categorization is that only about 80% of the MND patients could be categorized. This is a technique-immanent limitation as thresholds for the differentiation between patients and controls were defined in a data-driven approach. Due to an incomplete separation between ALS patients and controls (the sensitivity is about 80%), not all patients would be classifiable (Kassubek et al., 2014). The definition of optimized thresholds by repeated control scans or an increased number of control scans might increase the sensitivity and thus the percentage of categorized MND patients. A further limitation of present neuroimaging approaches is the lack of autopsy-confirmed data (Kassubek et al., 2018a); thus, the TOI-based analysis only provides a plausible surrogate pattern for *in vivo* "staging" for the pathology in the ALS cohorts. Finally, since DTI is a quantitative imaging technique, suboptimal acquisition, data processing and analysis approaches can affect the quality and reliability of DTI-derived metrices (Jones, 2010).

Summary

Many neurodegenerative diseases feature characteristic patterns of early neuronal and regional vulnerability, with increasing evidence that misfolded protein aggregates can spread by a self-perpetuating process, and novel neuroimaging techniques can help elucidating how these disorders spread across brain networks (Agosta et al., 2015b). Measurement of WM tract involvement seems to be a valid surrogate to assess the *in vivo* spreading of pathological proteins and seems to be a valid approach to provide insights into the trajectory of processes of neurodegeneration (Agosta et al., 2015b) in order to move neuroimaging "from snapshots to motion picture" according to Schuster and co-workers (Schuster et al., 2015).

In ALS as one of the neurodegenerative diseases with such a propagation pattern, the analysis of the neuropathologically defined structures demonstrated a characteristic alteration pattern of the involved WM pathways cross-sectionally as well as longitudinally (Kassubek et al., 2018a); at present, no direct neuroimaging marker for pTDP-43 exists, but previous neuropathological studies have shown the correlation between the degree of pTDP-43 aggregation and axonal loss (Geser et al., 2009). The DTI-based analysis of microstructural integrity is a different approach compared to analysis techniques like regional

volumetric studies that directly measure regional atrophy or intrinsic functional connectivity analysis (Filippi et al., 2015). Thus, the investigation of microstructural integrity by the DTI/TOI-based approach has potential to serve as a non-invasive *in vivo* neuroimaging marker.

The DTI-based techniques have the potential for future use in the work-up of individual patients, they potentially enlarge the spectrum of non-invasive biological markers as a neuroimaging-based read-out for clinical studies (Kassubek et al., 2018a). These studies also could be used for the identification of patients that could be elected for trials targeting at treating the specific histopathologic abnormalities causing MND (Kassubek et al., 2018a). DTI-based scores may provide a different target information to currently available scores for longitudinal screening, as a candidate read-out for future disease-modifying strategies on the transmission of TDP-43 in ALS (Kassubek et al., 2018b).

AUTHOR CONTRIBUTIONS

H-PM and JK conceived and designed the study, collected and interpreted the data, and wrote the manuscript.

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MINI REVIEW
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Biomarkers of Spinal and Bulbar Muscle Atrophy (SBMA): A Comprehensive Review

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Spinal and bulbar muscular atrophy (SBMA), also known as Kennedy's disease, is a rare, X-linked, late onset neuromuscular disorder. The disease is caused by a CAG trinucleotide repeat expansion in the first exon of the androgen receptor gene. It is characterized by slowly progressive lower motor neurons degeneration, primary myopathy and widespread multisystem involvement. Respiratory involvement is rare, and the condition is associated with a normal life expectancy. Despite a plethora of therapeutic studies in mouse models, no effective disease-modifying therapy has been licensed for clinical use to date. The development of sensitive monitoring markers for the particularly slowly progressing pathology of SBMA is urgently required to aid future clinical trials. A small number of outcome measures have been proposed recently, including promising biochemical markers, which show correlation with clinical disability and disease-stage and progression. Nevertheless, a paucity of SBMA-specific biomarker studies persists, delaying the development of monitoring markers for pharmaceutical trials. Collaborative efforts through international consortia and multicenter registries are likely to contribute to the characterization of the natural history of the condition, the establishment of disease-specific biomarker panels and ultimately contribute to the development of disease-modifying drugs.

Keywords: SBMA, biomarkers, clinical trials, multisystem involvement, outcome measures

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INTRODUCTION

Spinal and bulbar muscular atrophy (SBMA), also known as Kennedy's disease, is a rare, X-linked, adult onset, neuromuscular disorder (1) characterized by slowly progressive lower motor neuron (LMN) degeneration, skeletal muscle pathology and by a spectrum of multi-organ involvement (2–4). The disease is caused by a CAG repeat expansion in the first exon of the androgen receptor (AR) gene encoding for a poly-glutamine (polyQ) tract. A repeat number higher than 38 is considered pathogenic (5). PolyQ-AR toxicity is hormone-dependent and CAG repeat size inversely correlates with age of symptom onset but not with disease progression rates (6, 7). Heterozygous female carriers of the mutation only present subtle signs of neuromuscular involvement such as muscle

cramps and hand tremor (8, 9). The disease is rare, with an estimated prevalence of 3.5/100,000 male inhabitants in southern Europe (10, 11) but the presence of a founder effect is retained to cause considerable differences in the distribution of the disease in various geographical regions (12, 13). Subjects with minimal symptoms and the relatively limited awareness of the condition make it likely that the real prevalence of SBMA is underestimated.

Despite several promising therapeutic studies (14), no disease-modifying treatment currently exists for SBMA. Similarly to SMA, the lack of sensitive monitoring markers for the slow progression rates of SBMA is one of main the barriers to successful clinical trials (15, 16). The objective of this work is the systematic review of candidate biomarkers in SBMA and the appraisal of their potential in clinical management and pharmaceutical trials.

THE NEUROLOGICAL PRESENTATION

Limb weakness is present in 97% of SBMA cases. It usually appears at the of age of 35–40 and starts typically proximally in the lower limbs (2, 3, 6, 17). However, tremors, muscle cramps, myalgia, gynecomastia, and exercise intolerance are often reported long before the onset of frank limb weakness (17, 18). Clinical signs of LMN involvement, such as fasciculations, muscle cramps, and atrophy are invariably present. Proximal muscles are predominantly affected, leading to difficulties in climbing stairs and getting up from a sitting position. Motor impairment is usually slowly progressive (19) and survival is only slightly reduced (6, 17). In addition to limb muscle wasting, fasciculations, and decreased deep tendon reflexes, clinical features often include a high-frequency postural hand tremor and postural leg tremor (20).

Bulbar impairment occurs in about 10–30% of patients at the onset of the disease (17), but it is present in the majority of the patients at later stages. It slowly progresses over time and may lead to aspiration pneumonia, which is a frequent cause of death in SBMA (6). Dysphagia is due to impaired oro-pharyngeal phase of deglutition (21), and is associated with tongue's muscles weakness, fasciculations, and atrophy (21). Dysarthria is characterized by hypernasality secondary to incomplete soft palate elevation and is associated with dysphonia. Speech impairment can evolve into markedly reduced intelligibility. Facial weakness and asymmetry, perioral fasciculations, myokymia, and jaw drop are also common clinical features (21–23). Recurrent laryngospasms have been noted in up to 47% of SBMA patients (24).

The presence of a distal sensory neuropathy is a hallmark feature of the disease (25) which has been described in post-mortem studies (26), sural nerve biopsies (27), and neurophysiology (28). The sensory neuropathy may be asymptomatic or manifests in distal numbness and paraesthesia in the lower limbs and reduced sensation for vibration. Neurophysiological examination readily detects reduced or absent sensory action potentials (SAPs) (28, 29). Degeneration of small myelinated and unmyelinated fibers may explain the high incidence of neuropathic pain (30) in SBMA.

MULTISYSTEM INVOLVEMENT

Complex multi-organ involvement is a hallmark feature of SBMA. The core non-neurological features of SBMA include gynecomastia, testicular atrophy, reduced fertility and erectile dysfunction. Dysfunction of the AR protein leads to partial androgen insensitivity (31), manifesting in erectile dysfunction (3), gynecomastia and reduced fertility (31, 32). Testosterone and dehydro-epiandrosterone sulfate (DHEAS) are elevated in up to 38% of patients (32). The Androgen Sensitivity Index (ASI) (LH × testosterone), which reflects androgen resistance, is found to be increased in almost half of the patients (3, 32). DHEAS is thought to correlate with CAG repeat number as well as disease duration (32). Metabolic syndrome with increased BMI, elevated serum cholesterol, triglycerides, and fasting glucose is also a key feature of the disease (3, 31–33) and insulin resistance is associated with disease severity (34). Liver involvement with steatosis and sometimes inflammation has been described (33), but the risk of progression to liver fibrosis is unclear. Recurrent urinary symptoms and incomplete bladder emptying may affect more than the third of male SBMA patients even in the absence of benign prostatic hyperplasia, which is likely to be explained by pelvic floor and bulbuocanvernosus muscle dysfunction (3). While there is no evidence of a primary cardiomyopathy in SBMA (35), Brugada-like ECG abnormalities have been reported in almost half of the patients in a large Japanese cohort (36). Obstructive sleep apnea (OSA), poor sleep quality and periodic limb movements in sleep have also been reported (37).

BIOMARKERS IN SBMA

A biomarker is a parameter that can be measured accurately and reproducibly and used as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (WHO definition, 1998). An ideal biomarker should have a predictive value and capture subtle changes over relatively short periods of time. Additional requirements to biomarkers include cost-effectiveness, non-invasiveness, and reproducibility (38, 39). It is generally agreed that no single biomarker is suitable for diagnostic, prognostic and monitoring roles and a panel of several markers may be better suited as multirole indicators (40). SBMA is a rare and slowly progressing condition, therefore the development of sensitive outcome measures would enable smaller sample-size and shorter duration of pharmaceutical trials (41, 42).

BIOMARKERS OF NEUROLOGICAL INVOLVEMENT IN SBMA

In recent years, an unprecedented interest has developed in the standardized assessment of neuromuscular performance in SBMA, evaluation of novel therapeutic strategies (14) and in the launch of national SBMA registries (42, 43). Many of the commonly used instruments, such as the MRC score, respiratory function parameters, the modified Norris scale, ALSFRS-r, Quantitative Myasthenia Gravis Score etc. are non-specific to

SBMA, yet remain widely utilized. As these tools have been developed for other conditions, new batteries of tests have been recently proposed to specifically appraise disability in SBMA (**Table 1**).

6-Minute-Walk-Test (6MWT)

The 6-minute-walk-test (6MWT) was proposed as an accurate marker of disease progression (44). It measures the distance a person can walk within 6 min and is regarded as a composite proxy of cardiopulmonary and neuromuscular abilities (61). Due to its relative simplicity and cost-effectiveness it has been widely adopted as an outcome measure in several neuromuscular conditions, such SMA and myopathies (62, 63). The 6MWT is traditionally considered the most reliable marker of motor impairment in SBMA, it reliably captures a 10% decline over 1 year (44) and has been used as a primary outcome measure in clinical trials (45, 57). A shorter version of the test, the "2-MWT," also exists and is thought to be reliable (63).

Adult Myopathy Assessment Tool (AMAT)

The Adult myopathy assessment tool (AMAT) is a performance-based instrument composed of functional and endurance subscales (46). AMAT provides a comprehensive evaluation of motor function, and muscle fatigue, which is a key facet of disability in SBMA (64). One of the strengths of AMAT is that it can also be applied to non-ambulatory patients. It is widely used in both SBMA registers (43) and in clinical trials (47, 52).

SBMA Functional Rating Scale (SBMAFRS)

The SBMA functional rating scale (SBMAFRS) SBMAFRS is a recently validated scale (48, 49), which has been developed from the ALSFRS-r (65) and specifically adapted for the disability profile of SBMA. It is a questionnaire-based scale that measures physical function in activities of daily living (ADL) and consists of five main domains measuring bulbar, upper-limb, lower-limb, truncal, and respiratory function. The SBMAFRS has proven to be more sensitive than the ALSFRS-r in evaluating SBMA patients with moderate motor deficits (48).

1234-Scale

The 1234-scale is another questionnaire-based scale based on the ALSFRS-r, which focuses on SBMA-associated motor disability (50). It includes items such as the ability to do push-ups, ability to run and to stand up from a squatting position. The 1234-scale has shown good internal validity and high reliability (50), but its sensitivity as a monitoring marker has not been confirmed.

Quantitative Muscle Strength Assessment (QMA)

Manual muscle testing (MMT) is commonly used to describe muscle weakness in neuromuscular conditions even though it is highly evaluator-dependent (66). A number of more objective techniques are available to evaluate muscle strength quantitatively in the four limbs (67). Grip strength measured by a handheld dynamometer is one of the simplest and most reproducible QMA parameters. Significant changes in grip strength have been observed in a 3-year longitudinal study of SBMA (19), but progressive changes have not been captured over a 1-year follow-up (44). QMA of maximal voluntary isometric muscle strength has been repeatedly proposed as an outcome measure for clinical trials (46, 47, 52, 54), but its efficacy as a biomarker is limited by considerable inter-centers variability.

Videofluoroscopy (VF)

Videofluoroscopy (VF) is routinely used to evaluate dysphagia in a range of neurological conditions. In SBMA, VF can reliably detect the impairment of the oral phase of deglutition confirming large amount of oral barium residue (56). VF has been previously used in clinical trials (51, 55, 68), but the lack of standardization makes it less suitable for robust multicenter studies.

Fiber Endoscopic Evaluation of Swallowing

Fiber endoscopic evaluation of swallowing has also been assessed as a candidate biomarker of bulbar impairment, but the diagnostic and prognostic value of the technique is yet to be validated (21).

Tongue Pressure

Tongue pressure measurements using an electronic device has been proposed as a biomarker of dysphagia in SBMA, and has been shown to be a low-cost and reliable way of detecting tongue weakness early in the course of the disease (54). An important limitation is that it is susceptible to a ceiling effect in subjects with severe bulbar impairment. Nevertheless, it has been used successfully in a trial of head-lift exercises as a possible rehabilitation strategy in SBMA-associated dysphagia (55).

Electrophysiology

Standard electrophysiology measures are routinely used in the diagnostic work-up of SBMA, but they exhibit limited sensitivity to longitudinal changes (28). This is somewhat unexpected given the correlation between CAG repeat numbers and electrophysiological parameters (29). Quantitative Motor Unit Number Estimation (MUNE) techniques have emerged as a promising way of quantifying motor neuron loss in a number of motor neuron diseases (69, 70). Significant MUNE reductions have been shown in SBMA patients both in cross-sectional and longitudinal study designs, making it one of the most promising candidate outcome measures (58, 59). MUNIX is a more recent, non-invasive method of quantifying motor neuron loss, that has already been utilized in ALS (71), peripheral neuropathies (72), and more recently in adult SMA patients (16). The motor unit size index (MUSIX) (CMAP amplitude/MUNIX) is increasingly accepted as a measure of compensatory collateral sprouting. This technique has not been tested in SBMA yet, but is likely be a promising tool in the evaluation of longitudinal motor neurons loss.

Quantitative Muscle MRI

While quantitative muscle MRI would be an obvious candidate marker of disease progression in SBMA, there is a surprising scarcity of such studies. Existing studies have shown that muscle imaging can effectively detect muscle pathology in distal leg muscles which is less obvious on clinical assessment (60).

TABLE 1 | Research studies considering motor and bulbar skills-related outcome measures.

Primary outcome measure	Reference number	Authors	Other outcome measures in the study	Type of study	Number of patients	Duration of follow-up
MOTOR SKILLS-RELATED OUTCOME MEASURES						
6MWT	(44)	Takeuci et al.	Modified Norris score, ALSFRS-R, grip strength	Observational, longitudinal study	35 at baseline, 24 at follow-up	12 months
	(45)	Querin et al.	MMT, ALSFRS-R, FVC	Pilot, unblinded pharmacological trial (Clenbuterol)	20	12 months
AMAT	(46)	Harris-Love et al.	QMA, 2MWT, ADL assessment, SF-36v2	Observational, cross-sectional study	55	/
	(47)	Shrader et al.	QMA, STS test, Timed up and Go test, Balance tests, SF-36v2, Beck depression scale, serum CK, IGF-1 and testosterone	Randomized, evaluator-blinded pharmacological trial (Physical exercise)	50	12 weeks
SBMAFRS	(48)	Hashizume et al.	ALSFRS-R, Modified Norris Score	Observational, longitudinal study	80	12 months
	(49)	Querin et al.	MMT, 6MWT, ALSFRS-R	Observational, longitudinal study	60	8 weeks
1234 scale	(50)	Lu et al.	ALSFRS-R	Observational, longitudinal study	81	32 months
ALSFRS-R	(51)	Banno et al.	VF, MMT, FVC, serum CK, AST, ALT, Beck depression scale, 1C2-positive cells in scrotal skin biopsies	Randomized, double-blinded pharmacological trial (Leuprorelin)	50	48+96 weeks
QMA	(52)	Fernández-Rhodes et al.	AMAT, MMT, 2MWT, SF-36v2, IIEF, MUNE, CMAP VF, FVC, serum CK and testosterone	Randomized, double-blinded pharmacological trial (Dutasteride)	50	24 months
Hand grip strength	(53)	Hijikata et al.	Modified QMG score, ALSFRS-R, SBMAFRS, 15-foot timed-walk test, rise-from-bed test, swallowing questionnaires, FVC, Multidimensional Fatigue Inventory, urinary 8-OHdG	Randomized, double-blinded pharmacological trial (Creatine Monohydrate)	45	8 weeks
BULBAR FUNCTION-RELATED OUTCOME MEASURES						
Tongue pressure	(54)	Mano et al.	Modified Norris score, ALSFRS-R, QMA, grip strength, MMT, modified QMG score, VF, swallowing questionnaires, timed walk test	Observational, cross-sectional study (validity of tongue pressure as marker of dysphagia)	47	/
	(55)	Mano et al.	VF, modified QMG score, ALSFRS-R, serum CK and testosterone	Non-randomized, interventional study (head lift exercises)	6	12 weeks
VF	(56)	Hashizume et al.	ALSFRS-R, SBMAFRS, swallowing questionnaires, Limbs Norris score, Bulbar Norris score	Observational, longitudinal study	111	30 days
	(57)	Katsuno et al.	ALSFRS-R, 6MWT, modified QMG score, 1C2-positive cells in scrotal skin biopsies, serum CK and testosterone, ALSAQ-5 score	Randomized, double-blinded pharmacological trial (Leuprorelin)	204	12 months
FEES	(21)	Warnecke et al.	MMT, modified Rankin scale	Observational, cross-sectional study	10	/
INSTRUMENTAL OUTCOME MEASURES						
MUNE	(58)	Suzuki et al.	Limbs Norris score, Bulbar Norris score, ALSFRS-R, grip strength	Observational, longitudinal study	52	12 months
	(59)	Lehky et al.	CMAP, SMUP	Observational, cross-sectional study	54	/
CMAP and SNAPs	(29)	Suzuki et al.	Limbs Norris score, Bulbar Norris score, ALSFRS-R, spinal cord tissue specimens	Observational, cross-sectional study	106	/
Muscle MRI	(60)	Hamano et al.	/	Observational, cross-sectional study	3	/

ALSFRS-R, Amyotrophic Lateral Sclerosis functional rating scale-revised; MMT, manual muscle testing; FVC, forced vital capacity; QMA, quantitative muscle assessment, 2 or 6MWT, 2 or 6 minutes-walk-test; ADL, activity of daily living; DXA, Dual-energy X-ray absorptiometry, urinary 8-OHdG, 8-hydroxydeoxyguanosine; VF, videofluoroscopy; AMAT, adult myopathy assessment tool; IIEF, International Index of erectile function; MUNE, motor unit number estimate; CMAP, compound motor action potential; CK, creatine-kinase; QMC score, quantitative myasthenia gravis score; SMUP, single motor unit potential.

Spinal Cord Imaging

Spinal cord imaging has seen unprecedented advances in recent years and has been applied successfully to other motor neuron diseases such as ALS (73–75), and SMA (15) to characterize gray (76) and white matter pathology (77). There is an ongoing study to test its efficacy in SBMA patients (NCT02885870).

Quantitative Brain Imaging

Quantitative brain imaging studies demonstrated white matter alterations in the corticospinal tracts (CST), limbic system (78, 79), brainstem and cerebellum (80). Voxel-based morphometry (VBM) of SBMA cohorts revealed gray matter atrophy in the frontal lobes and in the brainstem (78–81). Frontal hypometabolism has been detected by positron-emission-tomography (PET) (82). These studies confirm the multisystem nature of SBMA-associated pathology, and that neurodegeneration is not limited to LMNs but involve the CSTs and widespread cerebral regions. Despite imaging evidence of extra-motor involvement, neuropsychological studies have only detected subtle frontal dysfunction in small study populations (83, 84) which were not confirmed in larger cohorts (85, 86).

BIOMARKERS OF MULTISYSTEM INVOLVEMENT IN SBMA

Increased Serum CK Levels

Increased serum CK levels have been reported by almost every SBMA study and support the hypothesis of a primary myopathy in SBMA (87, 88). Elevated serum CK levels can be detected prior to symptom onset (89) and may be most marked around disease manifestation (18, 19). Nevertheless, no correlation was found between serum CK levels and age of onset, CAG repeat numbers, disease duration or rate of progression (6, 19). As a result, CK levels are thought to be useful as part of the diagnostic workup, but of limited use in monitoring disease progression.

Transaminases Levels

Transaminases levels have also consistently been shown to be raised in SBMA including the pre-symptomatic phase of the disease (89), but they do not correlate with the progression of the neurological symptoms. The clinical significance of raised transaminases in SBMA is a topic of debate and its prognostic value remains to be established (33).

Serum Creatinine Level

Serum creatinine level has also been proposed as a potential biomarker (90) despite its lack of specificity to SBMA. It tends to be reduced in the pre-symptomatic and symptomatic phases of the disease (91) and correlate well with parameters of motor impairment (6, 19, 91).

Proxies of Metabolic Syndrome and Insulin Resistance

Proxies of metabolic syndrome and insulin resistance are considered closely associated with primary molecular disease mechanisms. The homeostasis model assessment of insulin resistance (HOMA-IR) index correlated significantly with motor function parameters in one study (34), but this relationship has

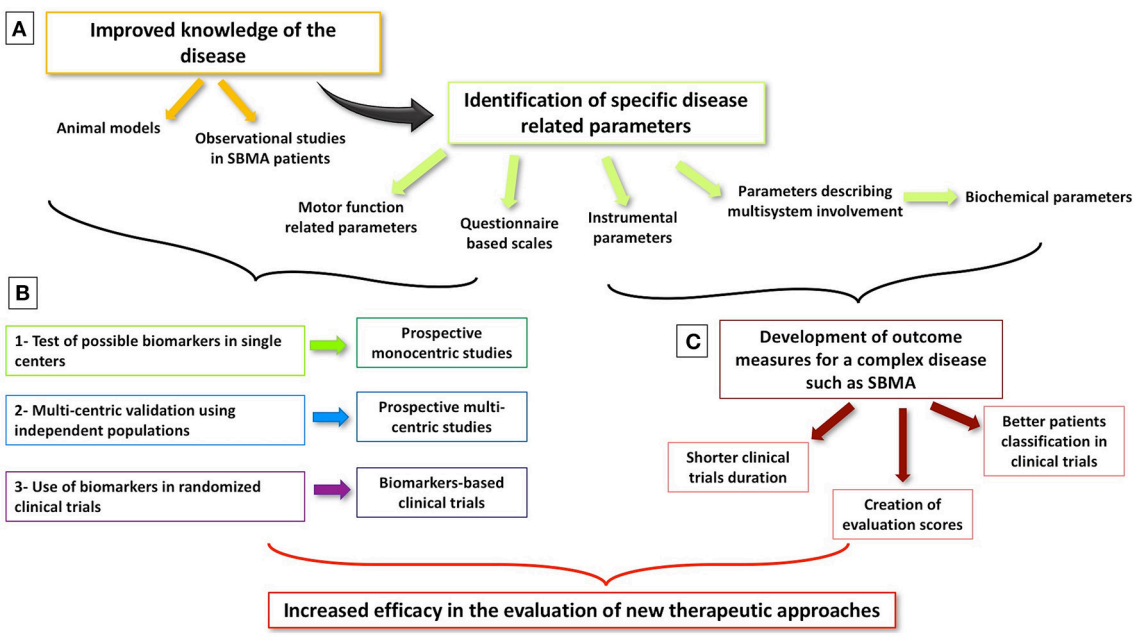

FIGURE 1 | Milestones of biomarker development in SBMA. **(A)** Better knowledge of SBMA through animal models and observational studies allows the identification of possible biomarkers of disease status and of its progression. **(B)** Different steps are needed to develop and validate a biomarker in order to make it a reliable outcome measure in clinical trials. **(C)** Considered the complexity of SBMA and its multi-system presentation, the development of global biomarkers, including both motor function and biochemical parameters, is warranted with the aim of improving the efficacy of upcoming clinical trials.

not been confirmed by others (32). **Hormones levels** and **ASI** (Androgen Sensitivity Index) have also been repeatedly proposed as markers of SBMA. Free testosterone levels correlate with muscle strength in one study (2) but it does not correlate with CAG repeat numbers or disease progression according to others (57). DHEAS levels have been linked to disease duration (91).

Skin Biopsies

Skin biopsies have been performed in some clinical trials to evaluate changes in the frequency of anti-polyQ antibody-positive cells after treatment (57). This index may be sensitive to changes during pharmacological treatment but the methodology is inherently invasive and poorly harmonized across different centers.

Adipose Tissue Quantification

A recent study proposed adipose tissue quantification using whole-body MRI and reported significant subcutaneous fat accumulation in SBMA patients. This correlated both with CAG repeat lengths, disease duration and progression rates (32). These data suggest that adipose tissue MRI may be an additional marker of multisystem involvement in SBMA.

DISCUSSION AND FUTURE PERSPECTIVES

Interest in SBMA biomarkers has grown steadily in recent years, fuelled both by accruing knowledge about pathogenesis and novel therapeutic strategies (14, 42). SBMA is now widely recognized as a multisystem syndrome (3). A multitude of studies focus on multi-organ involvement, and the systemic phenotype is now considered just as relevant as the neurological manifestations. It is increasingly recognized that non-neurological features of the disease have an equally important impact on the patients' quality of life (3, 31–34, 87, 88, 91, 92). Until now, clinical trials on SBMA focused almost exclusively on the treatment of motor symptoms (14, 45, 47, 51–53, 55, 57, 68, 92, 93), but a shift to targeted molecular therapies (94) and focus on systemic processes are likely to be witnessed in the near future. From a clinical trial perspective, ideal biomarkers should undergo robust validation, sensitivity and specificity profiling, and sampling and measurement harmonization across different centers. Crucially, candidate markers should be able to detect the subtle changes expected after the administration of a specific treatment (95). Given the particularly slow progression rates observed in SBMA, the definition of an effective outcome measures is challenging. The integration of neurological, metabolic, and endocrine indicators seems essential into composite biomarker panels in addition to functional scales. Serum creatinine levels appear to correlate strongly with motor impairment and HOMA-IR index with disease duration (34). The convincing validation of these parameters and their use as effective outcome measures in clinical trials will require robust multicenter study designs (96) (**Figure 1**).

Furthermore, the comparison of the specificity profile of candidate biomarkers seems essential to define their roles in clinical applications. The establishment of national and international SBMA registers is a clear priority which will be an invaluable resource for future SBMA research (42). As in other neurodegenerative conditions (95, 96), the integration of clinical, molecular, imaging and neurophysiological markers may be required for assessing the efficacy of disease-modifying interventions (95, 96). To conclude, we underline the relevance of considering both motor (muscle force evaluation, questionnaire based scales, and performed tasks) and biochemical parameters as possible outcome measures for a multi-system and complex pathology as SBMA. Beyond their monitoring roles, validated biomarkers will also aid patient stratification upon entry into pharmacological trials (97).

AUTHOR CONTRIBUTIONS

The paper was drafted by GQ and PB and has been reviewed for intellectual content by VM-P and P-FP.

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MINI REVIEW
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Clinical and Radiological Markers of Extra-Motor Deficits in Amyotrophic Lateral Sclerosis

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Amyotrophic lateral sclerosis (ALS) is now universally recognized as a complex multisystem disorder with considerable extra-motor involvement. The neuropsychological manifestations of frontotemporal, parietal, and basal ganglia involvement in ALS have important implications for compliance with assistive devices, survival, participation in clinical trials, caregiver burden, and the management of individual care needs. Recent advances in neuroimaging have been instrumental in characterizing the biological substrate of heterogeneous cognitive and behavioral deficits in ALS. In this review we discuss the clinical and radiological aspects of cognitive and behavioral impairment in ALS focusing on the recognition, assessment, and monitoring of these symptoms.

Keywords: amyotrophic lateral sclerosis, extra-motor involvement, cognition, behavior, neuropsychological deficits, neuroimaging

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is the most common form of motor neuron disease (MND), a progressive neurodegenerative condition defined by concomitant lower and upper motor neuron degeneration (1). Motor symptoms include muscle weakness, fasciculations, cramps, as well as spasticity and brisk reflexes that accrue to considerable limb and bulbar disability over time, and eventually respiratory failure (1). The identification of TAR DNA-binding protein 43 (TDP-43) positive ubiquitinated cytoplasmic inclusions in almost all patients with ALS and more than half of patients with frontotemporal dementia (FTD) has placed ALS on the so-called "ALS-FTD continuum," highlighting the considerable clinical, pathophysiological, and neuroimaging overlap between the two neurodegenerative conditions (2).

Although mentioned in early descriptions of ALS (3, 4), cognitive and behavioral deficits and frank dementia were previously considered atypical of ALS. It is not until the end of the twentieth century that clinical and research interest shifted to the extra-motor features of ALS and it has been gradually recognized as a genuine multisystem disease (5–8).

Neuropsychological deficits in ALS range from mild impairment to full-blown FTD. Up to 65% of ALS patients exhibit some cognitive or behavioral impairment (9–12) and 6–15% of sporadic ALS patients meet diagnostic criteria for FTD (10–13). While hexanucleotide repeat expansions in *C9ORF72* are often associated with ALS-FTD (14), extra-motor symptoms are not unique to this mutation and extra-motor neuroimaging findings can also be readily identified in a significant proportion of C9 negative patients (15, 16). The early recognition of extra-motor involvement in

ALS is crucial due to its impact on functional decline (17), survival (18), compliance with assistive devices (19), decision-making, and engagement in end-of-life and legal decisions (20).

COGNITIVE DYSFUNCTION

Much attention has been initially focused on executive dysfunction (21, 22) in ALS which has been gradually complemented by the characterization of language (23, 24), memory (25, 26), praxis (27), and theory of mind deficits (28) (**Table 1**). Population-based studies have confirmed distinct cognitive phenotypes without executive impairment (10, 11, 29).

Executive Dysfunction

Executive dysfunction is the most commonly cited facet of cognitive impairment in ALS. Executive function however is an umbrella term encompassing several relatively distinct higher-order processes, such as planning, organization, goal-directed activity, working memory, initiation, behavioral regulation, and inhibitory control, as well as situation-appropriate decision-making on the basis of projected positive and negative outcomes in novel, complex or ambiguous situations (30). In addition, tests of verbal (i.e., phonemic and semantic/category) and figural/design fluency are also often conceptualized as proxies of executive performance (31).

Verbal fluency impairment has been consistently reported in ALS (11, 22, 24, 27, 32–46). Coexisting phonemic and semantic fluency dysfunction or phonemic fluency deficits alone are often linked to executive dysfunction, while isolated semantic fluency deficits are associated with impaired semantic memory processing. Semantic (24, 34, 40, 44, 46, 47) and figural (34, 46) fluency are not typically impaired in ALS. A verbal fluency index has been proposed and is now widely utilized to account for patients' motor disability (32, 48). Other executive processes are also affected in ALS, such as concept formation and mental flexibility (24, 27, 33, 35, 36, 41, 49–53) which is typically examined by the Wisconsin Card Sorting Test or the Dellis-Kaplan Executive Function System Card Sorting Test (31). However, not all neuropsychology studies corroborate these findings (34, 38, 39, 45, 46, 54–56). Several studies have specifically evaluated mental set shifting ability in ALS using the Trail Making Test; most of them identifying considerable dysfunction (37, 42, 47, 55, 57), while others have not captured such deficits (58, 59). Response inhibition and attentional control are typically examined by the Stroop test, and are often impaired in ALS (11, 27, 35–37, 51, 53, 57, 60), but unaffected cohorts have also been reported (39, 40, 44, 49). ALS patients also often exhibit difficulties in maintaining, manipulating and retrieving information relying on working memory (27, 32, 34, 37, 43, 46, 61), but preserved working memory has also been observed (39, 44, 51, 54, 55, 58, 62). Subtle deficits in reasoning and coordinating rules have been found using ecologically valid measures of executive functions (44, 63).

Memory Deficits

Following inconsistent initial reports, memory dysfunction in ALS has received increasing attention recently (7, 64). While autobiographic memory seems to be preserved in ALS (65), semantic memory is often affected (66). Episodic memory is the most commonly evaluated memory domain in ALS, typically tested by list-learning tests, associate-learning tests, prose memory, as well as visual memory tests (7). Several studies have reported mild to moderate episodic memory impairments which are often interpreted as the corollary of underlying executive deficits (27, 35, 37, 39, 41, 67–69). Memory impairment in ALS is rarely identified in isolation (11), but using data-driven taxonomy approaches a subgroup of patients may show non-executive memory dysfunction (29). Several studies have found impaired encoding (37, 60, 68), retrieval (12, 17, 37, 60, 70) consolidation and recognition (26, 60), although recognition deficits in ALS are not universally recognized (11, 37, 41). Visual memory dysfunction has also been noted in ALS (12), although visual recall is typically less affected than delayed verbal recall (7). Neuroimaging studies have contributed to the characterization of ALS-associated memory impairment highlighting mesial temporal lobe involvement irrespective of frontal lobe pathology (64).

Language Deficits

Language deficits in ALS have traditionally attracted less attention compared to other cognitive domains and have been mostly appraised in association with ALS-FTD (7, 23, 71, 72). However, language dysfunction is increasingly recognized as a core feature of ALS and has been consistently detected in patients without executive dysfunction (24, 29, 73). Patients with ALS show impaired syntactic processing (74), deficits in verb naming and action verb processing (75, 76). Selective impairment in action knowledge (77) has been directly associated with motor cortex degeneration (78) suggesting a link between action execution and action conceptualization (79). Grammatical errors such as incomplete utterances (73, 74) and omission of determiners (73) have been reported in ALS and seem to be dissociable from the patients' motor and executive deficits (73). Phonemic and semantic paraphasias have also been reported (74, 80). Patients with ALS may find narrative discourse particularly challenging due to difficulties to establish (81) and adhere to the main topic of conversation (73, 81). Frequent pauses are another key characteristic of narrative speech in ALS in both demented and non-demented ALS cohorts (82). Syntactic comprehension deficits have also been detected in up to 72% of patients with ALS (83, 84).

Visuo-Perceptive and Visuo-Constructive Deficits

Visuo-perceptive and visuo-constructive functions are seldom specifically examined in ALS. Existing studies tend to focus on visuospatial memory measures and often fail to reach definite conclusions (37, 41, 46, 47, 85). Based on large meta-analyses, these domains are not significantly affected in ALS (7). The relative absence of visuo-perceptual deficits is further supported by the lack of reports on Balint's syndrome in ALS and is consistent with limited occipital involvement on neuroimaging (86) and pathology (87). While praxis deficits are also rarely

TABLE 1 | Most characteristic neuropsychological deficits in ALS categorised per cognitive domain.

Main cognitive domains	Target processes/main deficits	Representative studies (First author, year, sample size ALS/Control)
Executive functions	Verbal fluency	Ludolph, 1992 (21/12); Kew, 1993 (16/16); Abrahams, 1995 (12/6); Massman, 1996 (146/–); Abrahams, 1996 (12/6); Abrahams, 1997 (52/28); Frank, 1997 (74/56); Rakowicz, 1998 (18/24); Abrahams, 2000 (21/25); Lomen- Hanagasi, 2002 (20/13); Hoerth, 2003 (44/–); Abrahams, 2004 (28/18); Abrahams, 2005 (20/18); Pinkhardt, 2008 (20/20); Wicks, 2009 (41/35); Witgert, 2010 (225/–); Stukovnik, 2010 (22/21); Phukan, 2012 (160/110); Taylor, 2013 (51/35)
	Concept formation and mental flexibility	Abrahams, 1996 (12/6); Massman, 1996 (146/-); Abrahams, 1997 (52/28); Frank, 1997 (74/56); Evdokimidis, 2002 (51/28); Moretti, 2002 (14/15); Lomen-Hoerth, 2003 (44/-); Schreiber, 2005 (52/-); Libon, 2012 (41/25); Zalonis, 2012 (48/47); Taylor, 2013 (51/35)
	Mental set shifting	Hartikainen, 1993 (24/26); Hanagasi, 2002 (20/13); Kilani, 2004 (18/19); Witgert, 2010 (225/–)
	Response inhibition and attentional control	Abrahams, 1997 (52/28); Frank, 1997 (74/56); Hanagasi, 2002 (20/13); Moretti, 2002 (14/15); Lomen-Hoerth, 2003 (44/-); Sterling, 2010 (355/-); Christidi, 2012 (22/22); Phukan, 2012 (160/110); Zalonis, 2012 (48/47)
	Working memory	Abrahams, 1997 (52/28); Rakowicz, 1998 (18/24); Abrahams, 2000 (21/25); Hanagasi, 2002 (20/13); Abrahams, 2004 (28/18); Abrahams, 2005 (20/18); Lillo, 2012 (20/18)
	Reasoning and coordinating rules using ecologically valid measures	Meier, 2010 (18/18); Stukovnik, 2010 (22/21)
Memory	Episodic memory encoding	Hanagasi, 2002 (20/13); Mantovan, 2003 (20/20); Christidi, 2012 (22/22)
	Episodic memory retrieval	Hanagasi, 2002 (20/13); Ringholz, 2005 (279/129); Christidi, 2012 (22/22); Elamin, 2013 (186/120); Raaphorst, 2015 (26/21)
	Episodic memory consolidation/recognition	Machts, 2014 (40/40); Christidi, 2012 (22/22)
	Visual delayed recall	Ringholz, 2005 (279/129)
	Semantic memory	Hervieu-Begue, 2016 (15/-)
Language	Verb naming and action verb processing	Bak, 2001 (6/20); Grossman, 2008 (34/25); York, 2014 (36/13); Papeo, 2015 (21/14)
	Grammatical errors	Ash, 2015 (26/19); Tsermentseli, 2015 (26/26)
	Phonemic and semantic paraphasias	Roberts-South, 2012 (16/12); Tsermentseli, 2015 (26/26)
	Establishing and adhering to the main topic of conversations	Ash, 2015 (26/19); Bambini, 2016 (33/33)
	Narrative speech pauses	Yunusova, 2016 (85/33)
	Syntactic processing/comprehension	Yoshizawa, 2014 (25/–); Tsermentseli, 2015 (26/26); Kamminga, 2016 (35/23)
Praxis	Constructive apraxia	Abrahams, 1997 (52/28)
	Orofacial apraxia	Lobo, 2013 (1/–)
	Speech apraxia	Duffy, 2007 (7/–)
	Respiratory apraxia	Pinto, 2007 (1/–)
Social cognition	Theory of mind	Meier, 2010 (18/18); Girardi, 2011 (19/20); Burke, 2016 (59/59)
	Emotional processing and ability to recognize emotional facial expressions	Palmieri, 2010 (9/10); Girardi, 2011 (19/20); Crespi, 2014 (22/55); Savage, 2014 (29/30); Andrews, 2017 (33/22)
	Ability to describe intentions and feelings of others	Gibbons, 2007 (16/16); Staios, 2013 (35/30); Cerami, 2014 (20/56)
	Empathy	Girardi, 2011 (19/20); Cerami, 2014 (20/56)
	Social inferences	Staios, 2013 (35/30); Savage, 2014 (29/30)
Behavior	Apathy	Grossman, 2007 (45/–); Chio, 2010 (70/–); Witgert, 2010 (225/–); Girardi, 2011 (19/20); Radakovic, 2016 (83/83)
	Disinhibition	Grossman, 2007 (45/–); Terada, 2011 (24/–)
	Pathological crying and laughing	McCullagh, 1999 (18/10); Palmieri, 2009 (32/39); Olney, 2011 (35/–); Brooks, 2013 (9/–); Floeter, 2014 (22/28); Christidi, 2018 (56/25)

ALS, amyotrophic lateral sclerosis.

reported in ALS (27), orofacial (88), speech (89), and respiratory (90) apraxia have been sporadically reported.

Social Cognition Deficits

Social cognition refers to a diverse set of cognitive skills that allow humans to understand themselves, interact with and understand others and are crucial to adopt situation-appropriate, goal-directed behaviors in everyday social interactions (91). Despite considerable variations, deficits in theory of mind, empathy, social perception, social behavior are now recognized as key elements of the ALS-associated cognitive profile (7, 28, 92). It is however still unclear if these deficits are linked to

executive dysfunction (29, 93–98) or may be related to non-executive domains, such as episodic memory function and visuospatial abilities (99). Patients with ALS may also exhibit impaired emotional processing and ability to interpret emotional facial expressions, especially with comorbid FTD (96, 100–102). Impairments in complex facial affect recognition, affective prosody recognition and cross-modal integration have also been found in non-demented ALS cohorts (103). Multiple subcomponents of theory of mind seem to be affected in ALS, including the ability to describe the intentions and feelings of others (95, 98, 104), to recognize and provide explanations for social "faux pas" (63) and evaluate object preferences based on the interpretation of eye gaze direction (96, 105). Loss of empathy (96), impaired emotional empathy attribution (95), and erroneous social inferences (98, 100) have also been reported in non-demented ALS cohorts.

Behavioral Deficits

The clinical link between ALS and FTD is exemplified by overlapping behavioral changes which are similar to those observed in behavioral variant of FTD (106). These deficits are typically identified through a structured clinical interview with the caregivers or through validated questionnaires. Perseveration, apathy and disinhibition are the most commonly reported behavioral alterations, followed by loss of disease insight, indifference, loss of interest, aggression, irritability, and lability (107).

Apathy is the most commonly reported behavioral symptom in non-demented ALS (42, 45, 96, 108, 109), which used to be assessed by generic behavioral instruments, such as the Frontal Systems Behavior Scale (110) and the Frontal Behavioral Inventory (111), until the development of ALS-specific scales, such as the Dimensional Apathy Scale (112) which appraises initiation, executive and emotional apathy. Initiation apathy is thought to be particularly prevalent in ALS (113). ALS patients with apathy may require prompts to initiate or follow through with a task, including self-care, feeding, and taking medications. They may appear poorly motivated, aloof or uninterested. Apathy may impact of rehabilitation, hamper gait initiation, and curb communication efforts especially in the presence of bulbar impairment. It can be mistaken for low mood, depression and withdrawal by inexperienced observers. Disinhibition is more readily identified and reported by caregivers, and can precede (108) or follow (114) motor disability. Disinhibited behavior can manifest in rude, offensive, flirtatious comments, puns, "Witzelsucht" often violating social norms, personal space and may result in careless or impulsive decisions. Purchasing expensive items on a whim, hoarding, compulsive behavior, overeating, and developing a preference for sweets have also been reported (115).

Hallucinations have been reported by several groups (116–119) and are sometimes associated with the C9orf72 genotype. Symptomatic treatment includes the judicious use of small dose atypical antipsychotics, if necessary.

Patients with pseudobulbar affect or pathological crying and laughing exhibit sudden situation-inappropriate emotional responses (120–122) which may have a negative impact on their quality of life (123) and lead to social isolation or social stigma. It is most commonly associated with UMN-type bulbar dysfunction (124), but frontal abnormalities, executive dysfunction, basal ganglia pathology and impaired cerebellar gating mechanisms have also been linked this symptom (27, 122, 125–128).

INSIGHTS FROM NEUROIMAGING

Neuroimaging techniques provide optimal non-invasive tools to characterize extra-motor pathology in ALS underpinning cognitive and behavioral deficits and also permit exploratory correlations with clinical measures (129, 130).

Structural Imaging

Voxel based morphometry (VBM) and surface-based morphometry (SBM) are reproducible, validated and widely-used pipelines that use high resolution 3D T1-weighted MR images to identify focal GM alterations. Beyond the consensus on motor cortex atrophy (131), many studies also detect multifocal frontotemporal and parietal GM changes (132). GM abnormalities have also been identified in subcortical structures (133), such as the hippocampus (134–136), amygdala (137, 138), thalamus (134, 135, 139, 140), and insula (141, 142). Reduced GM density in occipital (139, 143–145) and cerebellar (139, 146) regions is less commonly reported. GM alterations in extra-motor areas have been linked to structure-specific cognitive and behavioral deficits in ALS (147, 148). Recent studies have highlighted extra-motor cortical changes in ALS patients without overt cognitive impairment (134, 135, 146, 149, 150). The anatomical patterns of extra-motor gray matter involvement in ALS further support the notion of the ALS-FTD continuum (72).

White matter integrity in ALS is most commonly evaluated by diffusion tensor imaging (DTI). Reduced fractional anisotropy and increased axial and radial diffusivity in the corticospinal tracts and corpus callosum are hallmark features of ALS (151, 152). Extra-motor white matter pathology has been consistently detected in frontal (139, 153–160), temporal (53, 154, 161), cingular (162), parahippocampal (25, 157, 160), insular (160), thalamic (141, 159, 163), and cerebellar regions (86, 146, 164). Similarly to gray matter analyses, extra-motor white matter involvement has also been identified in ALS patients without overt cognitive impairment (146).

Metabolic Imaging

MR spectroscopy in ALS has consistently revealed decreased N-acetyl aspartate (NAA)/choline and NAA/creatine ratios in motor regions (165–167), but whole brain spectroscopy also detected extra-motor NAA reductions in frontal, parietal, thalamic and occipital areas (168, 169).

Most positron emission tomography (PET) studies in ALS use 18F-FDG PET, but TSPO, $GABA_A$ (11C-flumazenil) and 5-HT1A receptor (11C-WAY100635) radioligands have also been utilized (170). Hypometabolism in motor regions is a characteristic FDG-PET finding in ALS (171–174), but extra-motor changes in dorsolateral prefrontal, orbitofrontal, anterior frontal, anterior temporal, fusiform, and occipital regions have also been reported (171–174). Frontotemporal hypometabolism

has been linked to cognitive performance (22, 39, 172), is thought to precede atrophy (175) and has been linked to shorter survival (176). There is also evidence of hypermetabolism in the hippocampus, amygdala midbrain, pons and cerebellum (173, 174, 177). PET imaging has identified microglial activation in frontotemporal, thalamic, midbrain, and pontine regions suggestive of extra-motor inflammation (178–181). Widespread reduction of 11C-Flumazenil binding to $GABA_A$ in sporadic ALS has been interpreted as inhibitory dysfunction (182) and is regarded as a one of cornerstones of ALS pathogenesis (183). Reduced serotonin receptor binding has also been reported in ALS using the 11C-WAY100635 radio-ligand (184).

Functional Imaging

Resting state fMRI enables the assessment of functional connectivity between different brain regions by evaluating synchronized neuronal activity at rest. Reduced (185–189) and increased (183, 190) functional connectivity have both been reported in sensorimotor networks of ALS patients which may be explained by the different sub-regions evaluated (191–193) and also by the inclusion of patients in different disease-stages. Similarly, both reduced and increased functional connectivity alterations have been reported in extra-motor areas which mediate cognitive and behavioral functions (187, 188, 193, 194). The functional connectivity of the default mode network (DMN) has been reported to be both decreased (187, 189, 193) and increased (193, 195). Increased functional connectivity has been detected in the DMN using graph theory-based analyses (196). Increased (193) and decreased (186, 189, 193) fronto-parietal network integrity has been both reported. Reduced "executive control network" (middle frontal cortex) and "salience network" (medial prefrontal cortex, insula) connectivity has been described in ALS cohorts without dementia (189). Increased connectivity in ALS has either been interpreted as evidence of attempted compensation for structural degeneration (197, 198) or proof of inhibitory dysfunction (183, 190, 199).

Task-based fMRI studies in ALS have consistently revealed the recruitment of pre- and supplementary motor regions when executing motor tasks. Additional activation has also been observed in areas associated with motor learning areas, such as the basal ganglia and cerebellum (200, 201). Despite difference in study protocols, an activation shift to premotor (202, 203), temporal and parietal regions (203–205) has been often noted. Cognitive paradigms have been particularly helpful in capturing frontotemporal network alterations. Impaired verbal fluency was linked to reduced frontotemporal, parietal, and cingulate activation in non-demented ALS patients (46). Impaired frontal inhibitory control was confirmed by a number of fMRI paradigms, such as Stroop, negative priming, antisaccade tasks, go/no-go tasks etc. Increased activation during the Stroop paradigm and decreased activation in negative priming conditions has been reported mostly in left hemispheric regions (206). Increased activation in supplementary and frontal eye fields and reduced activation in dorsolateral prefrontal cortex have been noted in antisaccade tasks (207). Furthermore, in go/no-go paradigms, ALS patients show increased inhibition-related activation in frontal and basal ganglia regions and increased execution-related activity in contralateral sensorimotor

TABLE 2 | ALS-specific instruments to screen for cognitive and behavioral changes at baseline and during the course of the disease.

Screening instrument	Duration of administration	Cognitive and behavioral domains examined	Parallel forms for longitudinal assessment	Validation in non-English speaking populations
Edinburgh Cognitive and Behavioral ALS Screen (ECAS)	15–20 min	Executive functions, Social cognition, Language, Visuoconstruction, Memory Behavioral changes (including psychotic symptoms)	Yes	American-English; Belgium; Chinese; Croatian; Czech; Dutch; French; German; Swiss-German; Greek; Hebrew; Italian; Japanese; Norwegian; Polish; Portuguese; Russian; Slovak; Slovenian; Spanish; Swedish; Welsh
ALS Cognitive and Behavioral Screen (ALS-CBS)	<10 min	Executive functions including attention, concentration, mental tracking and monitoring, verbal fluency Behavioral changes	Yes	Brazilian; Spanish; Greek
ALS Brief Cognitive Assessment (ALS-BCA)	5 min	Executive functions (working memory, set-shifting), Frontally-mediated language function, Delayed verbal recall, Behavioral changes	N/A	N/A
Beaumont Behavioral Inventory (BBI)	5–10 min	Frontal Behavioral symptoms; Executive functions; Language; Psychotic symptoms	N/A	N/A
Motor Neuron Disease Behavioral Instrument (MiND-B)	<10 min	Behavioral symptoms	N/A	N/A
ALS Frontotemporal Dementia Questionnaire (ALS-FTD-Q)	5–10 min	Behavioral symptoms (it also includes 3 items for memory, concentration and orientation in time)	N/A	N/A

regions (208). Few studies have specifically examined the functional correlates of social cognition to date. Patients with ALS tend to show increased activation compared to healthy participants in the right supramarginal, anterior cingulate and bilateral dorsolateral prefrontal cortex in response to socio-emotional stimuli (56, 209). The combined use of motor and memory tasks on fMRI enables the longitudinal characterization of divergent motor and extra-motor functional changes. Increased motor activation was found in ALS compared to controls at baseline, which has decreased on the follow-up assessment, suggestive of failing compensation. Contrary to the functional motor changes, hippocampal activation increased on follow-up when novel stimuli was presented (210).

RELEVANCE TO CLINICAL CARE

The detection (48), expert evaluation (11), categorization (211), and follow-up (17) of extra-motor deficits in ALS is crucially important for individualized patient care. While screening tests (**Table 2**) are useful for the detection of gross deficits, expert review by neuropsychologists is indicated for accurate patient classification. Adherence to treatment, compliance with assistive devices, participation in clinical trials, making informed financial and end-of-life decisions, choices in participating in non-licensed treatments are just some of the aspects of a patient journey which may be significantly affected by cognitive or behavioral deficits (19, 212). Cognitive impairment in ALS is widely regarded as a negative prognostic indicator and linked to reduced survival (17, 18, 213). Neuropsychological deficits in ALS are thought to be associated with increased caregiver burden (214, 215) and reduced quality of life (216). The recognition of the far-reaching effects of neuropsychological deficits on nearly all aspects of ALS care, caregiver support, resource allocation, and prognosis, led to the inclusion of specialist neuropsychologists as core members of ALS multidisciplinary teams worldwide (217, 218). The careful evaluation of motor deficits which are not directly linked to the corticospinal axis and are not reflected in the ALSFRS-R score, such as extra-pyramidal deficits are also crucial (219). Extra-pyramidal deficits may contribute to falls and gait impairment and are increasingly investigated in neuroimaging studies (220, 221). These symptoms may present early in the course of the disease, and contribute the clinical heterogeneity of the condition (220, 222). Postural instability and rigidity may be associated with other extra-motor deficits, and potentially linked to poor survival (205, 223). There is some controversy about the chronology of motor and extra-motor involvement in ALS. Extra-motor manifestations, such as dementia (224, 225), psychiatric features (226), and extra-pyramidal symptoms (227) have been reported to precede motor symptoms in some cases, and there is also compelling evidence of early extra-motor pathology in cognitively normal ALS patients (134, 135, 146).

RESEARCH OPPORTUNITIES AND FUTURE DIRECTIONS

Even though the high incidence of cognitive impairment and its impact on individualized patient care are now universally recognized, the neuropsychological aspects of ALS are seldom considered for patient stratification in clinical trials (228). Several ALS-specific cognitive screening tests have now been validated, but generic tests, such as MOCA and MMSE are still in use in some clinics. While neuropsychological scores are often adjusted for motor-disability and depression, medication-effects, fatigue, and hypoxia are seldom considered when interpreting cognitive performance on various instruments. Despite sporadic reports, the full spectrum of psychiatric manifestations and the precise incidence of psychosis remain to be established in ALS (119, 229, 230). Certain cognitive domains, such as memory and praxis have not been exhaustively characterized in ALS to date. Relatively little is known of the neuropsychological profile of ALS-causing mutation carriers before they develop motor symptoms (231–233). The gaps in our current understanding of extra-motor pathology in ALS shape future study designs. Novel technologies such as online assessments, internet-based data collection, mobile phone apps, and wearable devices are emerging research resources. Irrespective of specific neuropsychological instruments, the early detection, and careful of monitoring of cognitive deficits in ALS is pivotal for optimized patient and caregiver support and tailoring precision management strategies to individual patient needs.

AUTHOR CONTRIBUTIONS

The paper was drafted by FC, EK, and PB and has been reviewed for intellectual content by MR, NK, and IE.

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Biomarker Supervised G-CSF (Filgrastim) Response in ALS Patients

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Objective: To evaluate safety, tolerability and feasibility of long-term treatment with Granulocyte-colony stimulating factor (G-CSF), a well-known hematopoietic stem cell factor, guided by assessment of mobilized bone marrow derived stem cells and cytokines in the serum of patients with amyotrophic lateral sclerosis (ALS) treated on a named patient basis.

Methods: 36 ALS patients were treated with subcutaneous injections of G-CSF on a named patient basis and in an outpatient setting. Drug was dosed by individual application schemes (mean 464 Mio IU/month, range 90-2160 Mio IU/month) over a median of 13.7 months (range from 2.7 to 73.8 months). Safety, tolerability, survival and change in ALSFRS-R were observed. Hematopoietic stem cells were monitored by flow cytometry analysis of circulating $CD34^+$ and $CD34^+CD38^-$ cells, and peripheral cytokines were assessed by electrochemoluminescence throughout the intervention period. Analysis of immunological and hematological markers was conducted.

Results: Long term and individually adapted treatment with G-CSF was well tolerated and safe. G-CSF led to a significant mobilization of hematopoietic stem cells into the peripheral blood. Higher mobilization capacity was associated with prolonged survival. Initial levels of serum cytokines, such as MDC, TNF-beta, IL-7, IL-16, and Tie-2 were significantly associated with survival. Continued application of G-CSF led to persistent alterations in serum cytokines and ongoing measurements revealed the multifaceted effects of G-CSF.

Conclusions: G-CSF treatment is feasible and safe for ALS patients. It may exert its beneficial effects through neuroprotective and -regenerative activities, mobilization of hematopoietic stem cells and regulation of pro- and anti-inflammatory cytokines as well as angiogenic factors. These cytokines may serve as prognostic markers when measured at the time of diagnosis. Hematopoietic stem cell numbers and cytokine levels are altered by ongoing G-CSF application and may potentially serve as treatment biomarkers for early monitoring of G-CSF treatment efficacy in ALS in future clinical trials.

Keywords: amyotrophic lateral sclerosis, granulocyte-colony stimulating factor, cytokines, hematopoietic stem and progenitor cells, HSPC, treatment

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a life threatening neurodegenerative disorder characterized by premature loss of upper and lower motoneurons in the adult brain and spinal cord (1). The life time risk of ALS is below one in 400 individuals (2), the incidence is 2–3 per 100,000 in Europe (3). The unmet medical need in ALS patients is underlined by a median survival of 29.8 months from symptom onset, and of 15.8 months from diagnosis (4). Only modest treatment effects have been observed by riluzole (5) and edaravone (6).

In view of the great heterogeneity of disease etiology, neuronal damage likely results from many different pathologic changes, including neuroinflammation (3). Neurodegenerative processes with altered homeostasis, protein accumulation and cell death generates neuroinflammation, and central nervous system (CNS)-resident immune cells such as astrocytes and microglia trigger neuroinflammation and neurodegeneration (7). Inflammation may arise reactive to ALS-related CNS alterations, but also play an initial role and trigger both onset of disease and further accelerate progression of ALS (3). A complex, cytokine-mediated crosstalk between CNS and systemic immune cells regulates immune responses to either pro- or anti-inflammatory states, which evolve over time (7).

Granulocyte-colony stimulating factor (G-CSF) is a 20-kDa glycoprotein and a well characterized growth factor that plays a key role in production, mobilization, and differentiation of hematopoietic stem cells (8, 9). It is a widely used compound for treatment of neutropenia and for mobilization of $CD34^+$ hematopoietic stem cells prior to bone marrow transplantation. G-CSF enhances immunocompetence and has systemic anti-inflammatory effects (10). G-CSF is safe and well tolerated; most common side effects are moderate bone pain and musculoskeletal pain in 20–30% of patients, rarely splenomegaly and allergic reactions (11). Aside from hematopoietic functions, G-CSF acts as a neuronal growth factor in the CNS and possesses neuroprotective and -regenerative properties (12, 13). G-CSF passes the intact blood brain barrier, and its receptor is widely expressed within the CNS (12). G-CSF is thought to be neuroprotective through anti-apoptotic effects (12, 14), it induces neural differentiation, supports neurogenesis, contributes to re-endothelialization and arteriogenesis (12, 15). Systemic G-CSF induced hematopoietic stem cells may contribute on a direct cellular level in neurodegeneration by migration to the CNS (16, 17), where they may offer trophic support and modulate the local CNS immune system (17, 18). Observing G-CSF induced systemic hematopoietic stem cells may also shed light upon direct G-CSF effects on neural cells and stem cells as a surrogate system. Furthermore, G-CSF modulates monocyte function and attenuates the neuroinflammatory cascade (13). An interesting bone marrow-brain connection has been shown as G-CSF induced bone marrow derived cells migrate to CNS and express microglial phenotype in a mouse model of cranial irradiation. This was associated with a better functional outcome and suggested to facilitate neuroprotection by direct effects on resident CNS cells as well as modulation of cellular microenvironment in neurovascular niches (15). Angiogenic factors may promote neurogenesis through direct effects on neuronal cells (19) and indirectly by angiogenic support of the highly vascularized neurogenic zones. G-CSF improved motor function and survival in mouse models of ALS (20–22). Small trials with G-CSF treatment in ALS patients demonstrated excellent tolerability and safety (23–25), with modulation of immune parameters (26), and possible minor benefits detected by neuroimaging (27). In summary, G-CSF exerts multiple physiological effects within the CNS and may be a potent modulator of different functions relevant to ALS pathophysiology (13). Importantly, from *in vitro*, mouse model and human exploratory evidence the mode of action most relevant for potential treatment effects cannot with certainty be concluded.

Due to the paucity of available treatment options we provided individual, off-label G-CSF treatment to ALS patients. G-CSF, considering its multimodal systemic and CNS effects, may be a promising treatment option in view of the etiopathological and clinical heterogeneity of ALS. Biomarkers are measurable indicators of disease and/or intervention and may be useful in monitoring long-term degenerative or reparative processes within the CNS. In view of the above-discussed complexity of ALS, it seems unlikely that a single biomarker can sufficiently reflect treatment effects on disease progression. We therefore used a panel of pro- and anti-inflammatory blood parameters, angiogenic factors, as well as hematopoietic stem cell markers. Monitoring pro-differentiation and -mobilization effects on hematopoietic stem cells may serve as a proxy for G-CSF activity on neural stem cells in individual patients and/or reflect direct and indirect beneficial effects of mobilized hematopoietic stem cells. Observing a panel of peripheral cytokines may reveal system wide immune and inflammatory status relevant for peripheral-CNS crosstalk.

G-CSF is known to be a safe stem-cell mobilizing agent. We investigated whether the number of mobilized hematopoietic stem cells is different in G-CSF treated ALS patients of longer versus shorter survival. Secondly, we were interested in whether baseline cytokine levels are associated with survival of G-CSF treated ALS patients. Lastly, we sought to explore hematopoietic stem cells and cytokine level alterations during G-CSF treatment.

METHODS

Patients, Procedures and Ethics

Treatment with G-CSF was offered to 36 patients seen at the University of Regensburg with definite or probable ALS according to the revised El Escorial criteria (28). As this was not a prospective clinical trial, the use of formal exclusion criteria was not considered appropriate. However, neither patients with a current or past history of neurologic disease other than ALS, nor patients participating in any interventional study were offered this treatment option. Individual treatment of ALS patients and retrospective evaluation was done after written informed consent. The ethics committee of the University of Regensburg approved a retrospective analysis (ethics approval: 15-101-0106 and 14-101-0011). The principles of the Declaration of Helsinki (World Medical Association, revised version 2013) were strictly

adhered to. Survival was defined as the time between diagnosis and death from confirmed ALS-related complications, including suicide.

ALS patients were treated with subcutaneous injections of recombinant human G-CSF (Filgrastim) on an outpatient basis. Dose and application modes were adapted individually upon initiation and over time (**Figure 1**; **Table S1**). Adaption was made with the intent to maximize patient wellbeing and safety in the presence of any emerging safety signals, and with the aim of increasing efficacy as monitored by levels of mobilized hematopoietic stem cells, a potential individual marker of biological activity of G-CSF. This resulted in heterogeneous treatment schemes. The intervention and evaluation was initiated in January 2010 and is still ongoing. The data were analyzed up to March 2017. The treatment was provided by the hospital and not funded by a pharmacological company or other external source. No external or internal funding sources were involved in patient selection, study design, data analysis or interpretation.

Patient safety was analyzed at baseline (initiation of treatment) followed by monthly control visits with clinical examinations, blood counts, cytokines, blood smears and estimation of bone marrow function. We conducted baseline spleen sonography with follow-ups upon dose escalation. Clinical ALS progression was monitored using the established ALSFRS-R (29). If patients were not able to continue visits and treatment, patient survival was monitored by phone calls to patients, their families and general practitioners.

Changes in pro- and anti-inflammatory immune profiles were evaluated at baseline, at 3 months, and then every 6 months throughout treatment by multiplex electrochemoluminescence with the panel assay V-PLEX Human Biomarker 40-Plex Kit (MesoScale Discovery®, Maryland, USA). This industry standard panel has been validated in different immune related and non-immune diseases (manufacture's information). In patients receiving G-CSF on five consecutive days, evaluations of cytokine levels in the peripheral blood were conducted twice a month, before (day 0) and after G-CSF application (day 7).

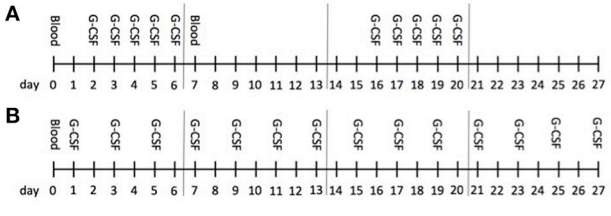

FIGURE 1 | Treatment course. The monthly protocol is illustrated; this schedule was repeated and individually adapted over the long-term treatment. G-CSF was administered subcutaneously. Mainly, patients received G-CSF either as a 5-day bolus **(A)** once (1st week) or twice (1st and 3rd week) or continuously **(B)** on single days up to every second day; G-CSF was administered in one or two doses á day. Blood was obtained before treatment onset at baseline (d0) and then once á month (d0) in patients receiving G-CSF continuously, and before (d0) as well as after a 5-day treatment (d7) in patients on bolus application. Cytokines were analyzed at d0 in both groups and at d7 in bolus treatment in the first month (baseline), then at the 3rd and 6th month during ongoing treatment.

In patients receiving G-CSF twice a week or every second day, analyses were conducted on a monthly basis 1 day after application. Peripheral blood serum was collected during regular visits at the hospital and immediately stored at −20°C for cytokine assays. For each assay, 25 µl of serum samples were used and test carried out in duplicates, according to the manufacturer's instructions.

We analyzed white blood cells including cell differentiation, platelet and red blood cell counts, and hemoglobin levels with an automatic cell counter (Sysmex®, Kobe, Japan). Peripheral blood smears were done on a 3-month basis by light microscopy. Peripheral blood $CD34^+$ and $CD34^+CD38^-$ hematopoietic stem and progenitor cells (HSPC) were analyzed by flow cytometry as earlier described by our group (25). In short, 1 ml donor blood was lysed in 9 ml NH_4Cl lysis buffer and cells were then stained for 30 min at 4°C with combinations of anti-CD45-FITC (clone HI30, BD Pharmingen, Franklin Lakes, NJ, USA), CD34-APC (clone 581, Biolegend, San Diego, CA, USA) and CD38-PE (clone HIT2, BioLegend) monoclonal antibodies. Analysis was performed on a Becton Dickinson CALIBUR flow cytometer (BD, East Rutherford, NJ, US).

Calculations and Statistics

Findings of immune parameters from three time points, baseline (initiation of treatment), 3 months and 6 months were selected for analysis. As patients did not always visit the outpatient clinic on the exact days of the given time points, the time points had to be defined as time periods. When assessing the ALSFRS-R at baseline, data from day of treatment initiation ±28 days were included. For baseline measures of blood counts, stem cell mobilization parameters and cytokines, only data obtained before the first G-CSF application were selected. The 3-month time point was defined as day 45–134 and the 6-month time point as ranging from day 135 to 224. If patients visited more than once during these time periods, the day closest to the intended time point was selected.

The immediate effects of G-CSF treatment on peripheral levels of cytokines, hematopoietic stem cells and blood counts were assessed by comparing respective levels 2 days before and 1 day after a 5-day treatment course with G-CSF. We then explored different patterns of immune responses depending on individual survival. Survival time was defined as time elapsed from day of diagnosis to day of death or day of last observation in the case of censoring. For this purpose, G-CSF treated patients were divided into two groups based on their survival being longer or shorter than 30 months from diagnosis, as this was a time point that separated the patients into two equal-sized groups. At the point of database closure, patients who were still alive were censored and included in the "long survival" group if they had been observed for over 30 months ($n = 7$). Patients who were alive and had not yet been observed for over 30 months were not considered for this analysis ($n = 3$). The same censoring was applied for correlation analysis. We then retrospectively analyzed baseline levels of cytokines, hematopoietic stem cells and blood counts in the long and short survivor groups and further correlated survival with cytokines upon treatment initiation.

R or GraphPad Prism 7 was employed for statistical analysis and graph design. Correlations were analyzed using two-tailed Pearson correlation and presented with correlation coefficient (r), coefficient of determination (R^2) and *p*-value. Comparisons were made with Mann-Whitney test and paired Wilcoxon test. Data were considered significant at $p \leq 0.05$. A trend was noted at $p \leq 0.1$. Comparisons were corrected for multiple testing by false discovery rate approach (FDR, two-stage step-up method of Benjamin, Krieger and Yekutieli with desired FDR (Q) at 10%) and considered a discovery at FDR-adjusted *p*-value (q) < 0.1. We used an Area Under the Curve (AUC) approach to estimate mobilization of hematopoietic stem cells after G-CSF treatment over time. Stem cell measurements before and after G-CSF dosing were available for patients on the 5-day treatment scheme. For better comparability regarding long and short survival times after diagnosis, we selected patients with ongoing 5-day treatment over the first 4 months. All patient measurements were used in the calculation. If patients had fewer data points, their mean AUC value calculated from all data points was applied (in the case of one patient). The AUC value was calculated with the *auc* function of the R-package "flux" (Jurasinski, Koebsch, Guenther and Beetz, 2014). The baseline value at day 0 or from any day prior to treatment start was used as threshold for the calculation.

RESULTS

Demographics, Intervention and Safety

36 caucasian ALS patients (25 male, 11 female, 28 limb onset, 8 bulbar onset, mean age 51.9 years, mean ALSFRS-R on initiation 38/48) were treated with G-CSF in addition to riluzole treatment. We here report on individual treatment on a named patient basis—consequently, treatment schemes were heterogeneous. Dose and application modes were adapted individually upon initiation and over time (**Table S1**). In summary, G-CSF was injected subcutaneously in a dose-range from 90 to 2160 Mio IU per month (900–21,600 μg/month), with a mean dose of 464 Mio IU/month (4,640 μg/month). Application modes ranged from once weekly to every second day in an ongoing individually tailored manner. The median duration of treatment was 13.7 months (mean 16.7 months; range from 2.7 to 73.8 months) (**Table 1**, **Figure 1**).

Long-term outpatient treatment with G-CSF was generally well tolerated in ALS patients and compliance was excellent. Minor adverse events were mild to moderate bone pain after G-CSF injection and leukocytosis. One patient experienced an episode with heat sensation, lightheadedness, and 15 min. of dyspnea on 1 day of drug application 39 months into G-CSF treatment. Due to the possibility of drug-related intolerance or mild allergic reaction, G-CSF was discontinued in this patient; antibodies against G-CSF were not detectable. This patient was switched from Filgrastim to Pegfilgrastim, a PEGylated form of recombinant human G-CSF, from his 46th to 53rd month after initiation, and then ended the off-label treatment without further adverse reactions. As expected, mild to moderate splenomegaly evolved during ongoing G-CSF treatment in most patients. Without any further symptoms or complications, the mean spleen width increased from 4.3 to 4.9 cm and length from 10.7 to 12.1 cm during treatment. There were no severe adverse events (SAE), and no signs for pre-malignant transformation in peripheral blood smears.

Baseline hematology showed no abnormalities in our patients. G-CSF mobilizes neutrophil leukocytes as well as $CD34^+$ and $CD34^+CD38^-$ hematopoietic stem and progenitor cells (HSPC) from the bone marrow into the peripheral blood. Leukocyte counts increased significantly in all treated patients, from an initial mean of $6.9 \times 10^3/\mu l$ to $48.2 \times 10^3/\mu l$ (range 8.3–118.7 $\times 10^3/\mu l$, $p < 0.0001$) after G-CSF application. A predicted increase in the average percentage of neutrophils (from 64.8 to 87.3%, $p < 0.0001$) was accompanied by a relative decrease in lymphocytes (from 24.1 to 7.0%, $p < 0.0001$), monocytes (from 8.8 to 4.7%, $p < 0.0001$) and eosinophils (from 1.8 to 0.7%, $p < 0.0001$) as well as a small decrease in red blood cell count (from 5.03 to $4.83 \times 10^3/\mu l$, $p < 0.0001$), hemoglobin level (from 14.9 to 14.4 g/dl, $p < 0.0001$) and hematocrit (from 44.2 to 43.6, $p = 0.0362$) during monitoring (all comparisons by paired *t*-test, two-tailed *p*-value. **Figure S1**). There were no significant changes in basophiles and platelet count during monitoring. The fold increase of $CD34^+$ and $CD34^+CD38^-$ HSP cells in peripheral blood served as an indicator of mobilization efficiency and was determined by comparing cells at baseline to cells after mobilization. The mobilization efficacy was heterogeneous with high intra- and inter-personal variability (data not shown).

G-CSF-Mediated Stem Cell Mobilization Was Associated With Survival of ALS Patients

Twenty-six of thirty-six G-CSF treated patients deceased between January 2010 and March 2017. 10 patients were alive, of which 6 were still treated with G-CSF. The patient who had suffered from a possible allergic reaction was regularly seen at the clinic. Three patients ended G-CSF treatment at days 82, 420 and 427, and were all lost to follow up. The overall median survival of *deceased* patients was 24.2 months from diagnosis (mean 25.5; range 3.9–56.6 months). For further analysis, patients were divided into two equally sized groups by survival being longer or shorter than 30 months from diagnosis. Patients, who were alive at the time of database closure, were considered for this analysis had they been observed for at least 30 months. The mean (median) survival differed in the two survival groups: 46.59 (39.55) months, SD 16.34 and 17.04 (18.30) months, SD 8.16 (two-tailed *p*-value $<$ 0.0001; Mann-Whitney *t*-test). The ALSFRS-R slope over time was significantly flatter in longer surviving patients (Wilcoxon test, $p = 0.00086$; **Figure 2**). Long survivors were younger (mean age 46.8 vs. 56.5 y, unpaired *t*-test, $p = 0.0163$) and had a longer latency between diagnosis and treatment onset (mean 333 vs. 163 days, unpaired *t*-test, $p = 0.0377$). Their clinical function upon treatment initiation was not significantly different (mean ALSFRS-R 38.6/48 vs. 37.3/48). Further, longer surviving patients were less frequently female (18.8 vs. 47.1%), but had similar occurrence of bulbar onset of disease (18.8 vs. 17.6%) (**Table 1**).

G-CSF is known to mobilize HSPC into the peripheral circulation. $CD34^+$ and $CD34^+CD38^-$ HSPC were evaluated in the sera of patients 2 days before (day 0) and 1 day after

TABLE 1 | Demographics and intervention in G-CSF treated ALS patients.

ALS patient	Age (years)	Gender	ALSFRS-R baseline	Site of onset	Time diagnosis to treatment (days)	Dose G-CSF (mean; range (MioIE/month))	Treatment duration (months)	Survival (months) from diagnosis
1	50	F	38	Limb	498	150 (150–150)	19	36.2
2	42	M	32	Limb	619	280 (150–300)	31	52.2
3	77	M	21	Limb	759	173 (150–240)	5	33.4
4	68	F	39	Bulbar	29	150 (150–150)	3	3.9
5	67	M	33	Limb	439	260 (150–300)	20	56.6
6	26	M	33	Limb	486	485 (150–1170)	74	89.7*
7	50	F	33	Limb	536	240 (240–240)	7	25.4
8	73	M	41	Limb	270	166 (150–240)	11	21.4
9	50	M	28	Limb	393	133 (90–150)	7	21.4
10	56	M	37	Limb	770	242 (150–300)	14	40.0
11	41	M	38	Limb	24	287 (150–300)	27	36.3
12	35	F	40	Bulbar	115	296 (240–300)	14	63.7*
13	48	F	46	Limb	38	216 (150–300)	14	29.7
14	43	M	44	Limb	61	561 (192–768)	45	47.3
15	65	F	32	Limb	81	192 (192–192)	14	18.3
16	51	F	42	Limb	101	225 (150–300)	3	6.4
17	60	F	38	Limb	21	192 (192–192)	9	11.5
18	58	M	44	Limb	45	311 (240–480)	25	25.4
19	46	M	46	Limb	249	150 (150–150)	26	34.7
20	50	M	-	Limb	1	198 (192–240)	8	8.0
21	27	M	44	Limb	53	301 (150–600)	39	71.3*
22	45	M	48	Limb	26	666 (240–1296)	37	39.1*
23	55	M	40	Bulbar	26	263 (150–300)	3	41.9*
24	61	M	44	Limb	66	375 (150–510)	5	9.2
25	60	M	40	Bulbar	135	602 (240–816)	19	23.0
26	65	F	30	Bulbar	122	563 (240–900)	7	11.3
27	43	F	41	Limb	338	628 (240–720)	14	35.7*
28	60	M	42	Limb	23	589 (480–720)	11	12.1
29	45	F	28	Limb	493	535 (150–720)	6	29.6
30	47	M	29	Limb	396	585 (450–720)	5	19.3
31	50	M	40	Limb	23	667 (240–720)	8	13.7
32	39	M	41	Bulbar	343	1015 (450–1170)	20	31.7*
33	56	M	42	Bulbar	525	744 (450–1056)	11	35.6
34	59	M	38	Bulbar	52	1044 (450–1440)	14	#
35	69	M	39	Limb	62	1344 (450–2160)	16	#
36	35	M	38	Limb	288	1141 (300–1440)	14	#
Mean (SD)	51.9 (12.2)	11 F 25 M	38/48 (6.1)	28 Limb 8 Bulbar	236.3 (231.4)	222.7 (104.1)	16.7 (14.4)	25.5 (14.3) in *deceased* patients

*Patients marked by # or * were alive upon closure of data admission. Patients who had been observed for less than 30 months at time of closure of data admission are marked by #. The sign * indicates patients, who at time of closure of data admission, had been observed more than 30 months from diagnosis. Baseline ALSFRS-R was not available in one patient, marked by -.*

(day 7) a 5-day treatment course with G-CSF at baseline, 3 months and 6 months. G-CSF led to a sustained increase of $CD34^+$ and $CD34^+CD38^-$ HSPC at all time points (**Figure 3**). In patients treated with ongoing 5-day courses of G-CSF *t*-tests displayed no significant reductions in mobilization of $CD34^+$ and $CD34^+CD38^-$ HSPC when comparing the respective levels after G-CSF treatment at baseline and after 3 and 6 months of treatment (mean number of $CD34^+$/ml at baseline 30307,

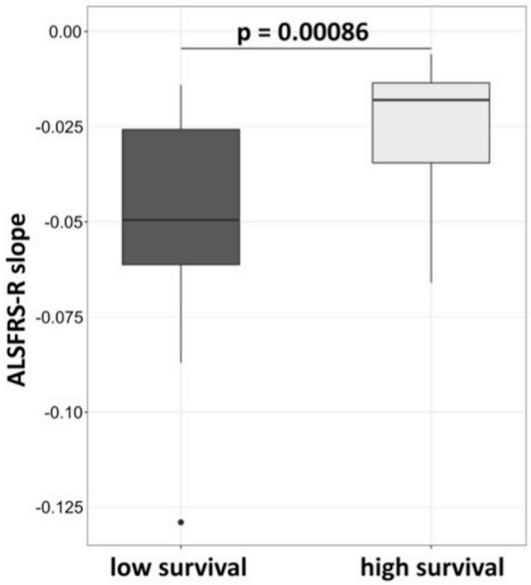

FIGURE 2 | ALSFRS-R decline is less rapid in patients who survive longer than 30 months. Patients were assigned to high survival group at survival longer or at 30 months, and to low survival group at survival below 30 months from diagnosis. The slope was calculated by robust calculation of the ALSFRS-R measurements. Median slope in the high survival group was −0.019 and −0.05 in the low survival group. Wilcoxon test, *p*-value 0.00086.

at 3 months 35250, at 6 months 22017; mean number of $CD34^+CD38^-$/ml at baseline 3092, at 3 months 2089, at 6 months 1632, all Wilcoxon paired *t*-test, all *p*-values not significant; **Figure S2**). However, we found a different capacity to mobilize hematopoietic stem cells in patients surviving longer or shorter than 30 months from diagnosis. This was analyzed by Area Under the Curve (AUC) approach to mobilized $CD34^+CD38^-$ cells within the first year of G-CSF treatment in 19 available patients, who all received ongoing 5-day treatment. Longer surviving patients displayed a significantly superior mobilization of $CD34^+CD38^-$ cells under G-CSF application at 1 year of treatment. At 4 months this difference was borderline significant (trend) (**Figure 4**).

Short and Long-Term Survivors Differed in Their Baseline Cytokine Levels

Survival in months from diagnosis was negatively correlated with baseline serum levels of the cytokine TNF-beta. MCP-1 and INF-gamma were, as a trend, negatively correlated with survival as well. IL-16 baseline levels displayed a positive correlation with survival. MDC, IL-8, IL-17A, and PIGF were, as a trend, positively correlated with survival (**Table 2**, **Figure 5**). We then dichotomized G-CSF treated patients according to their survival of either more or less than 30 months from diagnosis, and analyzed cytokines at baseline. Patients who survived longer than 30 months from diagnosis had significantly higher baseline levels of MDC and Tie-2. For IL-16, IL-17A, and PIGF we found similar trends. On the other hand, there were significantly higher baseline levels of TNF-beta and IL-7 in patients who survived less than 30 months from diagnosis. TNF-alpha and MCP-1 displayed similar trends. However, when correcting the cytokine comparisons in long and short survival for multiple testing, none of these findings remained significant [as assessed by the FDR-adjusted *p*-values (*q*-values) in **Table 2**].

G-CSF Treatment Modulated Serum Cytokine Levels of ALS Patients Over Time

The direct effects of G-CSF on cytokine levels were evaluated by comparing cytokine levels 2 days prior to and 1 day after ongoing 5-day G-CSF application in a subgroup of patients allowing this analysis. These immediate effects were determined at three different time points (baseline, 3 and 6 months after treatment initiation). Due to individual application modes, 5-day G-CSF applications with corresponding blood samples were available for 18 patients at baseline, for 17 patients at 3 months, and for 14 patients at 6 months of ongoing G-CSF treatment.

We found G-CSF to have an immediate effect on the level of various cytokines (**Table 3**, **Figure S3**). The serum level of IL-10 increased after 5 days of G-CSF treatment at baseline, 3 months and 6 months compared to its respective level before G-CSF application, however, at 3 months only as a trend. The levels of IL-16, Tie-2, TNF-alpha, MIP1-beta, IL-15, IP-10, VCAM, ICAM-1, and of Flt-1 were significantly increased after G-CSF treatment at all above-mentioned time points. The levels of TARC, IL-7, INF-gamma, and MCP-1 were decreased at all above-mentioned time points. There was an increase in SAA, IL-12/IL-23p40, CRP, and VEGF-A levels after G-CSF at baseline and 6 months, the latter at 6 months only as a trend. The levels of VEGF-C and PIGF were increased after G-CSF at 6 months, that of PIGF also at 3 months as a trend. There was a decrease of Eotaxin-1, Eotaxin-3 and VEGF-D after G-CSF application at baseline and 3 months. TNF-beta was decreased at baseline, at 6 months by a trend. MCP-4 was decreased at 6 months, at baseline by a trend. The level of bFGF was decreased after G-CSF application at 3 months and 6 months.

DISCUSSION

Our Main Findings

Long term and individually adapted off-label treatment with G-CSF in 36 ALS patients was well tolerated and safe. The number of G-CSF-mobilized hematopoietic stem cells, as measured by AUC, was associated with longer survival. Initial levels of serum cytokines, such as MDC, TNF-beta, IL-7, IL-16, and Tie-2 were significantly associated with survival, indicating the potential of prognostic application of these immune markers in G-CSF treated ALS patients. Continued application of G-CSF led to persistent alterations in various serum cytokines and ongoing measurements revealed the multifaceted effects of G-CSF.

ALS as a Neuroinflammatory Disease

ALS has been recognized as a multifactorial disease. Neurodegenerative processes trigger neuroinflammation

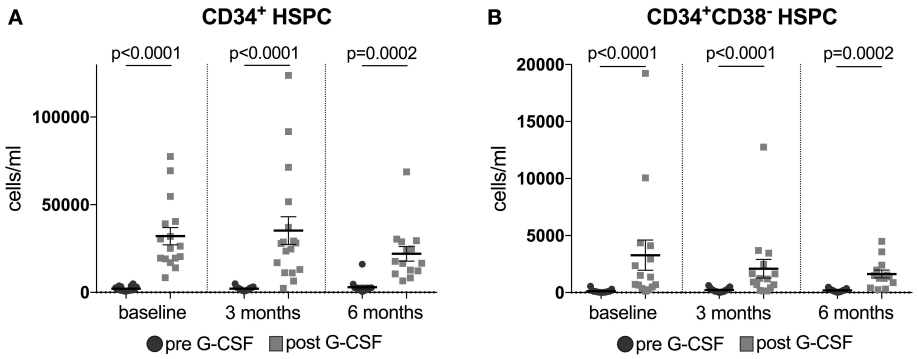

FIGURE 3 | (A,B) Mobilization of hematopoietic stem cells (HSPC) in G-CSF treated ALS patients. Plotted are $CD34^+$ (**Figure 2A**) and $CD34^+CD38^-$ HSPC (**Figure 2B**) in blood 2 days before (d0) and 1 day after (d7) daily application of G-CSF over 5 days in 16 (for $CD34^+$)/15 (for $CD34^+CD38^-$) patients at baseline, in 17 patients after 3 months, and in 14 (for $CD34^+$)/13 (for $CD34^+CD38^-$) patients after 6 months of treatment. Data are presented as scatter dot plot with mean + SEM. Paired Wilcoxon *t*-test, significance was taken at $p < 0.05$ (two-tailed). *T*-tests were corrected for multiple testing by FDR-adjusted *p*-values (*q*-values), discovery is indicated by $q < 0.1$. In $CD34^+$ and $CD34^+CD38^-$ HSPC at all time points: *q*-value = 0.0002.

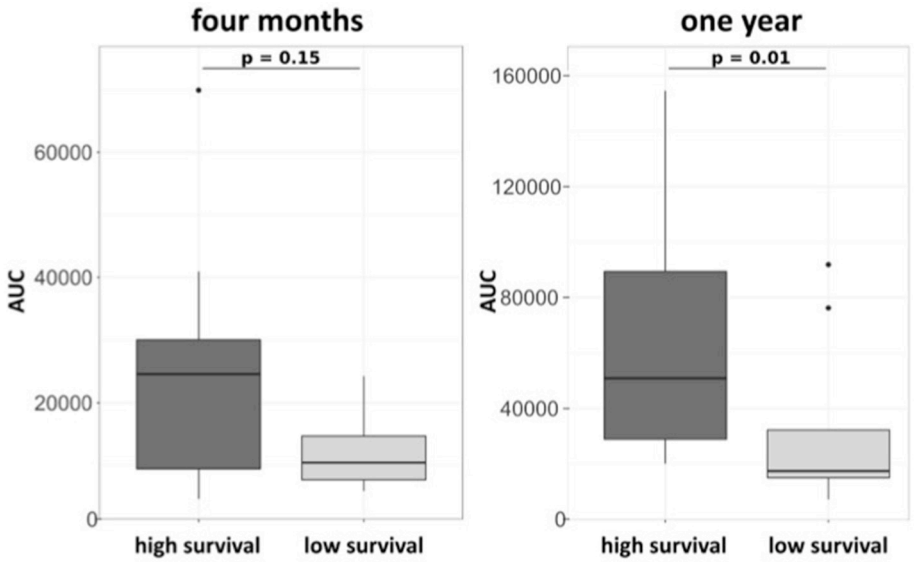

FIGURE 4 | Mobilization of $CD34^+CD38^-$ HSPC is associated with survival in ALS patients on a 5-day treatment scheme with G-CSF. Area under the curve (AUC) approach on blood HSPC over 4 months **(A)** and 1 year **(B)** in patients treated with 5-day application of G-CSF ($n = 20$). Patients were assigned survival groups dependent on survival being longer (high survival $n = 8$) or shorter (low survival) than 30 months from diagnosis.

and vice versa. Neuroinflammation with microglial activation, infiltration of peripheral immune cells into the CNS, and alterations in cytokine levels are pathological features in ALS. Cytokines are mediators of the immune communication that may cross the blood-brain barrier (BBB) and provide a mechanism by which the peripheral immune system may directly influence the CNS (30). In a recent study, we demonstrated a pro-inflammatory immune response with elevated inflammatory cytokines both in serum during disease and post-mortem in the CNS of ALS patients (31). However, immune response in ALS cannot be clearly dichotomized to a purely pro- or anti-inflammatory state, as cytokines are often pleiotropic, and their function may change over time and depend on concentration and specific disease context. Possibly, cytokine response in early ALS may be an attempt to restore homeostatic balance, whereas chronic exposure to pro-inflammatory cytokines might lead to cell destruction and loss of neuronal function. The latter supports a self-sustaining inflammatory process and possibly accelerates disease progression (7). Neuroinflammation and systemic inflammatory stimuli with their influence upon the CNS offer targets for therapeutic intervention in ALS (32). Analysis of peripheral blood is a feasible alternative for ongoing measurements of immune mediated and pathophysiological relevant parameters (33).

TABLE 2 | Cytokine levels at baseline in relation to survival.

Cytokine	Level in long survival	Correlation			Comparison (*t*-test)			
		r-value	R^2-value	*p*-value	Median long survival	Median short survival	*p*-value	*q*-value
ANTI-INFLAMMATORY								
MDC	↑	0.3269	0.1069	0.0726	939	227	**0.0494**	0.3088
PRO-INFLAMMATORY								
TNF-ß	↓	−0.4981	0.2481	**0.0043**	0.535	0.830	**0.0038**	0.1254
IL-7	↓	–	–	–	17	27	**0.0171**	0.2640
TNF-α	↓	–	–	–	2.5	3.0	0.0638	0.3088
MCP-1	↓	−0.3414	0.1166	0.0601	278	957	0.0544	0.3088
INF-γ	↓	−0.3264	0.1065	0.0731	–	–	–	–
IL-16	↑	0.4449	0.1979	**0.0122**	262	133	0.0655	0.3088
IL-8	↑	0.3492	0.1219	0.0542	–	–	–	–
IL-17A	↑	0.3749	0.1406	0.0710	2.58	0.68	0.0912	0.3421
ANGIOGENESIS								
Tie-2	↑	–	–	–	5762	4492	**0.0240**	0.2640
PlGF	↑	0.3277	0.1074	0.0719	33.8	31.7	0.0933	0.3421

Cytokine levels in pg/ml before first G-CSF application in ALS patients. Arrows indicate cytokine levels in patients with long compared to short survival. Then cytokine levels at baseline were correlated with survival. Next, Mann-Whitney test was calculated to assess differences in baseline cytokine levels in patients with survival longer or shorter than 30 months from diagnosis. Number of patients at baseline: 31 (16 long survival). Significance is indicated by bold marking when $p < 0.05$ (two-tailed p-value), trend when $p < 0.1$. T-tests were corrected for multiple testing by FDR-adjusted p-values (q-values), discovery is indicated by $q < 0.1$.

G-CSF in ALS

ALS is a multifactorial disease. Targeting common pathologic features such as neuro-inflammation and -degeneration may thus be beneficial for all ALS patients. Although G-CSF is an established, well-tolerated and safe growth factor for mobilization of hematopoietic stem and precursor cells (34), there is accumulating evidence that it is also a potent modulator of multiple CNS functions relevant to ALS (13). G-CSF modulates the immune response (35), it promotes anti-inflammatory and decreases pro-inflammatory mediators (36). Small clinical trials with G-CSF in ALS patients have delivered inconclusive results. Treatment with G-CSF was associated with a decrease in pro-inflammatory cytokine levels in serum and cerebrospinal fluid (CSF) of ALS patients (26), and minor benefits were detected by neuroimaging (27). But promising evidence for efficacy of G-CSF in ALS animal models has not yet been translated to ALS patients. It seems likely that a successful clinical translation requires higher dose, more frequent application and longer exposure to G-CSF as well as extended observation times (37). The latter is of crucial importance when aiming at structural and functional improvements or support of neurogenesis.

G-CSF Treatment in ALS Is Safe and Well Tolerated

Application of G-CSF in oncological indications is usually limited to treatment cycles, and the only clinical experience with life-long G-CSF therapy has accumulated with patients suffering from severe congenital neutropenia and cyclic neutropenia (38, 39). To our knowledge, we first reported on long-term G-CSF treatment in a CNS indication (25). We found G-CSF application to be generally well tolerated in ALS patients, with mild to moderate bone pain and leukocytosis after G-CSF applications as frequent minor adverse events. As this was off-label, experimental treatment of individual ALS patients, we had no control group for assessment of survival. If we only observe *deceased* patients and leave those still alive out, then the mean survival of these 26 patients at 25.5 months from diagnosis indicates no harm by G-CSF in ALS.

Stem Cell Mobilization Is Efficient and Associated With Longer Survival in G-CSF Treated ALS Patients

G-CSF is a well-known mobilizer of hematopoietic stem cells (8, 9). In all patients treated with G-CSF for five consecutive days, G-CSF increased mobilization of hematopoietic stem cells ($CD34^+$ and $CD34^+$ $CD38^-$) into the peripheral blood. Interestingly, we found an association between stem cell mobilization and survival. Patients who survived longer than 30 months from diagnosis mobilized more $CD34^+CD38^-$ hematopoietic stem cells than patients with shorter survival, as measured by Area Under the Curve after G-CSF treatment up to 1 year (**Figure 4**). Higher levels of circulating hematopoietic stem cells are associated with better clinical outcome and less structural damage after intracerebral hemorrhage in humans (40). The mechanism of how hematopoietic stem cells may contribute to neurodegenerative disease is yet unclear. Migration and

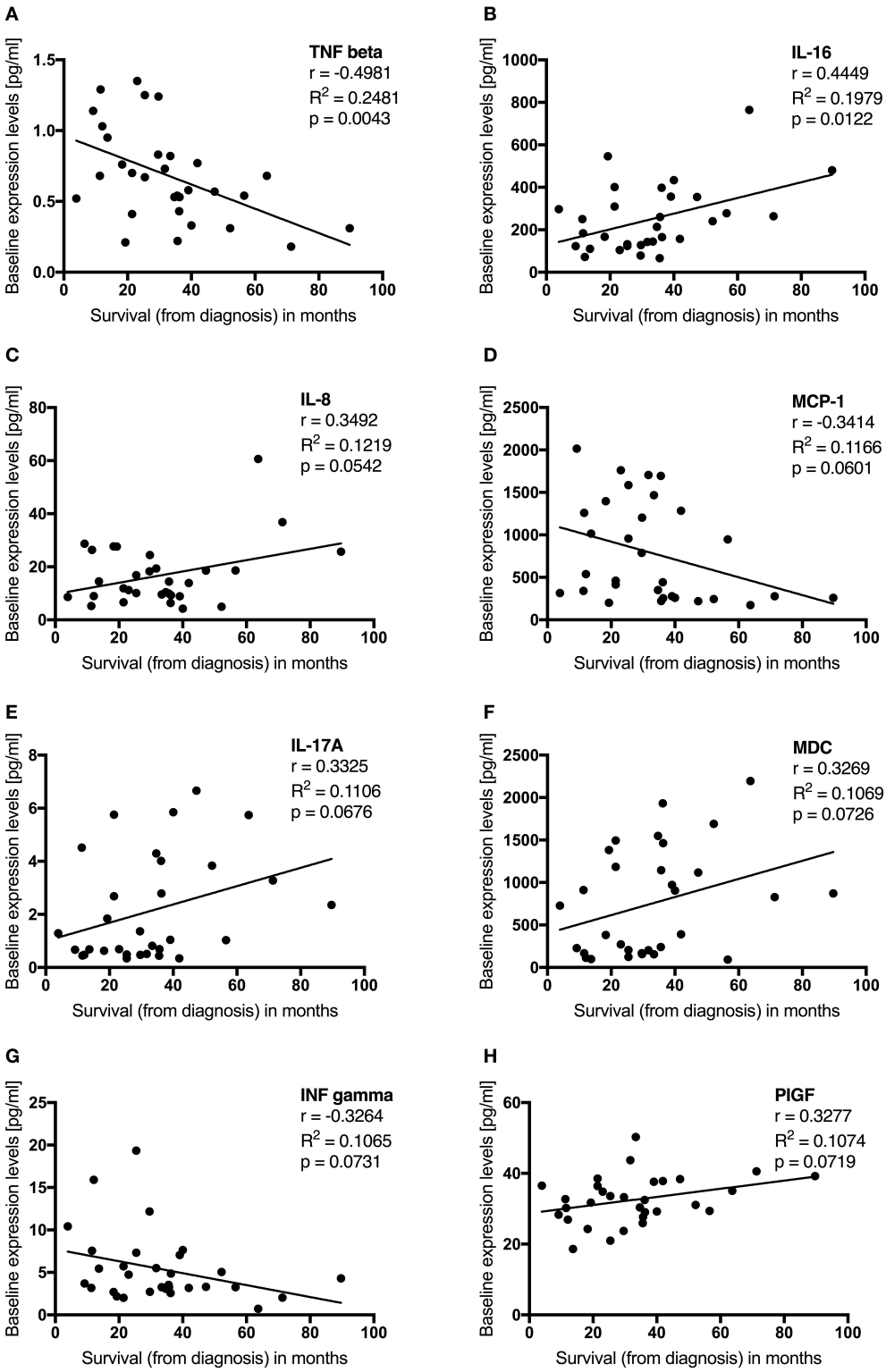

FIGURE 5 | (A–D) Baseline cytokine levels are associated with survival in G-CSF treated ALS patients. Plotted are initial cytokine levels of TNF beta **(A)**, IL-16 **(B)**, IL-8 **(C)**, MCP-1 **(D)**, IL-17A **(E)**, MDC **(F)**, ING gamma **(G)**, and PIGF **(H)** in pg/ml before first G-CSF application in 31 patients. Survival was assessed in months from diagnosis and censored upon data admission in living patients ($n = 7$). Displayed is Pearson r, the coefficient of determination (R^2), p-value (two-tailed) significant at $p < 0.05$, trend at $p < 0.1$.

TABLE 3 | Cytokine levels before and after G-CSF treatment at different time points.

Cytokine	Direction	Treatment start			3 months			6 months		
		Fold change d0-d7	*p*-value	*q*-value	Fold change d0-d7	*p*-value	*q*-value	Fold change d0-d7	*p*-value	*q*-value
ANTI-INFLAMMATORY										
IL-10	↑	1.95	**0.0016**	**0.0018**	1.24	0.0856	0.0371	2.02	**0.0004**	**0.0007**
PRO-INFLAMMATORY										
TNF-ß	↓	0.83	**0.0208**	**0.0112**	–	–	–	0.85	0.0591	0.0268
INF-γ	↓	0.81	**0.0214**	**0.0113**	0.78	**0.0182**	**0.0104**	0.62	**0.0009**	**0.0014**
IL-7	↓	0.66	**0.0003**	**0.0006**	0.80	**0.0011**	**0.0014**	0.80	**0.0107**	**0.0075**
MCP-1	↓	0.83	**0.0120**	**0.0080**	0.77	**0.0007**	**0.0012**	0.79	**0.0107**	**0.0075**
MCP-4	↓	0.94	0.0599	0.0268	0.92	**0.0150**	**0.0089**	–	–	–
TARC	↓	0.87	**0.0304**	**0.0148**	0.86	**0.0032**	**0.0030**	0.80	**0.0166**	**0.0097**
Eotaxin-1	↓	0.88	**0.0139**	**0.0085**	0.93	**0.0079**	**0.0059**	–	–	–
Eotaxin-3	↓	0.83	**0.0034**	**0.0030**	0.78	**0.0034**	**0.0030**	–	–	–
CRP	↑	4.45	**0.0010**	**0.0014**	–	–	–	3.99	**0.0085**	**0.0062**
SAA	↑	2.96	**0.0008**	**0.0013**	–	–	–	2.03	**0.0353**	**0.0168**
TNF-α	↑	1.77	**<0.0001**	**0.0003**	1.50	**0.0046**	**0.0038**	1.79	**0.0004**	**0.0007**
IP-10	↑	1.50	**0.0002**	**0.0004**	1.34	**0.0067**	**0.0052**	1.40	**0.0134**	**0.0085**
IL-15	↑	1.14	**0.0022**	**0.0022**	1.24	**0.0208**	**0.0112**	1.24	**0.0016**	**0.0018**
IL-12/IL-23p40	↑	1.24	**0.0047**	**0.0038**	–	–	–	1.19	**0.0052**	**0.0041**
IL-16	↑	3.14	**<0.0001**	**0.0003**	3.66	**0.0011**	**0.0014**	3.78	**0.0002**	**0.0004**
MIP1-ß	↑	3.38	**<0.0001**	**0.0003**	4.63	**0.0013**	**0.0015**	2.91	**0.0001**	**0.0003**
ANGIOGENESIS										
VEGF-A	↑	1.39	**0.0010**	**0.0014**	–	–	–	1.32	0.0580	0.0268
Tie-2	↑	1.27	**<0.0001**	**0.0003**	1.18	**0.0032**	**0.0030**	1.19	**0.0134**	**0.0085**
Flt-1	↑	1.45	**<0.0001**	**0.0003**	1.32	**0.0026**	**0.0026**	1.33	**0.0001**	**0.0003**
PlGF	↓	–	–	–	–	–	–	1.14	**0.0203**	**0.0112**
VEGF-C	↓	–	–	–		0.0984	0.0420	0.86	**0.0017**	**0.0017**
VEGF-D	↓	0.88	**0.0139**	**0.0085**	0.94	**0.0110**	**0.0075**	–	–	–
bFGF	↓	–	–	–	0.72	**0.0232**	**0.0121**	0.81	**0.0040**	**0.0035**
VASCULAR INJURY										
VCAM	↑	1.40	**<0.0001**	**0.0003**	1.28	**0.0267**	**0.0134**	1.41	**0.0001**	**0.0003**
ICAM-1	↑	1.30	**<0.0001**	**0.0003**	1.23	**0.0305**	**0.0148**	1.31	**0.0001**	**0.0003**

Paired Wilcoxon t-test. Arrows indicate direction, and fold change gives effect size of cytokine modulation when comparing respective levels 2 days before (d0) and 1 day after (d7) daily application of G-CSF over 5 days. Number of evaluable patients at baseline: 18, at 3 months: 17, and at 6 months: 14. Significance is indicated by bold marking when $p < 0.05$ (two-tailed p-value), trend when $p < 0.1$. T-tests were corrected for multiple testing by FDR-adjusted p-values (q-values), discovery is indicated by $q < 0.1$. Non-significant and non-trend findings are marked by -.

subsequent trans-differentiation of bone marrow derived cells within the CNS is controversially discussed (18). However, G-CSF increases the number of hematopoietic stem cells translocated to the damaged CNS (16, 17). There, hematopoietic stem cells modulate the immune system, they may interact with local cells, and produce neurotrophic factors, which promote growth of neural progenitors and survival (17, 18). A recent study in mice exposed to cranial irradiation demonstrated that G-CSF augments neurogenesis; bone marrow derived G-CSF-responsive cells migrate to the CNS, where they express macrophage and microglia phenotypes. The authors found that G-CSF treatment led to an improved functional outcome, thus arguing for the

neuroprotective mechanisms of G-CSF on brain repair (15). Human studies have demonstrated G-CSF to directly affect monocytes and to modulate monocyte cytokine secretion toward an anti-inflammatory polarization (41). A recent study applying G-CSF in healthy humans described expansion of a mature variant monocyte subtype displaying strong immunosuppressive properties (42). Next to neural cells, also neural stem cells have G-CSF receptors and G-CSF treatment induces a neural phenotype of these cells (12). Effects of G-CSF on hematopoietic stem cells may therefore serve as a proxy for biological cellular activity of G-CSF on neural cells.

Cytokine Levels Are Associated to Survival and Affected by G-CSF

Neuroinflammation contributes to the pathogenesis of ALS (3). Apart from CNS inflammation, peripheral cytokines and other inflammatory markers are affected in ALS, and cytokine levels may serve as biomarkers (43). We found that different cytokines at baseline were correlated with survival (**Table 2**, **Figure 5**). When dichotomizing patients depending on individual survival being longer or shorter than 30 months from diagnosis, we detected different peripheral cytokine levels at baseline (**Table 2**). In general, five-day treatment courses with G-CSF exerted immediate effects on cytokine levels and were able to partly counteract the harmful immune response in ALS (**Table 3**, **Figure S3**).

The initial levels of 11 cytokines were associated with survival, of which 8 were altered by G-CSF application. However, the correlation models, as indicated by the rather low R^2-values, could only explain smaller parts of the variance. Even though the cytokine comparisons in long and short survival did not withstand correction for multiple testing, we decided to explore the findings because they might help to generate hypotheses for further studies and show biologically important findings in spite of the small number of patients tested. Moreover, G-CSF led to change in many inflammatory cytokines, as well as cytokines involved in angiogenesis and vascular injury, of which all significant changes remained so after testing for multiple comparison (**Tables 2**, **3**).

Initial TNF-beta (LTA, lymphotoxin-alpha) levels negatively correlated with survival and were found at higher levels in shorter surviving ALS patients upon treatment initiation. G-CSF application led to reduction in TNF-beta, a pro-inflammatory cytokine and common cell death effector found to be increased in ALS sera (31). TNF-alpha was borderline increased in patients with shorter survival (trend) and G-CSF led to an increase in its serum levels. TNF-alpha is elevated in ALS (31, 43–46) and correlates with disease duration (47). But its role in ALS in unclear and the two TNF-alpha receptors, either associated with inflammation or neuroprotection, have opposing effects concerning survival in ALS (48). Dependent on subtype and context, activation can lead to neuroprotection and neurogenesis (49), reduced oxidative stress (50) and glutamate excitotoxicity (51). An increased occurrence of ALS after long-term use of TNF-alpha inhibitors in rheumatic arthritis, is suggested to be a consequence of deficient TNF-alpha mediated neuronal protection (52). Higher initial levels of IL-7 were associated with shorter survival, and reduced after ongoing treatment with G-CSF. IL-7 is considered a pro-inflammatory cytokine, and is increased in CSF (53) and serum (31) of ALS patients. MCP-1 (CCL2) was borderline correlated (trend) with shorter survival of ALS patients. We confirmed a reduction of MCP-1 levels in ALS after treatment with G-CSF (26). MCP-1 is a prominent pro-inflammatory cytokine that can enhance microglial recruitment to the CNS after injury, which may exacerbate ALS progression (54). MCP-1 correlates with faster disease progression (55) and ALS patients have elevated MCP-1 serum levels (31, 55, 56) and increased protein expression within spinal cord (31). INF-gamma was borderline negatively correlated with survival in our patients (trend). As known from healthy donors (57), INF-gamma levels were decreased by G-CSF. As a hallmark of proinflammatory cells, INF-gamma is proposed to contribute to motor neuron death in ALS (58). ALS patients have higher INF-gamma serum levels (47, 55, 59), that correlate with disease progression (47, 59) and shorter survival (55).

On the other hand, the pro-inflammatory marker IL-16 was positively correlated with survival and increased after G-CSF application. IL-16 also holds an immunomodulatory role by expansion of regulatory T cells (Treg) (60), that at lower levels in ALS, are associated with rapid disease progression and shorter survival (61). Thus, G-CSF related increase in IL-16 might be beneficial for ALS patients. Another pro-inflammatory cytokine, IL-17A, was borderline correlated with longer survival (trend) but not altered by G-CSF treatment. IL-17A has been reported elevated in serum (55, 62, 63) and CSF (64) of ALS patients. After G-CSF treatment, Chió et al. found a reduction of IL-17A in the CSF, but not in serum of ALS patients (26). IL-8 was borderline correlated with longer survival (trend), and not altered by G-CSF treatment. IL-8 is produced by several cells in response to inflammation, and higher plasma (44) and CSF levels (65) are known in ALS. MDC (CCL22) was associated with longer survival, however, not modulated by G-CSF treatment. MDC is an anti-inflammatory cytokine, and consistent with a proposed protective effect, ALS patients have lower MDC protein expression in the spinal cord (31). Further, angiogenic factors, such as Tie-2 and PIGF were associated with survival. Tie-2 was elevated in longer surviving patients and G-CSF led to an increase in it's serum levels. Angiogenesis is mediated by the angiopoietin-1/Tie-2 system (66), and stimulation of angiogenesis by another pro-angiogenic factor, VEGF, is found to increase neurogenesis (19). G-CSF treatment led to an increase in PIGF, and PIGF was as a trend both correlated with survival and elevated in longer surviving patients. PIGF supports angiogenesis (67), and may be a marker for the angiogenic niche.

The following 18 cytokines were significantly altered by G-CSF, however, not associated with survival. As known from healthy donors (68), IL-10 was markedly increased after G-CSF treatment. This anti-inflammatory cytokine is elevated in ALS-patients with mild symptoms or slow progression (53). G-CSF application led to reduced systemic levels of the pro-inflammatory cytokines MCP-4 (CCL13), TARC, Eotaxin-1 (CCL11), and Eotaxin-3 (CCL26). MCP-4 (31, 65), TARC (31)

and Eotaxin-1 (65) are elevated in ALS serum. The latter is further associated with Alzheimer's dementia (69), aging and inhibition of neurogenesis in mice (70). We also noticed increase in levels of the pro-inflammatory cytokines CRP, SAA, IP-10 (CXCL10), IL-15, IL-12/IL-23p40, and MIP1-beta after G-CSF application. The acute-phase proteins CRP and SAA have been described as elevated in ALS patients (31, 71). IP-10 is negatively correlated with disease progression rate (72) and increase after G-CSF treatment has been described (26). IL-15 (31, 55, 73) and MIP1-beta (31) are elevated in serum of ALS patients. MIP1-beta shares receptor (CCR5) with MIP-alpha, which is elevated and considered neuroprotective in ALS (74). MIP-1 beta is negatively correlated with disease severity and progression rate, and thus might exert neuroprotective effects in ALS (72). IL-12/IL-23p40 describes the p40 subunit shared by the cytokines IL-12 and IL-23, and is considered a pro-inflammatory marker. However, we noted no increase in cytokines induced by IL-12/IL-23p40, such as INF-gamma and IL-17A, after G-CSF treatment. Aside from neuroinflammation, impaired neurotrophic support is a hallmark of ALS. Levels of VEGF-A and Flt-1 were increased, whereas VEGF-C, VEGF-D, and bFGF levels were decreased after G-CSF application. VEGF-A and bFGF, two common neurotrophic and possibly protective factors in ALS (55), are both increased in ALS CSF (64). Further, VEGF-A supports neurogenesis and neural development and is an attractant for HSPC that has been associated with longer survival in ALS (55). We found an increase in ICAM-1 and VCAM-1 after G-CSF treatment. At the vascular endothelium these cellular adhesion molecules are involved in leukocyte transport (75), but their role in ALS is unclear.

In ALS, a short time delay for diagnosis is associated with inferior prognosis as these patients are likely to have a more aggressive disease (76). Accordingly, we observed a briefer latency between diagnosis and treatment initiation in patients with shorter survival, which might reflect a more rapid progression of disease in these patients. Hence, longer surviving patients presumably initiated treatment at a later pathophysiological stage of their disease. This might offer an explanation for the fact that levels of some pro-inflammatory cytokines such as IL-16, IL-17A, and IL-8 were associated with longer survival. However, the role of inflammatory markers in ALS is unclear and our findings may also indicate that inflammation does not only negatively impact the disease (71). The remaining relation between cytokines and survival seen in our cohort highlights the importance of these markers in predicting individual survival. Thus, different cytokines may be used as biomarkers for initial patient stratification, predicting later clinical course, monitoring treatment response and progression of disease.

Possible direct effects of G-CSF upon the CNS were not assessed, as we did not obtain post-mortem analysis of deceased patients. Neuroimaging studies conducted on our G-CSF treated patient cohort (77) did not directly address possible G-CSF related structural effects—we also had no patient control group without G-CSF treatment. One indirect mode of action by which G-CSF exerts neuroprotective effects may be through polarization of the immune system toward an anti-inflammatory state (13). We observed an increase in anti-inflammatory cytokines and neurotrophic factors as well as a decrease in pro-inflammatory cytokines. However, we also captured an increase in some pro-inflammatory cytokines, which might be due to the pleiotropic effects of G-CSF and possibly reflect an unspecific cytokine reaction after application. Overall, the effects of G-CSF on peripheral cytokine levels and ALS appear to be versatile and should be assessed in a prospective clinical study.

Strengths and Limitations

This retrospective analysis has several limitations. Firstly, we have not conducted a controlled clinical trial and thus, there was no placebo-arm. Rather, the aim of the intervention was to offer individual ALS patients a potentially beneficial off-label treatment with G-CSF. Evaluation of respiratory function was driven by clinical indication and not systematically assessed. Hence, we did not regularly screen for respiratory deficits upon treatment initiation. The same applied to assessment of cognitive function. In addition, we did not systematically analyze for ALS-specific gene mutations. Such factors have predictive value concerning prognosis (78), the lack of initial screening of respiratory and cognitive function as well as genetic background might impede interpretation of the data. Given the objective of evaluating safety of G-CSF and the absence of a control group, in this paper we assessed survival from time of diagnosis, and not from treatment initiation. The latency between symptom onset and diagnosis was not assessed in this report. This is a limitation, as quantification of diagnostic delay - being associated with longer survival (78), could have offered prognostic implications. During the experimental treatment, patients were routinely seen on an outpatient basis to monitor safety and blood samples were regularly obtained. This enabled a dynamic observation of alterations in neuroinflammation due to ALS disease and treatment with G-CSF over time. However, with only 36 G-CSF treated patients caution should be applied in trying to generalize our findings. Moreover, application and dosing schemes for G-CSF treatment were decided upon on an individual patient level and thus complicated the establishment of dose-effect relationships. When we analyze cytokine levels upon treatment initiation in our patient cohort retrospectively, we have to take into account that these patients differ concerning covariant factors such as age, gender, bulbar vs. spinal-onset, and functional status (ALSFRS-R). Given the small number of patients treated with G-CSF, a statistical evaluation of the predictive value of these subpopulations was not reasonable. There was also great heterogeneity in the latency between time of diagnosis and treatment initiation. Cytokine levels alter during progression of disease. Altogether, these aspects lead to a reduced statistical power, which may also provide an explanation for the variation and modest correlation seen between initial cytokine levels and survival. Moreover, we found that cytokine comparisons in long and short surviving patients did not withstand correction for multiple testing. These signals may be of biological relevance, as they were detected in spite of a small number of patients and great disease heterogeneity, and thus may assist in hypothesis generation for future studies.

CONCLUSION

Altogether, we found that long term G-CSF treatment is feasible and safe for ALS patients. G-CSF efficiently mobilized hematopoietic stem cell into peripheral blood, and the amount of mobilized stem cells was associated with longer survival. Thus, stem cell mobilization could be a potential biomarker to monitor treatment response to G-CSF. Peripheral cytokines are relevant in the course of disease in ALS. We identified TNF-beta, MDC, IL-16, IL-7, and Tie-2 as cytokines whose baseline levels may predict G-CSF treatment response and survival. Additionally, long term G-CSF treatment led to sustained alterations of multiple cytokines in peripheral blood. Thus, cytokines represent potential biomarkers for survival prediction and for early monitoring of G-CSF treatment in ALS, all of which need further validation in a prospective controlled randomized trial.

AUTHOR CONTRIBUTIONS

SJ care for ALS patients, conception of intervention, analysis, interpretation, wrote the manuscript. UB clinical responsibility for intervention. UB, WS-M, and VS care for ALS patients, conception of intervention and analysis, revision of the manuscript. T-HB conception of intervention and analysis, revision of the manuscript. BB contribution to analysis, revision of the manuscript. A-LM care for ALS patients, revision of the manuscript. TK organization of care and disposition of patient material. SP and EW performed cytokine experiments, revision of the manuscript. SI and JG performed and analyzed HSPC experiments, revision of the manuscript. AS and WH revision of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2018.00971/full#supplementary-material

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MINI REVIEW
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Emerging Magnetic Resonance Imaging Techniques and Analysis Methods in Amyotrophic Lateral Sclerosis

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Objective markers of disease sensitive to the clinical activity, symptomatic progression, and underlying substrates of neurodegeneration are highly coveted in amyotrophic lateral sclerosis in order to more eloquently stratify the highly heterogeneous phenotype and facilitate the discovery of effective disease modifying treatments for patients. Magnetic resonance imaging (MRI) is a promising, non-invasive biomarker candidate whose acquisition techniques and analysis methods are undergoing constant evolution in the pursuit of parameters which more closely represent biologically-applicable tissue changes. Neurite Orientation Dispersion and Density Imaging (NODDI; a form of diffusion imaging), and quantitative Magnetization Transfer Imaging (qMTi) are two such emerging modalities which have each broadened the understanding of other neurological disorders and have the potential to provide new insights into structural alterations initiated by the disease process in ALS. Furthermore, novel neuroimaging data analysis approaches such as Event-Based Modeling (EBM) may be able to circumvent the requirement for longitudinal scanning as a means to comprehend the dynamic stages of neurodegeneration *in vivo*. Combining these and other innovative imaging protocols with more sophisticated techniques to analyse ever-increasing datasets holds the exciting prospect of transforming understanding of the biological processes and temporal evolution of the ALS syndrome, and can only benefit from multicentre collaboration across the entire ALS research community.

Keywords: motor neuron disease, MRI—magnetic resonance imaging, event-based model, quantitative magnetization transfer imaging, neurite orientation dispersion and density imaging (NODDI)

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Neuroimaging modalities sensitive to the dynamics and patterns of tissue degeneration in amyotrophic lateral sclerosis (ALS) are required as objective biological markers of disease activity *in vivo*. Standard clinical assessment is usually adequate for diagnosis, however there is a pressing need for non-invasive neuroimaging biomarkers that may differentiate between the various phenotypes within the ALS syndrome, provide more accurate prognostic information, and monitor responses to therapeutic interventions. There is also a need for neuroimaging techniques which have the potential to interrogate the specific mechanisms of neurodegeneration, given that conventional MRI primarily aims to exclude alternative diagnoses (1). As such, it will be important to integrate new modalities of structural and functional imaging (including MRI and PET) with molecular

biomarkers of neuronal damage, and indicators of neuroinflammation if the therapeutic impasse for more effective disease treatments is to be broken. Diffusion MRI, particularly diffusion tensor imaging (DTI), has been extensively researched in patients with ALS to infer structural alterations within the brain and spinal cord by virtue of the movement of water molecules induced by magnetic field gradients. Fractional anisotropy (FA) is consistently reduced, often alongside increased mean or radial diffusivity (MD or RD, respectively), within the corticospinal tracts (CSTs) (2–15) and body of the corpus callosum through which pass the fibers connecting hemispheric motor areas (3, 5–8, 10, 12, 16, 17). Indeed, DTI changes are perhaps most reliably encountered within the posterior limb of the internal capsule (18, 19) which forms a common conduit for several descending motor pathways including the CST, cortico-rubro-spinal, and cortico-reticulo-spinal connections (20). Additional areas within the frontal, temporal (11, 21, 22), and parietal areas (11, 23) have shown reduced FA, all of which is consistent with the multisystem motor and extra-motor regions involved clinically and neuropathologically (24–26). Nevertheless, establishing the precise substrate or substrates underlying these changes observed on MRI is not straightforward and may be complimented by novel magnetic resonance imaging techniques and emerging big data analysis methods.

NEURITE ORIENTATION DISPERSION AND DENSITY IMAGING (NODDI)

Diffusion MRI is sensitive to the motion of water molecules at microscopic level. Nevertheless the signal it measures is averaged across volumes of 1–2 mm^3 (the so-called "voxel"). For this reason, any interpretation of the signal and its origin requires some degree of "modeling." More than one model has been proposed and each typically incorporates slightly differing mathematical assumptions to interpret and model the signal, thus providing only indirect inferences on anatomical configurations. For instance, DTI assumes that water movement will obey Gaussian properties and is widely accepted to lose consistency when neuronal fibers bend or fan out within a voxel, or where otherwise aligned fiber tracts are crossing each other (5) which is common to areas such as the centrum semiovale and even regions of the foliated corpus callosum (27, 28). Moreover, a reduction in FA signifies changes in both neurite density and orientation dispersion without distinguishing their individual contributions (28, 29). Therefore, variations on the diffusion tensor model have been created in an attempt to address these limitations. One such model is neurite orientation dispersion and density imaging (NODDI).

NODDI requires acquisition over a longer time than DTI and compartmentalizes non-Gaussian water diffusion into three geometric spaces encompassing isotropic (or free), hindered anisotropic and restricted anisotropic components. These are known as V_{ISO}, V_{IC}, and V_{EC} and each broadly correspond to free water/CSF, intra-neurite water (of axons and dendrites), and extra-neurite water (but potentially including glial cells and neuronal somata), respectively (29–31). The NODDI parameters ISO, NDI (neurite density index), and ODI (orientation dispersion index; a marker of the geometric complexity of neurites) can then be derived, the latter two of which are considered to provide a more structurally useful breakdown of single FA values (29) (see **Figure 1**). NODDI is able to better delineate white from gray matter, in which normal white matter displays higher NDI and lower ODI with the reverse in gray matter (33), and differentiate between different gray matter structures although might be more susceptible to changes in field strength in these areas (31). Compared to DTI, NODDI indices, particularly ODI, have been shown to correlate with histological measures of orientation dispersion in the spinal cord and might also display more inter-subject variability with implications for the sample sizes required for group analyses (33, 34). However, this may not necessarily be an inaccuracy in modeling rather a more accurate depiction of tissue composition (31). In addition, regions which might be expected to demonstrate considerable axon density and higher NDI values might counterintuitively show higher ISO due to the larger diameter axons enabling more freedom of water movement (31, 34).

NODDI has been used to demonstrate tissue alterations associated with normal aging (35–37) and in a range of conditions including focal cortical dysplasia (38), stroke (39), Wilson's disease (40), multiple sclerosis (33), neurofibromatosis type 1 (38, 41), and neurodegenerative diseases. Reduction in NDI and ODI of the contralateral substantia nigra pars compacta has been shown to correlate negatively with clinical severity of Parkinson's disease (42) whereas in pre-manifest Huntington's disease reductions in NDI and ODI are seen in a range of white matter tracts with reduced NDI in the corpus callosum correlating positively with markers of severity (43). In patients with young onset Alzheimer's disease reduction in NDI and ODI is seen corrected for reduced thickness within several relevant cortical areas, with lower NDI values in patients scoring less well on cognitive tests (44), while in a rodent model NODDI indices correlate more consistently than DTI parameters with the burden of tau pathology harbored by the cortex, corpus callosum, and hippocampus (45).

Use of NODDI imaging in ALS has only recently been undertaken. Whole brain analysis in patients with manifest disease has demonstrated a significant NDI reduction throughout the intracranial CSTs up to the subcortical matter of the precentral gyri and across the corpus callosum, with increased ODI in the anterior limb of right internal capsule and increased ISO adjacent to the right lateral ventricle relative to healthy controls (46). NDI within the right corona radiata and precentral subcortical white matter was decreased to a greater extent in those patients with both limb and bulbar involvement compared to limb alone, and longer disease durations correlated with reduced ODI in the precentral gyri, dorsolateral prefrontal cortices, and precuneus. Furthermore, at the statistical threshold used, FA was reduced as expected within the CSTs but less extensively than NDI, and changes were not observed within the corpus callosum, implying NODDI may be more sensitive than DTI. Indeed, combined NODDI and DTI has also been performed in pre-manifest C9orf72 mutation carriers alongside first degree relatives not possessing the pathological repeat

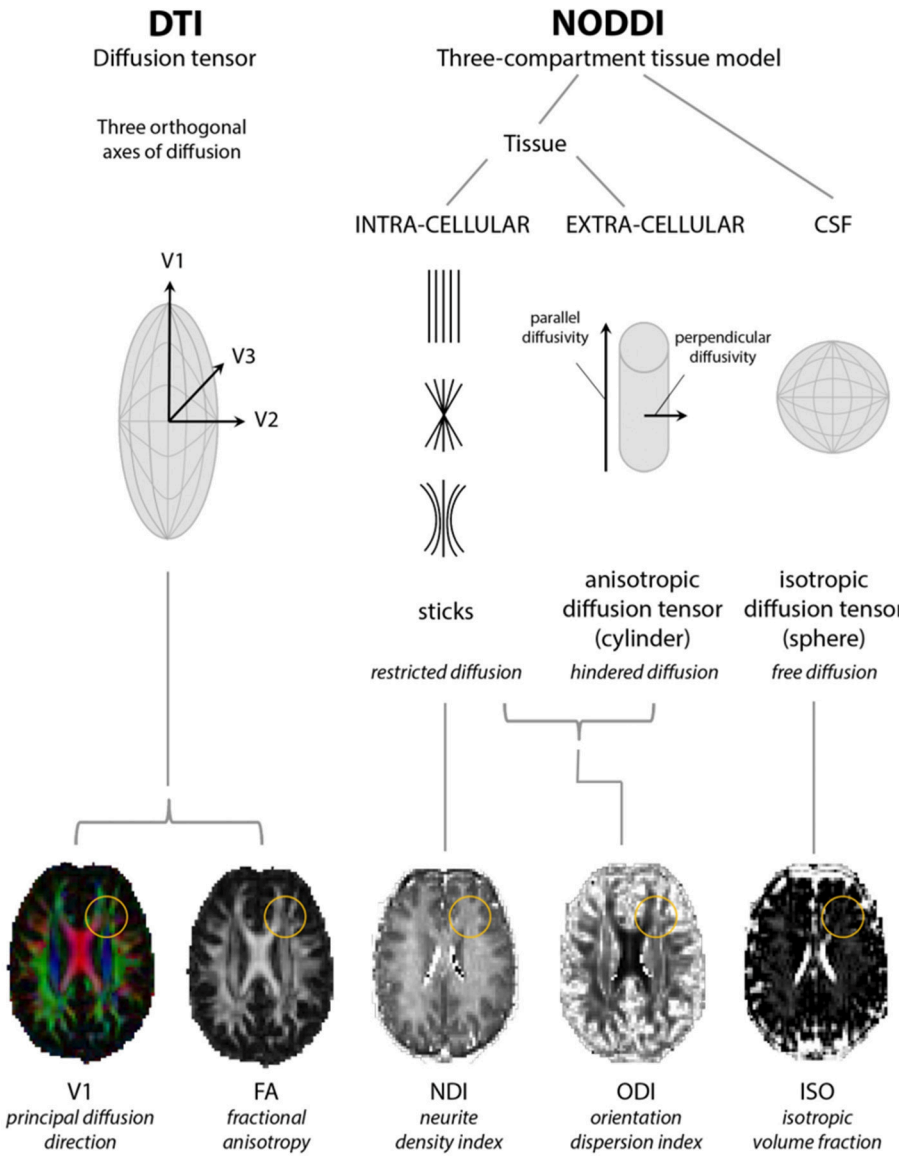

FIGURE 1 | Models of diffusion for neurite orientation dispersion and density imaging (NODDI) and diffusion tensor imaging (DTI). The diffusion tensor model of DTI is based upon three orthogonal axes of diffusion (V1, V2, and V3) yielding radial, axial, and mean diffusivity from which fractional anisotropy (FA) can be estimated. NODDI considers diffusion within three compartments: restricted diffusion in the intracellular compartment, hindered diffusion in the extracellular compartment, and free diffusion in cerebrospinal fluid (CSF), from which parameter maps representing neurite density (NDI), orientation dispersion (ODI), and isotropic fraction (ISO) indices can be estimated. Yellow circles highlight a region where changes in FA can be accompanied by changes in both NDI and ODI. Adapted from Rae et al. (32).

expansion (47). The effect size relating to detectable reductions of NDI within 7 of 11 white matter tracts, including the CSTs, is greater than that for DTI metrics (in this case increased axial diffusivity, RD, and MD rather than decreased FA) albeit statistically significant in just two. Therefore, the results appear to corroborate the implication that lowered FA (or increased diffusivity) in the CSTs and corpus callosum results from the loss of axon fibers rather than increased complexity or dispersion within tracts. Longitudinal NODDI scans have not yet been investigated although results from an ancillary imaging study to the Modifying Immune Response and Outcomes in ALS (MIROCALS) trial of low dose Interleukin-2 treatment are awaited.

In any case, neuroimaging techniques are constantly evolving with a raft of acronyms and employing different protocols aiming to reflect the true histological framework of gray and white matter. Although NODDI is considered non-inferior to other MRI modalities of high-angular resolution in this regard (48), it may be that acquisition protocols or MRI data modeling methods undertaken in NODDI, such as spherical (rather than linear) tensor encoding (49) along with tract-based (50), gray matter based (37), and gray matter surface based (51) spatial statistics

are further refined in due course to overcome its own recognized limitations.

QUANTITATIVE MAGNETIZATION TRANSFER IMAGING (QMTI)

Magnetization transfer imaging, unlike the NODDI model of diffusion MRI, essentially utilizes a "two pool" model in which hydrogen protons are either free or bound to macromolecules (lipids and proteins) within the semisolid tissue. The latter protons do not directly contribute to the MRI signal and are "silent" in diffusion sequences (increased radial diffusivity with DTI is not specific for demyelination) (52), but can be indirectly probed thanks to their interaction with the free protons following off-frequency radiofrequency pulses. The exchange in magnetization between the two compartments allows the state of the semisolid pool (saturated) to affect that of the free protons, resulting in partial saturation and in a decrease of its overall magnetization (53). The magnetization transfer (MT) effect can thereby produce a qualitative magnetization transfer tissue contrast (MTC) image and is already clinically utilized as part of MR angiography and gadolinium-enhanced T1-weighted sequences, for instance. Indeed, MTC T1 images in patients with ALS have shown hyperintensity along the CST (54, 55) and CC (54) in a proportion of cases (and more conspicuously than FLAIR) (55) compared to control subjects which was significantly related to the degree of reduced FA in the same regions and presumed to reflect damage to the white matter tracts, although with no clear association with clinical rating scales or disease duration (54). Acquiring a proton-density image with and without a MT pulse renders it possible to semi-quantify the MT effect and produce a voxel-wise magnetization transfer ratio (MTR) to reflect changes in macromolecular integrity. Accordingly, reduced MTR within the brain has been reported within the CSTs (56), the precentral and other frontal and extramotor gyri (57, 58), in patients with ALS compared to healthy controls, and independently of gray matter atrophy as measured by voxel-base morphometry (57). Significantly reduced average MTR within the spinal cord has also been reported with respect to controls (59–61), accompanied by diminished cord cross-sectional area and average FA (60), and with a longitudinal decline between sequential scans (59). More recent segmentation of the cord into gray and white matter areas, and using a particular adjusted MT protocol called inhomogeneous MT, has demonstrated localized reductions in MTR to the CSTs and dorsal columns in addition to the anterior horns at several non-contiguous cervical levels (62). However, the MTC and MTR are dependent on a range of imaging variables and their biophysical basis is undefined (53).

The development of mathematical models able to describe the MT-weighted signal as a function of the saturating pulses has enabled more biologically applicable parameters to be derived from quantitative magnetization transfer imaging (qMTi), including the macromolecular pool fraction [f; modeled to essentially represent myelin content], forward exchange rate of magnetization transfer [k_f], and transverse relaxation time of the free pool [$T_2{}^F$]. Although qMTi is yet to be explored in patients with ALS, studies in multiple sclerosis (MS) have demonstrated reductions in f and k_f, and increased $T_2{}^F$ in acute inflammatory lesions with a subsequent return to baseline over several months (63). Compared to healthy controls, normal appearing white matter (NAWM) has reduced f, kf, and MTR (64), and reduced MTR in chronic MS plaques and has been shown to correlate with greater disability (65). Incidentally, reduced MTR in the context of MS is generally considered to be a marker of demyelination, although a small study subdivided NAWM according to distance from a T2 hyper-intense plaque and degree of MTR reduction and found that, whereas at the edge of plaques reduced MTR correlates with reduced myelin content reduced MTR in NAWM may be result from to swollen microglia and, perhaps, axons (66), thus highlighting the uncertainty of its interpretation. MTR in normal appearing gray matter is also reduced in patients with relapsing-remitting MS (67–69) and may also correlate with disability, although variable results are reported (68). Acute increases in kf (but without change in f or T2f) on qMTi have also been induced within the insula in the context of a systemic inflammatory stimulus comprising intramuscular injection of typhoid vaccination and are associated with increased levels of reported fatigue, in addition to a co-localized increase in glucose metabolism measured by FGD-PET (70). Although the mechanisms underlying changes in magnetization transfer parameters are likely to be very different between diseases, it is plausible that qMTi would be sensitive to structural alterations in ALS given the likely role for the immune system in its pathogenesis (71, 72).

MULTIMODAL MRI

Furthermore, it may be that performing simultaneous qMTi with several other MR neuroimaging sequences, such as diffusion and (resting state) functional MRI, will be most helpful in building a better understanding how both tissue structure and function are affected by the disease process and, ultimately, the difference between certain phenotypes to guide more personalized treatments. Indeed, this is exemplified by the estimations of the myelinated fiber "g-ratio," the axon diameter divided by the diameter of its ensheathing myelin, which is estimated to ideally be around 0.7 in the central nervous system (73). As diffusion MRI is insensitive to myelin, the combination of intraneurite and isotropic fractions from NODDI and the *f* value from qMTi is required to calculate the g-ratio across the brain. Following adolescence, white matter g-ratio tends to steadily increase with age inferring myelin reduction and knock on effects with respect to the velocity of neuronal conduction (74) and premature increases in the g-ratio are accordingly seen within MS plaques (75, 76). Although ALS is not primarily a demyelinating disease, new insights into the secondary effects of the neurodegenerative process may be revealed with these techniques and correlate with clinical measures.

EVENT-BASED MODELING

Aside from interpreting the deviations of imaging parameters in terms of current tissue configuration, collecting longitudinal

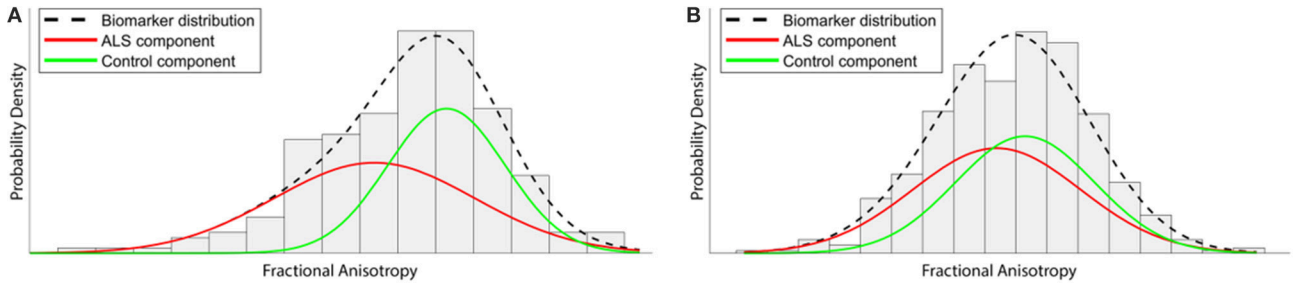

FIGURE 2 | Illustration of how the event-based model (EBM) aims to extract temporal information from a cross-sectional data set. Gaussian distributions of fractional anisotropy (FA) biomarker readings within a tract affected early in the course of the disease, such as the corticospinal tract, would be expected to demonstrate substantial separation between ALS and Control imaging data **(A)**. However, FA from another area affected at later stages demonstrates much less separation between distributions **(B)**. By exploiting and characterizing these differences across all biomarkers, the EBM attempts to order the change from "normal" to "diseased" across the entire disease course.

data is, at least conceptually, the most straightforward approach to understanding the temporal evolution of neurodegenerative pathology. However, patient tolerability for repeated MRI acquisition remains challenging in ALS, particularly, due to the rapid accumulation of symptoms and perhaps accounts for the relatively few studies conducted to date (5). Furthermore, it can be argued that participants who are included would be those harboring more slowly-progressing disease, and therefore may not be representative of the majority of patients with ALS.

Given these limitations, alternative methods such as "big data" analysis techniques and new modeling approaches have the potential to greatly increase our understanding of the mechanisms of disease progression. One such approach is the Event-Based Model (EBM) (77–79), a generative probabilistic model originally developed for use in Alzheimer's disease (AD) for which it has been validated in addition to Huntington's disease (80) and recently in ALS using oculomotor data (81). The EBM is designed to extract temporal information from cross-sectional data sets and, unlike traditional models of disease progression, does not rely on *a priori* staging of patients but instead extracts the event ordering directly from the data, thereby minimizing subjective bias.

The EBM defines a disease as a series of "events," where each event is the change of a biomarker reading from a "healthy" to a "diseased" state. Crucially, biomarker cut-off points are not determined beforehand, but are derived from the data during the modeling process. This not only reduces subjective bias, but also allows for much finer temporal characterization of disease progression than is possible under existing clinically-based staging systems. Healthy control data are used as a fixed reference, and each biomarker is modeled as a mixture of two Gaussian distributions (**Figure 2**). In order to perform temporal modeling, the EBM assumes that the disease progression is monotonic for individual biomarkers (i.e., the severity of disease burden can only increase). Thus, for biomarkers affected early on in the course of the disease, there will be larger differences between patient and control readings, while biomarkers that are affected late on will have smaller differences between patients and controls. Markov Chain Monte Carlo (MCMC) techniques can then be used to determine the most likely event order across the entire cohort (77).

As with any modeling approach, the EBM has strengths and weaknesses. The ability to extract fine-grained temporal information from cross-sectional data is exceptionally novel and valuable. Use of MCMC techniques also enables the model to quantify the positional variance of individual biomarkers across the cohort, thereby allowing a comparison of their relative importance and variability. In its current form, the EBM reveals aspects of disease progression that are common across the entire cohort (an "average" disease progression). The heterogeneity of ALS means that EBM analyses of stratified subgroups, based on genetic/prognostic factors, are an important future area for investigation.

The accuracy of the EBM output, as with any modeling process, will depend on the quality of the input biomarker data. As a consequence, ALS event-based modeling can require large quantities of data, particularly as individual mean cerebral CST FA values are known to have modest diagnostic power for ALS [found to have a pooled sensitivity and specificity of 0.68 and 0.73, respectively, in a meta-analysis (82)]. Current applications of the EBM to ALS data in progress include analysis of mean FA of white matter (WM) fiber bundles, modeling of patterns of cortical thinning, volumetric changes of brain structures, and oculomotor data. Future areas for development include the application of the EBM to multi-modal ALS biomarker data. Excitingly, the application of the EBM to higher order models of diffusion such as NODDI has the potential to give greater insight into ALS degeneration by simultaneously modeling the changes within ISO, NDI, and ODI parameters.

CONCLUSION

Ultimately, all modeling is an attempt to separate meaningful information from randomness. MRI techniques differentially model the signal to derive parameters that plausibly relate to tissue microstructure properties; these parameters can then be

modeled further using the EBM to reveal patterns that exist within the data, but which still require human assessment and interpretation (as well as clinical and histological validation). Although the innovative imaging and data analysis techniques presented here constitute a selection of available methods or protocols, their use singly and in combination has the potential to transform our understanding of the biological processes and temporal evolution of ALS, which is likely to benefit further from multicenter collaboration across the entire ALS research community.

AUTHOR CONTRIBUTIONS

AWB and MCG performed a review of the literature and drafted the paper. MC and PNL provided specialized expertise and critical appraisal of the article for submission.

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Developing a Neuroimaging Biomarker for Amyotrophic Lateral Sclerosis: Multi-Center Data Sharing and the Road to a "Global Cohort"

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Neuroimaging in Amyotrophic Lateral Sclerosis (ALS) has steadily evolved from an academic exercise to a powerful clinical tool for detecting and following pathological change. Nevertheless, significant challenges need to be addressed for the translation of neuroimaging as a robust outcome-metric and biomarker in quality-of-care assessments and pharmaceutical trials. Studies have been limited by small sample sizes, poor replication, incomplete patient characterization, and substantial differences in data collection and processing. This has been further exacerbated by the substantial heterogeneity associated with ALS. Multi-center transnational collaborations are needed to address these methodological limitations and achieve representation of rare phenotypes. This review will use the example of the Neuroimaging Society in ALS (NiSALS) to discuss the set-up of a multi-center data sharing ecosystem and the flow of information between various stakeholders. NiSALS' founding objective was to establish best practices for the acquisition and processing of MRI data and establish a structure that allows continuous data sharing and therefore augments the ability to fully describe patients. The practical challenges associated with such a system, including quality control, legal, ethical, and logistical constraints, will be discussed, as will be recommendations for future collaborative endeavors. We posit that "global cohorts" of well-characterized sub-populations within the disease spectrum are needed to fully understand the complex interplay between neuroimaging and other clinical metrics used to study ALS.

Keywords: amyotrophic lateral sclerosis, MRI, data-sharing, NiSALS, quality-control, harmonization, multi-center

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INTRODUCTION

Acknowledging the Inherent Heterogeneity in ALS

It is widely accepted that amyotrophic lateral sclerosis (ALS) is a multifactorial disease, with an etiology that extends far beyond the selective vulnerability of motor neurons. Heterogeneity stemming from site-of- and age-at-onset, survival, genetic predictors, and the presence of frontotemporal dementia has severely constrained therapeutic translation (1). Precision biomarkers provide frameworks for early detection, tracking, and patient stratification, ensuring that treatment effects are not occluded by phenotypic variability. Today, neuroimaging in ALS isn't

limited to merely structural-functional correlations and is on par with traditional "wet" biomarkers when it comes to group- and individual-level analyses (2, 3). Neuroimaging represents a crucial addition to the current repertoire of outcome metrics used in clinical trials; this includes respiratory measures, muscle strength, and the Revised Amyotrophic Functional Rating Scale (ALSFRS-R), the ambiguity of which has been previously reported (4).

NiSALS: Why Data-Sharing Is the Way Forward for ALS Research

Given the underlying complexity, low prevalence, and poor patient longevity, larger, multi-layered data sets are needed to capture the full spectrum of pathological signatures in ALS and develop population-specific markers. Such data sets can only be generated through well co-ordinated, multi-center efforts. In the wider neurodegenerative field, ventures like the Alzheimer's Disease Neuroimaging Initiative (ADNI) have demonstrated the analytical power of transnational collaborations. ADNI was launched in 2004 as a multi-site, longitudinal study to develop biomarkers for Alzheimer's Disease. To date, over 1,700 publications spanning several topics have resulted from ADNI data (5–7). ADNI has inspired similar initiatives in various neurodegenerative conditions, including ALS. "Sampling and Biomarker Optimization in ALS and other Motor Neuron Diseases" (SOPHIA) was the most comprehensive of these efforts and ran from 2012 to 2016, with ∼2.4 million EUR in funding (http://www.neurodegenerationresearch.eu/fileadmin/Project_Fact_Sheets/PDFs/Biomarkers/SOPHIA_Fact_Sheet.pdf). It was conceived with the goal of harmonizing optimal methodologies for biomarker identification, thereby providing a pan-European framework within which existing and future endeavors could integrate. By consolidating expertise from over 15 leading European centers, SOPHIA helped establish the Progeny database: a web-based sampling infrastructure for the streamlined collection of clinical, neurophysiological, imaging, and bio sample-based data. Furthermore, the development of a centralized repository system for MRI data as part of SOPHIA led to the establishment of The Neuroimaging Society in Amyotrophic Lateral Sclerosis (NiSALS). The first NiSALS meeting (Oxford 2010) recognized the need for quality-controlled and harmonized MRI data and led to the publication of consensus guidelines on data acquisition (8). Annual meetings have since cemented NiSALS' role as an international consortium fostering neuroimaging as a key tool for understanding ALS. Today, a growing number of centers across Europe, North America, and Australia are NiSALS members, and are actively contributing data and hosting symposiums.

Abbreviations: ADNI, Alzheimer's Disease Neuroimaging Initiative; ALS, Amyotrophic Lateral Sclerosis; ALSFRS-R, Amyotrophic Lateral Sclerosis Functional Rating Scale Revised; DICOM, Digital Imaging and Communications in Medicine; DTI, Diffusion Tensor Imaging; MRI, Magnetic Resonance Imaging; NiSALS, Neuroimaging Society in Amyotrophic Lateral Sclerosis; QC, Quality Control; SOPHIA, Sampling and Biomarker Optimization in ALS and other Motor Neuron Diseases.

Each year has brought its own set of hurdles and achievements, showing that large-scale efforts like NiSALS rather than being monolithic, have the capacity to continuously adjust to the needs of the scientific community (9, 10). This review, while not exhaustive, will use NiSALS to illustrate the stakeholders and processes involved in multi-center data sharing. We hope to demonstrate that the associated challenges, while not insignificant, are surmountable, and that only global cohorts can generate the volume and variety of data needed to understand complex disorders like ALS.

THE NiSALS ECOSYSTEM: A GENERAL OVERVIEW

NiSALS' primary goal was always to function as a self-sustaining entity that provides the ALS community with the tools needed to advance neuroimaging-based research. The establishment of a secure central repository and the institution of a formally elected steering committee (http://nisals.net/?page_id=159) were significant first steps. The committee is responsible for the democratic governance of NiSALS activities, including making timely project and data-transfer decisions, event management, and liaising with third-party stakeholders. The general flow of data and stakeholder-interactions is depicted in **Figure 1**. Participating centers can continuously upload MRI data into a designated repository slot. Folders are available for the collection of additional clinical data that can be integrated into the server architecture for appropriate dissemination. However, individual centers are responsible for (a) obtaining approval for data sharing from the relevant ethics committee or review board and (b) appropriate data coding. Contributing centers are provided with guides, accessible through the NiSALS webpage, that include recommended packages of established freeware imaging resources to ensure thorough data de-identification prior to upload. The uploaded data then undergoes an additional round of pseudonymization (discussed in Section Data De-identification) for complete legal compliance. Crucially, each center has exclusive read/write access to their own data, in addition to having read-only access to common information areas. The repository creates individual data root trees to prevent users from accessing data domains that aren't theirs. The exact repository content for each contributor is kept confidential to add credence to the NiSALS curation mechanisms.

Figure 1 shows that the centralized communication hub (overseen by the NiSALS co-ordinator and repository curator) is essential for the streamlined running of the platform. Given the dynamic data sharing that NiSALS entails, the hub serves as a liaison point for all stakeholders, especially since data generators have expressed a desire for continuous feedback on data content and usage. The co-ordinator is also responsible for organizing annual NiSALS meetings and collection of associated materials.

The NiSALS webpage (https://nisals.net/) is an indispensable platform tool. It is used for administrative duties, including member and event management, compiling support documentation, and regularly updating legal compliance notices. The website also serves as an entry-point for interested

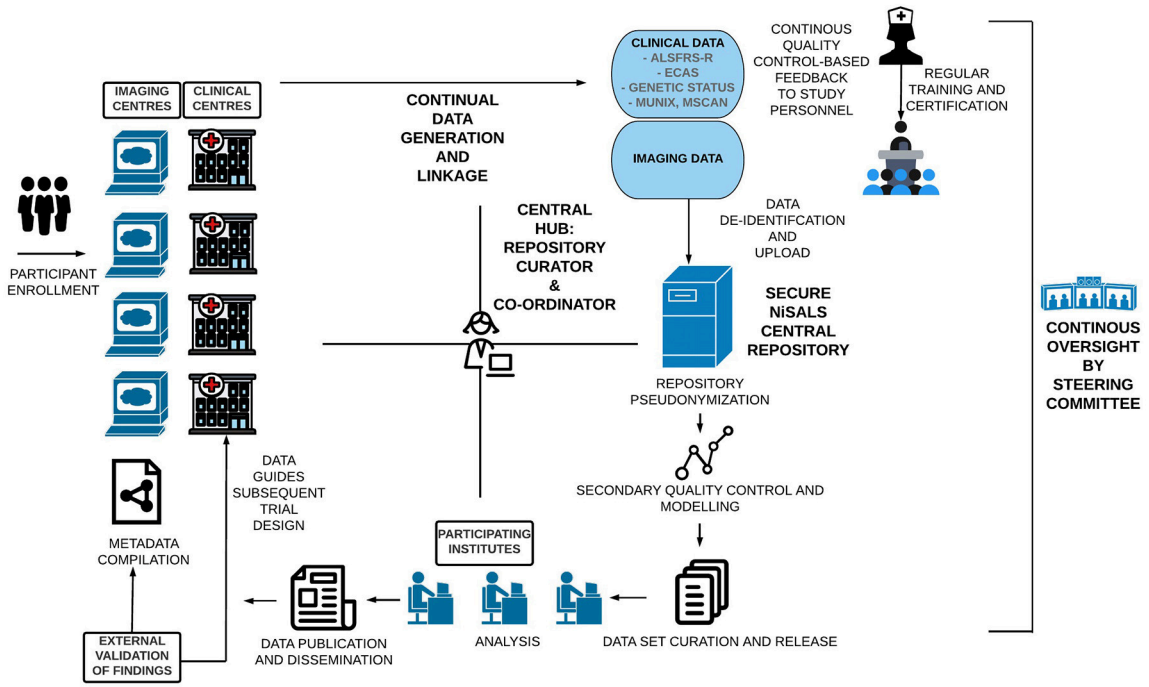

FIGURE 1 | Visual Schematic of the NiSALS ecosystem, including representations of future recommendations.

stakeholders, and is crucial for bolstering outreach. In the future, the webpage will contain teaching materials and enable center-specific repository content viewing.

LEGAL FRAMEWORKS AND DATA-SHARING

Central to any data-sharing effort is the cultivation of trust. All data-handling procedures are therefore in accordance with the NiSALS bylaws, which are designed to be collaborative and transparent. The bylaws recognize that all users need to be treated equally and should shoulder both the costs and benefits associated with embargo-free data sharing. Data sharing within NiSALS most closely resembles the "learned intermediary" model (11). Briefly, the model stipulates that an independent panel reviews applications and grants access to data primarily on the basis of applicant expertise and the quality of the proposed research. Within NiSALS, all applications are reviewed by the steering committee. Applicants must clearly detail (1) intended scientific analyses, (2) expected time-line to completion, and (3) specifications of required data in a project proposal. Successful applicants are bound by a stringent data-sharing agreement i.e., a legal mechanism to enforce NiSALS' core bylaws. Of note, are the following specifics:

1) Following publication, the released data set has to be destroyed
2) The released data set cannot be shared with third parties
3) Any additional analyses must first be vetted by the aforementioned application process.

Crucially, NiSALS recognizes that ownership of uploaded data permanently resides with the uploading center, regardless of which stage in the data-handling cycle the data is at. Thus, contributors also have the right to have their data removed from the repository upon written request.

As with any scientific undertaking, there arises the question of publications. NiSALS encourages collaborators to define and agree in writing to authorship roles prior to project commencement. Authorship credit should be in keeping with the guidelines developed by the International Committee of Medical Journal Editors. Responsible data generators should be offered contributory roles, regardless of the volume of data used. Finally, authors must reference NiSALS in resulting publications.

In summary, NiSALS operates with maximum practicability to ensure that (a) the immense benefits of sharing data outweigh the potential risks and (b) there is no disproportionate burden on data generators. Of note, when working with multiple stakeholders across geographical locations, it is unlikely that a "one size fits all" data-sharing agreement can be developed, as the judiciary requirements vastly vary between and within countries and institutions. Similar repositories should ensure that while their legal frameworks are exacting, they should be broad enough to facilitate the desired results.

DATA DE-IDENTIFICATION

As within other research domains, data sharing within neuroimaging is a constant balance between protecting confidentiality and sharing information to facilitate in-depth analyses. Multi-centre initiatives add further complexity, as

individuals have to be universally identifiable, with seamless linkage of their participation across various projects.

Substantial efforts in bolstering technical inter-operability in diagnostic imaging resulted in the establishment of the "Digital Imaging and Communications in Medicine" (DICOM) format. NiSALS adopted it for repository uploads, as the image-headers specify the parameters used during image acquisition. This information is needed for subsequent quality-control (QC) and harmonization procedures as it is essential for determining which parameters are most likely to have disturbed image quality or be most relevant during multi-center data comparison. However, all original DICOM-files also contain information that needs to be safeguarded to maintain participant confidentiality. De-identification within NiSALS is conducted in two basic steps explained below.

Basic DICOM Pseudonymization

DICOM files are first pseudonymized by removing information linked to participant identity. As mentioned above, individual contributing centers are responsible for ensuring this prior to uploading data. Further, private DICOM-header fields that are modality- and vendor-dependent must be removed (12, 13). NiSALS' internal naming conventions require that all uploaded files use local center-specific pseudonyms; this allows contributors to (a) keep track of uploaded data, (b) continuously provide additional data sets, and **(c)** link insights from the analysis process back to the original data set.

Internal Repository Pseudonymization

Data within the repository are also subjected to secondary internal checks prior to being released for analyses. These checks include the removal of identifiable facial structures (defacing) and auxiliary whole-DICOM header de-identification (14). The latter is always in keeping with the current recommendations by the National Electrical Manufacturers Association that regularly lists relevant public header fields (15). Any center-specific information is implicitly removed, as researchers using the data should be blinded to its source of origin. All study participants are allocated a unique NiSALS-generated internal pseudonym. As centers subsequently submit associated data, it is essential to maintain linkage through these layers. Therefore, NiSALS' requires all additional data to be submitted to the repository following the same pipeline of pseudonym generation, thus allowing integration with the individual participant.

QUALITY CONTROL PROCEDURES

As a first layer of QC, robust mechanisms are needed to prevent inclusion of corrupted MRI data in subsequent analyses. While being susceptible to obvious errors (e.g., extinction-artifacts), images in a multi-center set-up can also be compromised by scanner-hardware/software and modality-specific factors that may result in bias further downstream (16–18). Manual analysis and exclusion/inclusion of data sets by a trained rater is time- and labor-intensive, and contingent on rater expertise. Conversely, while automated QC procedures may overcome inter- and intra-rater variability, their applicability to one distinct data-set may not necessarily be transferable to new data acquired from different sites, thereby still necessitating visual checks by human operators (19).

Contributing centers are also responsible for complying with initial QC requirements prior to upload to minimize the risk of corrupted data entering the repository. Subsequently, modality- and analysis-specific QC approaches are applied to the stored data. Here, the challenge lies in identifying artefacts and correcting for scanner-specific factors prior to the data entering a multi-center analysis, whilst minimizing time expenditure and potential manual bias.

QC mechanisms that enable the processing of large multi-site data sets have been developed for T1 data. First, covariance screening of image parameters related to inhomogeneity or noise is conducted for outlier identification. Hereafter, software-based preprocessing algorithms for raw T1 images (e.g., as available with SPM; https://www.fil.ion.ucl.ac.uk/spm/) facilitate correction of scanner- and protocol-induced systematic artefacts, whilst minimizing alteration of disease-specific signatures. Mathematical models like Mahalanobis distance analysis can help minimize and eliminate software-bias and overcorrection when identifying technical artefacts in multi-center data sets. These models provide a meta-analysis of image quality parameters, indicating which data sets are similar and amenable to pooling as illustrated in **Figure 2**. Ultimately, all algorithmic solutions involve compromise between correction and the biological signal and therefore need to be continuously improved with feedback from all users, which is naturally extremely resource-intensive.

Similar QC procedures have been adopted by the NiSALS DTI Study Group; these include the automated exclusion of particular gradient directions in single DTI data sets (20, 21) and correction for acquisition-derived eddy-current-induced geometric distortions (20). The NiSALS DTI Study Group used these QC procedures to correct 442 single DTI data sets (from 8 contributing ALS centers) for artefacts like susceptibility-induced geometric warping, participant motion and chemical shift, prior to further analysis (10).

CROSS-PLATFORM MRI INTERPRETATION AND HARMONIZATION

As discussed above, multi-center-studies suffer from poor data comparability, owing to scanner-hardware/software differences. For instance, a study using MRI scans of the same subjects taken at different sites showed that DTI-derived values (e.g., fractional anisotropy) showed moderate reproducibility between different scanners, while particular higher field strengths and enlarged acquisition resolutions decreased inter-center variability (22).

Even if different sites use identical scanners, variance can still arise from differences in derived structural and functional imaging information; however, harmonization can improve comparability (23, 24). Processing procedures used at different sites can also contribute to variability. Therefore, as with ADNI, standardized MRI-data sets that rely on harmonized acquisition

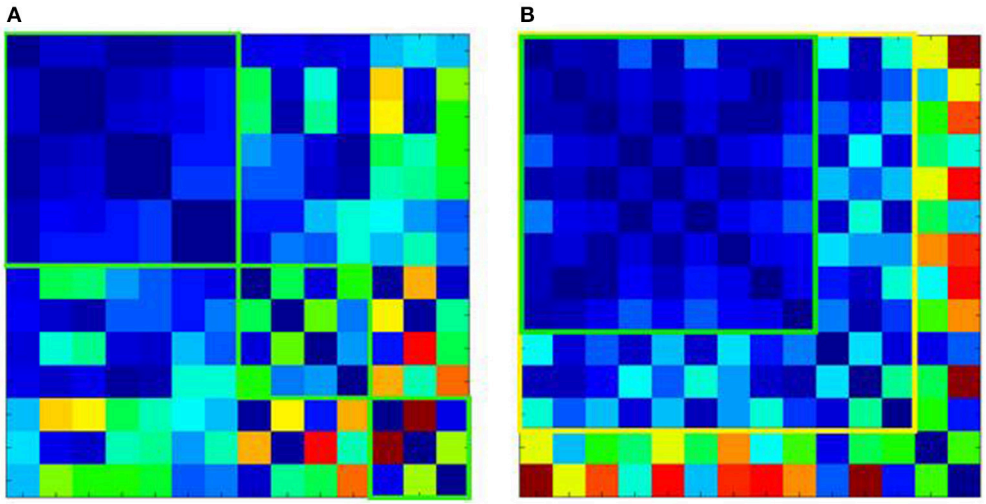

FIGURE 2 | Mahalanobis distance analysis of quality parameters for T1-weighted images of 14 ALS centers. **(A)** Shows the Mahalanobis distances of the raw T1 data, revealing 3 clusters of centers, which although internally homogenous (green squares) could not be pooled into one large data set. **(B)** Shows the effect of preprocessing. This allows pooling of T1 data from additional centers with good (green square) or acceptable homogeneity (yellow square). However, 2 centers although homogenous with each other, could not be pooled with the other data sets (shown in the last 2 rows or right-most columns, respectively).

schemes and have undergone QC are needed to support direct comparisons of different processing methods.

The majority of MRI-centric publications in ALS are offset by low sample sizes and high phenotypic heterogeneity within disease cohorts (3). One of NiSALS' core objectives was to define rules for MRI acquisition to help maximize accuracy and comparability and thereby enlarge study sample sizes. The published consensus guidelines therefore detail essential and desirable recommendations for T1, DTI, functional MRI and spectroscopy data acquisition (8).

ADNI uses a cross-platform calibration procedure that utilizes traveling phantoms for data harmonization (25). Certainly, implementing a comparable procedure for ALS centers on a global scale would require a substantially larger investment of financial and human resources, partly due to the lower prevalence of ALS (26). Therefore, NiSALS' current harmonization efforts focus on (a) ensuring that previously acquired neuroimaging data meets standards for multi-center analyses and (b) using feedback to maximize acquisition accuracy. Ultimately, MRI acquisition, and harmonization protocols need to be diligently updated to reflect the latest technical advances.

Although current uploads primarily include DTI and T1 data (∼1,000 participants for the latter), NiSALS welcomes the integration of multi-modal imaging techniques and combined structural-functional approaches and hopes to collect and disseminate data that reflects the full breadth of neuroimaging methods currently available. However, appropriate set-ups for the acquisition and use of these modalities also need to be concurrently developed if they are to be used for multi-site projects (9).

CLINICAL DATA LINKAGE

Owing to its complexity, ALS cannot be studied as a homogenous disease. In-depth multi-modal data are required for the classification of clinical, neuropsychological and imaging-based phenotypes of sporadic disease and genetic variants. This is particularly relevant when developing neuroimaging biomarkers. Incomplete patient characterization has limited several neuroimaging-based studies; the lack of clinical data constrains both accurate distinction of ALS from disease mimics and understanding of pathophysiology and progression. To fully understand the degree to which MRI and other modalities can assess disease activity and quantitate functional progression, they have to be placed within the framework of core clinical data and other biomarkers. The latter is crucial as individual biomarkers display different patterns across the disease course and in different clinical phenotypes; this has been well described for Alzheimer's Disease (27).

Naturally, this is contingent on available resources and NiSALS therefore advises contributing centers on clinical data to submit alongside MRI data sets; these have been previously published (https://www.encals.eu/wp-content/uploads/2016/09/2015-01-14-ALS-Core-clinical-dataset.pdf). In particular, NiSALS recognizes the importance of genotyping individuals and studying mutation carriers in presymptomatic disease phases to understand how genetic factors may influence the behavior of different markers (9).

Further, although data from healthy and disease controls is being continuously uploaded to the repository and requested in project proposals, both NiSALS and future efforts need to rigorously tackle the lack of longitudinal data from these subjects.

Although a detailed consideration of disease progression models is beyond the scope of this review, these are important tools for describing the disease course, particularly when clinical data cannot be collected at regular time-points for patients. Models can also help identify center-dependent and independent biological components. For instance, the newly developed D50 model enables random sampling of patients, comparisons between different progressor types and the placement of biomarker profiles within the functional time course of patients (28, 29).

FUTURE DIRECTIONS AND CONCLUSIONS

ALS, although highly heterogeneous, has the advantage of being measurably progressive. It is crucial to tap into neuroimaging's potential and use quantitative tools like MRI to describe the disease and understand its spread. Efforts like NiSALS can help the community develop and execute high-level data sharing, facilitate the replication of results and avoid unnecessary duplication of efforts. The ecosystem described here provides a structure for continuous QC and feedback that can help identify markers that are readily transferable to both the clinic and industry. Indeed, NiSALS hopes to establish well-defined collaborations with industrial partners looking to develop neuroimaging as an outcome metric for clinical trials. NiSALS can also offer its experience in implementing best practices, efficiently executing research, and disseminating results for the benefit of the neurodegenerative community. Future efforts must build on this momentum and endeavor to make the exercise more patient-centric by boosting engagement with them and communicating scientific results to them and the lay population. Stakeholders should also consider collecting meta-data on the outcomes of data sharing and how the process can be modified to better serve the community's needs.

Resources must also be directed toward building comprehensive, well-characterized multimodal biomarker panels. These can help expand the role of imaging beyond reductive clinico-structural correlations to a precision tool that can capture subtle pathological changes in population and individual-level analyses.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Biomarkers for Amyotrophic Lateral Sclerosis and Frontotemporal Dementia Associated With Hexanucleotide Expansion Mutations in *C9orf72*

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Now that genetic testing can identify persons at risk for developing amyotrophic lateral sclerosis (ALS) many decades before symptoms begin, there is a critical need for biomarkers that signal the onset and progression of degeneration. The search for candidate disease biomarkers in patients with mutations in the gene *C9orf72* has included imaging, physiology, and biofluid measurements. In cross-sectional imaging studies, C9+ ALS patients display diffuse reductions of gray and white matter integrity compared to ALS patients without mutations. This structural imaging signature overlaps with frontotemporal dementia (FTD), reflecting the frequent co-occurrence of cognitive impairment, even frank FTD, in C9+ ALS patients. Changes in functional connectivity occur as critical components of the networks associated with cognition and behavior degenerate. In presymptomatic C9+carriers, subtle differences in volumes of subcortical structures and functional connectivity can be detected, often decades before the typical family age of symptom onset. Dipeptide repeat proteins produced by the repeat expansion mutation are also measurable in the cerebrospinal fluid (CSF) of presymptomatic gene carriers, possibly throughout their lives. In contrast, a rise in the level of neurofilament proteins in the CSF appears to presage the onset of degeneration in presymptomatic carriers in one longitudinal study. Cross-sectional studies indicate that neurofilament protein levels may provide prognostic information for survival in C9+ ALS patients. Longitudinal studies will be needed to validate the candidate biomarkers discussed here. Understanding how these candidate biomarkers change over time is critical if they are to be used in future therapeutic decisions.

Keywords: *C9orf72*, cortical thinning, diffusion tensor imaging, dipeptide repeat proteins, functional connectivity, motor neuron disease, neurofilament proteins, biomarker

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INTRODUCTION

A repeat expansion mutation in the *C9orf72* gene is the most common cause of familial amyotrophic lateral sclerosis (ALS) in people of Northern European ancestry and accounts for 5-10% of sporadic ALS cases in Europe and the USA (1, 2). The *C9orf72* mutation (C9+) is also a common cause of familial frontotemporal dementia (FTD) (3). The clinical phenotype is

often mixed, and many C9+ ALS patients have some degree of cognitive impairment, ranging from mild executive dysfunction to frank FTD (4). Because mutation carriers can be identified by genetic testing many decades before symptoms begin, there is considerable interest in biomarkers to identify when degeneration begins and to monitor disease progression. Currently, development of such biomarkers is at the early stage of identifying measures that differ in group comparisons. This review will discuss the current status of studies of non-invasive biomarkers such as imaging and physiology, and minimally invasive biomarkers derived from biofluids.

IMAGING STUDIES

There is particular interest in neuroimaging as a biomarker because it offers a way to visualize pathological changes in the brains of living patients. In autopsy studies, brains from C9+ patients exhibited the neuronal loss, gliosis, and TDP-43 inclusions characteristic of sporadic ALS and some FTD patients (5), as well as the nuclear RNA foci and cytoplasmic aggregates of dipeptide repeat (DPR) proteins specific to the *C9orf72* mutation (5, 6). The distribution of these pathologic findings differs between C9+ ALS and C9+ FTD patient brains (7, 8). The story emerging from neuroimaging studies is that the diversity of clinical phenotypes reflects the extent to which the most affected brain regions contribute to networks that underlie cognitive, behavioral, motor, and language function (9, 10).

Structural MRI—Gray Matter Atrophy

In structural MRI scans, C9+ ALS patients displayed extensive, relatively symmetric volume loss and cortical thinning compared to similarly aged healthy subjects (1, 11–14). Compared to C9– ALS patients (i.e., without the *C9orf72* mutation), C9+ ALS patients had greater atrophy of extra-motor cortical regions, particularly parieto-occipital cortical areas, including the cuneus and precuneus (11–13), and relatively less atrophy of the precentral motor cortex (13, 14). Correlations between volumetric changes and cognitive testing measures have led several investigators to conclude that the predominant gray matter imaging pattern in C9+ ALS patients is associated with cognitive changes (11–14). A similar pattern of diffuse, relatively symmetric cortical volume loss is found in C9+ FTD patients (15–19).

Several studies report more atrophy of subcortical structures in C9+ ALS than in C9– ALS patients. The topographic specificity of connections between these subcortical structures and specific cortical regions can lead to discrete functional deficits. Nearly all volumetric studies to date have reported thalamic atrophy in C9+ carriers. Thalamic atrophy has been reported in C9+ ALS patients (11–13), C9+ FTD patients (15, 16, 18–22), and presymptomatic C9+ carriers (23–26). Although C9+ ALS patients may have more thalamic atrophy compared to C9– ALS patients with a similar degree of cognitive impairment (11), the association between thalamic atrophy and cognitive impairment can be seen in FTD patients with other gene mutations (27) and C9– ALS patients with cognitive impairment (28). Because there is topographic specificity of corticothalamic circuits, degeneration of particular thalamic nuclei should produce different functional impairments. However, most MRI studies measured the hemi-thalamus in its entirety. Using a more refined segmentation scheme in a cohort of C9+ FTD patients, Lee and colleagues (20) found atrophy specifically in the medial pulvinar nucleus of the thalamus, a multisensory nucleus with connections to posterior parietal, prefrontal, and cingulate cortical areas (29). Schonecker and colleagues reported greater atrophy of motor sub-regions of the thalamus in symptomatic C9+ carriers (30).

Atrophy of other subcortical structures has also been reported. The cerebellum has been of particular interest because high levels of DPR proteins (8, 31, 32) and RNA foci were found in cerebellar Purkinje and granule cells in C9+ patients (33), and levels of cerebellar DPR proteins in C9+ ALS were correlated with cognitive impairment (31). While a pathological study reported no appreciable neuronal loss in the cerebellum (15), cerebellar atrophy has been reported in some, but not all, imaging studies. Detection differences largely reflect whether the whole cerebellum or focal cerebellar regions were measured. Changes in focal cerebellar regions, such as in lobule VIIa/crus I, were found in several studies of C9+ ALS and C9+ FTD patients (11, 17, 21, 27, 34). This region of the cerebellum has been mapped in functional MRI studies to cortical association networks, including the dorsolateral prefrontal cortex and parietal association areas that play a role in executive function (35). Volume loss has also been reported in various nuclei of the basal ganglia in C9+ ALS and C9+ FTD patients (20, 28, 36), a finding associated with cognitive and behavioral scores across the spectrum of ALS and FTD, and thought to result from disruption of corticostriatal circuits (37). Two studies also reported hippocampal atrophy in C9+ ALS (11, 38), a finding consistent with the occurrence of hippocampal sclerosis in some C9+ ALS-FTD brains (5) and memory deficits.

The diffuse nature of the brain atrophy, involving cortical and subcortical structures, has led to the suggestion that changes in ventricular volume be used to follow longitudinal disease progression in C9+ carriers (13, 17, 34) (**Figure 1**).

Pathological Correlates

The distribution of atrophy in structural MRI scans of C9+ ALS and FTD patients mirrors the distribution of neuronal loss and TDP-43 pathology in brains of C9+ ALS-FTD patients (5) and sporadic ALS and FTD patients (39). However, the relationship between these hallmarks of degeneration–neuronal loss, gliosis, and TDP-43 inclusions—and the RNA foci and DPR protein aggregates specific for the C9+ genotype is still evolving. Unlike TDP-43 pathology, which closely parallels neurodegeneration, the distribution of RNA foci (33) and DPR protein pathology do not (6–8, 33, 40, 41), although reports on the latter have been somewhat conflicting. A moderate association between the amount of poly(GA) dystrophic neurites and degeneration in the frontal cortex was observed (40), and inclusions of poly(GR), which is especially toxic in *in vitro* models (42), correlated with TDP-43 pathology and neurodegeneration in C9+ FTD-ALS brains (7, 41). Nevertheless, the presence of DPR protein aggregates and RNA foci did not lead to TDP-43 accumulation

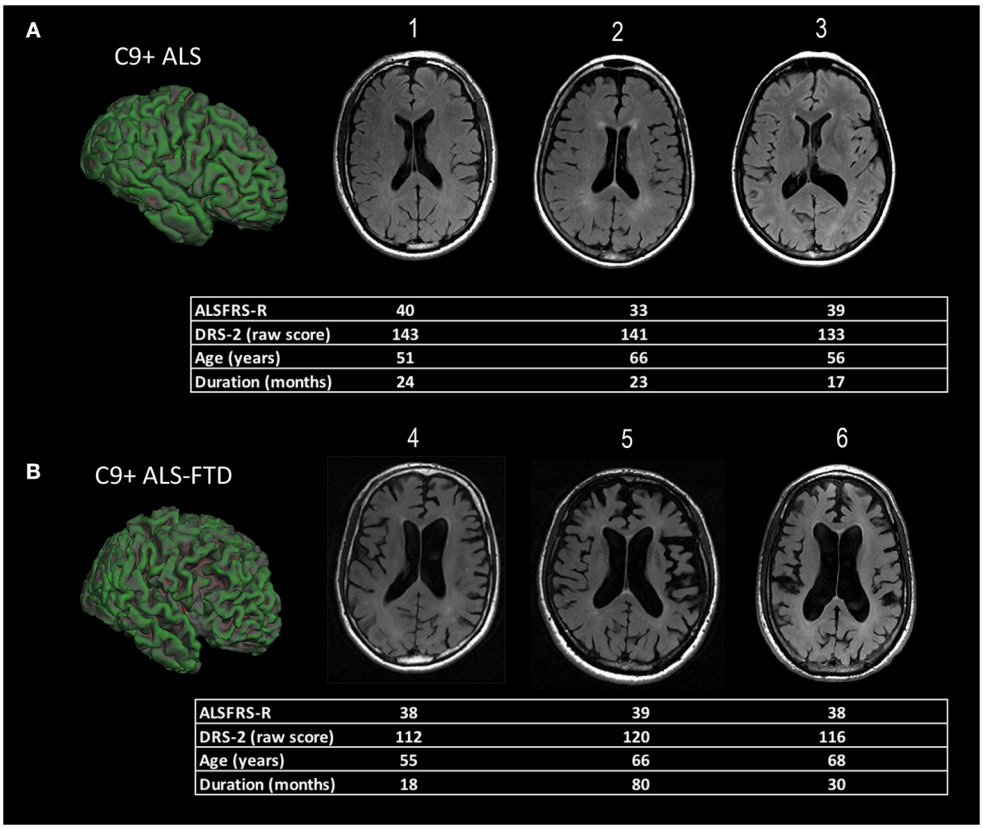

FIGURE 1 | Representative examples of diffuse cortical atrophy in MRI scans of ALS patients with *C9orf72* mutations. The demographic information and scores on motor and cognitive scales are listed below each patient's scan. **(A)** Compared to age-matched controls, mild ventricular enlargement was seen in C9+ patients 1, 2, and 3 who had ALS, but good cognitive function, as evidenced by their scores on the Mattis Dementia Rating Scale−2 (DRS-2). The surface rendering of one patient [left side of panel **(A)**] shows sulci in frontal lobe sulci are also mildly enlarged compared to the occipital lobe. **(B)** C9+ patients 4, 5, and 6 had ALS-FTD with a similar degree of motor dysfunction to those in panel **(A)**, as measured by their ALS functional rating scale revised (ALSFRS-R) scores, but marked cognitive impairment with low DRS-2 scores. There is marked enlargement of ventricles evident in axial slices, as well as enlargement of frontal and temporal sulci in the surface rendering at left of panel **(B)**.

in a neurologically healthy mosaic carrier (43), and DPR protein pathology with little, if any, TDP-43 pathology was observed in a c9FTD kindred with early intellectual disability (44) and three *C9orf72* mutation carriers who developed relatively rapid cognitive decline but died prematurely due to unrelated illness (45).

Diffusion Tensor Imaging of White Matter Tracts

In diffusion tensor imaging (DTI) studies, C9+ ALS patients showed more widespread loss of white matter integrity compared to healthy controls and C9− ALS patients, most commonly in the frontal white matter, as measured by decreased fractional anisotropy, increased radial diffusivity, or increased mean diffusivity (11, 12, 14, 38, 46). Several white matter tracts affected in C9+ ALS are not typically affected in cognitively intact C9− ALS patients, including the genu of the corpus callosum, anterior limbs of the internal capsule, thalamic radiations, and long association tracts such as the uncinate fasciculus, superior longitudinal fasciculus, and inferior longitudinal fasciculus (11, 12, 14, 38, 46). These frontal and association tracts were also affected in diffusion studies of C9+ FTD patients (17, 20, 36), and presymptomatic C9+ carriers in some studies (47). Motor tracts, including the corticospinal tract and motor segment of the corpus callosum, were affected in C9+ ALS patients compared to healthy controls (11, 46), but exhibited less disruption than in C9− ALS patients (14). In a group of C9+ carriers with a mixture of phenotypes, changes in diffusion indices of specific tracts correlated with clinical measures: frontal white matter correlated with lexical fluency and behavioral scores, and changes in motor tracts correlated with the ALS functional rating scale (46).

Unresolved Questions About Structural Imaging as a Biomarker

Several questions arise from the findings in structural MRI scans. First, does a genotype-specific C9+ MRI signature exist? To address this question, Westeneng and colleagues (38) identified a candidate "genotype-specific MRI signature" in a model comparing 92 volumetric and DTI variables in scans of 28 C9+ to 28 C9− ALS patients. Although 11

imaging variables identified a C9+ specific signature in the training dataset, nearly 20% of C9– ALS patients in a large validation dataset were classified as having the C9+ MRI signature. Misclassified patients scored more poorly on a measure of executive function, thus underscoring the close association between neuroanatomical atrophy patterns and clinical phenotypes. A second question is whether the volumetric differences in adult C9+ carriers arise during development or are a consequence of degeneration. This question was addressed in imaging studies comparing relatively young presymptomatic C9+ carriers (<age 40) to non-carriers from the same families. Although older presymptomatic C9+ carriers had clear evidence of atrophy compared to similarly-aged C9– family members, so did younger C9+ presymptomatic carriers when compared to C9– family members of the same age (24–26, 47, 48). Cortical and subcortical structures were smaller, particularly the thalamus, in younger C9+ carriers. The common genetic background of family members with and without the *C9orf72* mutation facilitated detection of small differences in these studies. Lee and colleagues found that smaller gray matter volumes occurred across a range of ages in presymptomatic C9+ carriers and had a similar age-related decline as in C9– controls, suggesting a developmental origin (47). Longitudinal studies in individual C9+ carriers before and after the onset of symptoms will be needed to truly determine whether congenitally small brain structures begin accelerated volume loss with the onset of degeneration in adulthood or whether the *C9orf72* mutation leads to slow, lifelong accumulation of subclinical pathology. Lastly, because the distribution of atrophy mirrors the distribution of TDP-43 in pathological studies (5), longitudinal structural imaging, in combination with clinical phenotyping, can be used to test hypotheses that TDP-43 pathology spreads through axonal connections. Pathological studies in sporadic ALS have led to the proposal that TDP-43 pathology spreads through corticofugal projections (49). In contrast, in behavioral-variant FTD, TDP-43 pathology has been proposed to spread from orbitofrontal cortex to posterior regions through axonal tracts (50).

Functional Connectivity

Changes in functional connectivity using task-based or resting state fMRI have been reported prior to development of clinical symptoms in patients with *GRN* or *MAPT* mutations at risk for FTD (51). Three studies examined changes in functional connectivity in resting state networks in C9+ carriers. One study in symptomatic carriers found that C9+ and C9– behavioral variant FTD patients had disruption of salience network connectivity that was associated with neuropsychiatric severity, as well as disruption of sensorimotor connectivity (20). The disruption of the salience network occurred with atrophy of different nodes within the salience network in individual patients (20). Disruption of the salience network and a network generated from a medial pulvinar nucleus seed was also observed in young presymptomatic C9+ carriers (47). Another study reported increased connectivity in the visual network of C9+ carriers with a mixture of motor and cognitive phenotypes compared to sporadic cases with similar phenotypes (11).

Proton Emission Tomography

Hypometabolism in the frontal lobes in FDG-PET studies is considered supportive of a clinical diagnosis of FTD (52). The few reports of PET imaging in C9+ carriers had slightly different findings. In one study, C9+ ALS patients had more widespread hypometabolism occurring in the cingulate, insula, caudate, and thalamus, with clusters of hypermetabolism in occipital, left precentral, left postcentral, and superior temporal cortex when compared to C9– ALS patients with or without FTD (53). In contrast, the other study reported that C9+ ALS and C9– ALS patients exhibited hypometabolism in peri-rolandic cortex; several prefrontal regions had hypometabolism in both groups, but C9+ ALS patients alone had focal hypometabolism in the thalamus and posterior cingulate cortex (54). One case study also reported frontal and temporal hypometabolism in a C9+ ALS patient who subsequently developed FTD (55). Another reported that amyloid imaging, but not FDG-PET, distinguished FTD from Alzheimer disease in a C9+ carrier (56).

PHYSIOLOGY

Physiological methods have been used to assess cortical function in C9+ carriers. Transcranial magnetic stimulation (TMS) is a non-invasive technique for assessing cortical excitability. Numerous TMS studies in sporadic ALS patients have provided evidence for hyperexcitability of the motor cortex early in disease (57), with loss of excitability at late stages (58). C9+ ALS patients were similarly found to have increased cortical excitability according to several different TMS indices, but presymptomatic C9+ carriers did not (59–61). Evoked potential measures have been used to explore particular cognitive functions in C9+ patients (62), but have not been routinely used to identify disease onset or severity. Electroimpedance myography (63) and Motor Unit Number Index (MUNIX) (64) are non-invasive methods that have been used to follow lower motor neuron dysfunction in ALS patients in clinical trials but, to date, have not been reported in C9+ ALS patients.

ENERGY METABOLISM

Patients with ALS develop defects in energy metabolism that include low body mass index (BMI), hypermetabolism, and hyperlipidemia (65, 66). While the contribution of dysregulated energy homeostasis to ALS pathogenesis remains to be resolved, such defects correlate with prognostic factors. For instance, weight loss and hypermetabolism are associated with faster disease progression and shorter survival in ALS (66–68). The cause of these metabolic changes is unknown, but may result from hypothalamic atrophy. Gorges et al. (69) have shown that the hypothalamus is atrophied in ALS patients and in presymptomatic ALS mutation carriers (the latter were comprised predominantly of C9+ individuals). Furthermore, they found a modest but significant correlation between hypothalamic volume and BMI, especially in patients with

TABLE 1 | Timeframes for detecting changes in selected candidate biomarkers in *C9orf72* carriers.

	Years prior to symptom onset	1 year prior to clinical symptoms	Early–mid stages of disease	Late stages of disease
CSF dipeptide repeat proteins	•	•	•	•
Functional connectivity salience network (fMRI)	•	•	•	•
Thalamic atrophy	•	•	•	•
CSF NfL		•	•	?
Cortical hyperexcitability (TMS)			•	?
Reduced integrity of frontal white matter and association tracts (DTI)		?	•	•
CSF pNfH		?	•	•
FDG-PET frontotemporal hypometabolism			•	•
Global loss of functional connectivity			•	•
Global volume loss–ventricular atrophy, subcortical atrophy			•	•
Diffuse cortical thinning			•	•
Diffuse loss of white matter integrity (DTI)			•	•

CSF, cerebrospinal fluid; DTI, diffusion tensor imaging; FDG-PET, fluoro-deoxyglucose proton emission tomography; fMRI, functional magnetic resonance imaging; pNfH, phosphorylated neurofilament heavy chain; NfL, Neurofilament light chain; TMS, transcranial magnetic stimulation. Question marks indicate measures needing further study.

familial ALS, and observed that anterior hypothalamic volumes correlate with age of disease onset (69). While these findings are not specific to C9+ carriers, they do suggest that hypothalamic atrophy, BMI, and disturbances in energy homeostasis could provide prognostic insight.

CSF AND BIOFLUID STUDIES

Fluid-based biomarker discovery efforts for ALS have most often been conducted using cerebrospinal fluid (CSF) due to its proximity to affected neuroanatomical regions. However, progress has been made using plasma and serum, and studies using urine and saliva are emerging (70). Among the more widely studied biomarker candidates are inflammatory mediators, metabolic markers and neurofilament proteins; the latter, however, have arguably garnered the most attention (70, 71). Neurofilament proteins, which include neurofilament heavy chain (NfH), neurofilament medium chain and neurofilament light chain (NfL), are abundantly and exclusively expressed in neurons where they form the neuronal cytoskeleton. Because neurofilament proteins are released from neurons upon axonal damage or degeneration, they are considered indicators of neuronal injury for various neurological disorders.

Neurofilament Proteins

In C9+ carriers, levels of CSF phosphorylated NfH (pNfH) were significantly higher in patients with ALS or FTD compared to asymptomatic individuals, and strongly associated with survival in patients with C9+ ALS (72). Notably, C9+ ALS patients had significantly higher pNFH levels than C9– ALS patients, which presumably reflected increased neurodegeneration, consistent with reports that patients with C9+ ALS develop greater brain atrophy, particularly in extra-motor regions, compared to C9– ALS patients (11–13). More diffuse degeneration may account for the shorter survival of C9+ ALS patients compared to C9– ALS patients (1, 72–75). Similar to pNfH, CSF NfL levels were elevated in symptomatic compared to presymptomatic C9+ carriers (76, 77), and higher NfL levels in symptomatic individuals correlated with greater disease severity and shorter survival (77). Furthermore, elevated CSF NfL in C9+ carriers was associated with lower gray matter volumes in the ventral and dorsomedial prefrontal cortex, ventral, and dorsal insula, anterior cingulate, caudate, medial thalamus, and other frontotemporoparietal regions (77).

These findings supporting CSF pNfH and NfL as prognostic markers for C9+ patients could significantly impact drug development. For instance, the heterogeneity of disease course in C9+ ALS could result in different proportions of fast and slow progressors in clinical treatment arms. Using pNfH and NfL levels as surrogates for progression rate could facilitate stratification of patients into balanced groups to reduce variability in treatment outcomes. Early evidence also suggests that NfL in CSF and serum can inform the potential phenoconversion of individuals from an asymptomatic to a symptomatic state (78). Through the study of individuals that carry a mutation in *C9orf72* or other ALS-associated genes, Benatar and colleagues found that NfL in asymptomatic mutation carriers was elevated above the range seen in healthy individuals as early as 12 months prior to the earliest clinical symptoms (78). Should these findings be validated in additional cohorts, NfL could provide insight on when neurodegeneration begins. This would facilitate the timely diagnosis of C9+ ALS, and increase the likelihood of enrolling patients in clinical trials at an early stage of disease when they are most likely to benefit from therapeutic intervention.

Dipeptide Repeat Proteins

In addition to prognostic biomarkers, markers of target engagement would improve the interpretation of clinical trials for C9+ ALS and FTD. As mentioned above, a characteristic neuropathological feature of C9+ ALS and FTD is the presence of neuronal inclusions formed of DPR proteins synthesized from

expanded *C9orf72* repeats. One of these proteins, poly(GP), is abundantly expressed in the brain of C9+ carriers and is detected in CSF (72, 77, 79, 80). While several studies observed that CSF poly(GP) did not associate with age at disease onset, survival, or markers of neurodegeneration (e.g., CSF pNfH or NfL, or measures of brain atrophy) (72, 77, 79), poly(GP) shows promise as a pharmacodynamic biomarker (81).

Since RNA transcripts of expanded *C9orf72* repeats are believed to play a key role in C9+ ALS and FTD (82), therapeutic strategies that target *C9orf72* repeat RNA are being developed. Given that levels of poly(GP) correlated with levels of repeat-containing RNA in the cerebellum of C9+ carriers (31, 83), poly(GP) was investigated as a marker of target engagement for repeat RNA-based therapies. Antisense oligonucleotides (ASOs), small molecules and genetic modifiers that target *C9orf72* repeat RNA attenuated poly(GP) levels in various preclinical models including yeast, worms, mice, and C9+ ALS patient cell lines (81, 84, 85). For example, poly(GP) was detected in CSF of mice expressing an expanded *C9orf72* repeat in the brain, and CSF poly(GP) was decreased following treatment with a repeat RNA-targeting ASO. Of note, CSF poly(GP) levels correlated with DPR protein pathology, repeat RNA levels and RNA foci burden in the brains of mice (81). These data suggest that monitoring CSF poly(GP) before and during treatment of patients participating in clinical trials presents a feasible approach to gauge target engagement.

SUMMARY

The search for biomarkers of disease onset and progression in *C9orf72* repeat expansion carriers has yielded promising candidate biomarkers (**Table 1**). Clinically, cognitive, behavioral, and motor impairment occur on a continuum in patients with the *C9orf72* mutation. Non-invasive imaging studies in C9+ carriers have identified structural and functional changes in critical components of the networks associated with cognition and behavior. Early thalamic involvement has been detected in structural, functional, and metabolic imaging studies in C9+ carriers across different clinical phenotypes, in both prospective and retrospective studies. Diffusion changes in frontal white matter may also occur early in disease. These non-invasive imaging measures warrant further study in asymptomatic carriers as early markers of degeneration. Among the minimally invasive biomarker measures, CSF pNfH or NfL may allow identification of disease onset in asymptomatic carriers and forecast survival in symptomatic carriers (72, 77, 78). Now that *C9orf72* mutation carriers can be identified by genetic testing many decades before symptoms begin, and efforts to develop gene-directed therapy are underway, it is possible to imagine that biomarkers will play important roles in future therapeutic decisions. For example, in the future, persons known to carry the *C9orf72* mutation could undergo periodic screening with non-invasive tests such as MRI or physiology, followed by minimally invasive testing to measure CSF or blood biomarkers when findings suspicious for neurodegeneration arise.

AUTHOR CONTRIBUTIONS

MF and TG drafted sections of the manuscript and proofread the entire manuscript.

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Conflict of Interest Statement: TG has a U.S. patent on methods and materials for detecting C9+ ALS and FTD using poly(GP).

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MINI REVIEW
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Metabolomics Biomarkers: A Strategy Toward Therapeutics Improvement in ALS

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Biomarkers research in amyotrophic lateral sclerosis (ALS) holds the promise of improving ALS diagnosis, follow-up of patients, and clinical trials outcomes. Metabolomics have a big impact on biomarkers identification. In this mini-review, we provide the main findings of metabolomics studies in ALS and discuss the most relevant therapeutics attempts that targeted some prominent alterations found in ALS, like glutamate excitotoxicity, oxidative stress, alterations in energetic metabolism, and creatinine levels. Metabolomics studies have reported putative diagnosis or prognosis biomarkers, but discrepancies among these studies did not allow validation of metabolic biomarkers for clinical use in ALS. In this context, we wonder whether metabolomics knowledge could improve ALS therapeutics. As metabolomics identify specific metabolic pathways modified by disease progression and/or treatment, we support that adjuvant or combined treatment should be used to rescue these pathways, creating a new perspective for ALS treatment. Some ongoing clinical trials are already trying to target these pathways. As clinical trials in ALS have been disappointing and considering the heterogeneity of the disease presentation, we support the application of a pharmacometabolomic approach to evaluate the individual response to drug treatments and their side effects, enabling the development of personalized treatments for ALS. We suggest that the best strategy to apply metabolomics for ALS therapeutics progress is to establish a metabolic signature for ALS patients in order to improve the knowledge of patient metabotypes, to choose the most adequate pharmacological treatment, and to follow the drug response and side effects, based on metabolomics biomarkers.

Keywords: ALS, metabolomics, pharmacometabolomics, therapeutic, creatinine

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is the most common adult-onset motor neuron disease, which ultimately leads to death due to respiratory failure usually 3–5 years after the appearance of first symptoms. ALS wandering diagnosis spreads ∼12 months after symptoms onset—this long delay being partly related to the lack of specific diagnostic tests. Today, only two pharmacological

treatments are approved for ALS: riluzole and edaravone, which only show small effects on survival and decline of functional impairment, respectively. Numerous clinical trials have been conducted on the identification of new therapies for ALS, but their findings are disappointing. One of the reasons of these failures could be the use of inappropriate methodology in the clinical studies, like poor design or lack of appropriate cohort enrichment strategies (1). Early diagnosis could also increase recruitment of patients in earlier stages of the disease to clinical trials (2). Moreover, the functional scales used to assess motor function in ALS patients (ALS Functional Rating Scale-Revised; ALSFRS-R, forced vital capacity, and muscular testing) may be insensitive to subtly follow drug response. Thus, the search and identification of reliable biomarkers for ALS diagnosis and prognosis is of utmost importance, as biomarkers follow-up could help in the identification of drug-response phenotypes, improving evaluation of treatment efficacy.

"Omics" research comprise systemic analyses (including transcriptomics, genomics, proteomics, lipidomics, and metabolomics) that advanced immensely in the field of biomarkers. For example, proteomics research identified a structural neuronal protein, the neurofilament, as a putative biomarker for ALS, especially for ALS diagnosis regarding its sensitivity and specificity (3). Neurofilaments also showed promising results in the field of prognostic prediction factors (4–6), but its application was not yet validated in the clinical practice.

Metabolomics studies identified several metabolites related to pathways implicated in the pathophysiology of ALS, both in animal models and in ALS patients, thus improving our knowledge about the disease mechanisms (7, 8). These metabolites could represent ALS biomarkers alone or in combination, by composing a metabolic signature for ALS. Furthermore, as identified metabolites are related to pathways that are modified in the disease, adjuvant therapy could target these pathways, and compensate their dysfunction. Identification of metabolic signatures also enables a personalized therapy and the direct follow up of drug effect in each patient—a proposition of a new field called pharmacometabolomics. In this review, we provide the main findings of metabolomics studies in ALS for biomarkers identification or for understanding ALS pathophysiology. Furthermore, we summarize recent evidence that support metabolomics applications in the clinical practice, as improvement of therapeutics and treatment follow-up. Here, we shed a light into other applications of metabolomics knowledge through the extension of its interest beyond the biomarkers research.

WHAT CAN METABOLOMICS ANALYSES TELL US?

Metabolomics is based on the global search for metabolites, defined as small molecules that represent the downstream products of ongoing biological processes in cells, tissues, and other biological samples (9). A particular metabolic profile—or "metabotype"—of a systemic biofluid (such as blood or the cerebrospinal fluid, CSF) reflects directly the metabolic status of different organs and tissues because of continuous exchanges of metabolites between tissues and fluids (7). To design a metabolic profile, metabolites are selected according to their polarity, mass, and concentrations using high-throughput techniques (10). After data pre-treatment, metabolites are analyzed by univariate analysis and multivariate analysis to identify the most important contributors to the discrimination between samples (11, 12).

Metabolomics research identified several individual metabolites and metabolic signatures (with or without identification of each metabolite composing such signature) that can discriminate ALS from non-ALS cases (10, 13–16). Metabolomics can also determine metabolic signatures that identify distinct subgroups of ALS patients according to their clinical characteristics or disease evolution (17–19). Altogether, the main objectives of metabolomics studies performed in ALS have been punctually reached. However, its application in the clinical routine or its extension to other aims (for example, for following drug responses) will depend on the ability to overcome several limitations of the method—for example, the differences in samples treatment, data analysis, and lack of external validation for many of these identified signatures.

METABOLOMICS STUDIES IDENTIFIED METABOLITES RELATED WITH PATHOPHYSIOLOGICAL MECHANISMS IN ALS

Although the exact mechanism that initiate ALS pathogenesis remain partially unknown, glutamatergic excitotoxicity, oxidative stress, and mitochondrial dysfunction have been reported as key contributors to the motor neuron degeneration (20). Metabolomics may provide a new light to evaluate these pathophysiological pathways by identifying metabolites directly associated with these pathways (8). Here we summarize the main findings of metabolomics studies linked with the most prominent pathophysiological alterations observed in ALS patients. Interestingly, these alterations were also observed in ALS models.

Glutamate

Glutamate plays a key role in ALS, as it is not only involved in excitotoxicity, but also in other mechanisms such as oxidative stress and metabolism disturbance (21). The only treatment approved that counteract the glutamatergic hyperactivation in ALS is riluzole, a non-competitive blocker of glutamatergic transmission (22–24). Glutamate remains the most cited metabolite increased in blood samples (12, 25, 26) and CSF (25, 27–29) from ALS patients, as reported by independent research groups. Recently, a metabolomics study proposed glutamic acid as a potential biomarker for ALS, after validating it in a healthy cohort (30). The increase of glutamate in CSF could be linked with the decrease in astrocytic glutamate transporter (GLT)-1 expression in motor cortex and spinal cord observed in ALS patients (17, 31, 32). Interestingly, ALS animal models also present alterations in glutamate levels (33–35). Rats expressing

the ALS-linked familial mutation Super Oxide Dismutase-1 (SOD1)-G93A showed a decrease in the astrocytic glutamate transporter expression in the spinal cord (36), as reported in ALS patients. Is important to note that astrocytes have been pointed as key elements in the pathophysiology of ALS, as is for their role in mediating glutamatergic activation or as for their metabolic support to neurons (37).

Antioxidants

Oxidative stress is also a well-known mechanism involved in ALS and is directly linked with glutamatergic toxicity that increases the production of reactive oxygen species (ROS) (38, 39). Astrocytes release ascorbic acid (an endogenous antioxidant) after glutamatergic stimulation, and the elevated level of ascorbate in the CSF of ALS patients may reflect a compensatory mechanism (11, 40). Another antioxidant metabolite, uric acid, was shown to be involved in ALS pathophysiology. Increased levels of uric acid were suggested to be associated with a slow progression of ALS (41, 42). Homocysteine, another endogenous antioxidant, was also pointed by metabolomics studies as a potential biomarker for ALS (30, 39).

Lipids

ALS patients usually present compromised energy homeostasis, including basal hypermetabolism, body weight loss, and abnormal metabolism of glucose and lipids (43, 44). In agreement with that, ALS patients present a 10-fold increase in the cholesterol esters C16:0 and C18:0 in the spinal cord, while in a mice model of ALS these substances are increased by 4- and 10-fold in the lower spinal cord during the presymptomatic and symptomatic phases, respectively (45). *Postmortem* analyses show that the spinal cord tissue from ALS patients presents a remarkable decrease in docosahexaenoic acid (DHA) levels and in n-3 polyunsaturated fatty acids (PUFA), in sharp contrast with the increase of DHA content found in the brain cortex (46).

Creatinine

Reduced levels of creatinine in the CSF or blood from ALS patients were reported from different research groups, including metabolomics studies (42, 47–49). Creatinine reflects skeletal muscle production and reduced levels of this metabolite are directly related to amyotrophy, a cardinal ALS symptom. Use of plasma creatinine levels as a biomarker in ALS was suggested for monitoring disease progression in clinical trials (50), and creatinine was the first metabolite already used to evaluate drug therapy response to dexpramipexole in a clinical trial (51).

Findings regarding metabolomics are promising but disappointing, as, to date, no biomarker was approved for diagnosis or prognosis use (10). To go further with this approach, well-designed and large cohorts studies would be essential for biomarker validation (52), and the improvement of analytical and statistical steps may improve the robustness of the strategy (16, 19). Importantly, all metabolomics studies published so far have identified metabolites linked to the same pathophysiological pathways, thus reinforcing the potential of metabolomics to explain pathophysiological mechanisms underlying ALS. In this context, we suggest that metabolomics analyses may be useful for other applications than identifying diagnostic or prognostic biomarkers, such as for example, monitoring disease course and identifying treatment outcomes and side effects in clinical trials.

METABOLOMICS-IDENTIFIED ALTERATIONS AS TARGETS FOR NEW THERAPEUTIC STRATEGIES

Disturbed pathways identified through metabolomics studies in cellular and animal models, as well as in ALS patients, hold the potential to be used for the discovery of new therapies in ALS (48). The application of metabolomics findings in preclinical and clinical studies to target glutamatergic toxicity (21) and energy metabolism dysfunction (44) were already reviewed. Thus, here we will summarize the ongoing therapeutics attempts that target alterations identified by metabolomics studies and with beneficial effects in ALS preclinical tests (**Table 1**).

As mentioned before, metabolomics and non-metabolomics studies demonstrated alterations in glutamate levels in CFS and blood of ALS patients. Several clinical trials tried to demonstrate the effect of anti-glutamatergic drugs—already approved for the treatment of other neurological diseases—for the treatment of ALS, but failed to show any improvements. This is the case for lamotrigine, topiramate, gabapentin, and talampanel (21). Current active clinical trials investigate the potential effect of memantine and perampanel in ALS, drugs used for Alzheimer's disease and epilepsy treatment, respectively (21).

Focusing on oxidative stress (as edaravone, the recent drug approved by the FDA for ALS treatment that is a ROS scavenger), a clinical trial is investigating the effect of inosine treatment for

TABLE 1 | Ongoing clinical trials with therapeutics interventions focused in alterations identified by metabolomics studies.

Target	Intervention	Clinical trials for ALS
Glutamatergic overactivation	Perampanel	Phase II, NCT03377309 (Lebanon); NCT03019419 (Japan); NCT03020797 (Unites States).
	Memantine	Ongoing (phase II, NCT02118727, Unites States). [No effect observed in phase II-III; (53)].
Oxidative stress	Inosine	Phase I, NCT02288091 (United States).
	CC100	Phase I, NCT03049046 (United States).
Hypermetabolism	Triheptanoin	Phase I-II, NCT03506425 (United States).
	High caloric fatty diet	NCT02306590 (Germany).
	Oral nutritional supplementation (high fat and protein)	NCT02152449 (France).

Information available in clinicaltrials.gov.

ALS. Inosine is a precursor of uric acid, an antioxidant molecule that is found altered in ALS patients. Furthermore, this clinical trial will follow therapy response by analyzing uric acid levels in treated individuals, applying metabolomics approaches both at treatment strategy and follow-up. CC100 (a synthetic form of the caffeic acid phenethyl ester) is another molecule with antioxidant properties that is currently being investigated in a Phase I clinical trial. The caffeic acid phenethyl ester is a natural compound with effects on lipid peroxidation and lipid metabolism (54).

Considering that energy metabolism is also altered in ALS patients, several studies focused in providing additional fuel to increase energy uptake (44). While preclinical studies successfully showed the beneficial effects of these treatments, clinical trials failed to show the same results. In the case of dexpramipexole (an improver of oxidative phosphorylation and thus of ATP synthesis), a Phase II clinical trial showed prevention of functional decline of ALS patients following a 12-month treatment (55). However, Phase III failed to show improvements (56). A Phase II clinical trial performed between 2009 and 2012 analyzed the beneficial effects of two hypercaloric (one high-fat and other high-carbohydrate) diets in ALS patients receiving enteral nutrition. Patients receiving a high-carbohydrate enteral formula presented less adverse effects compared to control subjects. They found that both diets were safe and tolerable, although they did not modify disease progression (57). Currently, ongoing clinical trials investigate the effect of high caloric fatty supplementation (Calogen®) and high caloric protein/fat supplementation (Fortimel®) in ALS patients.

Novel therapeutic strategies may focus on creatinine as a marker to identify the efficacy of drugs and follow-up of treatments aiming the inhibition of the muscular loss observed in ALS, or even in treatments aiming the increase of muscle mass in the patients. For example, in ALS animal models, inhibition of myostatin (a negative regulator of muscle growth) improved muscular mass and strength. Although myostatin treatment did not change the disease onset and progression, it improved the muscular function, especially in the diaphragm of the animals (58). If translated for the human disease, it could improve the quality of life of ALS patients during disease progression.

METABOLOMICS-DRIVEN THERAPEUTICS MANAGEMENT: THE ADVENT OF PHARMACOMETABOLOMICS

Metabotype information can be used to identify alterations in biochemical pathways in ALS patients that are modified or not by treatment. This new field, called pharmacometabolomics, allows clinicians to identify a metabolic state at baseline and after drug therapy, increasing information about treatment outcomes, especially drug-response phenotype (59).

Different studies revealed the potential of pharmacometabolomics to assess drug therapy response and identify distinct signatures of metabolites before and after treatment exposure in diverse pathologies, from cancer to cardiovascular diseases. For ALS, one study analyzed metabolites and lipids composition of plasma samples from individuals enrolled in a phase III clinical trial for Olexosime. This study identified a metabolic profile that distinguished the placebo from the Olexosime group, characterized mainly by alterations in the levels of glycine, citrulline/arginine, and kynurenine. Furthermore, clinical progression of ALS correlated with amino acids, lipids, and spermidine levels in the Olexosime group, and with glutamine levels in the placebo group (19). It is noteworthy to highlight that these metabolites are linked with some of the pathological pathways involved in ALS pathology (glutamatergic alteration and energy metabolism dysfunction), as described before.

In practice, pharmacometabolomics findings may improve the strategy of drug administration scheme, as a complementary tool of pharmacokinetics, and may provide new light on drug-response effect and downstream signaling pathways (60). This information may provide details on biochemical pathways involved in disease and in treatment effect in ALS patients in a narrowly controlled process.

METABOLOMICS RESEARCH IN ALS SHOULD IMPROVE THERAPEUTICS—CONCLUDING REMARKS

Metabolomics represent a new approach that is increasingly gaining importance as it helps to identify biomarkers and unravels pathways that contribute to the pathophysiology of ALS. Significant therapeutic advances are based on a deep knowledge of ALS pathogenesis and metabolomics holds great potential to play a key role in this objective. However, despite the efforts made by metabolomics researchers to identify biomarkers for ALS,

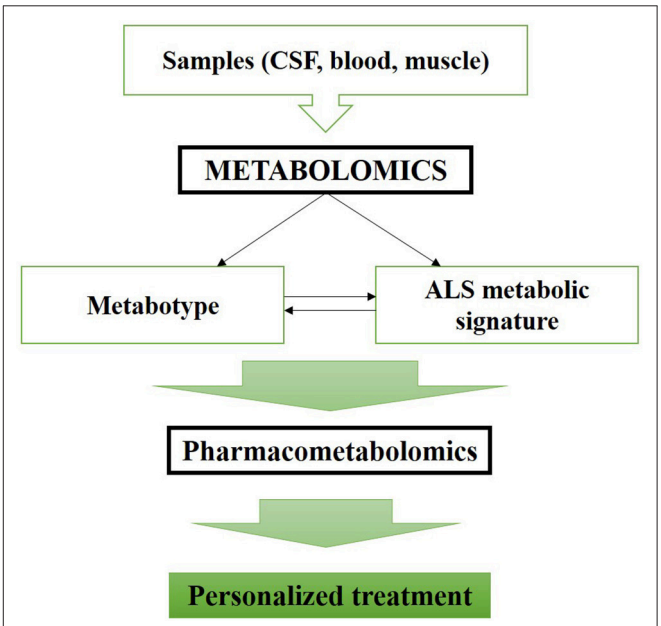

FIGURE 1 | Metabolomics applicability enhance ALS therapeutic management and allows a personalized medicine.

no biomarker was validated yet. Metabolomics studies should rather focus in identifying metabolic signatures then individual biomarkers for ALS. This would be a revolutionary step toward developing efficient strategies to evaluate not only disease progression, but also treatment responses to drug therapies (19).

This also point out the urgent need of metabolomics research to combine analysis and information (1) of different tissues in ALS patients, as CSF, blood and muscle samples; and (2) by combining different approaches (proteomics, transcriptomics, lipidomics, etc.) (52). Combination of "omics" approaches with clinical evaluation (for example, ALSFRS-R) could be the best practice for an early diagnosis of ALS (10). Importantly, omics analysis should be standardized between different research centers together with refinement of statistical analysis tools to analyze better the results obtained. Altogether, these efforts should readily improve metabolomics application in the daily clinical practice.

Metabolomics can also be applied to identify outcomes of pharmacological treatment. Usual parameters and endpoints used in clinical trials to evaluate drug efficacy are probably not enough sensitive to observe a slight effect. In this regard, metabolomics could identify biomarkers that are sensitive enough to detect even small effects of drugs tested in Phase II clinical trials, allowing them to be investigated into Phase III. Furthermore, pharmacometabolomics approaches provide help in evaluating drug effect as a primary or additional parameter. Metabolome may provide longitudinal, reproducible, and objective data that are crucial criteria to evaluate drug effect. Besides, adjuvant therapy based on metabolomics findings could compensate the identified altered pathways in a subtype of patients, allowing a personalized therapeutic strategy targeting specifically these pathways. Ongoing trials using this strategy are presented in **Table 1**. However, no study yet tried to approach several pathways at once, using a combined therapeutic strategy. This approach should be more relevant than focusing only on one altered pathway.

Metabolomics applied early in ALS management should improve therapeutic strategy and development. The major interest of metabolomics at disease onset is to build homogeneous subgroups of patients in order to apply a personalized therapeutic approach (**Figure 1**). Metabolomics complement data obtained from genomics, transcriptomics and proteomics, and combined with pharmacometabolomics approaches, they add the final piece of information to the study of disease pathophysiology and drug response (60). We propose to combine omics and clinical data to improve our comprehension about the specific metabolic pathways affected in each individual patient. Stratification of patients based on all these findings would considerably improve trials methodology and care management, as well as therapeutics strategies by providing a mean to a personalized medicine. To our knowledge, this review is the first to present diagnosis and prognosis biomarkers as an initial step to develop therapeutics. This new light on metabolomics application is promising for complex and heterogeneous diseases, like ALS, characterized by successive therapeutics failures.

AUTHOR CONTRIBUTIONS

DL and HB wrote the manuscript. DL, DRA, PC, PFP and HB critically revised the manuscript for important intellectual content. All authors read and approved the submitted version.

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Functional Biomarkers for Amyotrophic Lateral Sclerosis

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The clinical diagnosis of amyotrophic lateral sclerosis (ALS) relies on determination of progressive dysfunction of both cortical as well as spinal and bulbar motor neurons. However, the variable mix of upper and lower motor neuron signs result in the clinical heterogeneity of patients with ALS, resulting frequently in delay of diagnosis as well as difficulty in monitoring disease progression and treatment outcomes particularly in a clinical trial setting. As such, the present review provides an overview of recently developed novel non-invasive electrophysiological techniques that may serve as biomarkers to assess UMN and LMN dysfunction in ALS patients.

Keywords: amyotrophic lateral sclerosis, motor neuron disease, neurophysiological biomarkers, transcranial magnetic stimulation, cortical excitability

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a rapidly progressive neurodegenerative disease that was first described in the 1869 by Jean-Martin Charcot (1–3) although earlier detailed clinicopathological descriptions of a case of ALS, was published by Radcliffe and Clarke (4). Charcot postulated the importance of the upper motor neuron in its pathogenesis (3) and its associated degeneration of motor cortical Betz cells that has become a well-recognized pathological feature (5, 6). The diagnosis of classical amyotrophic lateral sclerosis (ALS) relies on the clinical identification of progressive dysfunction in both the cortical ("upper", UMN) and spinal ("lower", LMN) motor neurons involving multiple body regions, much of which is encompassed within the El Escorial criteria (7, 8). The clinical heterogeneity of ALS is a result of the variable mix of UMN and LMN signs (9), hence contributing to delay in diagnosis and difficulty in monitoring disease progression as well as treatment outcomes particularly in a clinical trial setting (6). As such, there is a critical need to devise objective biomarkers of disease progression in ALS that may facilitate both improvement in diagnosis as well as to provide meaningful outcome measures to monitor treatment (10).

The present review will provide an overview of recently developed neurophysiological biomarkers, with emphases on novel non-invasive electrophysiological techniques used to assess UMN and LMN dysfunction in ALS patients.

Biomarkers of UMN Dysfunction

An important component in the diagnosis of ALS relies on clinical features of UMN involvement in the presence of progressive LMN weakness (11), but often these signs of UMN impairment may be underappreciated in a limb that is concurrently affected by LMN loss especially in early stages of ALS (6, 12, 13). Upper motor neuron signs may initially be absent in approximately 7–10% of ALS patients (6, 14). As such, objective UMN biomarkers may be critical for the diagnosis of ALS, as

potential mimicking disorders such as multifocal motor neuropathy, Kennedy's disease and adult-onset spinal muscular atrophy (SMA), may present as pure LMN syndromes (6, 15, 16). Autopsy reports have also demonstrated UMN degeneration in 50–75% of patients with clinically pure LMN syndromes (5, 17, 18).

Transcranial Magnetic Stimulation

Since its original description more than 3 decades ago (19), Transcranial magnetic stimulation (TMS) has undergone significant evolution as a non-invasive technique for cortical stimulation, providing valuable insight into the functional integrity of brain pathways. Its main application has been in the investigation of complex neuronal networks of the primary motor cortex (M1), which is influenced by both inhibitory and excitatory mechanisms (20). Transcranial magnetic stimulation (TMS) biomarkers of cortical hyperexcitability appear to be useful biomakers of UMN dysfunction in ALS (21). In addition, TMS have provided insights into the underlying pathophysiological mechanisms in ALS, thereby allowing for the development of diagnostic and prognostic biomarkers in ALS (21).

The TMS technique utilizes a transient magnetic field to induce an electric current in the cortex (22). This magnetic field is generated through a stimulating coil held over a subject's head, which painlessly and non-invasively penetrates the skull without attenuation (**Figure 1**). Depending on stimulation intensity and coil type, the electromagnetic force can stimulate neurons at a depth of 1.5–3.0 cm beneath the scalp (23). There have been several theoretical models postulated to explain the exact effect of this electromagnetic field on biological tissue, with studies in both animals and humans conferring that TMS generates a corticomotoneuronal volley composed of direct (D) and indirect (I) waves occurs at intervals of 1.5–2.5 ms (24). Direct waves are thought to represent the activation of corticospinal axons and are only recruited at high intensities or with the TMS coil positioned such that induces currents in a lateral-medial direction. Indirect waves seem to be activated at lower intensities and are mediated by a more complex interaction between cortical excitatory and inhibitory neurons (25). TMS delivered over the primary motor cortex (M1) is thought to activate pyramidal neurons (Betz cells) trans-synaptically via I-waves (26), but the exact neural circuitries evoked remain to be determined. These complex neural circuits are critically dependent on both excitatory and inhibitory interneuronal systems, facilitated by cellular receptor and neurotransmitter interactions (27). Excitation is primarily mediated by glutamate/NMDA receptor interaction, while inhibition is facilitated by γ-aminobutyric acid (GABA)/$GABA_{A/B}$ receptor action (28).

Cortical hyperexcitability in ALS is heralded by reduced short-interval intracortical inhibition and CSP duration, in addition to increased intracortical facilitation and motor evoked potential amplitude (12, 29, 30). Furthermore, significant bilateral TMS abnormalities was observed in ALS patients at an early disease stage (31), consistent with previous studies that have reported functional abnormalities of the motor cortex as an early and specific feature of ALS, and preceding the onset of LMN dysfunction (6, 12, 29, 30, 32–34). More recent studies have demonstrated changes in TMS parameters indicative of cortical hyperexcitability, were more prominent over the dominant motor cortex and in particular, contralateral to the site of disease onset, suggesting a vulnerability of the dominant motor cortical neurons and supporting the importance of cortical processes in the pathophysiology of ALS as postulated first by Menon et al. (31).

Single-Pulse TMS

The resting motor threshold (RMT) is a reflection of the ease with which corticomotoneurons are excited, hence the corticomotoneuronal membrane excitability, as well as the density of UMN projections onto motor neurons (35). Through the α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA) receptors, RMT is influenced by the glutamatergic neurotransmitter system, such that excessive glutamate activity reduces RMT, and is susceptible to modulation by sodium channel blockers (28, 36). In ALS, the RMT is reduced early in the disease (indicative of cortical hyperexcitability) followed by progressive increase and eventual inexcitability with disease progression (32, 37–39). As motor threshold is modulated by glutamate activity (28), the reduced motor threshold supports the notion that cortical hyperexcitability being an early feature of ALS contributing to the ensuing lower motor neuron degeneration (21). The motor cortex is found to be inexcitable in approximately 20% of ALS patients and appears to be a late finding. In contrast, motor cortex inexcitability is a relatively frequent fidning in patients exhibiting the pure UMN phenotype termed primary lateral sclerosis [PLS] (40).

The central motor conduction time (CMCT) time is defined by the time interval between stimulation of the motor cortex and arrival of the corticospinal volleys at the spinal motor neurons, and is inferred from the motor evoked potential (MEP) onset latency (21). Prolongation of CMCT is an invariable finding in ALS being documented in 16–100% across different series (5, 21, 37, 41–44). In patients without clinically predominant UMN phenotypes, prolongation of CMCTs occurs in 50–71% of patients (41, 44). Although the mechanisms underlying CMCT prolongation are presently not fully elucidated, an increase in desynchronization of corticomotoneuronal volleys resulting from degeneration of the fastest conducting corticomotoneuronal fibers has been suggested (45, 46). Large discrepancies in sensitivity of this parameter reported by previous studies are likely attributable to technique-dependent variations associated with CMCT calculations.

The cortical silent period (CSP) refers to the interruption of voluntary electromyography activity in a target muscle after motor cortex stimulation (47), and the mechanisms that underly the CSP are complex but thought to be mediated primarily by the activation of inhibitory neurons acting via GABA-B receptors within the cortex (21, 48). The CSP duration has been consistently reduced in patients across all ALS phenotypes (21, 30, 32, 34, 43, 49–51). The decrease in CSP duration in ALS patients likely represent a combination of degeneration of

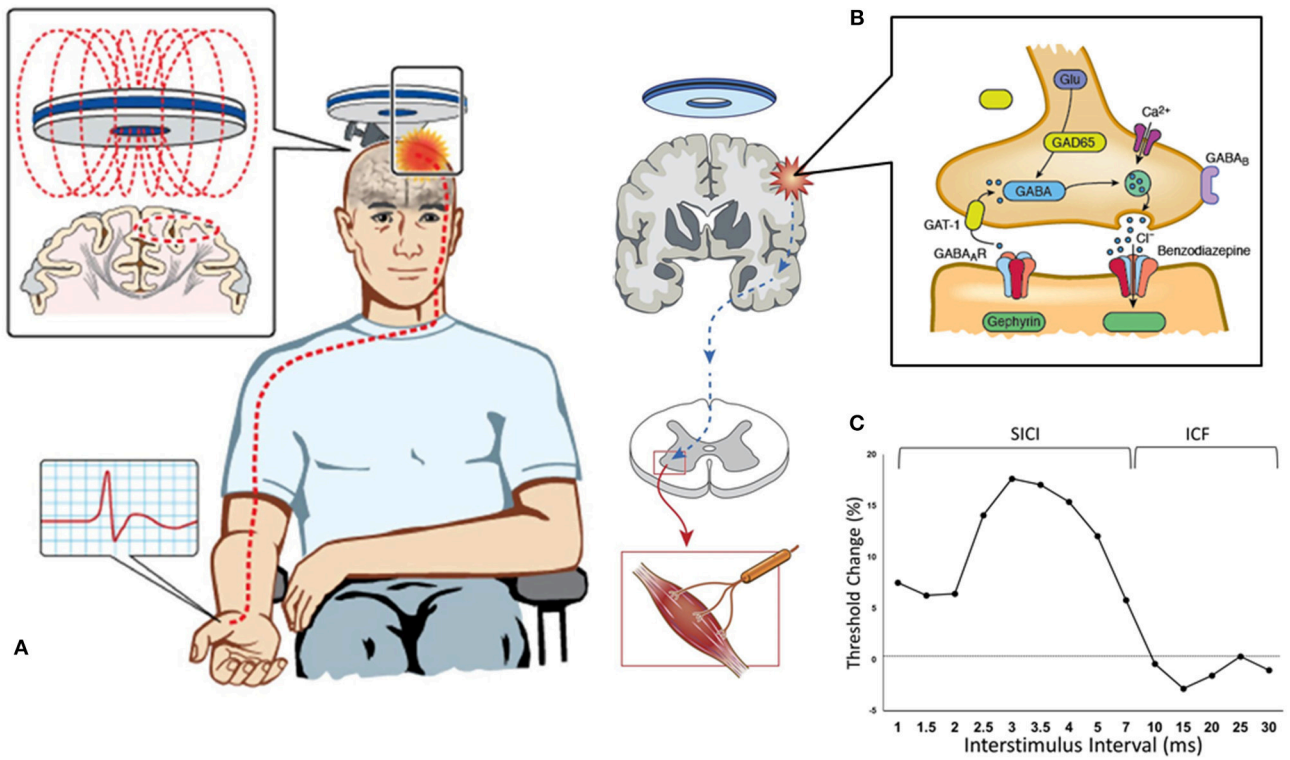

FIGURE 1 | Paired-pulse threshold tracking transcranial magnetic stimulation (TMS). **(A)** TMS coil placed over the vertex activates the primary motor cortex and the response (motor evoked potential, MEP) is recorded from the contralateral abductor pollicis brevis muscle. **(B)** TMS parameters are mediated by a complex interplay between intraneural circuits and cortical output cells, with cortical interneurons mediating inhibition by activation of GABAergic synapses leading to influx of chloride anions (Cl^-) and hyperpolarization of post-synaptic neurons. **(C)** Change in stimulus intensity required to achieve a target MEP of 0.2 mV (±20%) is used to quantify SICI (which is recorded with interstimulus intervals between 1–7 ms) and ICF (between 10–30 ms).

inhibitory interneurons as well as GABAB-mediated receptor inhibition dysfunction (21).

Paired-Pulse TMS

In the paired-pulse paradigm, a conditioning stimulus (CS) precedes and is utilized to modulate the effect of a second test stimulus (TS). By varying the time interval between the paired pulses (the interstimulus interval, ISI) a number of parameters can be determined, using either a constant stimulus method [in which the CS and TS are kept at a constant level and MEP amplitude is evaluated (52)] or the threshold-tracking (TT) TMS protocol (53). TT-TMS was developed to overcome the marked MEP amplitude variability seen when utilizing the earlier protocol and uses a fixed MEP response which is tracked by a varying TS (53, 54). By applying a subthreshold (set at 70% RMT) conditioning stimulus at predetermined time intervals prior to a suprathreshold test stimulus, the threshold-tracking TMS technique allows the short-interval intracortical inhibition (SICI) and intracortical facilitation (ICF) to be investigated (53, 55) (**Figure 1**).

Reduction or absence of SICI, which is a biomarker of cortical interneuronal inhibitory GABAergic function, has been established as an early feature of ALS (**Figures 2A,B**), correlating with biomarkers of peripheral neurodegeneration and at times preceding the onset of LMN dysfunction in sporadic ALS cohorts [(31, 53), etc]. Although there were no significant differences in the degree of reduction observed between the sides of the motor cortices, there was a trend for more changes observed over the dominant motor cortex, particularly contralateral to the side of disease onset (31). The changes were also similar regardless of the severity of LMN dysfunction, or site of onset (bulbar or limb) (12, 21, 32, 56).

The reduction in SICI has been a widely reported feature present in both familial and sporadic forms of ALS with the alterations observed as an early feature (21, 30, 34, 57–62). Further to this, longitudinal assessments of asymptomatic SOD-1 mutation carriers have identified cortical hyperexcitability developing prior to the clinical onset of ALS, therefore suggesting that cortical hyperexcitability underlies the process of neurodegeneration in familial ALS (34).

The use of threshold-tracking TMS may be able to uncover UMN involvement in ALS phenotypes without clinically evident UMN signs such as the flail limb variant of ALS or progressive muscular atrophy (PMA). Moreover, this technique was able to reliably distinguish between ALS and other neurological mimic conditions including multifocal motor neuropathy, spinal muscular atrophy, Kennedy's disease, peripheral nerve hyperexcitability disorders, Hirayama disease,

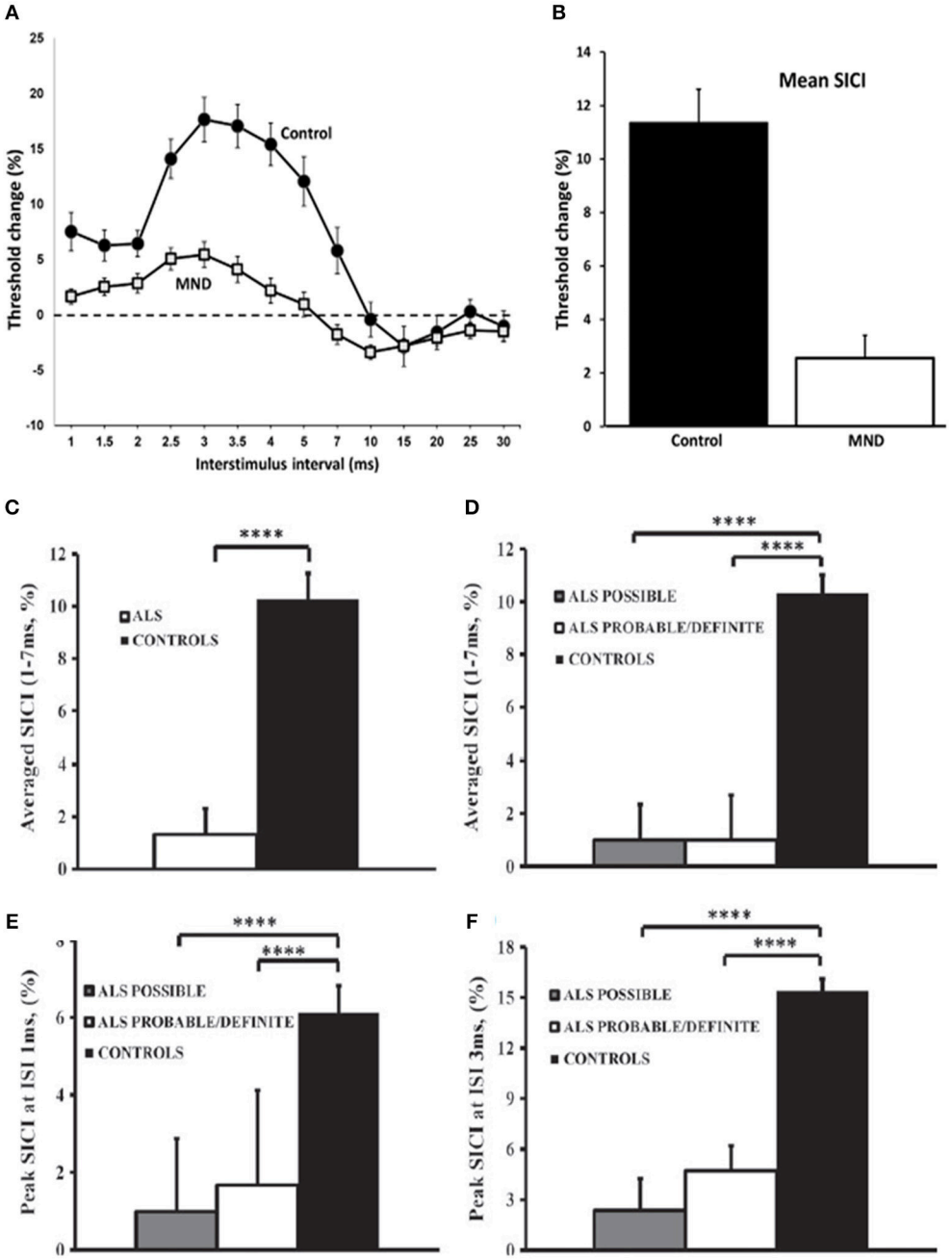

FIGURE 2 | Cortical excitability in motor neuron disease (MND). Paired-pulse subthreshold conditioning transcranial magnetic stimulation demonstrating **(A)** reduction in short-interval intracortical inhibition (SICI, above dotted line) and intracortical facilitation (ICF, below dotted line) and **(B)** significant reductions in averaged SICI (between interstimulus intervals of 1–7 ms) in MND patients compared with controls **(C)** Averaged short-interval intracortical inhibition (SICI), between interstimulus interval (ISI) 1–7 ms, was significantly reduced in amyotrophic lateral sclerosis (ALS). **(D)** The reduction of averaged SICI was comparable in Awaji subgroups. Peak SICI at ISI **(E)** 1 ms, and **(F)** 3 ms was significantly reduced in Awaji subgroups. $^{****}P < 0.0001$. Reproduced with permission license no. 4457360494951 (1) and license no. 4457440155614 (12).

CIDP, lead neuropathy, hereditary spastic paraparesis, as well as hereditary motor neuropathy with pyramidal features (63–68).

SICI abnormalities using the threshold-tracking technique, appear to be the most robust diagnostic parameter that is indicative of UMN dysfunction in ALS patients (12, 29, 69). Using either an abnormal SICI or an inexcitable cortex, this TMS method demonstrated a sensitivity of approximately 73% and a specificity of 81% (69). Moreover, an absent SICI was associated with a 97% sensitivity (33). TMS abnormalities

were observed in 77% of patients with ALS, with frequency of abnormalities that were similar across all Awaji diagnostic groups, using the established cut-off SICI of <5.5% (63) resulting in 88% of Awaji-criteria possible patients being reclassified as Awaji-criteria probable or definite (12). More specifically, an abnormally reduced SICI was demonstrated in 56% of Awaji-criteria possible patients (12) (**Figures 2C–F**).

More recent studies have also documented increasing cortical hyperexcitability with advancing disease indicating that intracortical inhibitory neurons become progressively dysfunctional in ALS (**Figure 3A**) (70). Reduced SICI was also reported to be an independent prognostic biomarker in ALS patients within the first 2 years of disease onset (71) (**Figure 3B**). Separately, SICI was shown to partially normalize with treatment by riluzole (72), an anti-glutamatergic agent exhibiting modest clinical effectiveness in ALS (73, 74). Paralleling the clinical efficacy Riluzole, the modulating effects last about 3 months (75), and may be related to overexpression of efflux pumps located at the blood brain barrier during the disease course (76). Regardless of the underlying mechanisms, studies of riluzole have suggested a utility of threshold-tracking TMS in assessing biological effectiveness of compounds at an early stage of drug development. Taken together, these results suggest that non-invasive *in vivo* monitoring of cortical function and particularly, SICI may also be an effective biomarker used to monitor the effects of novel therapeutics in a clinical trial setting.

Biomarkers of LMN Dysfunction

Objective assessment of LMN dysfunction, utilizing neurophysiological techniques, appear to be more sensitive than clinical assessments (77, 78). Conventional neurophysiological techniques, such as nerve conduction studies which measure the compound muscle action potential (CMAP) amplitude, may be relatively insensitive in assessing LMN degeneration due to the process of reinnervation (79).

Estimation of Motor Unit Numbers

As such, various methods to approximate the number of motor units innervating individual muscles, including motor unit number estimation (MUNE), and motor unit number index (MUNIX), may potentially represent valuable biomarkers of LMN degeneration. Since the development of the first MUNE technique in 1971 (80), there have been numerous other MUNE techniques introduced (81–85). The basic principle of MUNE techniques is the dividing of the maximal CMAP amplitude by the average surface-recorded motor unit potential (86). The original MUNE technique utilized incremental stimulation whereby the stimulus intensity at one point on the nerve was gradually increased from subthreshold until 10 increments in the motor response was recorded, but this technique relied on the assumption that the smallest recorded potential using the surface electrode over a target muscle following minimal stimulation represented a single motor unit potential (**Figure 4A**). Consequently, the variance in the result MUNE was considerable and resulted not uncommonly in artificially lower MUNE counts (86, 88).

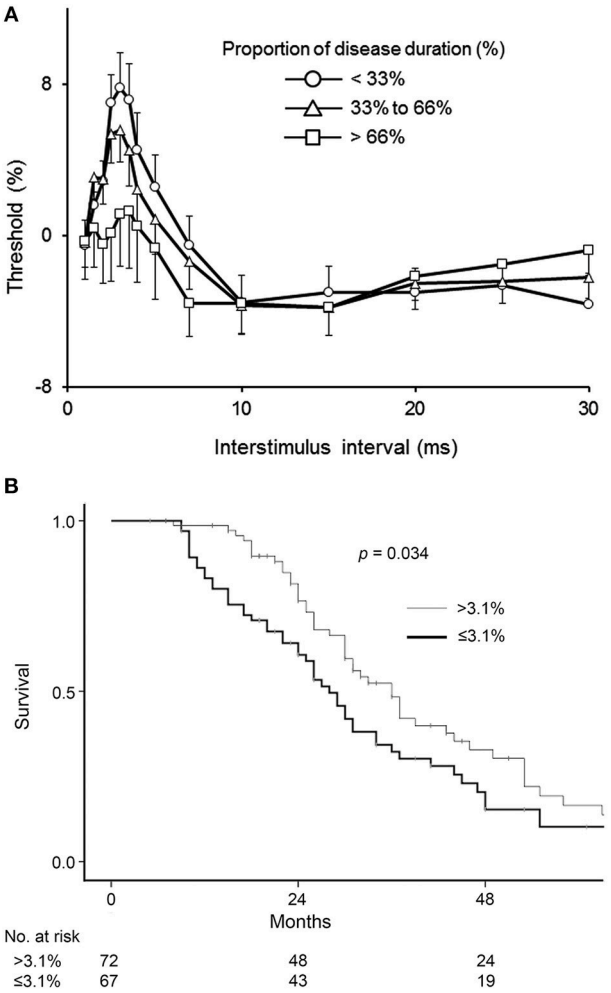

FIGURE 3 | (A) Cortical excitability changes with disease progression. Patients were divided into three groups according to disease stage. The duration of the illness from onset to death was normalized between zero and one and expressed as a percentage (%), with data averaged by proportion of disease duration. Early stage (Circle) was defined as the proportion of disease duration <33%, mid (Triangle) was 33–66%, and late (Square) was >66%. ALSFRS-R of patients in early stage was 42.3 ± 0.6, that in mid was 40.2 ± 0.7, and that in late was 34.8 ± 2.0. SICI at ISI 1–7 ms decreased with disease progression. Data are given as mean ± SE. Reproduced with permission license no. 4456860473754 (70). **(B)** Kaplan-Meier plots of survival probabilities according to averaged short-interval intracortical inhibition (SICI) values. Amyotrophic lateral sclerosis patients with a disease duration under 2 years were divided into 2 groups according to values in average SICI, interstimulus interval 1–7 ms. Patients with SICI ≤3.1% demonstrated reduced survival compared to patients with SICI >3.1% ($p = 0.034$). Estimated median survival was 28 months in patients with reduced SICI and 36 months in patients with higher SICI. Reproduced with permission license no. 4456870994973 (71).

The motor unit index (MUNIX) technique is a method designed to express the number of functioning motor units within a muscle as an index, instead of providing a direct measure of their absolute numbers. It is based on patients performing a voluntary contraction at various intensity levels and surface interference patterns being captured and decomposed to obtain

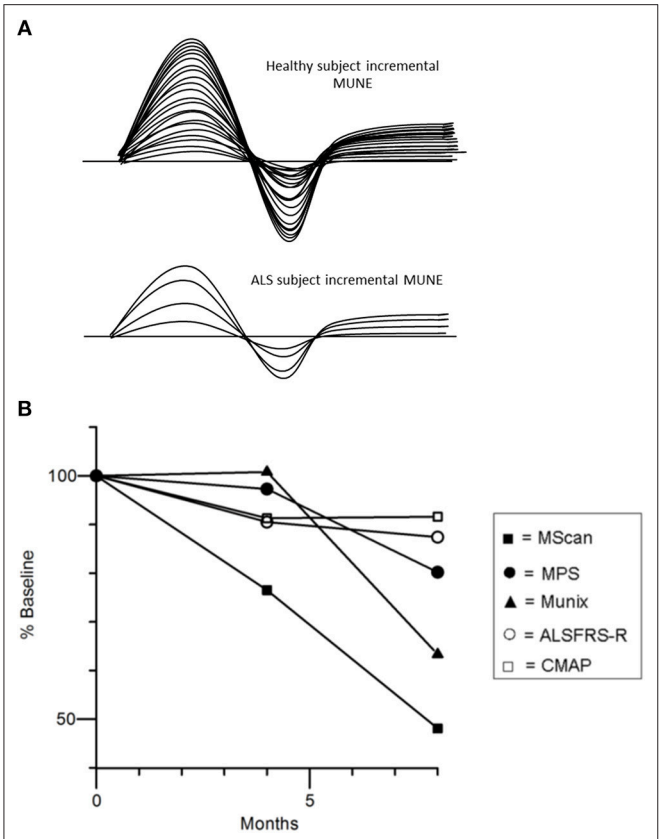

FIGURE 4 | (A) Incremental MUNE in healthy and ALS subjects, demonstrating a large number of "steps" with increasing stimulus intensity consistent with a large number of functioning motor units within measured muscle, whilst there were only four steps in the ALS subject indicating only four functional motor units remaining in muscle. **(B)** Percentage changes in MUNE values (geometric means) and mean ALSFRS-R and CMAP amplitude at 4 and 8 months. Reproduced with permission license no. 4457481173441 (87).

a normalized motor unit size, which is then in turn divided into the maximal CMAP value to obtain the MUNIX (86, 89, 90).

Recent studies using different MUNE methods have demonstrated potential utility for assessing disease progression in ALS patients as reflected by a progressive linear decline in MUNE counts (87, 91–94). Interestingly, a recently developed MUNE technique, termed MScan, appeared to be the most sensitive MUNE method in detecting ALS disease progression (**Figure 4B**) (87). Additionally, MUNIX was able to detect disease progression in presymptomatic muscles in ALS (95, 96), and changes longitudinally in these muscle groups appeared more sensitive to those changes in the revised ALS Functional Rating Scale (ALSFRS-R) (93).

Neurophysiological Index

The neurophysiological index (NI) is a potential electrophysiological biomarker in assessing lower motor neuron loss in ALS (97). Using a simple formula, The NI has the advantage of using routine CMAP amplitude, F-wave frequency, and distal motor latency of the ulnar-nerve innervated abductor digit minimi (ADM) muscle and is more sensitive than the CMAP amplitude alone in demonstrating longitudinal lower motor neuron loss in ALS. NI was able to detect motor neuron loss in muscles of the presymptomatic limb in ALS patients as well as successfully tracking disease progression, demonstrating continued loss of functional motor units during this presymptomatic period, when weakness, atrophy, or fasciculations were not detectable to both patients and evaluating clinicians (78). The validation of NI as a clinically meaningful parameter in disease progression of ALS patients was also demonstrated longitudinally in the symptomatic muscles of patients that correlated with their ALSFRS-R decline (97, 98). Additionally, NI was able to detect deterioration that occurred over a short period of 4 weeks in ALS patients, hence enabling the utility of this index in a clinical trial setting (77). NI has favorable reproducibility and low intraindividual variability but amongst its limitations, the index is only restricted to the ADM muscle (which is less affected compared to other intrinsic hand muscles such as the APB and FDI, in keeping with the split hand pattern of wasting and weakness) (99) and requires persistent F-waves (that can be frequently absent in ALS) (78).

Split-Hand Index

The split-hand sign is documented as an early and specific clinical feature in patients with ALS that is not characteristic in other commonly encountered clinical mimics (99, 100). It refers to the preferential wasting and weakness of the thenar complex muscles (APB and FDI) with relative preservation of the hypothenar muscle, ADM (99), and appeared to have a cortical origin with the corticomotoneuronal input to the thenar complex in ALS patients preferentially affected (101, 102). This clinical observation provided an opportunity to develop a simple neurophysiological biomarker to aid the diagnosis of ALS using conventional nerve conduction studies. The split-hand index (SI) was derived by multiplying the CMAP amplitude of the APB muscle by the FDI CMAP amplitude and then dividing the product by the ADM CMAP amplitude. It was demonstrated that a reduction in the split-hand index was consistent across ALS phenotypes but appeared most pronounced in those with limb-onset, and that a cut-off value $\leq$5.2 reliably differentiated between ALS and other neurological disorders (103).

Electrical Impedance Myography

Electrical impedance myography (EIM) is a novel non-invasive form of testing to provide quantitative information on neuromuscular disorders that may be useful and reliable in assessing longitudinally the severity of a disease process (104–107). EIM utilizes a small, high-frequency electrical current applied across two electrodes positioned over a muscle, and the resulting surface voltages are measured between a second pair of electrodes, from which the resistive and capacitive properties of the tissue are obtained (86, 105). The advantage is that this technique does not rely on inherent electrical activity of the tissue (which conventional neurophysiological techniques do), but rather on how the tissue impacts the applied current, rendering the technique sensitive to structural and compositional changes in muscle such as denervation, reinnervation, myofiber

atrophy and fat replacement within the muscle that occur in ALS (104). EIM values have been shown to correlate with standard clinical approaches including handheld dynamometry and MUNE (106, 107), and may be able to provide more than a five-fold reduction in sample size requirements for ALS clinical therapeutic trials over standard outcome measures such as the ALS functional rating scale-revised (ALSFRS-R) (108). Although EIM can detect changes early in the disease course of ALS as well as in clinically unaffected muscle groups (105), a limitation of EIM is that identified changes may not be able to differentiate ALS from other neuromuscular conditions (109, 110).

CONCLUSION

Amyotrophic lateral sclerosis remains a devastating neurodegenerative disorder with a poor prognosis, much of which is attributable to frequent delays in diagnosis, an incomplete understanding of the underlying pathophysiological mechanisms, and the current lack of effective disease-modifying treatment available. As such, there is a critical need to devise accurate and reliable biomarkers to address the above shortfalls in current ALS management. The current review has presented recent developments in novel neurophysiological biomarkers that are able to effectively interrogate upper and lower motor neuron dysfunction and characterize their change over time with disease progression, thereby exhibiting the potential to improve diagnosis, as well as facilitating in the prognosis and monitoring of the effects of future therapeutic agents in a clinical trial setting.

AUTHOR CONTRIBUTIONS

WH drafted manuscript and made all necessary edits and revisions as well as obtained required copyright permissions for figures. TD, SV, and MK revised and edited manuscript.

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MINI REVIEW
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Imaging Cerebral Activity in Amyotrophic Lateral Sclerosis

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Advances in neuroimaging, complementing histopathological insights, have established a multi-system involvement of cerebral networks beyond the traditional neuromuscular pathological view of amyotrophic lateral sclerosis (ALS). The development of effective disease-modifying therapy remains a priority and this will be facilitated by improved biomarkers of motor system integrity against which to assess the efficacy of candidate drugs. Functional MRI (FMRI) is an established measure of both cerebral activity and connectivity, but there is an increasing recognition of neuronal oscillations in facilitating long-distance communication across the cortical surface. Such dynamic synchronization vastly expands the connectivity foundations defined by traditional neuronal architecture. This review considers the unique pathogenic insights afforded by the capture of cerebral disease activity in ALS using FMRI and encephalography.

Keywords: amyotrophic lateral sclerosis, motor neurone disease, biomarker, neuroimaging, neurophysiology, cortex

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INTRODUCTION

Neurodegenerative disorders are increasingly understood as a disintegration of complex cerebral functional networks (1). Amyotrophic lateral sclerosis (ALS) is characterized by loss of upper and lower motor neurones of the corticospinal tract, brainstem, and spinal anterior horns, resulting in progressive weakness of downstream muscles. In addition to protean upstream causes (2, 3), there is firmly established clinical, pathological, and genetic overlap of ALS with frontotemporal dementia (FTD). The diagnosis remains a clinical one, with a lack of biomarkers being a significant barrier to the development of highly-effective disease-modifying therapy. Advanced neuroimaging, in combination with histopathological insights, has brought the brain to the forefront of biomarker development (4).

The earliest studies of cerebral blood flow in ALS employed positron emission tomography, and demonstrated a widened region of cortical activation in response to a simple motor task (5). Among the hypotheses for this "boundary shift effect" was loss of local inhibitory GABA-ergic interneuronal circuits [reviewed in (6)]. A consistent pathological feature of ALS has been the observation of increased cortical excitability, possibly reflecting reduced local inhibitory influences, measured using short-interval paired transcranial magnetic stimulation (TMS) (7, 8). Through the characterization of monogenetic associations, ALS research has expanded to include the study of what is now thought to be a long presymptomatic phase (9), in which cortical functional abnormalities may be the among the earliest detectable manifestations (10).

Blood oxygenation level-dependent (BOLD)-based functional (FMRI), with the major advantages of avoiding ionizing radiation and the greater availability of MRI technology, confirmed a profound alteration in cortical activity inherent to the pathogenesis of ALS. Tools to study cortical

neurophysiology in real-time have also undergone significant development in both sensitivity and analysis. This review will consider the unique insights that FMRI and encephalography bring to the understanding of the pathogenesis of ALS at the systems level, which is increasingly of greatest relevance to the long-term goal of neuroprotective therapy.

FUNCTIONAL MRI

FMRI has been extensively used to characterize network dysfunction in ALS in cross-sectional, longitudinal, and presymptomatic study designs. The practical advantages of FMRI in ALS include the widespread availability of MRI platforms, the large number of freely available analysis suites and the ability to provide crucial biological insights in relatively simple, cost-effective, and non-invasive study designs (11). Despite some inconsistencies in the literature (12), two main themes have consistently emerged from the application of FMRI to ALS: (i) the characterization of compensatory changes, such as the recruitment of additional cortical (13–16), subcortical (14, 17, 18), and cerebellar (17, 19) regions to execute motor and cognitive tasks, and ii) the concept of inhibitory dysfunction as a key facet of ALS-associated pathophysiology (6, 20).

Methodological Considerations

Functional MRI has been extensively used to characterize network dysfunction in ALS in cross-sectional, longitudinal, and presymptomatic study designs (21) (**Table 1**), but method-associated limitations are rarely articulated. The protracted hemodynamic response to neural activity hampers the temporal resolution of fMRI. Blood oxygen-level dependent (BOLD) signal typically peaks only 5–6s after focal activation therefore careful study designs are indispensable for meaningful temporal inferences (22, 23). Spatial distortions and signal dropout due to susceptibility gradients near air-tissue interfaces lead to decreased BOLD signal in lateral parietal, orbitofrontal and dorsolateral prefrontal regions necessitating meticulous sequence optimization before data acquisition (24–26). Scanner noise may also impact on the interpretation of BOLD signal, particularly in the default-mode network (27), and careful experimental designs are required to minimize the influence of background noise (28). While fMRI findings are often presented by overlaying activation maps upon high-resolution structural images, the inherent spatial resolution of fMRI is limited by the signal-to-noise ratio profile of consecutive, rapid whole-brain imaging. In the majority ALS studies, the voxel size of fMRI protocols is two to four times larger than what is used for structural acquisitions (21).

Motor Paradigms

Pioneering FMRI studies in ALS relied initially on hand movement paradigms (15, 16), which were gradually complemented by innovative bulbar studies (13, 29, 30). In motor-task FMRI studies, different strategies have been utilized to control for limb weakness, motor effort and lower motor neuron involvement for the interpretation of cerebral activation. Motor imagery (31) has attracted considerable attention, not only for emerging brain-machine interface applications (32) but also as an FMRI paradigm for a condition like ALS in which patients typically develop severe motor disability (33). The execution and imagination of specific movements manifest in similar activation patterns in ALS and controls (15) suggesting that this approach may be particularly pertinent to patient cohorts with mixed disability profiles. Some ALS studies however report divergent activation maps in motor imagery and execution (34). Similarly to motor imagery, action observation is also thought to result in comparable cortical activity to action execution which has been used to study the mirror-neuron system in ALS (35, 36). Another approach to control for motor disability and establish ALS-specific activation patterns is the inclusion of disease-controls, i.e., non-ALS patients with motor disability (16, 37). Very few FMRI studies to date have specifically evaluated functional changes in other rarer motor neurone disorders such as the upper motor neurone-only primary lateral sclerosis (PLS) (38, 39) and lower motor neurone-dominated Kennedy's disease (30) using motor paradigms. Patient stratification into separate study groups based on motor disability is another strategy to interpret functional alterations in the context of disability (14). In light of the fundamentally divergent study designs, the inclusion of patients in different stages of their disease and small sample sizes, the inconsistent findings of motor activation studies are not surprising. Whilst, hypo- (29, 30, 40) and hyper-activation (13, 14, 16, 35, 36) of the somatosensory cortex have both been reported in response to motor tasks, the recruitment of premotor areas is a relatively consistent finding. An integrative explanation of the seemingly divergent findings is that the initial hyper-activation represents an early-stage adaptive process to execute movement (14), which gradually gives place to hypo-activation as progressive structural changes ensue (41, 42). Robust multi-timepoint longitudinal studies are required to clarify the timeline of functional changes in ALS as very few task-based longitudinal FMRI studies have been published to date (33, 43). One longitudinal study identified reduced motor activation on 3-month follow-up which was interpreted as compensatory failure due to progressive neural loss (43), while another study reported increased precentral gyrus activity 6-month after initial scanning as evidence of ongoing adaptation (33). In addition to compensatory processes in motor, premotor and supplementary motor areas (44), evidence also exist that the basal ganglia (17, 18, 45, 46), the ipsilateral motor cortex (14, 47), and the cerebellum (17, 19, 47, 48) also contribute to adaptive network reorganization.

Extra-Motor Studies

With the increasing recognition of cognitive impairment in ALS (49, 50), a series of elegant language (51), executive (52), theory-of-mind (36), and memory (43, 53, 54) task-based activation studies have also been published. In addition to the cognitive activation paradigms, visual, auditory and somatosensory stimulation studies have further characterized the spectrum of extra-motor involvement in ALS (55, 56). Other innovative non-motor activation studies in ALS include an anti-saccade study with concurrent eye tracking to investigate dorsolateral prefrontal cortex (DLPFC) function (57). Similar to the divergent findings of motor-task studies, increased activation

TABLE 1 | Selected motor task-based and resting-state fMRI studies in ALS.

Authors	Year	Study design	ALS (n)	Controls (n)	Main study findings/interpretation
Li et al. (29)	2009	Motor task: swallowing, Cross-sectional study	10	10 HC	Reduced somatosensory cortex activation in patients with dysphagia
Mohammadi et al. (30)	2009	Motor: tongue movement, Cross-sectional study	22	22 HC 5 DC (SBMA)	ALS patients with bulbar symptoms showed decreased cortical and thalamic activation
Palmieri et al. (53)	2010	Emotional attribution and recognition task, Cross-sectional study	9	10 HC	Altered emotional processing similar to patents observed in FTD.
Lule et al. (55)	2010	visual, auditory and somatosensory stimulation, Cross-sectional study	14	18 HC	Decreased response in secondary visual areas in ALS, delayed response in secondary auditory areas, reduced response to somatosensory stimulation
Goldstein et al. (52)	2011	Cognitive task, Cross-sectional study	14	8 HC	Increased left temporal and decreased precentral and left medial frontal activation: altered inhibitory processing in ALS
Kollewe et al. (13)	2011	Motor task: hand and tongue movement, Cross-sectional study	20	20 HC	Decreased cortical activation during tongue movements in patients with bulbar symptoms. Increased activation during hand movements. Different functional reorganization in limb and bulbar impairment.
Mohammadi et al. (14)	2011	Motor task, Cross-sectional study	22	22 HC	Patients stratified into three groups based on disability, Increased activation in early-stage, decreased activation in later stage disease.
Poujois et al. (15)	2013	Hand motor task Motor imagery, Cross-sectional study	19	13 HC	Motor execution and imagery yields to similar activation patterns. Increased contra- and ipsilateral somatosensory cortex activation.
Passamonti (59)	2013	Emotional processing task, Cross-sectional study	11	12 HC	Increased activation in prefrontal areas and altered amygdala-prefrontal cortex connectivity in ALS, suggestive of limbic system dysfunction
Witiuk et al. (57)	2014	Antisaccade task with eye tracking, Cross-sectional study	12	12 HC	ALS patients make more antisaccade direction errors and exhibit reduced DLPFC activation compared to controls i.e. deficits in automatic response inhibition are associated with impaired DLPFC activation
Stoppel et al. (43)	2014	Go/No-Go paradigm, Longitudinal design	14	14 HC	Increased motor activation compared to controls with subsequent decline on follow-up scanning suggestive of failing adaptive compensation
Mohammadi et al. (61)	2015	Movement inhibition task (go/no-go), Cross-sectional study	17	17 HC	Increased motor inhibition and execution related activation in patients with ALS compared to controls.
Jelsone-Swain et al. (36)	2015	Action Observation and Execution task, Cross-sectional study	19	18 HC	Increased activation during action-execution and observation in ALS patients in opercular, premotor and primary motor regions. Mirror neuron system mediated compensation.
Li et al. (35)	2015	Action observation paradigm, Cross-sectional study	30	30 HC	Action observation activates similar networks to action execution. Increased activation observed in the DLPFC and supplementary motor regions of ALS patients.
Aho-Ozhan (60)	2016	Cognitive task Cross-sectional study	15	14 HC	Impaired processing of negative emotions such as fear and disgust in ALS
Vellage et al. (54)	2016	Cognitive task: working memory Cross-sectional study	14	14 HC	Reduced hemodynamic responses in the left occipital cortex and right prefrontal cortex in ALS patients compared to healthy controls
Keller et al. (58)	2018	Cognitive task: ToM and executive task, Cross-sectional study	65	33	Increased activation in all ALS patients compared to HC. High performing patients exhibit more activation than those with neuropsychological deficits suggestive of compensation.
Mohammadi et al. (66)	2009	Resting-state fMRI, Cross-sectional study	20	20 HC	ICA analyses: decreased DMN activation in the anterior and posterior cingulate and parietal regions
Jelsone-Swain et al. (84)	2010	Resting-state fMRI, Cross-sectional study	20	20 HC	ROI analyses: decreased functional connectivity between the right and left motor cortices
Douaud et al. (20)	2011	Resting-state fMRI, Cross-sectional study	25	15 HC	Increased SMN, premotor, prefrontal and thalamic functional connectivity, interpreted as compensation and inhibitory dysfunction

(Continued)

TABLE 1 | Continued

Authors	Year	Study design	ALS (n)	Controls (n)	Main study findings/interpretation
Agosta et al. (42)	2011	Resting-state fMRI, Cross-sectional study	26	15 HC	Increased SMN, cingulate, cerebellar connectivity interpreted as compensation.
Fekete et al. (76)	2013	Resting state fMRI, Cross-sectional study	40	30 HC	Widespread motor, cerebellar and basal ganglia functional connectivity alterations in the ALS cohort. Accurate subject classification using multiple kernel learning.
Zhou et al. (82)	2013	Resting-state fMRI, Cross-sectional study	12	12 HC	Positive correlation between disability and functional connectivity
Agosta et al. (65)	2013	Resting-state fMRI, Cross-sectional study	20	15 HC	ICA analyses: Increased parietal connectivity is associated with cognitive deficits which may represent compensation
Welsh et al. (67)	2013	Resting state fMRI, Cross-sectional study	32	31 HC	Machine learning (support-vector machine) based on fMRI metrics achieves over 71% accuracy for disease state classification
Zhou et al. (73)	2014	Resting state fMRI, Cross-sectional study	12	12 HC	Decreased regional brain synchrony in the superior medial SMN detected by regional coherence measures
Meoded et al. (38)	2015	Resting state fMRI, Cross-sectional study		14 HC 16 PLS	Increased functional connectivity between the cerebellum and cortical motor areas and between the cerebellum and frontal and temporal cortex in primary lateral sclerosis
Schmidt et al. (77)	2014	Resting state fMRI, Cross-sectional study	64	27 HC	A strong positive correlation exist between changes in SC and FC averaged per brain region; suggesting that structural and functional network degeneration in ALS is coupled
Chenji et al. (75)	2016	Resting state fMRI, Cross-sectional study	21	40 HC	Increased DMN and reduced SMN connectivity associated with greater disability interpreted as inhibitory dysfunction
Zhou et al. (69)	2016	Resting state fMRI, Cross-sectional study	44	44 HC	Increased cerebellar, occipital and prefrontal degree centrality (DC) and decreased DC in the primary motor cortex and sensory motor regions of ALS patients
Menke et al. (79)	2016	Resting state fMRI, Presymptomatic study design	12	12 psALS 12 HC	Increased FC between the cerebellum and precuneus-cingulate-frontal lobe network in asymptomatic mutation carriers compared to controls
Trojsi et al. (72)	2017	Resting state fMRI, Cross-sectional study	21	15	Decreased FC in DMN, salience and fronto-parietal network. More significant SLN connectivity changes observed in bulbar onset patients compared to those with spinal onset.
Zhang et al. (74)	2017	Resting state fMRI, Cross-sectional study	38	35 HC	Impaired interhemispheric functional connectivity eidenceed by voxel mirrored homotopic connectivity (VMHC) reductions, correlations with CC diffusivity metrics
Zhang et al. (166)	2017	Resting state fMRI, Cross-sectional study	25	25 HC	Reduced occipital surface-based local gyrification index (LGI) is associated with decreased functional connectivity in the bilateral precuneus.
Lee et al. (80)	2017	Resting state fMRI, Presymptomatic study design		13 psALS 46 HC	Connectivity deficits detected in salience, sensorimotor, default mode and thalamic networks in presymptomatic *C9orf72* carriers
Li et al. (68)	2018	Resting state fMRI, Cross-sectional study	38	35 HC	Graph theory method (functional connectivity density FCD) Decreased FCD in the primary motor cortex, increased long-range FCD in the premotor cortex in ALS patients.
Bueno et al. (167)	2018	Resting state fMRI, Cross-sectional study	20	15 HC	Focus on Papez circuit integrity. Decreased functional connectivity in ALS patients between hippocampal, parahippocampal and cingulate regions.
Menke et al. (39)	2018	Resting state fMRI, Longitudinal study	13	3 PLS	Multi-timepoint structural-functional study, ICA and DRA, decreased FC between SMN and frontal pole, increased FC between primary motor cortex and fronto-parietal network

HC healthy control, DC Disease Control, DLPFC DorsoLateral PreFrontal Cortex, FC functional connectivity, SC Structural Connectivity, SBMA Kennedy's disease, FTD FrontoTemporal Dementia, PLS Primary Lateral Sclerosis, DMN Default Mode Network, SMN SensoriMotor Network, ToM Theory of Mind.

(36, 52, 58, 59) and impaired activation (51, 60) have both been noted on cognitive tasks, which is likely to represent stages of successful and failing adaptation. More often however a pattern of coexisting hypo- and hyper- activation is reported (37, 54, 61).

Resting-State Studies

The analysis of task-free BOLD signal in the so-called resting-state (rsFMRI) benefits from fast acquisition times with a data-driven, more consistent experimental design, making them an attractive add-on to high-resolution structural

protocols. With the establishment of the internationally collaborative Neuroimaging Society in ALS (NiSALS) (62) and successful multi-site initiatives (63), there is interest in FMRI sequence harmonization and potential for multicentre data pooling (12, 64). rsFMRI studies differ considerably in their analysis approaches and their methods span from independent component analysis (65–67), to graph theory (68, 69) and amplitude of low frequency fluctuation (ALFF) (70, 71). rsFMRI studies in ALS identified decreased frontotemporal (72), sensorimotor (70, 73–75), and cortical-subcortical (76) network integrity and increased default mode network (75), and cerebellar (38, 69) connectivity. Large combined structural-FMRI studies suggest that patterns of structural degeneration overlap with functionally impaired regions and that a strong positive correlation exists between functional and structural connectivity alterations (77). Longitudinal rsFMRI studies indicate declining functional connectivity in sensorimotor, thalamic, and visual networks and increasing connectivity in fronto-parietal and temporal circuits (39). Multimodal, structural-functional, multi-timepoint longitudinal studies (39) are best suited to characterize the natural history of progressive neurodegenerative changes (78). Data from presymptomatic carriers of ALS-causing gene mutations revealed increased cerebello-cerebral functional connectivity (79) and decreased salience, sensorimotor, default-mode, and thalamic networks connectivity (80). Despite the controversy around direct clinico-radiological correlations (81), some studies in ALS have reported significant associations, most often with functional measures (73, 82–84), disease duration (59, 73), and progression rates (20, 40, 85).

Practical Limitations

For a condition in which accumulation of physical disability is accompanied by ventilatory compromise with orthopnoea, supine MRI limits longitudinal assessment to those with slower rates of progression (39). The application of such a biomarker as an outcome measure in a small-scale clinical trial would then entail costly statistical compromises, since no ideal solution exists for the imputation of data points selectively lost from those patients with more aggressive disease (86).

ENCEPHALOGRAPHY

Cortical processes, and the diseases that impact on them, are inadequately described without reference to dynamic neural communication (87, 88), but this necessitates temporal precision, without the dispersive effects of the haemodynamic response function that smears neural signals across several seconds (89). Surface electroencephalography (EEG) as a biomarker in ALS is appealingly practical, well tolerated and non-invasive.

Methodological Limitations

Even a high-density array of surface EEG electrodes still sacrifices spatial resolution owing to the attenuation and mutation of neural signals as they pass through several tissue layers with varying electrical conductivity (89). Magnetoencephalography (MEG) permits recording of tiny (femtoTesla) fluctuations in the magnetic field external to (and undispersed by) the scalp (90). Yet reconstruction of cortical sources remains a mathematically "ill-posed" problem—any given recorded signal could in theory be generated by multiple neural sources and the analytical choice to address this (for example "beamforming") necessitates certain assumptions (91). MEG's improvement in spatial precision is also offset by expenses and susceptibility to artifact from ferromagnetic interference, albeit mitigated by acquisition and analysis standardization (92, 93). The resulting data is feature-rich, subsequent analysis may necessarily be restricted to a frequency-band of interest or a selected connectivity metric, these choices may in turn influence study conclusions (94) (**Table 2**).

Evoked Potentials

Small-scale EEG studies have addressed the utility of somatosensory, visual and brainstem evoked potentials in ALS (95–100). Reflecting the inconsistency of reported results, these well-established and standardized assessments have failed to find any routine clinical application in ALS, although they may yet find a role in multimodal assessment (101, 102).

To better reflect the pathological burden in ALS studies have therefore moved toward either motor or cognitive activation paradigms, initially appraising cortical processes via evoked response potentials [ERPs, previously reviewed in (103)]. The "Bereitschaftspotential," a classical lateralized change in cortical electrical potential, easily recordable during movement preparation, appeared robustly decreased in ALS (104, 105). More recent studies have considered the implications of abnormal movement-related cortical potentials (MRCPs) in ALS in terms of clinical and structural correlates. While a study of 21 ALS patients demonstrated higher MRCPs overall, the effect was shown to be driven by patients with a low burden of clinically detectable UMN morbidity (106). The inference that increased MRCPs reflect cortical compensatory mechanisms was born out by longitudinal study of a sub-set in whom MRCPs declined over 10 months. A comparable study of finger movement in 32 ALS patients revealed reduced MRCPs only in patients with a high UMN burden, alongside evidence of ipsilateral premotor activation to suggest a compensatory "boundary shift" (107). MRCPs are also elicited during imagined movements, but only a limited study in ALS has thus far been performed (108), mandating replication before application of these measures in control and communication devices is to be seriously considered.

Motor Paradigms

Motor events (including self-generated movement) are reflected in frequency-specific changes to continuous "background" neuronal oscillations (109). As might be expected, the neurodegeneration associated with ALS results in distinct alteration to pre-central sensorimotor rhythms. While studies are yet to be widely replicated, they show promise both in terms of relevance to daily motor tasks, and sensitivity to detect early cortical dysfunction in patients still capable of performing the task in question. The results may also contribute to the ongoing efforts to characterize a presymptomatic phase to ALS and have implications for the development of brain-computer interfaces

TABLE 2 | Selected motor task-based and resting-state encephalographic studies in ALS.

Authors	Year	*n*	EEG/MEG (channels)	Protocol	Main measure	Phenotype correlations
Westphal et al. (104)	1998	16 ALS 16 HC	EEG (11c)	Self-paced R fist closure	Reduced BP	Spasticity correlation
Thorns et al. (105)	2010	13 ALS 13 HC	EEG (19c)	Cued R or L index finger button press	Reduced BP	N/A
Inuggi et al. (107)	2011	32 ALS 12 HC	EEG (29c)	Self-paced R thumb extension	Reduced MRCPs (only in UMN+ ALS)	Ipsilateral MRCP correlation with movement speed
Riva et al. (119)	2012	16 ALS 15 HC	EEG (29c)	Self-paced R thumb extension	Reduced beta ERS; Unaltered beta ERD	ERS correlation with CST damage via MRI and TMS
Gu et al. (108)	2013	4 ALS 7 HC	EEG (15c)	Imaginary R wrist extension	Slower MRCP rebound	N/A
Bizovičar et al. (120)	2014	21 ALS 19 HC	EEG (30c)	Self-paced R index finger flexion	Reduced beta ERD; Lateralised ERS	None
Proudfoot et al. (122)	2017	11 ALS 9 PLS 12 Presymp 10 HC	MEG (306c)	Cued R or L index finger extension	Excess beta ERD; Delayed ERS	Altered ERS lateralisation in PLS
Proudfoot et al. (126)	2018	17 ALS 11 HC 5 Presymp	MEG (306c)	Cued R and L hand grips	Reduced beta CMC; Reduced inter-hemispheric beta FC	CMC unaltered in Presymp
Mai et al. (143)	1998	18 ALS 14 HC	EEG (18c)	Resting	Reduced central alpha power	Alpha correlation with MRC and Norris scales
Santhosh et al. (144)	2005	12 ALS 12 ALS	EEG (8c)	Resting	Reduced alpha power	N/A
Jayaram et al. (145)	2015	6 ALS 32 HC	EEG (124c)	Resting	Reduced central theta power; Widespread increased high gamma power	Gamma reduced only in patient with ALSFRS=0
Iyer et al. (147)	2015	18 ALS 17 HC	EEG (128c)	Resting	Increased FC especially within salience and default-mode networks	N/A
Nasseroleslami et al. (148)	2017	100 ALS 34 HC	EEG (128c)	Resting	Increased FC especially interhemispheric theta and fronto-parietal gamma	FC correlation with structural MRI degeneration
Fraschini et al. (150)	2018	21 ALS 16 HC	EEG (61c)	Resting	Widespread reduced alpha FC	N/A
Proudfoot et al. (151)	2018	24 ALS 24 HC 15 Presymp 9 PLS	MEG (306c)	Resting	Widespread increased broadband FC	Similar changes in PLS. More subtle changes in Presymp.
Sorrentino et al. (154)	2018	50 ALS 25 HC	MEG (163c)	Resting	Broadband increased FC with disorganized topology	Advanced ALS associated with a more centralized, "vulnerable" network

HC healthy control, FC functional connectivity, Presymp asymptomatic ALS-causing gene carriers, BP Bereitschaftspotential, MRCP movement related cortical potential, ERD event related desynchronization, ERS event related synchronization PLS primary lateral sclerosis, CMC cortico-muscular coherence UMN+ above average quantity of upper motor neuron signs, CST cortico-spinal tract, ALSFRS ALS functional rating scale (disability metric).

aiming to facilitate environmental control by patients with advanced ALS (110).

Movement is accompanied by reliable and well-characterized fluctuations in neural signal power, particularly within the beta (15–30 Hz) band, with recognizable anatomical localization to motor cortex. Beta-band power is reduced (event related desynchronization, ERD) prior to and during movement execution; movement termination is followed by an equally reliable increase in power well above baseline levels (synchronization, ERS or post-movement beta-rebound) (111). Temporally corresponding to fluctuations in cortical excitability (112), ERD and ERS are adjusted to meet task requirements [including force (113), speed (114), and complexity (115)], are sensitive to pharmacological manipulation [particularly synaptic GABA levels via benzodiazepines (116) or tiagabine (117)] and may be disrupted by other disease states including Parkinson's (118).

Motor Studies (EEG)

Two independent EEG studies have demonstrated attenuation of ERS in ALS. The first involved 16 patients efficiently performing self-paced thumb extensions (119). The degree of

ERS attenuation was shown to correlate with corticospinal pathological burden as measured by both mean diffusivity on structural MRI and diminished motor evoked potentials in APB in response to TMS stimulation. The second study included 21 patients performing both sniffing and right index finger flexion (120). Although the patients had detectable weakness in terms of both maximal grip strength and sniff nasal-inspiratory pressure, there were no group differences in the precise pressure produced during the task performance. Neural data from the sniff task were heavily contaminated by facial muscle artifact, but the finger flexion task resulted in reliable ERD/S. The ALS patients were observed to have diminished beta ERD, interpreted as a consequence of pyramidal cell degeneration. Both motor preparation and execution timepoints were affected, while the lateralization of beta ERS was also altered. The study failed to establish clinical correlations with these measures, nor was there any successful correlation with F-wave elicitability (an imperfect measure of corticospinal tract integrity in any case).

Motor Studies (MEG)

The neural signal acquired by MEG is far less susceptible to distortion as it passes through skull and scalp, source modeling is therefore likely to be more accurate than EEG, and an expanding range of MEG studies have specifically appraised sensorimotor rhythms (121). A MEG study involving 11 ALS patients, 9 with PLS, and 12 asymptomatic genetic mutation carriers, investigated sensorimotor rhythms during a laterally-cued motor preparation task requiring speeded index finger extension of either hand (122). Whole-brain source-space data were analyzed pre, during, and post movement, specifically focusing on beta-band frequencies. Although the task was behaviorally performed comparably by ALS patients, the neural data revealed larger beta ERD, 500 ms after cue presentation, during the period of maximal motor preparation, particularly within contra and ipsilateral gyri. Beta ERS, after movement termination, was delayed in both patient groups. The asymptomatic carriers produced excessive beta ERD during motor execution. Conceptually the results are concordant with cross-modality support for cortical hyperexcitability in ALS (123, 124).

The integrity of upper motor neurone pathways can also be non-invasively appraised using MEG. Cortico-muscular coherence (CMC), by which neural oscillations and surface electromyography correlate temporally (particularly during sustained contraction), principally reflects direct corticospinal drive to the peripheral musculature (125). A MEG study of 17 ALS patients was designed to measure CMC during a bilateral forearm grip task (126). As expected, source-space beta CMC was distinctly strongest from the contra-lateral precentral gyrus, but this frequency specific peak was markedly attenuated in the ALS group, despite adequate grip production and without any correlation to force production. The analysis also took advantage of MEG spatial precision to consider motoric cortico-cortical communication during the same task performance. Interhemispheric functional connectivity, in terms of beta band amplitude envelope correlation, was reduced in ALS patients. The inference of reduced CMC, a measure that in health indexes the quality of motor performance (127), is that beta coherence may serve as a novel UMN specific biomarker at the disposal of future therapeutic efforts (128).

Extra-Motor Studies

Taking advantage of the high temporal resolution of encephalographic data, component steps in the complex cognitive dysfunction associated with the ALS-FTD syndrome may be examined. The mismatch negativity (MMN) paradigm considers the attentional modulation of auditory perception. An early EEG study failed to show any abnormalities within ALS patients (129). However, using MEG, plus subtle experimental design adjustments in 12 participants all with bulbar symptoms, MMN response amplitudes were shown to increase relative to healthy controls (130). Given the previously demonstrated sensitivity of MMN responses to ketamine administration, the authors tentatively linked their findings to the glutamatergic excitotoxicity ALS pathogenesis theory. This rare example of "gain of function" was not consistently replicated in two later EEG studies, which interpreted delayed MMN responses as evidence of sub-clinical extra-motor dysfunction (131, 132).

Less well-replicated methodologies have also been applied to ALS patients to consider neural processes underlying working memory (133, 134), selective attention (135, 136), and executive control (137, 138). Broadly, these studies have provided further evidence in favor of sub-clinical disruption to "frontal" cognitive processes in keeping with the extended non-motor ALS phenotype (139). Parietal cortex dysfunction was also implicated in an EEG study involving the Wisconsin Card Sorting Test. While 26 ALS patients did not differ in performance of a "set-shifting" task, even patients without mild cognitive impairment failed to produce the expected enhancement of parietal ERPs during a task-switch (140). Although the attenuation of the "switch potential" failed to correlate with neuropsychological indices, the authors speculated whether such sub-clinical deficits could predict future behavioral disorder.

A study requiring cognitive task performance during data acquisition took a very different analytical approach, using 200 s of data to measure "transfer entropy" between scalp electrodes rather than the millisecond granularity of evoked potentials. The directionality of functional connectivity was appraised via EEG in 18 ALS patients, revealing only feedforward (parietal to frontal) connectivity to increase across a broad frequency band (141). As the patients engaged in a spelling task with a view to brain-computer interfacing, sensory (visual) stimuli were hypothesized to be more readily processed in compensation for the diminished proprioceptive input resulting from physical disability, but an alternative explanation in terms of failing cortical inhibition was also acknowledged (20).

Resting-State Studies (EEG)

The earliest EEG investigations of ALS reflected the emerging concept of cognitive dysfunction within the ALS clinical spectrum, with slowed cortical rhythms noted in non-demented patients (142). A more systematic study of 18 ALS patients conversely revealed sparse differences to healthy controls (143). Only at central electrodes, and only within the alpha band (8–13 Hz), was the power of neural oscillations reduced in ALS. The reduction was interpreted to reflect selective neuronal loss within

the sensorimotor cortices. A comparable result was described in a subsequent smaller study (144), and increases in the gamma band (30–90 Hz) power beyond central regions was also reported (145).

Further ALS electrophysiology studies have reflected a growing interest in the so-called "dynome" (146), the extent to which the organization of cortical function is reflected in particular patterns of active connectivity. High-density (128 channel) surface EEG was used to calculate connectivity between both scalp points and projected source nodes in an initial study of 18 patients (147). Fronto-central areas were shown to have increased connectivity, and this was explored across a broad range of measures. A subsequent study expanded this work to 100 patients, including some longitudinal analyses (148), and confirmed EEG-derived connectivity changes in ALS to be more striking than limited group differences in the scalp-recorded power spectrum. This more parsimonious analysis appraised only sensor-space, deriving coherence estimates within 8 consecutive frequency bands. Widespread increases in connectivity were again demonstrated relative to healthy controls, particularly theta band interhemispheric sensorimotor connectivity and gamma band fronto-parietal connectivity. As 59 of the ALS patients had undergone contemporaneous structural MRI, mathematically derived structural "degeneration modes" (accounting for the large-scale gray and white matter changes typical in ALS) were shown to correlate with EEG change, conceptually aligned with the concept of progressive network decline overlying structural disintegration.

Network structures can also be summarized using graph theory metrics, this was explored in sensor-space in 21 patients, demonstrating a more "de-centralised" organization (149). The connectivity metric chosen in this study was phase-based, thus insensitive to any group differences in spectral power, and furthermore was significantly correlated with disability between individuals. This group later re-analyzed the same data reconstructed into source-space (150) and filtered into 3 classical frequency bands to show spatially distributed decreases in connectivity, albeit restricted to the alpha band spectrum.

Resting-State Studies (MEG)

A resting-state MEG study explored functional connectivity in 24 ALS patients using source-space data acquired after co-registration with structural MRI (151). Ten minutes of continuous data was parcellated into 39 regions of interest and the broad-band (3–40 Hz) signal used to calculate "edge" strength between these 39 "nodes," In keeping with many FMRI studies, functional connectivity was broadly increased in ALS patients relative to age-matched healthy controls, particularly affecting communication links to the posterior cingulate cortex. This finding was aligned with the hypothesis of loss of cortical inhibitory neuronal influences underlying cortical excitability in ALS (5). Comparable posterior non-motor connectivity changes were described using FMRI (152). Nevertheless, the diversity of reported results and interpretations serves to highlight a need for replication and standardization between centers and where possible across modalities (153). A further study of 50 patients, using a different (phase-based) connectivity measure, also described widespread connectivity increases in ALS (154). The increases were not restricted to specific frequency bands and the extracted graph theory metrics suggest global network hyper-centralization to accompany disease progression.

FUTURE DIRECTIONS

MEG is providing broader insight into cognitive mechanisms underpinning higher cortical function in health (155), and comparable results may eventually prove achievable using surface EEG (156). The next generation of wearable sensors may yet dramatically expand MEG's application (157). The spinal cord is a core but functionally understudied aspect of the motor system disintegration that characterizes ALS. Spinal FMRI is in its infancy (158), but a number of promising studies have already been published in animal models (159, 160), healthy populations (161, 162) and other clinical cohorts (163, 164). The goal of non-invasively studying the integrated activity of upper and lower motor neurone pools looks more feasible with the success in studies involving the dorsal pathways (165). Cerebral FMRI parameters are likely to take an increasing role in emerging machine-learning and classification studies both in diagnostic and prognostic applications (67, 76). Future studies need robust longitudinal design and to capitalize on the growing infrastructure for multicentre studies. This will permit the testing of pathogenic hypotheses within larger cohorts of clinically more homogeneous ALS patients, and define the earliest markers of pathology in presymptomatic individuals essential for the assessment of future neurotherapeutic interventions.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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MINI REVIEW
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Diagnostic and Prognostic Performance of Neurofilaments in ALS

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There is a need for biomarkers for amyotrophic lateral sclerosis (ALS), to support the diagnosis of the disease, to predict disease progression and to track disease activity and treatment responses. Over the last decade multiple studies have investigated the potential of neurofilament levels, both in cerebrospinal fluid and blood, as biomarker for ALS. The most widely studied neurofilament subunits are neurofilament light chain (NfL) and phosphorylated neurofilament heavy chain (pNfH). Neurofilament levels are reflecting neuronal injury and therefore potentially of value in ALS and other neurological disorders. In this mini-review, we summarize and discuss the available evidence about neurofilaments as diagnostic and prognostic biomarker for human ALS.

Keywords: amyotrophic lateral sclerosis, frontotemporal dementia, neurofilament, cerebrospinal fluid, serum, plasma, NfL, pNfH

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder primarily affecting the motor system network, giving rise to progressive muscle weakness in the limbs, the bulbar region, but also of the respiratory muscles. Survival is typically between 2 and 5 years after disease onset, but in about 15% of patients a slower disease progression is present (1). The most important extramotor manifestations of the disease include behavioral changes, executive dysfunction and language problems, reminiscent of frontotemporal dementia.

As of today, the diagnosis of ALS remains based on clinical judgement and requires a combination of signs of upper and lower motor neuron involvement in a patients with progressive muscle weakness, without alternative explanation for the presenting symptoms and signs (2). Despite efforts to make the diagnostic criteria more sensitive (3, 4), the diagnostic delay remains about 10–12 months after symptom onset (5). The current clinical criteria also do not discriminate between different subtypes of ALS, although they may have very different disease trajectories. Combinations of clinical parameters allow to predict disease progression and survival in ALS patients, but they do not reflect the underlying biological processes (6).

Biomarkers, which reflect hallmarks of the disease, may not only aid in the diagnostic algorithm of ALS, but could also be of value in defining homogeneous subgroups of patients. Potentially, they could also be helpful to track disease progression and treatment responses (7). Neurofilaments (NF) have been studied extensively in different neurological conditions, and are considered to

be useful as marker of acute and chronic neuronal injury (8). Neurofilaments are intermediate filaments of 10 nm in neurons, composed of heteropolymers of different subunits, neurofilament light chain (NfL), neurofilament medium chain (NfM), and neurofilament heavy chain (NfH) (9). Phosphorylation and O-glycosylation are believed to be important for NF assembly (9) and especially NfM and NfH undergo these posttranslational modifications. NF are highly expressed in neurons, provide structural support for neurons and determine axon caliber and conduction velocity (10). Mutations in the genes encoding NfH and NfL can cause the inherited neuropathy Charcot-Marie-Tooth disease (11), inframe deletions or insertions in the side arm domain or C-terminal tail domain of NfH have also been linked to ALS (12). Neurofilamentous abnormalities and elevated NF levels are not restricted to ALS. However, NF have been implicated in the pathogenesis of ALS for more than 2 decades (13). In post mortem spinal cord of ALS patients, accumulations of NF are seen in the perikaryon and axons of motor neurons (14) and motor neurons display reduced NfL mRNA levels (15). Overexpression of NfH causes a motor axonopathy with NF inclusions in mice, which can be rescued by NfL overexpression (16), suggesting that an imbalance between the relative expression levels of the different NF subunits may be important. In line with this hypothesis, reducing the NfL levels and overexpression of NfH levels in the SOD1 mouse model of ALS, increased the lifespan of these animals (17, 18). In this model of ALS, the degeneration of motor neurons is accompanied by a progressive rise in blood NF levels, and these levels have been shown to be able to capture treatment responses (19, 20).

In this review, we will give an overview of the current knowledge about the diagnostic and prognostic value of NF levels in cerebrospinal fluid and blood for human ALS.

AVAILABLE METHODS TO MEASURE NEUROFILAMENTS LEVELS

Numerous studies employed in house developed assays or commercial "for research use only" ELISAs for NF measurements (20–27). Although the precision and recovery profile of such kits was acceptable (**Table 1**), the analytical sensitivity in terms of limit of detection and limit of quantification was insufficient to precisely detect NF levels in CSF of controls or in blood of most patients with ALS (30). Using the same antibodies against NfL, novel technologies including electrochemiluminescence (ECL) and Single Molecule Array (SIMOA) enabled to precisely and sensitively quantify NfL in CSF and blood (22, 29, 40). Furthermore, an improved ELISA assay allowed to accurately quantify pNfH in blood and CSF of patients with ALS (39).

DIAGNOSTIC VALUE OF NEUROFILAMENTS

It is already known for more than 2 decades that NF levels are roughly 5–10 times higher in ALS patients compared to healthy controls (41). Numerous studies since then, have shown that NF levels are increased in patients with ALS, not only in CSF, but also in serum or plasma (42). As NFs are produced by neurons, the serum/plasma levels are 10 fold lower compared to CSF levels.

Several studies showed that NfL and pNfH are elevated in CSF and serum/plasma in patients with ALS (20, 23, 30–32, 35, 37–39, 43–55). There is a good correlation between NF levels in CSF and in blood, and this is the case for NfL and pNfH (34, 39, 40). Nevertheless, the diagnostic performance was found to be better in CSF compared to blood (39, 54). Most studies compared ALS patients to healthy controls, only few studies tested the diagnostic performance in comparison to ALS mimicking disorders (23, 30, 31, 39). The sensitivity and specificity for ALS was better for pNfH than for NfL in studies comparing both neurofilament subunits (23, 32, 39). Even though there is considerable elevation in NF in some of the ALS mimicking disorders, the diagnostic accuracy to detect ALS is still good. The diagnostic performance of NfL and pNfH assays is shown in **Table 1**. One study suggested that the discrimination from disease controls improved by using the CSF pNfH/complement C3 ratio (24). For implementation in the routine clinical practice, assay standardization, and characterization, and independent validation of the cut-offs are required. Indeed, the development of reference methods for NF measurements, e.g., by means of mass spectrometry (56, 57), and of certified reference materials for traceability of the calibrators and to demonstrate commutability among the different assays should be encouraged (58). Independent evaluation of the performance characteristics of the NF assays enables the public availability of data on the analytical quality of the different commercially available assays. Furthermore, automation of immunoassay facilitates single measurements with similar precision profiles as duplicate measurements in manually performed ELISAs, the former significantly reducing the implementation costs for patients (59). As the range of NF levels in ALS mimicking disorders is rather wide, the robustness of reported cut-offs might be challenged by the rather low number of ALS mimicking disorders included in most studies (23, 39). Multicenter studies are warranted to establish universally applicable cut-offs for NF.

Importantly, the increase in NF is already measurable early in the disease course (23, 31, 40). A recent study showed that NfL levels increase already several months prior to symptom onset in *SOD1* mutation carriers (60). NFs are elevated in sporadic and familial ALS patients, although slightly lower in confirmed *SOD1* cases (43) and higher in *C9orf72* positive patients (51).

The neuroanatomical correlate of elevated NF levels in ALS is not entirely clear. Both NfL and pNFH correlate with the extent of clinical upper and lower motor neuron involvement (23), although pNfH levels correlate better with lower motor neuron involvement and NfL levels better with upper motor neuron involvement (23, 34). An imaging study revealed that NfL levels in CSF correlate with the extent of corticospinal tract involvement on DTI (48).

TABLE 1 | Analytical and diagnostic performance of NfL and pNfH assays.

	Capture antibody epitope	Calibrator	Analytical performance					Diagnostic performance (ALS vs. mimics)					
			LOD	LOQ	CV_{wr}	CV_{rr}	Recovery	Sens	Spec	PPV	NPV	LR+	LR-
NfL			pg/mL		%	%	%	%	%	%	%		
Gothenburg (Blennow lab) ELISA (21)	Core domain (aa 93-396) of full length recombinant NfL (Origene)	Not disclosed	-	78[a,b]	8[a,b]	13[a,b]	80 - 109[a,c]	Not addressed					
NF-light ELISA (22) (Uman Diagnostics)	Core domain (aa 92–396) (NF-L mAb 47:3 and NF-L mAb 2:1 having nonsterical overlapping epitopes) (28)	Purified bovine spinal cord NfL (Progen Biotechnik GmbH)	–	78[(29),c,e]	1.5 [a,c]	17.4[a,c]	-	78[(23),c] 77[(30),c] 89[(31),c] 96.2[c,(32)]	85 88 89 56.3	92 95 - -	64 56 - -	3.9 - - -	- - - -
Simoa NF-light digital immunoassay (29) (Quanterix)			0.038[a,o]	0.62[(29),c,e]	6.6	17.0	118.5[c,o]	100[d,(31)]	84	-	-	-	-
							90.7[d,o]	85.5[d,(33)]	77.3	91.4	65.4	-	-
NFL ECL assay (29)			-	15.6[c,e]	9.2 3.6[p]	14.8 6.6 [p]	-	79[(34),d]	84-90[f]	98	34	-	-
pNfH													
Nijmegen pNfH ELISA (35)	Low to highly phosphorylated NFH (Anti-SMI35 (or 03-44) Ab via Sternberger Monoclonals Incorporatedand later on via Covance Research Products) (36) Cross-reacting for 7.8% with NFM (25)	Bovine NFHp35 standard (ICN, Burlingame, CA)						72[c]	80 [c]	-	-	-	-
London pNfH ELISA (25)		HPLC-purified bovine NfH (Affiniti Research	200[c,g]		10.6 [c] 7.9[c,(26)]	23[c] 9.76[h]- 14.08[l,c,(26)]	119[(26)]		CSF levels of NfHSMI35 were five times higher in patients with ALS (1.7 ng/mL) than in controls (37)				
pNfH ECL assay (38)		Bovine NfH (USBiological)	(0.6) 24 [g]		4.8[c]	8.4[c]	84.5- 93.2[c]	-	-	-	-	-	-
Gainesville pNfH ELISA (26, 27)	220 kDa Form of NFH isolated from bovine Spinal cord [affinity purified chicken anti pNfH via EnCor Biotechnology (Alachua, Florida)], no obvious Reacting with of NFM or other lower molecular weight material (27)	In house purified bovine NfH	400[g]		2.96 [c,h,]	6.67[c,h,j]	-	-	-	-	-	-	-
Euroimmun pNfH ELISA		Purified bovine pNfH (EnCor Biotechnology Inc., Gainesville, Florida, USA)	69[c,n]	150[c,n]	5.50[a,o] 6.90 [c,n]	5.95[a,o] 9.17 [c,n]	-	92.9[c,(39)]	96.0	98.7	80.0	23.3	-
								100[c,m,(32)]	68.8	-	-	-	-
								71.8[d,(39)]	85.2	93.8	48.9	4.8	-

(Continued)

TABLE 1 | Continued

NfL	Capture antibody epitope	Calibrator	Analytical performance					Diagnostic performance (ALS vs. mimics)					
			LOD pg/mL	LOQ	CV_{wr} %	CV_{rr} %	Recovery %	Sens %	Spec %	PPV %	NPV %	LR+	LR-
Jacksonville pNfH ELISA (20)	Bovine pNfH purified from spinal cord (purified AH1 monoclonal antibody)	Bovine pNhH purified from spinal cord	31.25[g,k]			8.5-12.5	-	First paper to show higher levels of plasma pNfH in ALS than in healthy controls					
Biovendor pNfH ELISA (24)	Not publically available		23.5[g,o]	-	4.5[a,b,o]	-	98.8[c,o] 95.8[d,o]	84.4[c,m] 83[c,(30)] 90.7[c,(23)]	93.5 [c,m] 80 88.0	- 93 95.8	- 62 75.9	- - 7.6	- - -

a, protocol not (fully) disclosed/available; b, matrix not disclosed; c, in CSF; d, in serum; e, value below lowest calibrator reaching accuracy of 80-120% and CV% ≤ 20% (which is most closely related to the definition of LOQ); f, <6 m and >6 m disease duration, respectively; g, signal of blank + 3 times the standard deviation, considered as limit of blank within current review; h, high pNfH sample; i, low pNfH sample; j, 9 consecutive days for 12 samples; k, serially diluted bovine pNfH; l, standards run 16 times on each of 7 days; m, in disease controls also including ALS mimics; n, in house data (not published) via the "Verification of Performance for Precision and Trueness; Approved Guideline-Second Edition". CLSI document EP15-A2,; o, manufacturer's data - p, low NfL levels ranging from 9.91 pg/mL to 72.8 pg/mL. aa, amino acids; CVwr, within-run or intra assay coefficient of variance; CVrr, between-run or inter assay coefficient of variance; ECL, Electrochemiluminescence; ELISA, Enzyme-Linked Immunosorbent Assay; LOD, limit of detection; LOQ, limit of quantification; LR, likelihood ratio for abnormal (+) or normal (-) result; NFM, neurofilament medium chain; NPV, negative predictive value; PPV, positive predictive value; sens, sensitivity; spec, specificity.

PROGNOSTIC VALUE OF NEUROFILAMENTS

The levels of both NfL and pNfH have been shown to correlate with parameters of disease severity, such as the decline on the ALS functional rating scale-revised or ALSFRS-R (23, 30, 34). They also predict survival of ALS patients, with higher NF levels being unfavorable. In Cox regression analyses both NfL and pNfH have been shown to be independent predictors of survival, when taking other prognostic factors into account (30, 34, 45, 61). Patients with very long survival typically have low levels of NFs (23, 53). The predictive value of NFs is present when using both CSF and blood samples. As higher NF levels are associated with a faster disease progression in typical ALS patients, NF levels could theoretically be used to stratify patients in clinical trials. However, data on this topic are currently lacking.

The difference in disease progression between different clinical subtypes of ALS is not always reflected in NF levels. Patients with *C9orf72* ALS have been reported to have higher pNfH CSF levels (51), but further studies on NF levels are needed in different motor neuron disease subtypes. In patients with primary lateral sclerosis (PLS), the levels can also be increased, but mostly to a lesser extent (30, 31, 34). ALS patients with cognitive/behavioral impairment or comorbid FTD have a worse outcome (62, 63), but if this is reflected in NF levels requires further study (64). The unfavorable outcome of patients with bulbar onset or respiratory onset ALS may not be reflected in NF levels.

VALUE OF NEUROFILAMENTS TO TRACK TREATMENT RESPONSE?

NFs may not only have value to help with the diagnosis and prediction of disease severity in ALS, they may also become of value to track the response to treatments. As marker of neuronal injury it is anticipated that neuroprotective treatments would result in lower NF levels. For ALS, there are no studies in patients that report a treatment response on NF levels at present. Whether the effect of riluzole on survival can be captured by measuring NF levels remains unknown. On the other hand, a recent study using rodent mutant SOD1 models, showed a clear survival benefit of treatment with antisense oligonucleotides, which was accompanied by a reduction in serum pNfH levels (65). In addition, in other neurological disorders, such as multiple sclerosis, NFs levels reflect the effect of disease-modifying therapies (66).

In patients with ALS, it is know that NFs levels are relatively stable during the course of the disease in many patients (51, 67). However, there is some evidence that the levels may increase during the first phase of the disease (53). This is backed up by data from a recent study in *SOD1* mutation carriers, which showed that the levels slowly increase up to 12 months prior to symptom onset and can continue to rise the months following symptom onset (60). The NF levels also correlate with the number of body regions affected by ALS and the ALS progression

rate (23, 34), suggesting that they reflect the extent and rate of motor neuron degeneration. Several cross-sectional studies have reported a negative correlation of NF levels with survival (30, 34, 53). This may suggest that the levels drop slightly in later disease stages, although there certainly is a bias introduced by the enrichment for patients with a longer survival at later time points. Longitudinal sampling shows a tendency to lower levels upon follow up, especially in fast-progressing patients (67).

CONCLUSION

Evidence is emerging that NF levels can become valuable biomarker for ALS, both for diagnosing ALS, for predicting outcome, and potentially for the monitoring of treatment effects. The CSF pNfH level seems to be the most accurate diagnostic marker, but both pNfH and NfL serum or plasma measurements perform good to predict survival and disease progression. Further research is needed to establish the value of NF levels for stratification and for disease monitoring in clinical trials.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Diagnostic Challenge and Neuromuscular Junction Contribution to ALS Pathogenesis

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Amyotrophic lateral sclerosis (ALS) represents the major adult-onset motor neuron disease. Both human and animal studies reveal the critical implication of muscle and neuromuscular junctions (NMJs) in the initial phase of this disease. Despite the common efforts, ALS diagnosis remains particularly challenging since many other disorders can overlap yielding similar clinical phenotypic features. A combination of further research on the NMJ parameters that are specific for this disease and laboratory tests are crucial for the early determination of specific changes in the muscle, as well as in motor neuron and the prediction of ALS progression. Also, it could provide a powerful tool in the discrimination of particular ALS and ALS-mimic cases and increase the efficacy of therapeutic treatments.

Keywords: amyotrophic lateral sclerosis (ALS), axonopathy, neuromuscular junction (NMJ), dying back hypothesis, ALS-mimic diseases

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AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic Lateral Sclerosis (ALS) is a disease characterized by a progressive degeneration of upper motor neurons (MNs) in the motor cortex and lower motor neurons in the brainstem and the spinal cord. The death of these neurons leads to spasticity, weakness, and atrophy of the muscles, progressing to paralysis. The incidence of ALS in Europe is 2–16 per 100,000 each year (1), with respiratory failure being the predominant mode of death in patients within 3 years of diagnosis (2). The onset of the disease occurs prevalently during adulthood (peak age of 58–63 years) (3), though with a small proportion of early-onset disease in certain patients (before 35 years of age). ALS also shares neuropathological and genetic features with another neurodegenerative disorder, frontotemporal dementia (FTD) (4, 5), with many ALS patients showing some cognitive or behavioral changes. This has led to consider ALS and FTD as the ends of the same spectrum of disease (6).

Although the majority of ALS cases occur sporadically (sALS), there is a Mendelian inheritance in about 10% of the cases (familial ALS, fALS), mainly in an autosomal, dominant fashion (7). The two are clinically indistinguishable and a variety of genetic defects in more than 20 genetic loci have been linked with the ALS phenotype (8), with new genes constantly being identified in subsets of ALS patients (9–11). Four major genes which mutations are known to cause ALS are the f ollowing: chromosome 9 open reading frame 72 (C9orf72), superoxide dismutase 1 (SOD1), transactive response DNA-binding protein (TARDBP) and fused in sarcoma (FUS) (12–15). C9orf72 has an important role in membrane trafficking and autophagy (16), and SOD1 primary function is thought

to be as a cytosolic and mitochondrial antioxidant enzyme, converting superoxide to molecular oxygen and hydrogen peroxide (17). TARDBP and FUS encode nucleic acid-binding proteins that reside in the nucleus, and are involved in multiple aspects of RNA processing, such as transcription and splicing [reviewed in (18)].

NMJ INVOLVEMENT IN ALS

Despite the progress in our understanding of the molecular pathogenesis linked to these genes, it is still unclear where the motor neuron dysfunction begins and the extrinsic factors that accelerate motor neuron degeneration. This led to the consideration of ALS as either a *dying forward* process that proposes an anterograde degeneration of motor neurons by glutamate excitotoxicity from the cortex, or a *dying back* phenomenon in which motor neuron degeneration starts distally at the nerve terminal or at the neuromuscular junction (NMJ) and progresses toward the cell body (3, 19). The NMJ is a tripartite synapse composed by the presynaptic motor neuron, the postsynaptic muscle and the synapse-associated glial cells (terminal Schwann cells, TSC) and allows the transmission of action potentials from motor neurons to muscles [reviewed in (20)]. In this complex structure, besides motor neuron degeneration, glial cells, and muscle fibers play also a major role in ALS onset and progression.

The muscle contribution in ALS development, through NMJs disassembly, is still a matter of debate. Nonetheless, increasing evidence points to the critical role of NMJ defects in the early stage of the disease in ALS patients [reviewed in (21)] and a variety of animal models have permitted important advances into exploring this hypothesis.

The human $SOD1^{G93A}$ transgenic mouse, the first and most studied ALS model, is the one that has yielded the majority of information about the muscular deficits in ALS (22). Spatiotemporal analysis of NMJs in $SOD1^{G93A}$ mouse revealed end-plates denervation before the appearance of clinical symptoms and neuron cell body loss (23), with the fast-fatigable synapses being more vulnerable to denervation (24). Because of its high expression in ALS muscle biopsies, the neurite outgrowth inhibitor Nogo-A was proposed as a factor responsible for motor nerve terminals repulsion and destabilization at the NMJ at very early asymptomatic stages (25, 26). This hypothesis was then confirmed in $SOD1^{G93A}$ mouse model, where genetic ablation of Nogo-A in muscle led to marked reduction of muscle denervation and prolonged survival (27). Morphological observation of NMJs in $SOD1^{G93A}$ also contributed to reinforce the dying back hypothesis, showing more detailed NMJ alterations prior to functional symptom onset (28). A detailed overview of the findings concerning neuromuscular defects in the $SOD1^{G93A}$ mouse model has been reviewed by Dupuis and colleagues (22).

Despite the predominant use of rodent models for studying pathomechanisms and potential therapeutic targets in ALS, the use of smaller animal models, like *Drosophila melanogaster* and zebrafish (*Danio rerio*), is continually increasing. Their advantages lie in their fast development allowing quick generation of lines, their availability and the ease in manipulating gene expression and in drug screening. In drosophila, studies showed locomotor defects, reduced life span, and anatomical defects at the NMJ, causing impairments in synaptic transmission, in loss and gain of function models of TARDBP (29, 30). Similar results were found for FUS. Gene deficiency and overexpression of FUS in Drosophila models caused decreased synaptic transmission, reduced number of presynaptic active zones, altered postsynaptic glutamate receptor subunit composition at the NMJ, motor neuron degeneration and impaired motor behavior (31, 32). Zebrafish studies have highlighted gain and loss of function mechanisms for TARDBP and FUS, demonstrating shorter axonal projections from motor neurons, premature and excessive branching, impaired synaptic transmission at the NMJ leading to swimming defects (33–35). C9orf72 gene has also been modeled in zebrafish in a loss-of-function model that displayed behavioral and cellular deficits related to locomotion (36).For more details about the different models, all the ALS gene mutations that have been modeled are summarized in a recent review by Van Damme et al. (37).

Altogether, fundamental research supports the crucial role that NMJ could play in ALS pathogenesis and its possible employment as efficient early marker of the disease.

ALS DIAGNOSTIC CHALLENGES

The difficulty to diagnose ALS resides mainly in the existence of several mimic syndromes, unrelated to ALS but which present similar clinical features (38, 39).

Motor neuron diseases (MNDs) are classified in four main groups in which ALS represents the most common form (**Table 1**). Although these diseases affect people in different ways, they share several symptoms due to motor neuron loss of function. All of them present progressive weakening of skeletal muscles, which eventually affects the ability to speak, swallow and breathe. ALS diagnosis is even more difficult if we add to the list other neurological conditions unrelated to MNDs which can mimic its early symptoms. Moreover, increasing evidences point to a possible direct implication of muscle in the early stage of the disease, adding myopathies to the list of ALS-mimic pathologies (**Table 1**).

Standard diagnostic criteria for ALS have been established in 1991 [El Escorial criteria [EEC] (40)] and were revised in 1997 [AirlieHouse criteria [AHC] or El Escorial Revisited (41)]. Even though the essential requirements for ALS diagnosis were defined by these criteria, many neurologists and neuromuscular clinicians were missing the diagnosis, proving the low clinical accuracy of these diagnostic roles (42).

In 2008, electrodiagnostic studies, known as the Awaji criteria (43), were included in the clinical procedure to allow earlier and more accurate assessment of ALS diagnosis. However, the

TABLE 1 | General overview of neuromuscular diseases and ALS-mimic pathologies.

MOTOR NEURON DISEASES (MND)	
Amyotrophic Lateral Sclerosis (ALS)	
Primary Lateral Scerosis (PLS)	
Progressive Muscular Atrophy (PMA)	
Progressive Bulbar Palsy (PBP)	
OTHER NEUROLOGICAL CONDITIONS THAT CAN MIMIC ALS	
Mithochondrial Disorder (MID)	
Psedobulbar Palsy	
Spinal Muscular atrophy (SMA)	
Primary lateral sclerosis (some subtupes not related to ALS)	
Progressive spinal muscular atrophy (some subtype not related to ALS)	
Spinobulbar muscular atrophy (SBMA or Kennedy's disease)	
Autoimmune Syndromes Monoclonal	
Myopathies	
Cachectic myopathy	
Polymyositis Sarcoid myositis	
Carcinoid myopathy	
Nemaline myopathy	
Inflammatory myopathies	Polymyositis (PM)
	Dermatomyositis
	Inclusion-body myositis (IBM)

Neuromuscular Disorders (NMD) implicate deficits and degeneration of nerves (motor and sensory neurons) and muscles (skeletal muscles) of the central and peripheral nervous system, leading to muscles weaken and waste away (atrophy). NMDs are classified in 4 categories, with Amyotrophic lateral sclerosis representing the main one. ALS-mimic pathologies is a vast group of diseases characterized by weakness and wasting away of muscle tissue, with or without the breakdown of nerve tissue, thus mimicking ALS symptoms. Currently, no cure exists for NMDs and the treatments aim to relieve the symptoms and delay disease progression.

application of those sets of defined features are still insufficient to rule out other similar and related diseases (44, 45).

Methods for Diagnosis

Although the main ALS evaluation remains the clinical one, laboratory testing, based on advanced techniques of electrodiagnosis, neuroimaging, immunobiochemistry, and neurogenetics, is required for accurate ALS diagnosis.

Tests to rule out other neuromuscular conditions may include:

Electromyogram (EMG)

The needle EMG is the most important study in determining diagnostic certainty of ALS (46). During this test, a needle electrode is inserted through the skin into various muscles, starting with the most severely involved limb (**Figure 1**). The examination then progresses through four anatomical region: bulbar, cervical, thoracic, and lumbar. At least three anatomical regions have to be positive to this test to define ALS. The fasciculation potential (FP) has been included in Awaji criteria as a hallmark of ALS muscular denervation. In general, a decreased number of motor unit recruitment, with long duration of the motor unit potential, and abnormal spontaneous activity, are measured at the EMG in ALS patients [reviewed in (47)].

Nerve Conduction Study (NCS)

This test measures how fast an electrical impulse moves through the nerve (**Figure 1**). During the test, one electrode placed on the skin stimulates the nerve of interest with a very mild electrical impulse. Variations in time spent to reach a second electrode can help in identifying a nerve damage. Whereas, EMG measures the electrical activity in the muscles, the nerve conduction study is specific for nerves and helps to localize the disorder among nerve, neuromuscular junction, and muscle. NCS is a powerful tool to discriminate ALS from axonal demyelination or conduction block impairments (48). NCS parameters are generally normal in ALS, albeit the presence of prolonged distal motor latency and slowed conduction velocity could be consistent with the diagnosis of ALS (49, 50). These changes suggest loss of large myelinated fibers, but also motor axons regeneration phenomena (50).

Magnetic Resonance Imaging (MRI)

This technique is able to produce detailed images of the brain and spinal cord, the latter with the advantage of simultaneously investigating the upper and lower motor neurons. During several years, its application was related to the exclusion of other disorders, as tumors or hernias that can display certain of the ALS-mimic symptoms (51). The evolution and improvement of this multimodal tool has recently become essential for the diagnosis of ALS. MRI scans can show cerebral degeneration and gray/white matter atrophy [reviewed in (52)], and also detect abnormalities in ALS muscle, likely due to denervation atrophy process (53).

Blood and Urine Tests

Testing hematological factors is helpful to exclude diseases that are capable of mimicking ALS symptoms. Recently, a population-based study, proposed serum albumin, creatinine levels, and lymphocyte count as markers for ALS, indicating muscle waste and inflammation respectively (54). Other markers potentially related to a better ALS outcome have been proposed: LDL/HDL levels, which are elevated in ALS plasma and represent a general unexplained hypermetabolism (55, 56); serum uric acid levels, which are decreased among ALS patients, further demonstrating the possible role of oxidative stress in the induction and propagation of the disease (57); serum ferritin levels which are elevated in ALS patients and could reflect perturbation in iron metabolism (58); concentrations of certain amino acids, which are decreased in ALS (59); levels of serum proinflammatory cytokines, such as IL-6, which are increased in ALS (60). Finally, high level of circulating AChE and metalloproteinases (MMP) have been reported in ALS plasma (61, 62) and although the exact source of these two classes of enzymes remains uncertain, it could in part reflect a disruption of extracellularly bound AChE at the NMJ and early change in the nerve-muscle integrity.

Spinal Tap (Lumbar Puncture)

Using this particular test, a small amount of cerebrospinal fluid (CSF) is taken from the lower back of the patient for laboratory

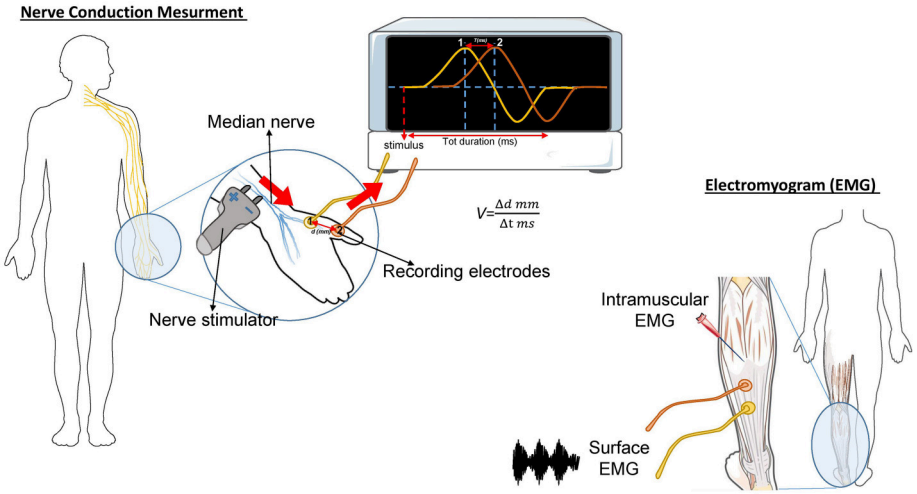

FIGURE 1 | Schematic representation of the nerve conduction and muscle contraction studies. *Nerve Conduction Velocity (NCV - left)* measures the velocity and the quality of conduction of the electrical signal in a nerve. During the test, your nerve is stimulated, with an electrode attached to your skin. One or two more electrodes patches are placed on the skin over your nerve. The electrical impulse of the stimulated nerve pass from the stimulator to the other receiving electrode. The time (in milliseconds) spent by the impulse to move from a point to another, on the order of millimeters, represent the Velocity. In ALS, the impulse conduction is slower respect with control cases and is worsened by the increase of axonal degeneration. The *electromyogram (EMG-right)* measures the electrical activity of the muscles at rest and during contraction. There are two kinds of EMG: surface EMG and intramuscular EMG. In the first one the muscle activity is recorded by one or more electrodes patched on the skin and it asses the contractile response of superficial muscles. This approach presents several limitation since the result signal is influenced by the depth of the subcutaneous tissue at the site of the recording and by the discharges of adjacent muscles. With the intramuscular EMG, specific deep muscle activity is recorded by using one needle electrode inserted into the muscle. EMG and NCV tests are often done together to give more complete information.

tests. Thanks to its proximity to the central nervous system, the CSF is considered one preferred tissue to search for ALS biomarkers [reviewed in (63)]. Several markers for ALS have been identified in CSF such as Tau, TDP43, Nefl, and MMP levels [reviewed in (64)]. In particular, MMPs with their ability to digest collagen, proteoglycan, and laminin (65), may reflect ongoing destruction of the matrix which wraps synapses (66) and pathological changes at the brain-blood barrier (62).

Muscle Biopsy

With this technique, a small portion of muscle is removed by needle biopsy and sent to a laboratory for histopathological analysis. Rarely performed because of its painful and invasive nature, this tool is useful when ALS diagnosis is in doubt. Generally, ALS muscles present signs of active denervation/reinnervation and an increased number of atrophic fibers (67).

Genetic Testing

People with familial ALS (fALS) background can get an efficient diagnosis through genetic testing (68, 69). This technique may help ALS patients to understand the basis of their condition, and improve the genotype-specific treatments (70). Unluckily, there is a lack of consensus among clinicians above the definition of fALS, since newly genes related to ALS are continuously found (71). Nowadays, genetic testing is not wildly used because of its high cost and the belief that ALS genetics is not well-enough understood to provide a better treatment plan, as reported in 2017 in a study which involved 167 clinicians from 21 different countries around the world (71).

EXAMPLES OF NMJ PATHOLOGIES ALS-MIMIC

Here we report some examples of ALS-mimic pathologies. The *Spinal muscular atrophy (SMA)* is an inherited MND that prevalently affects children. Its incidence is 1 per 11,000 live births (72). All forms of SMA are caused by the loss of SMN1, a gene implicated in axonal mRNA transport and snRNP biogenesis (73). Studies involving mice and fly mutants demonstrated that the probable origin of this pathology resides in the early loss of sensory information from proprioceptive neurons (74), which in turn causes degeneration of α motor neurons. In consequence, progressive muscle weakness, and, in severe cases, respiratory failure appear (75). Despite being considered a child's illness, the SMA type 4, that has an adult onset, overlaps with ALS diagnosis (76). Furthermore, like in ALS, several studies reveal the early implication of NMJs in SMA, with synaptic pathology prior to the appearance of clinical symptoms (77–81). However, the first evidence of neuromuscular pathology occurred at different time points of the disease progression, with presynaptic pathology preceding morphological changes at the endplate in ALS, and simultaneous pre and post-synaptic pathologies in SMA, suggesting the possibility to study this particular zone in diagnosis (81). Histochemical skin biopsy comparison was suggested as a powerful diagnostic tool in differentiating ALS and SMA, since

the small collagen fibrils and the increased amount of amorphous material, which are characteristic of ALS, are not in SMA (82).

The *Spinobulbar muscular atrophy [(SBMA) Kennedy's disease]* is a X-linked hereditary lower motor neuron disease, where the expanded trinucleotide repeat (CAG > 37) in the androgen receptor gene (AR) causes its nuclear inclusions and impairment of its function (83, 84). The disease affects 1 per 200,000 males in Europe and Asia each year (85). In this pathology, degeneration of anterior horn cells of the spinal cord, where androgen receptors are widely expressed, is observed (86, 87). Although SBMA patients exhibit facial weakness as first sign of the disease, they progressively develop myopathic features, such as muscle atrophy and necrotic myofibers (88). Like ALS, SBMA disease reveals mixed pathological findings, with both myopathy and neurogenic atrophy features, which is the cause of misdiagnosis at the early stage of the disease.

Among the autoimmune syndromes, *myasthenia gravis (MG)* overlaps ALS syndrome. The annual incidence ranges from 3 to 30 per 1,000,000 people (89). In fact, the binding of autoantibodies to components of the NMJ in MG causes a characterized muscle weakness and fatigability (90). Even if acetylcholine receptor antibodies are considered to be highly specific for the diagnosis of MG, ALS patients can also present these autoantibodies at the blood test (91, 92). In these cases, it is very difficult to define the false positive cases and an experimental treatment with AChE inhibitors is necessary to differentiate MG from ALS (93).

The skeletal muscle disorders are represented with the term *Myopathies*. Myopathies hold a list of pathologies (**Table 1**) where muscle weakness can begin in the hands and feet (distal muscles) as well as in the muscles near the center of the body (proximal muscles) sometimes mimicking ALS features, confusing the diagnostic and the treatment decision. Among them, *Inclusion Body Myositis (IBM)* (94) is the most common ALS-mimic disease. It is the most common adult myopathy in 50 year-old persons and older, and its incidence is 3.5 per 100,000 (95). It is characterized by inflammatory cells surrounding and invading non-necrotic muscle fibers, rimmed vacuoles, congophilic inclusions, and protein aggregates in muscle (96, 97). In this case, the unique way to exclude ALS is the muscle biopsy combined with quantitative electromyographic analysis, especially in those patients where disease progression is slow and atypical (98).

CONCLUSION

Amyotrophic Lateral Sclerosis (ALS) and MNDs are not yet curable. However, accurate diagnosis is crucial to provide adequate counseling and information about the prognosis and disease course, and to avoid inappropriate therapy. Moreover, a good diagnosis could furnish a more equal stratification of cases and be important in the choice of additional medical support, as for example nutritional intervention strategies or physical therapy.

Currently, there is not a common consensus in the use of laboratory analysis for ALS diagnosis. Basically, clinicians decide for the application of certain techniques based on their experience, expertise and hospital practice. Progress in molecular genetics and identification of specific biomarkers is ongoing, which will translate to a refined diagnostic certitude. Therefore, there is the emerging need to establish a widely accepted protocol for laboratory tests to discriminate the majority of cases that present clinical features resembling ALS.

Increasing human and animal evidence proposed NMJ impairments as possible biomarkers for detection and discrimination of ALS and mimic diseases in an early, preclinical stage. However, further studies are needed to understand how these impairments could be monitored and specifically treated.

AUTHOR CONTRIBUTIONS

M-LC and A-RB contributed to conception and drafting. EK provided useful comments and reviewed the manuscript.

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Clinical Measures of Bulbar Dysfunction in ALS

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Bulbar impairment represents a hallmark feature of Amyotrophic Lateral Sclerosis (ALS) that significantly impacts survival and quality of life. Speech and swallowing dysfunction are key contributors to the clinical heterogeneity of ALS and require well-timed and carefully coordinated interventions. The accurate clinical, radiological and electrophysiological assessment of bulbar dysfunction in ALS is one of the most multidisciplinary aspects of ALS care, requiring expert input from speech-language pathologists (SLPs), neurologists, otolaryngologists, augmentative alternative communication (AAC) specialists, dieticians, and electrophysiologists—each with their own evaluation strategies and assessment tools. The need to systematically evaluate the comparative advantages and drawbacks of various bulbar assessment instruments and to develop integrated assessment protocols is increasingly recognized. In this review, we provide a comprehensive appraisal of the most commonly utilized clinical tools for assessing and monitoring bulbar dysfunction in ALS based on the COnsensus-based Standards for the selection of health Measurement INstruments (COSMIN) evaluation framework. Despite a plethora of assessment tools, considerable geographical differences exist in bulbar assessment practices and individual instruments exhibit considerable limitations. The gaps identified in the literature offer unique opportunities for the optimization of existing and development of new tools both for clinical and research applications. The multicenter validation and standardization of these instruments will be essential for guideline development and best practice recommendations.

Keywords: amyotrophic lateral sclerosis, Bulbar ALS, outcome assessment (Health Care), dysphagia, dysarthria, COSMIN

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INTRODUCTION

ALS is a relentlessly progressive neurodegenerative disease with considerable clinical heterogeneity compared to other neurodegenerative conditions. Bulbar impairment (oro-motor, dysarthria and dysphagia) is a hallmark feature of the disease and has been associated with the condition since its earliest descriptions (1). While only approximately 30% of patients exhibit bulbar symptoms at onset, the majority of patients develop speech and swallowing difficulties with disease progression.

Bulbar signs and symptoms play an important role in the diagnosis of ALS and pose unique management challenges. Bulbar presentation has been associated with shorter survival (2, 3), faster functional decline (4), reduced quality of life (5–7) and increased multidisciplinary support needs (8, 9). Dysarthria has been consistently associated with low mood, withdrawal from activities and social isolation (10, 11). Dysphagia in ALS may lead to weight loss, malnutrition, dehydration, aspiration pneumonia, hospitalization and reduced quality of life (12, 13). Despite these important sequelae, bulbar impairment in ALS is relatively understudied, and the research literature is sparse (14). Proxies of bulbar impairment are underrepresented among outcome measures in clinical trials (15). Validated diagnostic, monitoring and prognostic markers of bulbar dysfunction are lacking and clinical assessment practices vary considerably across various centers (16).

Assessment measures are broadly classified as "diagnostic" when their primary purpose is to confirm the diagnosis, exclude mimics, or classify individual patient according to disease-onset. Some measures have been optimized to characterize symptom severity, while other indices are primarily used to monitor longitudinal change. Depending on the primary purpose of a measure, it is subject to a specific set of requirements defined by the COnsensus-based Standards for the selection of health Measurement INstruments (COSMIN) guidelines (17). These require that, in order to reliably integrate assessment tools into clinical practice, their measurement properties need to be firmly established relative to their primary purpose. All tests need to be assessed for validity and reliability (reproducibility). Diagnostic and screening tests should also be evaluated for their detection abilities (i.e., sensitivity/specificity). Discriminative measures need to be able to detect group differences and measures proposed to track longitudinal change need to be assessed for their ability to capture progressive changes.

The objective of this paper is to provide a review of established bulbar measures in ALS from a diagnostic, screening and disease monitoring perspective. This work is not intended as an exhaustive review of all available measures of bulbar impairment in ALS but as a summary of the current state of the field and its most pressing needs.

Abbreviations: AAC, augmentative alternative communication; ALS, Amyotrophic Lateral Sclerosis; ALSFRS-R, Amyotrophic Lateral Sclerosis—Functional Rating Scale—Revised; ALSSS, ALS Severity Scale; CNS-BFS, Center for Neurologic Study-Bulbar Function Scale; COSMIN, COnsensus-based Standards for the selection of health Measurement Instruments; CNE, Cranial nerve exam; DDK, dysdiadochokinesis; EAT-10, Eating Assessment Tool-10; EIM, electrical impedance myography; EMG, electromyography; FLAIR, Fluid-attenuated inversion recovery; FDA, Frenchay Dysarthria Assessment; FEES, fiberoptic evaluation of swallowing; FSE, Videofluoroscopic swallowing evaluation; FT9, Fine'til 9; GRE/SWI, gradient recalled echo/susceptibility weighted imaging; IOPI, Iowa Oral Performance Instrument; KPa, kilopascal; LMN, lower motor neuron; MAIP, maximum anterior isometric pressure; MiToS, Milano-Torino staging; MR, magnetic resonance; MRI, magnetic resonance imaging; MUNE, Motor Unit Number Estimation; MTP, maximum tongue pressure; MUNIX, Motor unit number index; MUPs, motor unit potentials; NdSSS, Neuromuscular Disease Clinical Status Scale; OSS, Oral Secretion Scale; SCM, sternocleidomastoid; SLPs, speech-language pathologists; SSS, Sialorrhea Scoring Scale; SIT, Sentence Intelligibility Test; syl/sec, syllables per second; T1 W, T1 weighted; UMN, upper motor neuron; WPM, words-per-minute.

TOOLS FOR DIAGNOSING AND SCREENING FOR BULBAR ALS

Table 1 provides a summary of tools primarily used for the diagnosis of bulbar dysfunction in ALS highlighting their main advantages and limitations.

Cranial Nerve Exam (CNE)

Clinical evidence of upper motor neuron (UMN) and lower motor neuron (LMN) degeneration is required for the diagnosis of ALS. With regards to bulbar impairment, clinical UMN signs include pathological reflexes (e.g., brisk jaw jerk, gag, and other facial reflexes) (18) and LMN signs encompass muscle weakness, atrophy and fasciculations in the jaw, face, tongue and palate (33). Although the clinical neurological examination remains "the best way to localize neurodegeneration *in vivo* and to follow the process in real time," (34) and the reliability of CNE has been evaluated in various neurologic populations (21, 35), the quantitative psychometric profile of CNE i.e., inter and intra-later reliability, sensitivity, specificity, and responsiveness, have not been systematically evaluated in ALS to date. This represents a research priority for the standardization of assessments.

Needle EMG

The role of electromyography (EMG) in ALS is the confirmation of acute and chronic denervation. The former may be evidenced by fibrillations, positive sharp waves and fasciculation potentials, which in the tongue are not readily detectable since complete relaxation is difficult to achieve (22). Polyphasic motor unit potentials (MUPs) with prolonged duration, increased amplitude and decreased recruitment are suggestive of chronic denervation. Quantitative motor unit action potential analysis in subclinical bulbar involvement is thought to be superior to peak ratio interference pattern analysis (36). Depending on local protocols, the genioglossus is the most commonly assessed muscle (37), but the evaluation of the sternocleidomastoid (38), masseter, temporalis, frontalis (39), mentalis (40), and trapezius (22) muscles have also been proposed to resolve diagnostic uncertainty. While Motor Unit Number Estimation (MUNE) techniques (41, 42), such as MUNIX (43) have been extensively utilized to quantify motor neuron loss in the limbs, they have only been relatively recently adopted to assess the denervation of the tongue (44) and further development is required for their acceptance to clinical practice.

Clinical Neuroimaging

While brain imaging is not required to establish the diagnosis of ALS, MRI is commonly used as part of the diagnostic work-up to rule out alternative neurological conditions which may mimic ALS (45, 46). In bulbar onset patients the careful evaluation of the brain stem for structural, neoplastic, vascular, inflammatory and infiltrative processes is particularly important. Pathological processes superior to the brainstem; demyelination, neurovascular syndromes, neurosarcoidosis, leukodystrophies, malignancies, and neurodegenerative conditions may also manifest in bulbar symptoms if involving the corticobulbar tracts or the bulbar segments of the motor cortex. A number

TABLE 1 | Tools for diagnosis of bulbar signs or ALS.

Tool	Description	Advantages	Limitations	Recommendations
1. Cranial Nerve Examination	Bulbar motor UMN and LMN signs: weakness, fasciculations, atrophy, tone, reflexes	Well established; (18) easy to perform; reliability of items established in stroke, (19) structural disorders (20) and mixed neurological populations; (21) validated with respect to detection of dysphagia with VFSE	Not sufficiently standardized, subjective; measurement properties have not been evaluated in ALS	Individual items and the test as a whole require further standardization and better testing of measurement properties
2. Needle EMG, Genioglossus, Sternocleidomastoid (SCM), Trapezius	Indicates acute and chronic denervation in selected muscles, LMN changes	Well established, helpful in the exclusion of mimics able to detect subclinical involvement; can be quantitative	Invasive, not well standardized across clinics, requires substantial training, low sensitivity due to difficulty with relaxation (tongue<SCM<trapezius); (22) commonly qualitative	Requires further standardization and establishment of quantitative measures
3. Clinical MRI of bulbar regions (e.g., brainstem, bulbar region of the PMC)	The clinical role of MRI is to rule out neurological mimics, tongue and pharyngeal pathology	Widely available, noninvasive, potentially sensitive to prodromal stages of bulbar disease	Qualitative, not formally assessed with respect to clinical utility in the diagnosis of early stages of bulbar disease	Requires further research effort in establishing clinical utility
4. Auditory perceptual assessment of dysarthria types	Detection of UMN vs. LMN signs in speech/ voice tasks; rating specified dimensions of voice/ speech quality on a Likert scale	Well-established method in dysarthria assessment in SLP	Specific set of ALS-relevant items is not established; (23, 24) may not be equally sensitive to all dysarthria severities; requires specialized training; not well utilized in neurology; lengthy; low reliability for some items (25)	Items require further identification and standardization in ALS as well as better testing of measurement properties
5. Frenchay Dysarthria Assessment	Comprehensive assessment of bulbar structure and function (goals and item overlap with #1 and #4)	Well-established method in dysarthria assessment in SLP; reliability is established (26)	Relatively lengthy; not specific to ALS; validation is limited	Items require further identification and standardization as well as better testing of measurement properties
6. Videofluoroscopic Swallowing Exam (VFSE)	"Gold standard" dysphagia assessment to directly visualize swallow safety and efficiency	Well established in ALS; showed sensitivity to prodromal stage of dysphagia and sensitivity to change (27–29)	Requires expensive instrumentation and highly trained personnel; involves radiation exposure (minimal); need for a separate and additional test	Recommended clinically in patients demonstrated high risk of dysphagia to diagnose and test impact of strategies for treatment planning
7. EAT-10	Screening tool for dysphagia; 10 items; self-administered, symptom-based	Validated and reliability assessed in a large non-ALS cohort; differentiated safe vs. unsafe swallowers in ALS; cut-off of 8 or higher indicates high likelihood of dysphagia (3.1 times); sensitivity 86%, specificity 76%; (30) quick and easy, low administrative burden for scoring	Subjective; may not be sensitive to early disease stages	Recommended for use in clinic as a screener for the presence of dysphagia in ALS.
8. 3oz Swallow Test	Screening tool for dysphagia; 3oz of water given to patient in a cup; Pass / Fail; fail includes inability to drink without stopping, cough or throat clear, "wet" voice	Validated in general patient populations with very high sensitivity but poor specificity; quick and easy to use	Not validated in ALS; may miss patients with sensory deficits (silent aspirators) and early signs of dysphagia, (31) could also overestimate dysphagia risk due to "maximum performance" nature of test	Requires further standardization as well as better testing of measurement properties
9. Voluntary Cough	Screening tool for airway defense physiologic capacity using airflow spirometry or peak cough flow meter	Objective, instrumental; validity, reliability, sensitivity (up to 90%) and specificity (up to 82%), depending on the measure, relative to VFS, have been established in ALS (32)	Requires instrumentation and a trained examiner; voluntary cough is mediated differently neurologically to a reflexive cough; effort dependent	Recommended for use in clinic as a screener to index airway defense capacity to expel tracheal aspirate or secretions in ALS

of extrapyramidal and cerebellar conditions may also present with localization-specific (ataxic, hypokinetic, hyperkinetic) dysarthria and imaging has a role to rule out gross striatal, nigral and cerebellar pathologies. The incidental identification of tongue tumors on MRI in patients with suspected ALS has also been reported (47). A number of radiological cues have been associated with ALS, such as high signal along the pyramidal tracts on T2 weighted or FLAIR imaging, low signal in the precentral gyrus on GRE/SWI, isolated motor cortex atrophy on T1W, but these qualitative visual cues are not specific to ALS and are not sensitive for diagnostic or monitoring purposes (48). Quantitative imaging studies of ALS on the other hand have successfully captured the cortical (UMN) components of bulbar dysfunction in a somatotopic distribution (49, 50) and characterized the pathological substrate of pseudobulbar affect (51, 52). With relentless methodological (53) and conceptual advances in neuroimaging (54), the establishment of multicenter data repositories (55) and the increasing availability of 7 Tesla systems (56), the anatomical underpinnings of bulbar dysfunction are likely to be characterized in further detail.

Auditory-Perceptual Dysarthria Evaluation and Frenchay Dysarthria Assessment

"The Mayo Clinic" method of dysarthria categorization involves auditory-perceptual evaluation of specific voice and speech features during a passage reading, phonation of /a/, and oral dysdiadochokinesis (DDK) with /pa, /ta/, /ka/, and /pataka/ (57–59). The identification of "harsh," "strained," or "strangled" voice quality, slow speaking rate and "excess and equal" stress pattern during passage reading and DDK are typically linked to UMN dysfunction and "spastic dysarthria." "Breathy" or weak voice, hypernasality, nasal emissions, and articulatory imprecision without changes in speaking rate are classically associated with LMN dysfunction and "flaccid dysarthria." ALS is typically characterized by mixed spastic-flaccid dysarthria presenting with articulatory imprecision, hypernasality, harshness, slow rate and prosodic abnormalities. Although the reliability of observational assessments have been repeatedly questioned (10), protocol standardization, assessor training, and reference samples are thought to improve assessment reliability (60). Despite these efforts, auditory-perceptual assessment remains surprisingly underutilized, requiring standardization of practices, psychometric evaluation and multi-center validation in ALS.

Tools like the Frenchay Dysarthria Assessment (FDA) (26) are particularly well-suited for diagnostic purposes as they can comprehensively assess both structure and function of the bulbar musculature through a combination of CNE items and the auditory-perceptual dysarthria assessment. However, FDA was not specifically developed for ALS, and the evaluation of its measurement properties in ALS is lacking. DDK, which is included in CNE, FDA and perceptual dysarthria assessments, is commonly used to track disease progression, and has shown high sensitivity but low specificity for detecting bulbar signs in the prodromal phase of bulbar ALS (61, 62). With further optimization, DDK may have a diagnostic potential, particularly if certain performance constrains are imposed or its complexity is increased (63, 64).

Dysphagia Diagnosis and Screening

Videofluoroscopic swallowing evaluation (VFSE) remains the gold standard of dysphagia assessment in most neurological conditions allowing the direct visualization of swallowing safety and efficiency i.e., aspiration and the presence of residue, respectively. In ALS however, VFSE is underutilized (16) due to a number of factors such as the presumed lack of therapeutic relevance, lack of access to equipment or perceived patient burden etc. A number of screening tools have been recently evaluated for the early identification of those at risk for dysphagia in ALS. Currently, the Eating Assessment Tool-10 (EAT-10) demonstrated good sensitivity and adequate specificity for detecting aspiration in ALS (30), while instrumental measures of airflow during voluntary cough showed excellent sensitivity and specificity to detect aspiration (32). The bedside 3oz water swallow test is also extensively utilized, but its measurement properties in ALS are still unknown. There is a general consensus among SLPs that patients who fail dysphagia screening should be further evaluated by instrumental techniques to directly visualize the swallowing process using VFSE or fiberoptic evaluation of swallowing (FEES) techniques (65). This is an important consideration given the high incidence of "silent" aspiration in this patient population. Instrumental assessments, not only confirm the diagnosis of dysphagia, but inform on swallowing safety, help to identify the specific etiology of dysphagia, and guide therapeutic strategies that can be tested during the instrumental exam by directly visualizing their impact.

TOOLS FOR DISEASE MONITORING—STAGING AND LONGITUDINAL TRACKING

Certain bulbar measures have been optimized to track the decline of bulbar function in individual patients and entire cohorts. **Table 2** summarizes proposed bulbar monitoring tools in ALS.

Bulbar Monitoring (Overall)

A recent clinical practice survey of ALS care in the United States revealed that the Revised ALS—Functional Rating Scale (ALSFRS-R) bulbar sub-score, clinician or patient administered, represented the only measure routinely used to evaluate bulbar dysfunction in the clinical setting (16). It contains only 3 questions to address changes in speech, swallowing and salivation that are each merely rated on a four-point ordinal scale. While the total ALSFRS-R score is thought to have excellent reliability, the measurement properties of the individual sub-scores (e.g., bulbar) have not been specifically evaluated to date (66, 67, 91, 92). The Center for Neurologic Study-Bulbar Function Scale (CNS-BFS) is a 15-item questionnaire of bulbar involvement which has recently been validated against the ALSFRS-R and "timed" speech and swallowing tasks, and has already been successfully utilized in a clinical trial (71, 72). However, the CNS-BFS still needs to be validated against VFSE.

The Appel scale is one of the best characterized tools to track ALS-associated impairment and functional decline (73). Other clinician-administered instruments include the Norris (74), Tuffs (93), and Charing Cross (94) scales, but their original

TABLE 2 | Tools to measure bulbar dysfunction severity and disease progression.

Tool	Description	Advantages	Limitations	Recommendations
1. ALSFRS-R, bulbar sub-score	Tracks bulbar disease progression; 3 "bulbar" questions; 0 (no function)—4 (normal function)	Quick and easy to perform; patient and caregiver versions available; well validated as a total score; (66) recent studies suggest using "domain-specific" subscores instead of a total score; (67, 68) declines linearly; an accepted end point in clinical trials	Limited assessment of bulbar dysfunction; symptom report; may underestimate sisease severity; (69) changes relatively late in the disease; skewed to detection of the LMN impairment (70)	Recommended for use in clinic and clinical trials but caution due to limited nature of bulbar assessment
2. Center for Neurologic Study-Bulbar Function Scale (CNS-BFS)	Reports solely on bulbar symptoms, 21 questions regarding speech, swallowing and salivation	Validated-high criterion and construct validity, (71) good reliability, responsive to change over time and improved in a clinical trial (72)	Symptom report; potentially not sensitive to early phases of the disease; need further validation against VFSE	Recommended for bulbar evaluation in clinic and clinical trials
3. Appel scale	Includes 5-point ratings of functional status of speech and swallowing (scores 6–30) and bulbar disease progression	Reliable; responsive to disease progression (linear decline); the composite score distinguishes slow from fast progressors, predicts survival, provides bases for clinical classification with management recommendations depending on severity (73)	Validation is limited to date; includes only 2 questions related to bulbar function-—1 speech and 1 swallowing	Requires further evaluation of measurement properties; limited for the assessment of bulbar dysfunction
4. Norris scale	34-item ranking system; (74) 6 items in the "bulbar" category (i.e., chew, swallow, speak, jaw jerk, atrophy face/ tongue, lability) on a 3-point scale for each item	Quick and easy to administer; includes a range of bulbar items; has been used in clinical trials (75)	All items (functional and non-functional) rated equally; 3-point scale might be too coarse to detect change; limited information on the development and validation of the tool; responsiveness not established	Requires further evaluation of measurement properties; limited for the assessment of bulbar dysfunction
5. ALS Severity Scale (ALSSS)	10-point staging scale; was designed to supports management/rehabilitation practices in ALS; includes 1 speech and 1 swallowing item (0 to 10)	Easy to perform; clear description of each stage; adequate reliability; sig correlations with timed tests and speech intelligibility; responsive to change over time (76)	Ordinal scale; includes only 1 speech and 1 swallowing item	Requires further evaluation of measurement properties
6. Neuromuscular Disease Clinical Status Scale (NdSSS)	8-stage dysphagia severity scale to track the development of symptoms of dysphagia over time	Quick and easy to administer by a trained clinician; reliability, concurrent validity relative to other scales and responsiveness reported (77) and adequate	Focused predominantly on description of intake/ diet; not validated against VFSE	Although promising, requires further evaluation of measurement properties in other cultures
7. Oral Secretion Scale (OSS)	5-point scale to evaluate the severity of sialorrhea in ALS	Quick and easy to administer; validated against ALSFRS-R bulbar subscore and SSS; adequate reliability; can be used by different professions (78)	Floor effect in the more severely involved individuals; responsiveness not assessed; not linked to dysphagia outcomes	Recommended for evaluation of severity of sialorrhea in clinic and in clinical trials
8. Sialorrhea Scoring Scale (SSS)	9-point scale to evaluate the severity of sialorrhea	Quick and easy to administer; validated against ALSFRS-R bulbar subscore and OSS; adequate reliability; can be used by different professions; better spread of scores across the severity range compared to OSS (78)	Responsiveness not assessed; reliability was somewhat lower than for OSS; not linked to dysphagia outcomes	Recommended for evaluation of severity of sialorrhea in clinic and in clinical trials

(Continued)

TABLE 2 | Continued

Tool	Description	Advantages	Limitations	Recommendations
9. Sentence Intelligibility Test—Speech Intelligibility and Speaking rate	% of words understood by a listener during a sentence transcription task, and number of words produced per minute (WPM)	Easy to perform; supported by software; validated in multiple studies with respect to ALSSS (79) and ALSFRS-R; decline in rate to 125 WPM predicts intelligibility drop and is used to time AAC interventions; WPM changes linearly with disease progression (80, 81)	Requires a trained SLP; requires a trained transcriber; low sensitivity to early bulbar disease; (61) declines over 12% in sentence intelligibility and 37 WPM are outside of measurement error (82)	Speaking rate is recommended to be tracked during clinic in order to plan AAC interventions for those at risk for loss of speech intelligibility
10. Timed tests: Speech and pause durations in a passage*	A passage reading task (e.g., Bamboo) (83) allows a separation between speaking and pause events; gives a detailed picture of the components of speaking rate	Easy to perform; allows practice to minimize reading errors; distinguished patients with ALS with bulbar and respiratory signs; (84) showed sensitivity to change in a drug trial (85)	Currently requires time consuming, by-hand measurements; requires training; measurement properties (e.g., responsiveness, measurement error) are not well established; bulbar effects need further differentiation from respiratory and cognitive effects	Requires further standardization as well as better testing of measurement properties; subsequently would benefit from automation
11. Times tests: DDK*	A syllable repetition task (pa; ta; ka; pa-ta-ka) in syllables per second (syl/sec) that is used to detect slowing of the oral movements	Easy to perform; clinicians are familiar with the task; easily measured instrumentally; free of cognitive-linguistic effects; distinguishes slow from fast progressors; (86) cut off 4.6 syl/s 91% sensitivity and 54% specificity in detecting bulbar signs in pre-symptomatic patients (61)	Requires training/ modeling and maximum effort from patients; measurement properties are not fully established (e.g., responsiveness; error of measurement)	Requires further standardization as well as better testing of measurement properties; subsequently would benefit from automation
12. Maximum Tongue Pressure (MTP)*	A measure of tongue strength using a commercially available devices	Affordable easy to use clinical tool; validated against ALSFRS-R bulbar subscore and VFSE; cut off <21 KPa has sensitivity 80% and specificity 100% for detecting bulbar dysfunction on ALSFRS-R (87) and oral dysphagia; adequate reliability; independent prognostic factor of survival (88)	Requires training of the clinician and patient prior to measurement—results are placement dependent; (89) requires max effort; insufficient data on responsiveness; (90) not associated with dysarthria and speech intelligibility loss	Requires further standardization as well as testing of measurement properties

May be used for diagnostic purposes.

development, optimization and validation studies can be difficult to acquire and subsequently, their performance is relatively difficult to judge. The ability of these instruments to represent specific stages and their potential to track progressive bulbar impairment is largely unknown. A number of global ALS staging systems have been developed recently, such as the King's clinical staging system, the Milano-Torino (MiToS) functional staging, the Fine'til 9 (FT9) framework (95, 96), but bulbar impairment is just a small component of these instruments. Among the staging tools, the ALS Severity Scale (ALSSS) is particularly noteworthy, as it uses a 10-point scale for two bulbar functions, speech and swallowing. It was designed to guide rehabilitation efforts in ALS and, and pending formal psychometric evaluation, it may prove to be particularly useful (97).

Functional Monitoring of Dysphagia and Oral Secretion Scales

The Neuromuscular Disease Clinical Status Scale (NdSSS), which focuses solely on dysphagia, underwent one of the most rigorous psychometric evaluations to date. This tool exhibited excellent inter- and intra- rater reliability and correlated well with the functional oral intake scale (77). It has not been validated against VFSE yet, and given the potential for considerable geographical differences in oral intake, it is unclear how this tool may be validated around the world. While there are several tools to assess sialorrhea in ALS, such as the Oral Secretion Scale (OSS) and Sialorrhea Scoring Scale (SSS) available (78), these also need comprehensive psychometric evaluation and validation.

Functional Monitoring of Dysarthria

"Speech intelligibility" refers to the degree to which a speaker is understood by a listener, and "speaking rate" refers to speaking speed. Although both of these measures can be assessed on a 5 or 7-point Likert scale (49), the Sentence Intelligibility Test (SIT) is often preferred by SLPs, as it provides a more fine-grained estimate of speech intelligibility (i.e., percent of words transcribed correctly) and speaking rate (i.e., number of words produced per minute) (98). Speech intelligibility is considered abnormal when it falls below 97%, and speaking rate is considered abnormal below 160 words-per-minute (WPM) (99, 100). Speech intelligibility is a general indicator of the severity of dysarthria and it declines relatively late in the course of the disease (101). Speaking rate typically declines prior to significant changes in speech intelligibility, and it changes more linearly with symptom duration than speech intelligibility. Therefore, speaking rate is particularly useful in monitoring bulbar impairment longitudinally (102, 103). A speaking rate of 125 WPM or less is the recommended cut off for to trigger referral to the augmentative and alternative communication services (99).

Digital speech recordings and automated analyses can provide new opportunities for in-depth, observer-independent evaluations, especially during a passage reading and syllable repetition (DDK) tasks. In passage reading tasks, such as the Bamboo Passage, which has been specifically developed to support automatic analyses, certain phrases are semi-automatically identified, and speech duration and pause intervals can be accurately quantified (83). The measures derived from this analysis e.g., percentage pause time, mean phrase duration etc. have been identified to be sensitive to the prodromal stages of bulbar dysfunction (61) and also showed to detect response to pharmaceutical interventions such as dextromethorphan/quinidine (Nuedexta) therapy (85). A recent longitudinal study suggested that the main advantage of the DDK tasks may be in their ability to reliably distinguish slow- and fast-progressors (86).

Physiological Monitoring

Muscle strength testing in ALS has been initially performed using force transducers (strain gauges) (104, 105) and later with pressure bulbs via the Iowa Oral Performance Instrument (IOPI) (IOPI Medical LLC) or TPM-01 (JMS, Hiroshima). Lingual pressure testing using the IOPI revealed adequate reliability of a maximum tongue pressure estimate (MTP, or maximum anterior isometric pressure, MAIP) but not for the measure of endurance (89). Only one study assessed longitudinal changes in MTP in ALS to date (90) and reported its decline in patients with bulbar onset within 3 months and for those with spinal onset within 6 months. Tongue strength has also been shown to be an independent predictor of survival (88); however, formal psychometric evaluation is awaited to determine the MTP's utility to measure progressive changes over time.

DISCUSSION

In order to firmly establish the clinical utility of specific bulbar instruments and their potential as outcome measures in clinical trials, their measurement properties need to be comprehensively characterized. Among the diagnostic dysphagia instruments, screening tools, such as EAT-10 and voluntary cough (30, 32) have been well evaluated. Among speech measures, only DDK rate came close to demonstrating diagnostic utility (61). The remaining tools require extensive evaluation with regards to their diagnostic accuracy. While a large number of novel assessment tools have been proposed to track the progression of bulbar impairment, only the ALSFRS-R, the CNS-BFS and some bulbar staging systems (e.g., NdSSS, OSS, SSS) meet at least basic measurement requirements. Most existing disease monitoring tools lack the ability to capture subtle progressive changes, which is indispensable for disease tracking tools. Robust systematic psychometric evaluation is needed to improve the currently available clinical, academic and pharmacological-trial assessment tools.

Despite the gaps in the current literature and the limitations of current clinical trial designs, we are likely to witness considerable advances in standardized bulbar assessments and the emergence of purpose-designed, disease-specific, well-validated bulbar assessment tools. Emerging technologies such as quantitative neuroimaging, muscle ultrasound, electrical impedance myography (EIM), high-resolution manometry, videomanofluoroscopy, and speech acoustic monitoring are likely to soon complement our armamentarium of clinical tools. A number of promising imaging techniques have already been utilized to characterize the pathological substrate of bulbar impairment in ALS including diffusion tensor imaging (106, 107), cortical thickness measurements (50, 108, 109), morphometry-type analyses (49, 110), magnetization transfer

ratio imaging (106), MR spectroscopy (111), MRI intensitometry (112), and task-based functional MRI (113, 114). Despite these advances, MRI-derived metrics remain underutilized in the clinical setting and as outcome measures in pharmacological trials. This is in sharp contrast with clinical trials in Multiple Sclerosis, where MRI plays an established role as a key outcome measure in phase III clinical trials (115). Muscle ultrasound may capture tongue fasciculations in the absence of fasciculation potentials on EMG and the combination of ultrasound and EMG may help the detection of early denervation (116). Likewise, EIM shows promise in detecting changes in the structural composition of the tongue in ALS and may evolve into an important tool to detect early bulbar involvement (117, 118). High-resolution manometry and videomanofluoroscopy may provide unique insights into the dynamics of bolus movement and swallowing pressures enabling early detection of bulbar dysfunction and thus, timely interventions (119, 120). Acoustic analysis of speech has been proposed as a means for the objective assessment of bulbar impairment for over two decades, but until recently extracting these measure has been extremely time consuming. Recent developments in automatic audio and video analysis methods and smart phone technologies make speech analysis technologically feasible, enabling observer-independent multiparametric analyses (121–123). These emerging methodologies will need careful development, optimization and evaluation according to established methodological guidelines (e.g., COSMIN framework).

CONCLUSIONS

Recent advances in neuroimaging, development of staging systems, patient-reported outcome measures and the emergence of novel instrumental speech and swallowing assessment techniques promise novel insights into bulbar dysfunction in ALS. However, in order for these methods to be integrated into routine clinical practice and pharmacological trials, they have to be rigorously evaluated with respect to their measurement properties, diagnostic performance and longitudinal tracking abilities. The establishment of large international collaborations and relentless biomarker research efforts give cause for optimism for the development of validated bulbar assessments, which in turn will contribute to best practice recommendations, enable well-timed clinical interventions and facilitate accurate patient stratification in clinical trials.

AUTHOR CONTRIBUTIONS

YY and PB reviewed references, drafted the manuscript, and generated the tables. EP provided expert opinion on dysphagia assessments and edited the drafts of the manuscript. JG provided expert opinion on dysarthria assessments and edited the drafts of the manuscript. CB provided expert opinion on measurement development and edited the drafts of the manuscript.

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REVIEW
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Diaphragmatic Neurophysiology and Respiratory Markers in ALS

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The main reason for short survival in amyotrophic lateral sclerosis (ALS) is involvement of respiratory muscles. Severe compromise of diaphragmatic function due to marked loss of motor units causes poor inspiratory strength leading to symptomatic respiratory fatigue, and hypercapnia and hypoxemia, often firstly detected while sleeping supine. Weakness of expiratory muscles leads to cough weakness and poor bronchial clearance, increasing the risk of respiratory infection. Respiratory tests should therefore encompass inspiratory and expiratory function, and include measurements of blood gases during sleep. Non-volitional tests, such as phrenic nerve stimulation, are particularly convenient for investigating respiratory function in patients unable to perform standard respiratory function tests due to poor cooperation or facial weakness. However, SNIP is a sensitive test when patients with bulbar involvement are able to perform the necessary maneuvers. It is likely that central respiratory regulation is disturbed in some ALS patients, but its evaluation is more complex and not regularly implemented. Practical tests should incorporate tolerability, sensitivity, easy application for regular monitoring, and prognostic value. Impending respiratory failure can cause increased circulating inflammatory markers, but molecular assessment of respiratory distress requires further study. In future, home-monitoring of patients with accessible devices should be developed.

Keywords: amyotrophic lateral sclerosis, diaphragm physiology, progression, respiratory function tests, survival

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INTRODUCTION

Respiratory insufficiency (RI) in ALS usually emerges in the late stage of disease progression, although it may sometimes be the presenting feature (1, 2). Respiratory complications, especially hypoventilation (2), reduced bronchial clearance and lung infection (3) account for the majority of deaths in ALS. Mild respiratory involvement causes fatigue in daily-life activities and disruption of sleep, with negative impact on quality of life (4, 5) and hypoxemia may impair cognitive function (6), especially important in a population with a lower cognitive reserve.

ALS affects both inspiratory and expiratory muscles, as well as upper airway muscles (7). Cough, an essential reflex for airway protection and clearance, depends on effective glottis closure and efficient expiratory muscle function. Bulbar muscle dysfunction impairs the former and, for this reason, cough effectiveness is not always correlated with expiratory muscle weakness (8). Weakness

of pharyngeal and laryngeal muscles increases the risk of aspiration and lung infection. The latter is more critical when associated with marked cough deficiency. For active inspiration the diaphragm is the most important muscle, although other muscles function as accessory muscles of inspiration, e.g., sternocleidomastoid, scalenus, trapezius, external intercostal, pectoralis, and paraspinal muscles. These are particularly important when the diaphragm is weak and during exercise. Severe diaphragm weakness leads to hypoxemia and carbon dioxide retention, since the work capacity of these accessory muscles is not sufficient to compensate. Furthermore, these muscles are themselves progressively involved in the disease process (2). In ALS, the major reason for frank respiratory failure is involvement of the diaphragm (2). The phrenic nerve motor nuclei in the cervical spinal cord are located in a region early affected in ALS, shown by early morphometric changes in these neurons (9). Dyspnea in ALS is closely correlated with diaphragmatic dysfunction (10). Indeed, diaphragm weakness as assessed by the evoked response to transcutaneous phrenic nerve stimulation is predictive of hypoventilation (11) and survival (12). It is therefore relevant to assess the physiology of the diaphragm in people with ALS.

DIAPHRAGM PHYSIOLOGY

The diaphragm is the most important muscle of ventilation. It is a dome-shaped muscle that separates the thoracic and abdominal cavities. It has a musculo-fibrous structure, formed by a central non-contractile fibrous region, and contractile muscle fibers that radiate circumferentially from the central tendon to attach peripherally to the upper three lumbar vertebrae posteriorly (crural diaphragm) and onto the inner surface of the lower six ribs and costal cartilages antero-laterally (costal diaphragm). In humans, the diaphragm comprises approximately equal numbers of type I and type II fibers, but these muscle fibers are smaller than in the expiratory muscles. They have a rich capillary supply and are resistant to aging (13). Muscle spindles are present only in small numbers in the diaphragm (14), so muscle stretching does not much modulate phrenic neuronal excitability. Diaphragm is well adapted to the rhythmic continuous periodical inspiration of ventilation and to ocassional more forceful contractions, as in deep breaths and coughing. The mean diaphragm thickness at the point of functional residual capacity is 2.29 $\pm$ 0.4 mm, as measured by ultrasound (15) but is variable over its surface, and also dependent on body position. Diaphragmatic thickness can increase two-fold during full inspiration (16).

The motor innervation of the diaphragm is almost exclusively from the phrenic nerve (C3–5), which branches to innervate the entire muscle. Contraction of the diaphragm causes axial descent of the dome of the muscle, decreasing intrapleural pressure, and increassing intrabdominal pressure, thus exerting an expansive force on the lower thorax (17). This negative intrathoracic pressure causes an inflow of air to the lungs, promoting inspiration. The diaphragm is a very mobile muscle. With full inspiration it flattens, expanding the thorax down to the level of costal margin anteriorly, and during forced expiration it rises anteriorly to the level of the fourth or fifth intercostal space.

There is appreciable force reserve in the diaphragm. In humans the maximum transdiaphragmatic pressure is about 11 kPa, which more than 10 times the value measured during eupnea (18). Indeed, normal respiration activates fatigue-resistant slow-units (19). However, coughing and sneezing are demanding maneuvers requiring very strong diaphragmatic contraction, close to 50% of the maximum transdiaphragmatic pressure, which implies activation of fast-fatigable motor units (19).

During calm breathing at rest expiration, unlike inspiration, is a passive phenomenon resulting from the relaxation of the inspiratory muscles and reduction of lung compliance. However, active forced expiration relies on recruitment of expiratory muscles, namely the internal intercostals and the abdominal ventro-lateral muscles (20). Generation of an adequate expiratory flux is needed for coughing, sneezing or vomiting. This is only possible with strong inspiration, closure of the glottis, and a sudden increase of intra-abdominal and intra-thoracic pressures. Effective peak cough flow (PCF) in healthy subjects exceeds 360–400 L/min (21). Peak flow values >160–200 L/min are needed for effective mucus expectoration (22) and values above 250–270 L/min are required to prevent aspiration pneumonia in patients with neuromuscular disorders (23).

The inspiratory pace-maker is located in the pre-Bötzinger Complex in the medulla (24). Its activity, both during inspiration and expiration, is modulated by inhibitory pre-motor neurons and by the Bötzinger Complex (18). Although expiration is a passive movement, active expiration involves a rostral generator, the retrotrapezoid nucleus (25). Synaptic drive to phrenic nerve nuclei is derived from pre-motor neurons located in the ipsilateral ventrolateral and dorsomedial medullary tracts, which respond to central chemoreceptors, sensitive to hypercapnia, and peripheral chemoreceptors, especially the carotid bodies, that are sensitive to hypoxemia. These premotor neurons are also sensitive to sleep-wake state modulation (18). Spinal interneurons can modulate phrenic motoneuronal activity, in particular via intercostal muscle afferents signaling strain of the chest wall (26). Voluntary control of breathing depends on fast, direct corticospinal inputs, which are also critical for respiratory control during speech (18). This pathway can be investigated by magnetic stimulation of cortical areas.

ASSESSMENT OF RESPIRATORY DYSFUNCTION IN ALS

In ALS inspiratory and expiratory muscles, as well as upper airway muscles are progressively involved. Studies of a possible dysfunctional central respiratory drive are few, but it is likely this could be affected in some patients with ALS (27). As such, different tests are necessary to provide a global view of the respiratory function of diseased subjects. The American (28) and the European (29) guidelines agree that a first respiratory evaluation should be made at the baseline clinical assessment and then periodically thereafter. Nevertheless, this must be adjusted individually, according to the rate of progression of

the disease and when there are intercurrent events, such as infection, that may affect respiratory function. A summary of the available tests, their utility and limitations is provided in **Table 1**.

Global Respiratory Evaluation

Forced vital capacity (FVC) is a non-invasive respiratory test that has long been used in ALS. It assesses both the inspiratory and expiratory loops, requiring expiration done forcefully after a maximal inspiration, as opposed to slow vital capacity (SVC). This test is sensitive to change and predictive of hypoventilation and survival in ALS (30). The change of FVC is an adequate test to follow ALS patients (30, 31), since its decline rate tends to be linear (∼3.5/month), there is a high interpatient variability (32) but this rate is a strong predictor of survival (33). FVC can be an unreliable measure of ventilatory function in patients with bulbar involvement due to orofacial weakness, due to air leakage around the mouthpiece (2). FVC is more sensitive in detecting diaphragmatic weakness when performed in the supine position (34), but this position is often poorly tolerated due to secretions or to the extent of diaphragmatic weakness. In addition, it is not a very sensitive test to detect hypercapnia, since gas exchange is well maintained until FVC values are very low (35). SVC is easier to perform in patients with bulbar involvement, because the air is exhaled slowly, with less air-leakage around the mouthpiece. SVC has been preferred in a number of recent trials, as it is very strongly correlated with FVC (and with other respiratory tests such as Maximal Inspiratory Pressure and Maximal Expiratory Pressure), as well as with ALSFRS-R (36). It is a predictor of progression, the need for positive pressure ventilation, and survival in ALS (37, 38).

Maximal voluntary ventilation (MVV) assesses respiratory function on maintained efforts. The patient is asked to breathe in and out, through a mounthpiece, as deeply and quickly as possible during 12 s, for at least two trials (39). The value is extrapolated for 1 min. The test is demanding for ALS patients, due to their respiratory fatigue. It can be a sensitive measure of disease progression (30), but only in the early stages of the disease (39). This test is rarely performed in daily practice.

Nocturnal pulse oximetry (NPO) is a useful, non-invasive, inexpensive, and convenient method, which accesses respiratory function in a demanding state—when patients are lying and sleeping. It can be used individually or during polysonography, the latter allowing for clear characterization of possible central and/or peripheral apnea. NPO assesses percutaneous oxygen saturation (maximum, median, and minimum values), in relation with heart rate. Further, the pattern of the oxygen saturation curve overnight can be explored. NPO has been shown to be predictive of survival in ALS (40, 41). In addition, it can indicate central drive dysfunction in patients with normal respiratory muscles, a factor that is probably more common in spastic patients (42). NPO is a mandatory method to follow non-invasive ventilation adaptation in patients, which permits home-telemonitoring and distance alteration of ventilatory settings (43). Transcutaneous capnometry (PtcCO2) is a more modern approach to evaluate respiratory function in ALS and other neuromuscular disorders (44). PtcCO2 recordings show strong correlation with arterial measurements. A value higher than 49 mmHg during ≥10% of the total recording time indicates respiratory insufficiency (44). Transcutaneous capnography has been strongly recommended for detection of nocturnal hypoventilation in patients with ALS (45). In patients on non-invasive ventilation, PtcCO2 can be helpful to monitor a proper ventilation, in particular to differenciate between hypoventilation and hypoxemia related to other reasons like as ventilation/perfusion mismatch, as well as in detecting hyperventilation (46). Both techniques have some limitations, for example they cannot discriminate other causes of sleep disturbances, such as obstructive sleep apnea, drug-effect, or associated lung disorder. Nonetheless they are very convenient as a screening method.

Blood gas measurements provide information about CO_2 retention and hypoxemia when respiratory failure is severe. Because respiratory assessment is desiged to evaluate early changes, this test is not extensively used in ALS; however, it can provide relevant information for respiratory management in some patients.

Sleep studies have been investigated for a long time in ALS. In this disorder, reduction of the rapid eye movement (REM) sleep stage is typically observed, in particular when the diaphragm is markedly affected and accessory respiratory muscles are weak (47). It has been speculated that disturbed REM sleep might protect patients from hypoventilation (48). However, in patients with preserved diaphragmatic function, signs of sleep hypoventilation are observed as frequently in REM and non-REM phases (27), probably due to reduced respiratory drive (42). Arnulf et al. (48) found that ALS patients with upper motor neuron involvement to respiratory muscles tended to have abnormal REM sleep and poor prognosis. There is a strong link between severity of respiratory function impairment, poor quality of sleep, and daytime somnolence, in ALS (49).

Evaluation of Inspiration

Maximal inspiratory pressure (MIP) and nasal inspiratory pressure during a maximal *sniff* (SNIP) are inexpensive and non-invasive respiratory measures that access maximal inspiratory muscular strength, the first against a mouth occlusion and the second using a plug inserted in one nostril (50–52). In both, it is necessary to secure cooperation from patients to breath forcefully against a resistance. While 3 consistent measures are necessary to determine MIP (53), the number rises to 10 for SNIP, 5 in each nostril (53, 54), as the result improves with practice. Fatigue is a limiting factor for both techniques. MIP is more sensitive than FVC in detecting hypoventilation (55). However, its marked early decline (floor effect) limits its use in following patients and it is difficult to perform in patients with orofacial weakness (56) or with spasticity. SNIP is a sensitive tool especially suited for ALS patients with orofacial weakness. It is predictive of survival (57) and of the onset of significant hypoventilation in spinal-onset patients (5). There is some uncertainty about the best technical approach to test ALS patients in order to obtain reliable values (58). SNIP seems to depend more on diaphragm force and MIP

TABLE 1 | A summary of the most relevant respiratory tests in ALS.

	Tolerability	Simplicity	Reliability	Sensitivity	Rate of change	Technical difficulty	Cost*	Ease for monitoring**	Experience in trials
GLOBAL									
FVC	++	++	++	+	++	Volitional. Limited by orofacial paresis and dyscognition.	++	++	+++
SVC	++	+++	++	+	++	Volitional. Limited by orofacial paresis and dyscognition.	++	++	+++
MVV	+	+	+	+?	+?	Volitional. Needs motivation; Limited by orofacial paresis, fatigue, and dyscognition.	++	+?	0
NPO	+++	+++	+++	++	+	Limited by cold hands or poor sleep.	+	+++	0
TCP	+++	+++	+++	++	+?	Limited by cold hands and poor sleep.	++	+++	0
Sleep studies	+	0	+	+++	+	Limited by poor sleep.	+++	+	0
INSPIRATORY TESTS									
MIP	+	+	++	+++	+++	Volitional. Limited by orofacial paresis, fatigue, and dyscognition; early floor effect.	++	+	0
SNIP	++	++	++	++?	++	Volitional. Limited by orofacial paresis and dyscognition.	+	++	+++
Diaphragm US	+++	+++	+++	++?	++	Limited by dyscognition.	++	++	0
Phrenic stimulation	+	++	++	+	++	Limited by electrical stimulation intolerance.	++	++	
EXPIRATORY TESTS									
PEF	++	++	++	+?	++	Volitional. Limited by orofacial paresis and dyscognition.	++	++	0
PCF	++	+++	++	+?	++	Volitional. Limited by orofacial paresis and dyscognition.	+	++	0
MEP	+	+	++	+++	+++	Volitional. Limited by orofacial paresis, fatigue, and dyscognition; early floor effect.	++	+	0
CENTRAL DRIVE FUNCTION									
P01	++	+	+	+?	+?	Volitional. Limited by orofacial paresis, fatigue and dyscognition.	++	+	0

FVC, forced vital capacity; SCV, slow vital capacity; MVV, maximal voluntary ventilation; NPO, nocturnal percutaneous oximetry; TCP, percutaneous capography; MIP, maximal inspiratory pressure; US, ultrasound; PEF, peak-expiratory flow; PCF, peak-cough flow; MEP, maximal expiratory pressure; P01, mouth occlusion pressure (100 ms).

** Cost (greater number of plus symbol means higher cost) was estimated taking into account equipament price and the requirement of a technician.*

*** Ease for monitoring was estimated considering patient confort and technical complexity.*

more on the sternocleidomastoid muscle power, making these tests complementary (59).

Transdiaphragmatic pressure (Pdi) can be assessed by inserting balloon catheters in the stomach and mid-esophagus and measuring the differential pressure during active maximal inspiration (60) or following stimulation of the phrenic nerve (61). This is an uncomfortable test that is not suited to clinical application.

Diaphragmatic ultrasound (US) is a non-invasive technique that assesses diaphramatic dynamics, and measures the muscle thickness at tidal volume and on maximal inspiration, as well as the ratio between baseline and maximal inspiration, useful measures to detect diaphragm involvement (62, 63). Significant correlations have been found between these measurements and FVC, SNIP, and the amplitude of the motor response of the phrenic nerve (62–64). However, ultrasound studies are less sensitive than phrenic nerve motor responses in assessing early deterioration of the diaphragm in ALS (65).

Phrenic nerve stimulation by percutaneous electrical or magnetic stimulation in the neck to elicit diaphragm motor responses is an objective, non-volitional test (66, 67) that can

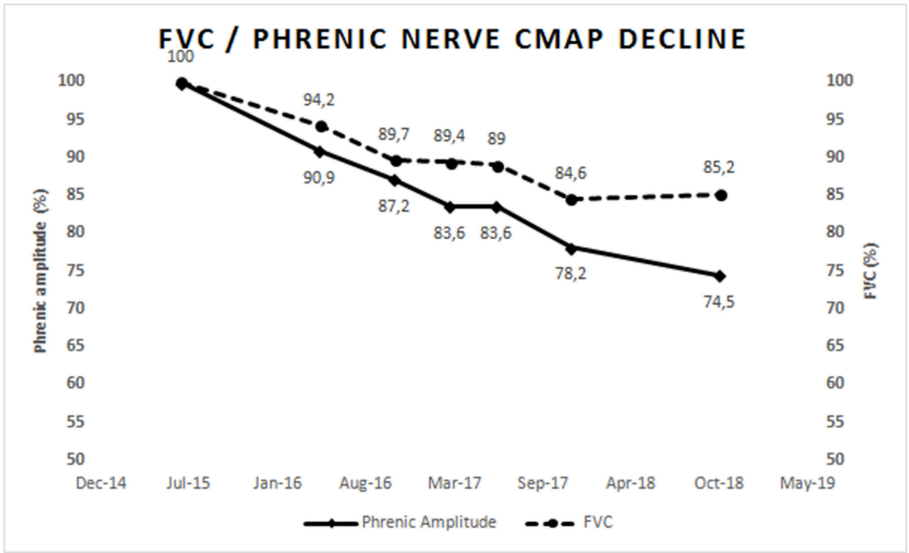

FIGURE 1 | Represents the progressive parallel decline of FVC and phrenic nerve compound muscle action potential amplitude in an ALS patient with slow progression. Values were normalized to 100% of normal at first assessment. This figure is simply for representative purposes and not intended to present research findings.

be used to assess the number of functional motor units in the diaphragm (68). Abnormal amplitude (or area) of the motor response has good predictive value for hypoventilation in both bulbar- and spinal-onset patients, and is correlated to FVC (11). This technique is useful in patients with marked facial weakness or in those unable to cooperate, for example those with fronto-temporal dementia. The amplitude of the motor response declines significantly over 3–6 months, and correlates with FVC and SNIP change (69); it is predictive of survival in ALS (12). **Figure 1** represents the progressive and parallel decline of FVC and phrenic nerve compound muscle action potential in an ALS patient.

Evaluation of Expiration

The efficiency of the expiratory muscles can be easily addressed by evaluating the peak expiratory flow (PEF) and the peak cough flow (PCF), and maximal expiratory pressure (MEP) evaluates the strength of these muscles. These three volitional tests are simple to perform, inexpensive, and non-invasive. Although they measure expiratory muscle function, they depend on central motor control as well as on the efficiency of the inspiratory muscles. Abnormally reduced values indicate inability to expel bronchial secretions, leading to a high risk of respiratory infections (39), leading to increased morbility and mortality (70). MEP values are measured by asking the patient to exhale forcefully against an ocluded mouthpiece. Abnormal values are common in ALS patients (8, 71), and correlate with inspiratory involvement. PEF and PCF use peak flow meters, coupled with a face mask for PCF testing, and assess the ability to exhale forcefully after a maximal inspiration (72, 73) and to cough after a submaximal inspiration (60). Coughing can also be assessed by the gastric pressure generated during a maximal cough, which is a sensitive method to assess expiratory muscle strength, but this is an invasive and poorly tolerated test (74).

Evaluation of the Central Respiratory Drive

Both NPO and sleep studies can detect respiratory center dysfunction, in particular in patients with normal respiratory muscles in whom nocturnal hypoventilation is detected without obstructive apnea (27). Inspiratory mouth occlusion pressure at 100 ms during quiet breathing (P0.1) is considered an indicator of respiratory drive. Spastic patients with normal diaphragm function tend to show abnormal P0.1/FVC values, associated with a poor prognosis for survival (42). P01 values are similar in bulbar and spinal-onset patients at presentation (56), suggesting that impaired central drive does not depend on the region of onset. The observation of "respiratory apraxia" in ALS patients highlights the complexity and importance of the cortical control of respiration and its potential involvement in ALS (75).

CONCLUSIONS

There are many tests available to evaluate different features of respiratory function in ALS. In general, most centers follow a conventional approach by evaluating SVC and FVC, which are are often applied in clinical trials. Patients may also be asked to undergo maximal pressure measurements, expiratory peak flows and nocturnal oximetry, sometimes associated with EEG recordings. Less commonly, diaphgram ultrasound or phenic nerve motor responses to percutaneous cervical electrical stimulation of the nerve are tested. Percutaneous capnography is emerging as a relevant technique. Disparity in patients'tolerability and technical limitations would recommend to apply more than one single test to assess respiratory function in ALS patients.

A future study combining most of these tests in a single set of ALS patients would provide more information about diagnostic accuracy, sensitivity, realibility, and convenience for monitoring disease progression. This would have major potential implications in clinical trials, since changing the rate of respiratory decline is critical for improving survival and functional capability.

The identification of a molecular marker of respiratory impairment in ALS would be a convenient and valuable test. Some research indicates that respiratory insufficiency can precipitate an inflammatory response (76, 77), and this is a new avenue yet to be fully explored. User-friendly devices for in-home respiratory evaluation is another future step. New tests to directly evaluate strength of respiratory muscles will require a better understanding of their physiology.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Machine Learning in Amyotrophic Lateral Sclerosis: Achievements, Pitfalls, and Future Directions

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Background: Amyotrophic Lateral Sclerosis (ALS) is a relentlessly progressive neurodegenerative condition with limited therapeutic options at present. Survival from symptom onset ranges from 3 to 5 years depending on genetic, demographic, and phenotypic factors. Despite tireless research efforts, the core etiology of the disease remains elusive and drug development efforts are confounded by the lack of accurate monitoring markers. Disease heterogeneity, late-stage recruitment into pharmaceutical trials, and inclusion of phenotypically admixed patient cohorts are some of the key barriers to successful clinical trials. Machine Learning (ML) models and large international data sets offer unprecedented opportunities to appraise candidate diagnostic, monitoring, and prognostic markers. Accurate patient stratification into well-defined prognostic categories is another aspiration of emerging classification and staging systems.

Methods: The objective of this paper is the comprehensive, systematic, and critical review of ML initiatives in ALS to date and their potential in research, clinical, and pharmacological applications. The focus of this review is to provide a dual, clinical-mathematical perspective on recent advances and future directions of the field. Another objective of the paper is the frank discussion of the pitfalls and drawbacks of specific models, highlighting the shortcomings of existing studies and to provide methodological recommendations for future study designs.

Results: Despite considerable sample size limitations, ML techniques have already been successfully applied to ALS data sets and a number of promising diagnosis models have been proposed. Prognostic models have been tested using core clinical variables, biological, and neuroimaging data. These models also offer patient stratification opportunities for future clinical trials. Despite the enormous potential of ML in ALS research, statistical assumptions are often violated, the choice of specific statistical models is seldom justified, and the constraints of ML models are rarely enunciated.

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Conclusions: From a mathematical perspective, the main barrier to the development of validated diagnostic, prognostic, and monitoring indicators stem from limited sample sizes. The combination of multiple clinical, biofluid, and imaging biomarkers is likely to increase the accuracy of mathematical modeling and contribute to optimized clinical trial designs.

Keywords: amyotrophic lateral sclerosis, machine learning, diagnosis, prognosis, risk stratification, clustering, motor neuron disease

1. INTRODUCTION

Amyotrophic Lateral Sclerosis (ALS) is an adult-onset multi-system neurodegenerative condition with predominant motor system involvement. In Europe, its incidence varies between 2 or 3 cases per 100 000 individuals (Hardiman et al., 2017) and its prevalence is between 5 and 8 cases per 100 000 (Chiò et al., 2013b). An estimated 450 000 people are affected by ALS worldwide according to the ALS Therapy Development Institute. While no unifying pathogenesis has been described across the entire spectrum of ALS phenotypes, the incidence of the condition is projected to rise in the next couple of decades (Arthur et al., 2016) highlighting the urgency of drug development and translational research. Given the striking clinical and genetic heterogeneity of ALS, the considerable differences in disability profiles and progression rates, flexible individualized care strategies are required in multidisciplinary clinics (den Berg et al., 2005), and it is also possible that precision individualized pharmaceutical therapies will be required.

Depending on geographical locations, the terms "ALS" and "Motor Neuron Disease" (MND) are sometimes used interchangeably, but MND is the broader label, encompassing a spectrum of conditions, as illustrated by **Figure 1**. The diagnosis of ALS requires the demonstration of Upper (UMN) and Lower Motor Neuron (LMN) dysfunction. The diagnostic process is often protracted. The careful consideration of potential mimics and ruling out alternative neoplastic, structural, and infective etiologies, is an important priority (Hardiman et al., 2017). ALS often manifests with subtle limb or bulbar symptoms and misdiagnoses and unnecessary interventions in the early stage of the disease are not uncommon (Zoccolella et al., 2006; Cellura et al., 2012). Given the limited disability in early-stage ALS, many patients face a long diagnostic journey from symptom onset to definite diagnosis which may otherwise represent a valuable therapeutic window for neuroprotective intervention. Irrespective of specific healthcare systems the average time interval from symptoms onset to definite diagnosis is approximately 1 year (Traynor et al., 2000). ALS is now recognized as a multi-dimensional spectrum disorder. From a cognitive, neuropsychological perspective, an ALS-Frontotemporal Dementia (FTD) spectrum exists due to shared genetic and pathological underpinnings. Another important dimension of the clinical heterogeneity of ALS is the proportion of UMN / LMN involvement which contributes to the spectrum of Primary Lateral Sclerosis (PLS), UMN-predominant ALS, classical ALS, LMN-predominant ALS, and Progressive Muscular Atrophy (PMA), as presented in **Figure 1**.

The genetic profile of MND patients provides another layer of heterogeneity. Specific genotypes such as those carrying the *C9orf72* hexanucleotide expansions or those with Super Oxide Dismutase 1 (*SOD1*) mutations have been associated with genotype-specific clinical profiles. These components of disease heterogeneity highlight the need for individualized management strategies and explain the considerable differences in prognostic profiles. Differences in survival due to demographic, phenotypic, and genotypic factors are particularly important in pharmaceutical trials so that the "treated" and "placebo-control" groups are matched in this regard.

With the ever increasing interest in Machine Learning (ML) models, a large number of research papers have been recently published using ML, classifiers, and predictive modeling in ALS (Bede, 2017). However, as these models are usually applied to small data sets by clinical teams, power calculations, statistical assumptions, and mathematical limitations are seldom discussed in sufficient detail. Accordingly our objective is the synthesis of recent advances, discussion of common shortcomings and outlining future directions. The overarching intention of this paper is to outline best practice recommendations for ML applications in ALS.

2. METHODS

Machine learning is a rapidly evolving field of applied mathematics focusing on the development and implementation of computer software that can learn autonomously. Learning is typically based on training data sets and a set of specific instructions. In medicine, it has promising diagnostic, prognostic, and risk stratification applications and it has been particularly successful in medical oncology (Kourou et al., 2015).

2.1. Main Approaches

Machine learning encompasses two main approaches; "supervised" and "unsupervised" learning. The specific method should be carefully chosen based on the characteristics of the available data and the overall study objective.

"Unsupervised learning" aims to learn the structure of the data in the absence of either a well-defined output or feedback (Sammut and Webb, 2017). Unsupervised learning models can help uncover novel arrangements in the data which

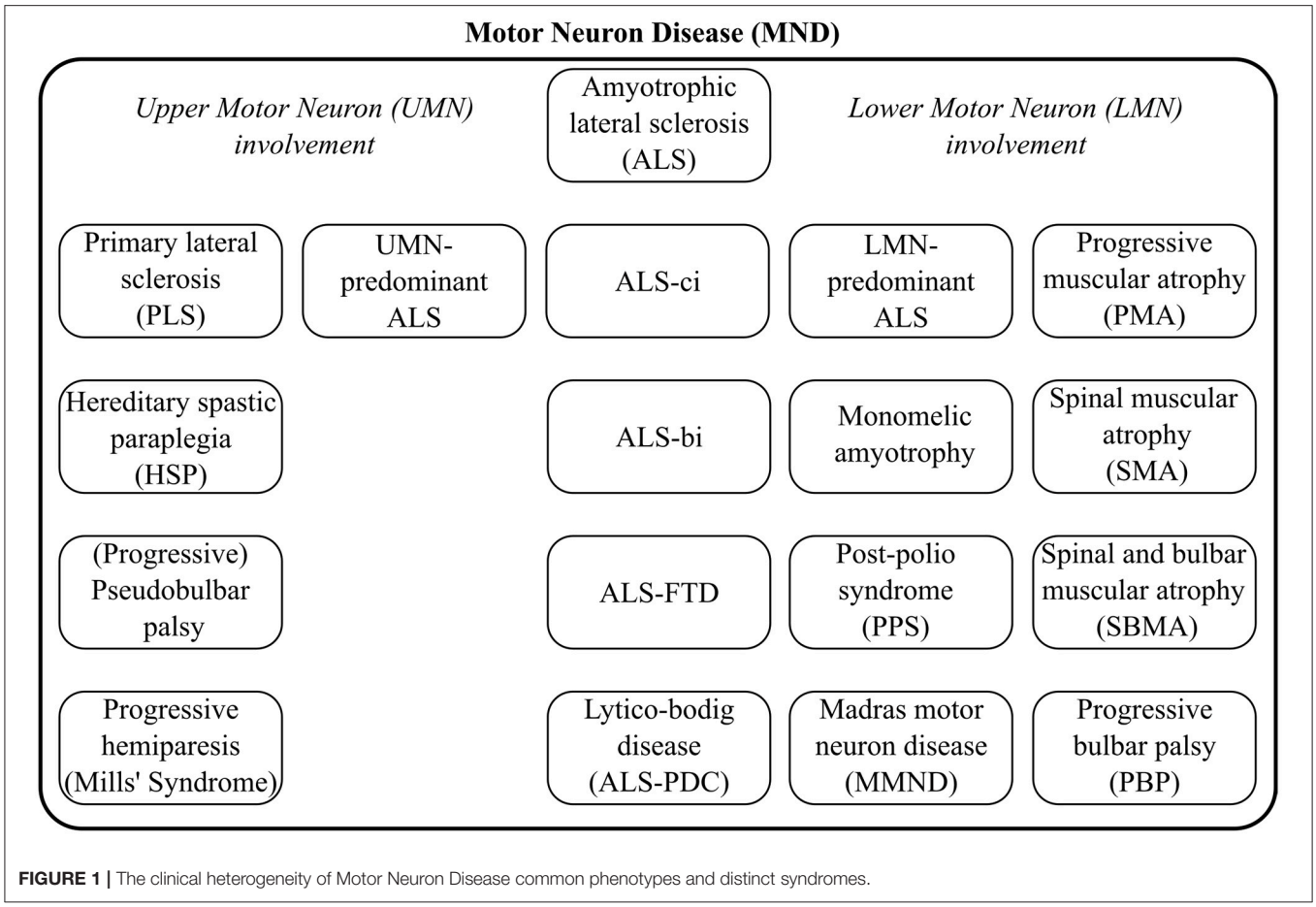

FIGURE 1 | The clinical heterogeneity of Motor Neuron Disease common phenotypes and distinct syndromes.

in turn can offer researchers new insights into the problem itself. Unsupervised learning can be particularly helpful in addressing patient stratification problems. Clustering methods can be superior to current clinical criteria, which are often based on a limited set of clinical observations, rigid thresholds, and conservative inclusion/exclusion criteria for class membership. The K-means algorithm is one of the most popular methods. It recursively repeats two steps until a stopping criterion is met. First, samples are assigned to the closest cluster, which are randomly initialized, then cluster centers are computed based on the centroid of samples belonging to each cluster. Unsupervised learning methods have been successfully used in other fields of medicine (Gomeni and Fava, 2013; Marin et al., 2015; Beaulieu-Jones and Greene, 2016; Ong et al., 2017; Westeneng et al., 2018). **Figure 2** represents an example of a patient stratification scheme using an unsupervised learning algorithm.

Supervised learning focuses on mapping inputs with outputs using training data sets (Sammut and Webb, 2017). Supervised learning problems can be divided into either classification or regression problems. Classification approaches allocate test samples into specific categories or sort them in a meaningful way (Sammut and Webb, 2017). The possible outcomes of the modeled function are limited to a set of predefined categories. For example, in the context of ALS, a possible classification task is to link demographic variables, clinical observations, radiological measures, etc. to diagnostic labels such as "ALS," "FTD," or "healthy." Schuster et al. (2016b), Bede et al. (2017), Ferraro et al. (2017), and Querin et al. (2018) have implemented diagnostic models to discriminate between patients with ALS and healthy subjects. Regression problems on the other hand, deal with inferring a real-valued function dependent on input variables, which can be dependent or independent of one another (Sammut and Webb, 2017). For instance, in the context of prognosis, a possible regression task could consist of designing a model which accurately predicts motor decline based on clinical observations (Hothorn and Jung, 2014; Taylor A. A. et al., 2016). When a regression task deals with time-related data sequences, often called "longitudinal data" in a medical context, it is referred to as "time series forecasting." The core characteristics of the data, which are most likely to define group-membership are referred to as "features."

2.2. Common Machine Learning Models

While a plethora of ML models have been developed and successfully implemented for economic, industrial, and biological applications (Hastie et al., 2009; Bishop, 2016; Goodfellow et al., 2017), this paper primarily focuses on ML

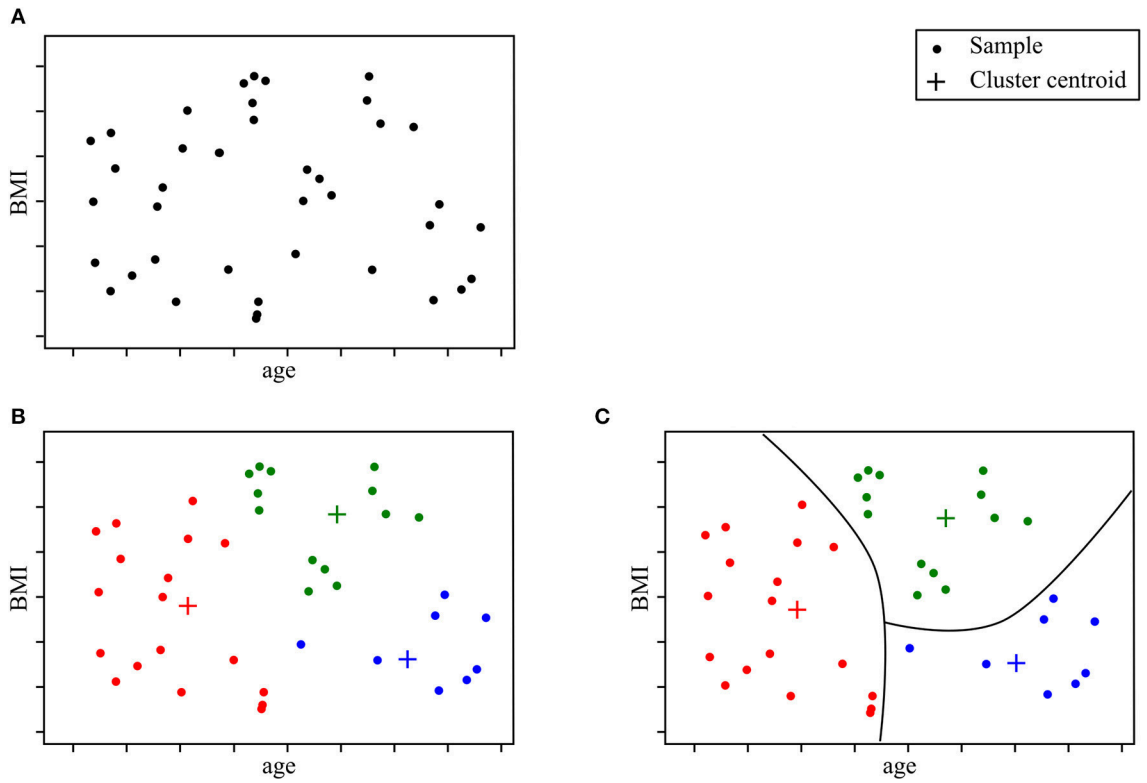

FIGURE 2 | Clustering model for patient stratification. The available data consist of basic clinical features; age and BMI. Given this specific ALS patient population, the objective is to explore if patients segregate into specific subgroups. After running a clustering algorithm, we obtain clusters and cluster memberships for each patient. Further analysis of shared traits within the same cluster can help identify novel disease phenotypes. **(A)** Initial data samples without output. **(B)** Identify cluster and cluster membership. **(C)** Stratify samples based on shared feature traits.

methods utilized in ALS research. These include Random Forests (RF) (Hothorn and Jung, 2014; Ko et al., 2014; Beaulieu-Jones and Greene, 2016; Sarica et al., 2016; Taylor A. A. et al., 2016; Ferraro et al., 2017; Fratello et al., 2017; Huang et al., 2017; Jahandideh et al., 2017; Seibold et al., 2017; Pfohl et al., 2018; Querin et al., 2018), Support Vector Machines (SVM) (Srivastava et al., 2012; Welsh et al., 2013; Beaulieu-Jones and Greene, 2016; Bandini et al., 2018; D'hulst et al., 2018), Neural Networks (NN) (Beaulieu-Jones and Greene, 2016; van der Burgh et al., 2017), Gaussian Mixture Models (GMM) (Huang et al., 2017), Boosting methods (Jahandideh et al., 2017; Ong et al., 2017), k-Nearest Neighbors (k-NN) (Beaulieu-Jones and Greene, 2016; Bandini et al., 2018). Generalized linear regression models are also commonly used (Gordon et al., 2009; Taylor A. A. et al., 2016; Huang et al., 2017; Li et al., 2018; Pfohl et al., 2018), but will not be presented here. Please refer to Bishop (2016) for additional information on linear modeling. Our review of ML model families does not intend to be comprehensive with regards to ML models utilized in other medical subspecialties. Additional models with successful implementation in neurological conditions include Latent Factor models (Geifman et al., 2018) and Hidden Markov Models (HMM) (Martinez-Murcia et al., 2016) which have been successfully implemented in Alzheimer disease cohorts.

2.2.1. Random Forests

Tree-based methods partition the input space into sets that minimize an error function, impurity, or entropy (Hastie et al., 2009). A decision tree is a tree-based method that can be described as a series of bifurcations with yes/no questions. To compute the output of a data sample, one needs to start at the top of the tree, and iteratively decide where to go next based on the answer. **Figure 3** illustrates an example of a decision tree for diagnosis modeling in ALS.

"Random Forest" (RF) is a ensemble method based on decision trees. By relying on multiple learning algorithms to combine their results, ensemble methods obtain a more efficient prediction model. Each tree in the RF is built on a random subset of the training data and available features. This increases robustness to outliers and generalizability. The final estimation is the average or majority of the trees' estimation depending on whether the target is a regression or classification task (Louppe, 2014). Most RFs contain more than a hundred decision trees and decision tree length and width can also be sizable depending on the number of input features. In ML, the term "interpretability" refers to the degree to which the machine's decision is comprehensible to a human observer (Miller, 2017). While global model interpretability is de facto rather low, RFs evaluate feature importance with regards to its discriminatory power.

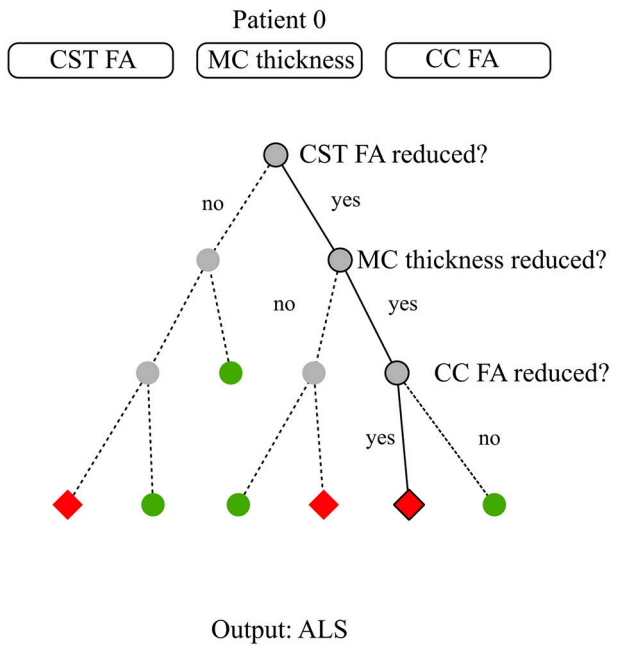

FIGURE 3 | Decision tree model for diagnosis. The available data consist of three basic neuroimaging features: average Corticospinal Tract (CST) Fractional Anisotropy (FA), Motor Cortex (MC) thickness, and average Corpus Callosum (CC) FA. For patient 0, these features are reduced CST FA, reduced MC thickness, reduced CC FA. The target is to classify subjects between healthy and ALS subjects. Establishing a diagnosis requires to run through the decision tree till there are no more questions to answer. At step 1, the closed question directs to the right node due to patient 0's CST pathology. At step 2, the closed question directs to the right node due to patient 0's MC pathology. At step 3, the closed question directs to the left node due to patient 0 CC involvement. Step 3 is the last step as there is no more steps below. The diagnosis for patient 0 is the arrival cell value which is ALS.

Feature relevance is appraised based on the error function upon which the decision trees were built. Extremely Randomized Trees (Extra Trees) have shown promising results for discriminating patients suffering from Progressive Supranuclear Palsy (PSP) and Multiple System Atrophy (MSA) using speech analysis (Baudi et al., 2016). Please refer to Breiman (2001) for a more thorough description of decision trees and RFs and to Rokach (2016) and Shaik and Srinivasan (2018) for a general overview of forest models and their evolution. **Figure 4** illustrates a possible diagnostic application of RF in ALS.

2.2.2. Support Vector Machines

Support Vector Machines (SVM) map input data into high dimensional spaces, called feature spaces, using a non-linear mapping function (Vapnik, 2000). They define a hyperplane that best separates the data. While traditional linear modeling is performed in the input space, SVMs perform linear modeling after projecting the data into another space. The features which discriminate in the projected space, also known as "feature space," derive from input features but these are not readily interpretable. The feature space hyperplane is defined by a limited set of training points called support vectors, hence the name of the method. The chosen hyperplane maximizes the margins between the closest data samples on each side of the hyperplane, which is why SVMs are also referred to as "large margins classifier." These vectors are identified during the "learning phase" after solving a constrained optimization problem. SVMs work as a "black box" as the logic followed by the model cannot be directly interpreted. SVM were state-of-the-art models before being outperformed by NN architecture. That being said, SVM models can adjust well to imaging specific tasks such as anomaly detection using one class SVM. Medical applications of one class SVMs have addressed the issues of tumor detection (Zhang et al., 2004) or breast cancer detection (Zhang et al., 2014). Please refer to Bishop (2016) for more information on SVMs. **Figure 5** illustrates an example of a SVM used to predict prognosis in ALS.

2.2.3. Neural Networks

A "perceptron," also called "artificial neuron," is a simplified representation of a human neuron. It is defined by its afferents (inputs), the inputs' respective weights and a non-linear function. The perceptron's output is the linear combination of its inputs onto which the non-linear function is applied. The linear combination consists of the sum of the multiplications of each input and their respective weight. Perceptrons can be compiled, the output of one perceptron providing the input of the next perceptron. The resulting structure is called a "multi-layer perceptron" which is the most common Neural Network (NN) framework. The contribution of each input to the neuron is modulated by its respective weight which is commonly regarded as a "synapse." NN structures are chosen based on manual tuning and model weights are selected using iterative optimization methods. The stochastic gradient descent method is one of the most popular approaches. Specific model architectures are optimally-suited for specific data types such as "Recurrent NNs" (RNN) for time series or "Convolutional NNs" (CNN) for images. Deep learning models are NN models with significant depth or number of layers (hence the name deep learning) and extensive height or number of nodes per layer, which strongly limits their direct interpretability, similarly to SVMs. Deep learning models are currently state-of-the-art in multiple domains, specifically those which deal with imaging data. Substantial achievements were reached in the field of oncology with regards to melanoma (Esteva et al., 2017), breast cancer and prostate cancer detection (Litjens et al., 2016). Advanced neural network architecture such as the Generative Adversarial Networks (GAN) (Goodfellow et al., 2014) have been tested in a medical imaging synthesis (Nie et al., 2017) or patient record generation (Choi et al., 2017) contexts. Please refer to Goodfellow et al. (2017) for additional material on NNs, Amato et al. (2013) for NN applications in medical diagnosis, Lisboa and Taktak (2006) for NN models in decision support in cancer and Suzuki (2017). **Figure 6** provides a schematic example of NNs to aid prognostic modeling in ALS using a two layer multi-layer perceptron.

2.2.4. Gaussian Mixture Models

Gaussian Mixture Models (GMM) are probabilistic models which can be used in supervised or unsupervised learning. The model hypothesis is that the data can be modeled as

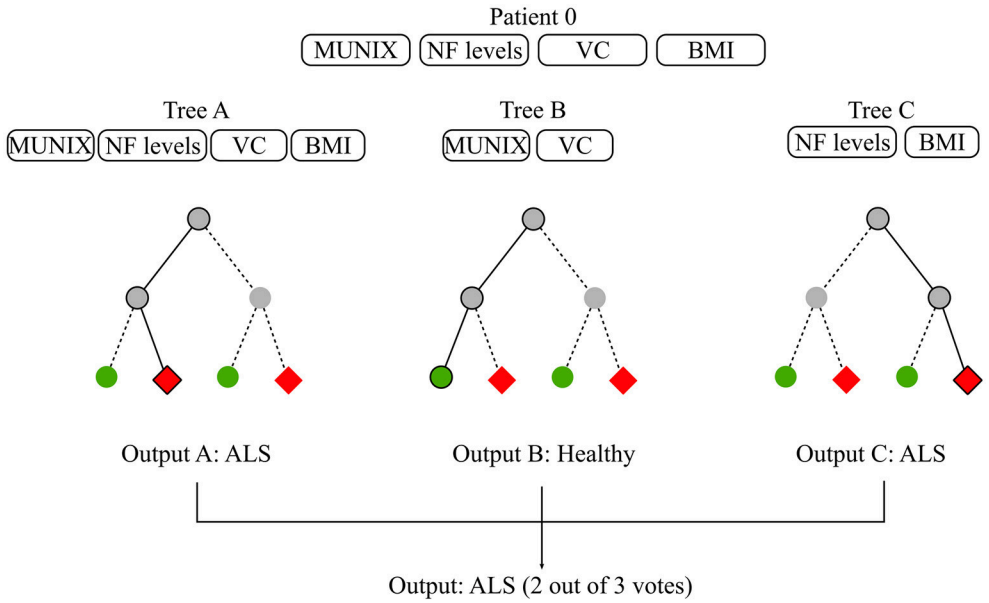

FIGURE 4 | Random forest for diagnosis. The available data consist of basic biomarkers features which are MUNIX, CSF Neurofilament (NF) levels, Vital Capacity (VC), and BMI. The objective is to classify subjects between healthy and ALS patients. The RF contains 3 decisions trees which use different feature subsets to learn a diagnosis model. Tree A learns on all available features, Tree B learns on MUNIX and VC, Tree C learns on NF levels and BMI. Each tree proposes a diagnosis. RF diagnosis is computed based on the majority vote of each of the trees contained in the forest. Given that two out of three trees concluded that patient 0 had ALS, the final diagnosis suggested by the model is ALS.

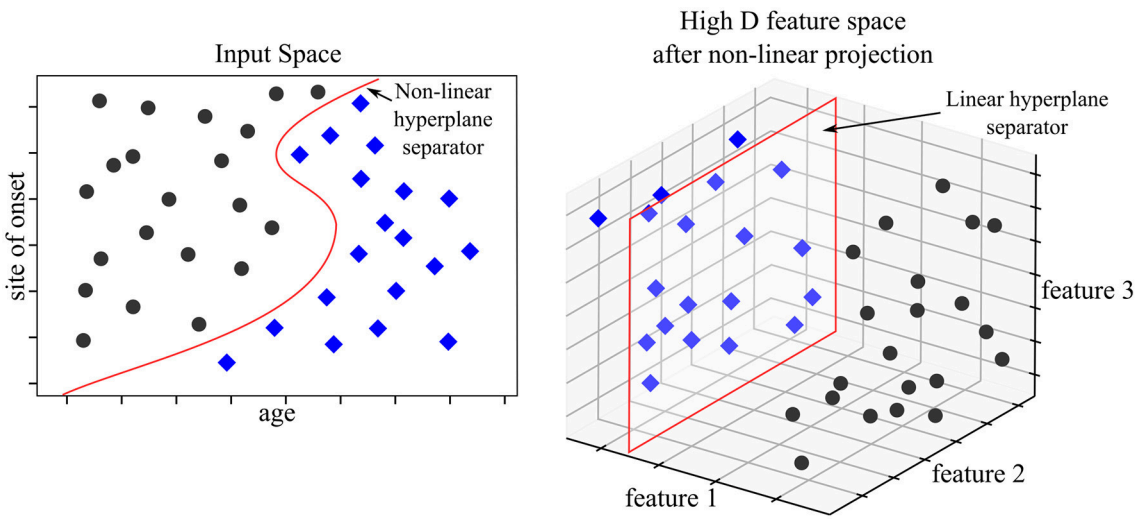

FIGURE 5 | SVM model for prognosis. The available data consist of basic clinical and demographic features; age and site of onset. The objective is to classify patients according to 3-year survival. In the input space (where features are interpretable), no linear hyperplane can divide the two patient populations. The SVM model projects the data into a higher dimensional space—in our example a three dimensional space. The set of two features is mapped to a set of three features. In the feature space, a linear hyperplane can be computed which discriminates the two populations accurately. The three features used for discrimination are unavailable for analysis and interpretability is lost in the process.

a weighted-sum of finite Gaussian-component densities. Each density component is characterized by two parameters: a mean vector and a covariance matrix. Component parameters are estimated using the "Expectation Maximization" (EM) algorithm based on maximizing the log likelihood of the component densities. Inference is performed by drawing from the estimated mixture of Gaussian densities. GMM has achieved good results in medical applications, including medical imaging (de Luis-García et al., 2011) and diagnosing of PD (Khoury et al., 2019). Please refer to Rasmussen (2005) for additional material on GMMs,

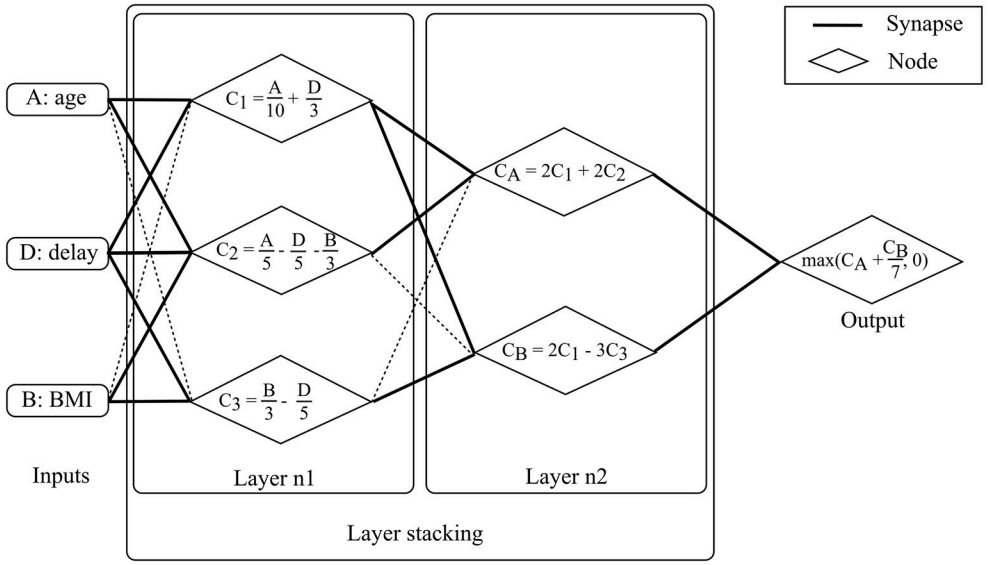

FIGURE 6 | Neural Network model for prognosis. The available data consist of basic demographic and clinical features: age, BMI and diagnostic delay. For patient 0, these features are 50, 15kg/m^2, and 15 months, respectively. The objective is to predict ALSFRS-r in 1 year. The multi-layer perceptron consists of two layers. Nodes are fed by input with un-shaded arrows. At layer 1, the three features are combined linearly to compute three node values, C_1, C_2, and C_3. C_1 is a linear combination of age and delay, C_2 is a linear combination of age, delay and BMI, and C_3 is a linear combination of BMI and delay. For patient 0, computing the three values returns 10, 2, and 2 for $C1$, $C2$, and $C3$, respectively. At layer 2, outputs from layer 1 (i.e., C_1, C_2, and C_3) are combined linearly to compute two values, C_A and C_B. C_A is a linear combination of C_1 and C_2 while C_B is a linear combination of C_1 and C_3. For patient 0, computing the two values gives 24 and 14 for C_A and C_B, respectively. Model output is computed after computing linear combination of C_A and C_B and applying a non-linear function (in this case a maximum function which can be seen as a thresholding function which accepts only positive values). The output is the predicted motor functions decline rate. For patient 0, the returned score is 26.

Moon (1996) for more information on the EM algorithm and Roweis and Ghahramani (1999) for a global overview of Gaussian mixture modeling.

2.2.5. k-nearest Neighbors

k-Nearest Neighbors (k-NN) is an instance-based model. Inference is performed according to the values of its nearest neighbors. The advantage of the model is that limited training is required: all of the training data is kept in memory and is used during the prediction phase. Based on a selected distance function, the K most similar neighbors to the new sample are identified. The new sample's label is the average of its nearest neighbors' label. An advanced version of the method is called Fuzzy k-NN (Fk-NN) which has been used to diagnose PD based on computational voice analyses (Chen et al., 2013). Please refer to Bishop (2016) for more information on k-NN models and Aha et al. (1991) for a review on instance-based ML models.

2.2.6. Boosting Methods

Boosting algorithms are ensemble methods: they rely on a combination of simple classifiers. In contrast to RF models, which are made up of decision trees and output a result based on the average or majority vote of the decision belonging to the RF mode, boosting algorithms are based on simple classifiers. The concept behind boosting is combining multiple "weak" (performance wise) learning models. This combination provides a more robust model than working with a simple base model. Model learning is based on finding the right weighting of the weak learners which make up the model to learn an efficient global model. Recent applications of boosting models include analysis of genetic information to inform on breast cancer prognosis (Lu et al., 2019) and cardiac autonomic neuropathy (Jelinek et al., 2014). Please refer to Bishop (2016) for more information on boosting methods and (Schapire, 2003) for a general overview of boosting methods.

As opposed to relying on a single ML model, models have been increasingly used in combination. For example, NN has been combined with a RF in Beaulieu-Jones and Greene (2016) where the NN output is fed into the RF model. Learning sub-models on specific feature sets have been used to feed sub-model outputs to another ML model as in Fratello et al. (2017) which trained two RF models on different imaging data sets (functional and structural MRI features) and combined intermediate outputs as the final model output. Model combination and model integration can significantly enhance overall performance, but the complexity of both approaches is often underestimated. ML model constraints are even more stringent when used as part of combined or integrated models.

2.3. The Limitations of Machine Learning Approaches

ML models have considerable advantages over traditional statistical approaches for modeling complex datasets. Most ML models, including the six approaches presented above, do not require stringent assumptions on data characteristics.

They offer novel insights by identifying statistically relevant correlations between features and, in the case of supervised learning, of a specific outcome. Despite the pragmatic advantages, the application of ML models requires a clear understanding of what determines model performance and the potential pitfalls of specific models. The most common shortcomings will be discussed in the following section. Concerns regarding data analyses will be examined first, which include data sparsity, data bias, and causality assumptions. Good practice recommendations for model design will then be presented, including the management of missing data, model overfitting, model validation, and performance reporting.

2.3.1. Data Sparsity

"Data sparsity" refers to working and interpreting limited data sets which is particularly common in medical applications. Medical data is often costly, difficult to acquire, frequently require invasive (biopsies, spinal fluid), uncomfortable (blood tests), or time consuming procedures (Magnetic Resonance Imaging). Other factors contributing to the sparsity of medical data include strict anonymization procedures, requirements for informed consent, institutional, and cross-border data management regulations, ethics approvals, and other governance issues. The processing, storage, and labeling of medical data is also costly and often requires specific funding to upkeep registries, DNA banks, brain banks, biofluid facilities, or magnetic resonance repositories (Turner et al., 2011; Bede et al., 2018b; NEALS Consortium, 2018; Neuroimaging Society in ALS, 2018). Multicenter protocols are particularly challenging and require additional logistics, harmonization of data acquisition, standardized operating procedures, and bio-sample processing, such as cooling, freezing, spinning, staining, etc.

Most ML models have originally been intended, developed, and optimized for huge quantities of data. Accordingly, the generalizability of most ML models depends heavily on the number of samples upon which it can effectively learn. Additionally, there is the "curse of dimensionality." The number of samples required for a specific level of accuracy grows exponentially with the number of features (i.e., dimensions) (Samet, 2006). If the number of samples is restrictively low, then the features lose their discriminating power, as all samples in the dataset seem very distinct from one another (Pestov, 2007). ML models learn the underlying relationship between data samples through feature correlations. This requires the ability to discriminate between similar and dissimilar samples in the dataset. Calculating the Sample to Feature Ratio (SFR), i.e., the number of samples available per feature, is a simple way to assess whether the sample size is satisfactory for a given model. An "SFR" of around 10–15 is often considered the bare minimum (Raudys, 2001), but this is based on historical statistical models and may be insufficient for working with complex ML models. Working with a low SFR can lead to both model "underfitting" or "overfitting." These concepts will be introduced below.

2.3.2. Data Bias

Discussing data bias is particularly pertinent when dealing with medical data. Most ML models assume that the training data used is truly representative of the entire population. The entire spectrum of data distribution should be represented in the training data, just as observed in the overall population, otherwise the model will not generalize properly. For example, if a model is presented with a phenotype which was not adequately represented in the training data set, the model will at best label it as an "outlier" or at worst associate it to the wrong category label. Medical data are particularly prone to suffer from a variety of data biases which affect recorded data at different analysis levels (Pannucci and Wilkins, 2010). The four most common types of bias include: study participation bias, study attrition bias , prognostic factor measurement bias, and outcome measurement bias (Hayden et al., 2013). In ALS, study participation bias, - a.k.a. "clinical trial bias," is by far the most significant. It affects prognostic modeling in particular, as patients in clinical trials do not reflect the general ALS population: they are usually younger, tend to suffer from the spinal form of ALS and have longer survival (Chio et al., 2011). Unfortunately, very little can be done to correct for participation bias *post-hoc*, therefore its potential impact needs to be carefully considered when interpreting the results. Study attrition bias also influences ALS studies as data censoring is not always systematically recorded. "Censoring" is a common problem in medical research; it refers to partially missing data, typically to attrition in longitudinal studies. Prognostic factor measurements can be influenced by subjective and qualitative medical assessments and by "machine bias" in imaging data interpretation. The single most important principle to manage these factors, especially if limited data are available, is overtly discussing the type of bias affecting a particular study, and openly reporting them.

2.3.3. Causality Assumption

ML models identify strong (i.e., statistically significant) correlations between input features and the output in the case of supervised learning. Models can only capture observed correlations which are fully contained within the training data. Causality between features and the output cannot be solely established based on significant correlations in the dataset, especially when working with small and potentially unrepresentative population samples. Causality is sometimes inferred based on ML results which can be misleading.

2.4. Good Practice Recommendations

2.4.1. Feature Selection

Identifying the most appropriate features is a crucial step in model design. In "sparse data" situations, the number of features should be limited to achieve an acceptable SFR and to limit model complexity. Various feature selection and engineering approaches exist, which can be chosen and combined depending on primary study objectives. It can be performed manually based on a priori knowledge or using a RF model which ranks data features based on feature importance. This method is commonly used in medical contexts as it easily gives a broad overview of the feature set. Dimension reduction is another option, with linear methods such as Principal Component Analysis (PCA) or Independent Component Analysis (ICA) and non-linear methods such as manifold learning methods. Automated

feature selection methods, such as the "wrapper" or "filtering," undergo an iterative, sometimes time-consuming process where features are selected based on their impact on overall model performance. Finally, provided that sufficient data are available, NN Auto Encoders (AE) models can also reliably extract relevant features. To this day, feature selection and engineering cannot be fully automated and human insight is typically required for manual tuning of either the features or the algorithms performing feature selection. Please refer to Guyon et al. (2006) for further information on feature selection strategies, Fodor (2002) for an overview of dimension reduction techniques and (Lee and Verleysen, 2007) for additional material on non-linear dimension reduction.

2.4.2. Missing Data Management

While most ML models require complete data sets for adequate learning, medical data are seldom complete and missing features are also common. Missing data may originate from data censoring in longitudinal studies or differences in data acquisition. One common approach to missing data management is the discarding of incomplete samples. This has no effect on model design provided there is sufficient data left and that sample distribution is unaltered after discarding. This strategy usually requires large volumes of data with only a small and random subset of missing records. This condition however is rarely met in a clinical setting, where data is sparse, and missing data patterns are typically not random. Missing data can often be explained by censoring or specific testing procedures. Discarding data in these situations may increase data bias as it alters the sample distribution. The first step to missing data management is therefore to explore the mechanisms behind missing data features. Features can be "missing completely at random," without modifying the overall data distribution, "missing at random," when missing feature patterns are based on other features available in the dataset or "non-missing at random" for the remaining cases. Depending on the type of missing data, an appropriate imputation method should be selected. Basic data imputation methods, such as mean imputation, work well on "missing completely at random" cases but induce significant bias for "missing at random" scenarios. In this case, advanced imputation methods such as "Multiple Imputation using Chained Equations" (MICE) (van Buuren, 2007) or "Expectation Maximization" (EM) (Nelwamondo et al., 2007) algorithms operate well. Recently, missing data imputation has been managed using Denoising Auto-Encoders (DAE) models (Nelwamondo et al., 2007; Costa et al., 2018), which have a specific NN architecture. MICE and EM algorithms are statistical methods which substitute missing feature values with feature values from the most similar records in the training set. DAE models build a predictive model using the data available with no missing features to assess substitution values."Non-missing at random" patterns are usually dealt with missing at random imputation methods, but this induces bias in data which needs to be specifically acknowledged. Please refer to Little (2002) for general principles on missing data management and (Rubin, 1987) for missing data imputation for "non-random missing" patterns.

2.4.3. Model Overfitting

Each model design is invariably associated with a certain type of error. "Bias" refers to erroneous assumptions associated with a model, i.e., certain interactions between the input and the output may be overlooked by the model. 'Variance' refers to errors due to the model being too sensitive to training data variability. The learnt model may be excessively adjusted to the training data and poorly generalizable to the overall population if it has only captured the behavior of the training dataset. "Irreducible error" is inherent to model design and cannot be dealt with *post-hoc*. "Bias" and "variance" are interlinked, which is commonly referred to as the "bias-variance trade-off." A high level of bias will lead to model "underfitting," i.e., the model does not represent adequately the training data. A high level of variance will lead to model "overfitting," i.e., the model is too specific to the training data. Overfitting is critical, as it is easily overlooked when evaluating model performance and with the addition of supplementary data, the model will not be able to accurately categorize the new data. This severely limits the use of "overfitted" models. Complex models tend to "overfit" more than simpler models and they require finer tuning. Carefully balancing variance and bias is therefore a key requirement for ML model design. Please refer to Bishop (2016) for more information on overfitting.

2.4.4. Validation Schemes

Working with an optimal validation scheme is crucial in ML. Validation schemes usually split available data into "training" and "testing" datasets, so that performance can be assessed on novel data. Training and testing data should share the same distribution profile, which in turn should be representative of the entire population. Overfitting is a common shortcoming of model designs and carefully chosen validation schemes can help to avoid it. Several validation frameworks exist, "hold out validation" and "cross validation" being the two most popular. The former splits the initial dataset into two sets, one for training the other for testing. The latter performs the same splitting but multiple times. The model is learned and tested each time and the overall performance is averaged. Nevertheless, caution should be exercised in a sparse data context as validation schemes do not compensate well for poorly representative data. Please refer to Bishop (2016) for additional considerations regarding validation schemes.

2.4.5. Harmonization of Performance Evaluation and Reporting

Formal and transparent performance assessments are indispensable to compare and evaluate in ML frameworks. To achieve that, standardized model performance metrics are required. In classification methods, model evaluation should include sensitivity and specificity, especially in a diagnostic context. Sensitivity (or "recall") is the true positive rate, and specificity is the true negative rate. "Accuracy" and Area Under

the "Receiver Operating Curve" (ROC) metrics can be added but should never be used alone to characterize model performance. Accuracy is the average of sensitivity and specificity. ROC is used to represent sensitivity and specificity trade-offs in a classifier model (Fawcett, 2004). The ROC space represents the relationship between the true positive rate (i.e., sensitivity) and the false positive rate (which is 1 - specificity). Given a threshold sensitivity rate, the prediction model will return a specificity rate, adding a data point to the ROC. Multiple thresholding enables the generation of the ROC curve. Perfect predictions lead to 100% sensitivity and 100% specificity (i.e., 0% false positives) which leads to an Area Under the ROC (AUC) of 1. Random predictions will return a 50% accuracy rate which is represented by a continuous straight line connecting the plot of 0% sensitivity with 100% specificity and the plot of 100% sensitivity with 0% specificity, which leads to an AUC of 0.5. Accuracy can hide a low specificity rate if there is a class imbalance and AUC can be misleading as it ignores the goodness of fit of the model and predicted probability values (Lobo et al., 2008). In regression approaches, Root Mean Squared Error (RMSE) (also referred to as Root Mean Square Deviation) and R^2, the coefficient of determination, are good metrics. R^2 represents the ratio of explained variation over the total variation of the data (Draper and Smith, 1998). The closer this index is to one, the more the model explains all the variability of the response data around its mean. Hence the model fits the data well. It is advisable to report multiple performance index for model evaluation as each metric reflects on a different aspect of the model. Using confidence intervals when possible is another good practice, as it conveys the uncertainty relative to the achieved error rate. General reporting guidelines for model design and model evaluation are summarized in the Transparent Reporting of a multivariate prediction model for Individual Prognosis or Diagnosis, or TRIPOD, statement (Moons et al., 2015).

Both "supervised" and "unsupervised" learning approaches have a role in clinical applications, the former for diagnosis and prognosis, the latter for patient stratification. There are a large number of ML models available, but recent work in medicine has primarily centered on three models: RF, SVM, and NN models. The advantages and drawbacks of the specific models are summarized in **Table 1** (Hastie, 2003). The following factors should be considered when implementing ML models for a specific medical project:

Data limitation considerations:

- SFR assessment
- Data bias assessment
- Causality assumptions

Model design considerations:

- Feature selection with regards to SFR
- Missing data management
- Overfitting risk assessment
- Validation framework selection
- Performance metric selection
- Comprehensive model performance reporting.

3. RESULTS

Diagnostic, prognostic, and risk stratification papers were systematically reviewed to outline the current state of the art in ML research efforts in ALS. Consensus diagnostic criteria, established monitoring methods, and validated prognostic indicators provide the gold standard to which emerging ML applications need to be compared to.

3.1. Current Practices in ALS

3.1.1. Current Practices in ALS for Diagnosis

The diagnosis of ALS is clinical, and the current role of neuroimaging, electrophysiology, and cerebrospinal fluid (CSF) analyses is to rule out alternative neurological conditions which may mimic the constellation of symptoms typically associated with ALS. Patients are formally diagnosed based on the revised El Escorial criteria (Brooks, 1994; Brooks et al., 2000; de Carvalho et al., 2008) which achieve low false negative rates (0.5%), but suffer from relatively high false positive rates (57%) (Goutman, 2017). As most clinical trials rely on the El Escorial criteria for patient recruitment, erroneous inclusions cannot be reassuringly ruled out (Agosta et al., 2014). Additionally, misdiagnoses are not uncommon in ALS (Traynor et al., 2000) and these, typically early-stage, ALS patients may be left out from pharmaceutical trials.

3.1.2. Established Prognostic Indicators

Providing accurate prognosis and survival estimates in the early-stage ALS is challenging, as these are influenced by a myriad of demographic, genetic and clinical factors. There is a growing consensus among ALS experts that the most important determinants of poor prognosis in ALS include, bulbar-onset, cognitive impairment, poor nutritional status, respiratory compromise, older age at symptom onset, and carrying the hexanucleotide repeat on *C9orf72* (Chiò et al., 2009). Functional disability is monitored by the revised ALS Functional Rating Scale (ALSFRS-r) worldwide (Cedarbaum et al., 1999), which replaced the AALS scale (Appel ALS) (Appel et al., 1987). The ALSFRS-r is somewhat subjective as it is based on reported abilities in key domains of daily living, such as mobility, dexterity, respiratory and bulbar function. Despite its limitations, such as being disproportionately influenced by lower motor neuron dysfunction, the ALSFRS-r remains the gold standard instrument to monitor clinical trials outcomes. Prognostic modeling in ALS is typically approached in two ways; either focusing on survival or forecasting functional decline.

3.1.3. Current Practices in ALS for Patient Stratification

Current patient stratification goes little beyond key clinical features and core phenotypes. These typically include sporadic vs. familial, bulbar vs. spinal, ALS-FTD vs. ALS with no cognitive impairment (ALSnci) (Turner et al., 2013). A number of detailed patient classification schemes have been proposed based on the motor phenotype alone, as in Mora and Chiò (2015) and (Goutman, 2017): "classic," "bulbar," "flail arm," "flail

TABLE 1 | Overview of model pros & cons, updated from Hastie (2003).

Characteristics	Neural network	SVM	Decision tree	RF	Generelized linear model	Gaussian mixture model	k-NN	Boosting
Model complexity	High	High	Low	Fair	Low	High	Low	Fair
Sensitivity to data sparsity	High	High	Low	Fair	Low	High	High	Fair
Sensitivity to data bias	High	High	High	High	High	High	High	High
Interpretability	Poor	Poor	Fair	Poor	Good	Poor	Good	Poor
Predictive power	Good	Good	Poor	Good	Poor	Good	Poor	Good
Ability to extract linear combinations of features	Good	Good	Poor	Poor	Poor	Poor	Poor	Poor
Natural handling of missing values	Poor	Poor	Good	Good	Poor	Good	Good	Good
Robustness to outliers in input space	Poor	Poor	Good	Good	Fair	Good	Good	Good
Computational scalability	Poor	Poor	Good	Good	Good	Poor	Poor	Good

SVM, Support Vector Machine; RF, Random Forest; k-NN, k-Nearest Neighbors.

leg," "UMN-predominant," "LMN-predominant," "respiratory-onset," "PMA,""PLS," "Mills' syndrome," etc. Patients may also be classified into cognitive phenotypes such as ALS with cognitive impairment (ALSci), ALS with behavioral impairment (ALSbi), ALS-FTD, ALS with executive dysfunction (ALSexec) (Phukan et al., 2011), as presented in **Figure 1**. Diagnostic criteria for these phenotypes tend evolve, change and are often revisited once novel observations are made (Strong et al., 2017). Irrespective of the specific categorization criteria, these classification systems invariably rely on clinical evaluation, subjective observations, choice of screening tests, and are subsequently susceptible to classification error (Goutman, 2017). Adhering to phenotype definitions can be challenging, as performance cut-offs for some categories, such as cognitive subgroups (i.e., ALSbi/ ALSci) may be difficult to implement (Strong et al., 2009; Al-Chalabi et al., 2016). Al-Chalabi et al. (2016) used muscle bulk, tone, reflexes, age at onset, survival, diagnostic delay, ALSFRS-r decline, extra-motor involvement, symptom distribution, and family history as key features for patient stratification. ALS and FTD share common aetiological, clinical, genetic, radiological and pathological features and the existence of an ALS-FTD spectrum is now widely accepted. Up to 15% of patients develop frank dementia (Kiernan, 2018) and 60% show some form of cognitive or behavioral impairment (Phukan et al., 2011; Elamin et al., 2013; Kiernan, 2018). The presence of cognitive impairment is hugely relevant for machine-learning applications because neuropsychological deficits have been repeatedly linked to poorer survival outcomes (Elamin et al., 2011), increased caregiver burden (Burke et al., 2015), specific management challenges (Olney et al., 2005), and require different management strategies (Neary et al., 2000; Hu et al., 2009).

Clinical staging systems

One aspect of patient stratification is to place individual patients along the natural history of the disease by allocating them to specific disease phases or "stages." The utility of staging in ALS is 2-fold; it guides the timing of medical interventions (non-invasive ventilation, gastrostomy, advance directives) and also allows the separation of patients early in their disease trajectory from "late-phase" patients in clinical trials. Three staging systems have been recently developed; Kings' (Roche et al., 2012), MiToS (Chiò et al., 2013a), and Fine Till 9 (FT9) (Thakore et al., 2018). While the MiToS stage can be directly calculated based on ALSFRS-r scores, the Kings' stage is a derived measure. It is noteworthy, that the stages and the ALSFRS-r score are highly correlated (Balendra et al., 2014a). Both staging systems have been cross-validated, compared and they are thought to reflect on different aspects of the disease (Hardiman et al., 2017). The MiToS system is more sensitive to the later phases of the disease, while Kings' system reflects more on the earlier phases of ALS. The FT9 system is not partial to earlier or later stages. The FT9 framework defines stages based on ALSFRS-r subscores, using 9 as a threshold after testing different values on the PRO-ACT dataset. One of the criticism of MiToS, is that stage reversion is possible and that it does not directly capture disease progression (Balendra et al., 2014b). Ferraro et al. (2016) compared MiToS and King clinical staging systems and Thakore et al. (2018) compared all three systems on PRO-ACT data.

Current diagnostic approaches in ALS are suboptimal and often lead to considerable diagnostic delay. Prognostic protocols are not widely validated and current patient stratification frameworks don't represent the inherent heterogeneity of ALS. Accordingly, machine-learning approaches have been explored to specifically address these three issues.

3.2. Results in Diagnosis

3.2.1. Advances in Biomarker Research

The majority of ML research projects focus on the development, optimization, and validation of diagnostic biomarkers. These typically include clinical, biofluid, and neuroimaging indicators. Diagnostic model performance depends on the feature's ability to describe how the disease affects the subjects. Optimal diagnostic biomarkers should not only discriminate between ALS patients and healthy controls but also between ALS patients and patients with mimic or alternative neurological conditions (Bede, 2017). Ideally, an optimal diagnostic model should have outstanding

early-stage sensitivity and specificity so that patients can be recruited into clinical trials early in their disease.

Clinical biomarker research

MUNIX (Fathi et al., 2016) is a non-invasive neurophysiological method which is extensively used in both clinical and research settings. It may also have the ability to capture pre-symptomatic motor neuron loss (Escorcio-Bezerra et al., 2018), therefore it has the potential to confirm early-stage disease in suspected cases. An earlier diagnosis would in turn enable the earlier initiation of neuroprotective therapy with established drugs and more importantly, earlier entry into clinical trials.

Biological biomarker research

Cerebrospinal Fluid (CSF) Neurofilaments (NF) are regarded as one of the most promising group of "wet" biomarkers in ALS (Rossi et al., 2018; Turner, 2018). Typically, research studies assess both Neurofilament Light (NF-L) chain and phosphorylated Heavy (pNF-H) chain levels that are released due to axonal degeneration and can be detected in the CSF and serum. Studies have consistently shown increased CSF pNF-H levels in ALS and up to ten times higher levels than in patients with Alzheimer disease (Brettschneider et al., 2006) or other neurological conditions (Gresle et al., 2014; Steinacker et al., 2015). Even though ALS studies have consistently detected raised pNF-H concentrations, these values vary considerably in the different reports. CSF NF-L levels were linked to reduced pyramidal tract Fractional Anisotropy (FA) and increased Radial Diffusivity (RD) (Menke et al., 2015) and NF-L levels are also thought to correlate with progression rates (Tortelli et al., 2014). Other biological biomarkers include proxies of oxidative stress, such as CSF 4-hydroxy-2,3-nonenal (4-HNE) (Simpson et al., 2004) or 3-nitrotyrosine (3-NT) (Tohgi et al., 1999). Neuroinflammation is another important feature of ALS, and several studies have detected an increase in inflammation-associated molecules, such as interleukin-6 (IL-6) and TNF alpha ($TNF-\alpha$) (Moreau et al., 2005) and galectin-3 (GAL-3) (Zhou et al., 2010). Increased levels of CSF Chitotriosidase-1 (CHIT1) is thought to indicate increased microglial activity (Varghese et al., 2013). Raised levels of CSF hydrogen sulfide (H_2S) was also reported in ALS, which is released by astrocytes and migrolia and is known to be toxic for motor neurons (Davoli et al., 2015). These are all promising wet biomarkers, indicative of disease-specific pathological processes and it is likely that a panel of several biomarkers may be best suited for diagnostic purposes.

Genetic biomarker research

A shared pathological hallmark of neurodegenerative conditions is protein aggregation. The accumulation of the Transactive Response DNA Binding Protein 43 (*TDP-43*) is the most consistent pathological finding in approximately 95% of ALS cases (Neumann et al., 2006). Given the widespread aggregation and accumulation of *TDP-43* in FTD-ALS spectrum, *TDP-43* detection, measurement or imaging is one of the most promising biomarkers strategies. A recent meta-analysis evaluated the diagnostic utility of CSF *TDP-43* levels in ALS (Majumder et al., 2018) and found that increased levels may be specific to ALS, as *TDP-43* levels are significantly raised compared to FTD as well. Reports on *SOD1* levels in the CSF of ALS patients have been inconsistent; some studies detected increased levels (Kokić et al., 2005) whereas others have identified decreased levels (Ihara et al., 2005) or levels comparable to controls (Zetterström et al., 2011).

Proteomics biomarker research

Beyond the interpretation of clinical and imaging data, ML models have an increasing role in genetics, RNA processing and proteomics (Bakkar et al., 2017). Using IBM Watson 5 new RNA-Binding Proteins (RBPs) were identified which were previously not linked to ALS; Heterogeneous nuclear ribonucleoprotein U (*hnRNPU*), Heterogeneous nuclear ribonucleoprotein Q (*SYNCRIP*), Putative RNA-binding protein 3 (*RBMS3*), ell Cycle Associated Protein 1 (*Caprin-1*) and Nucleoporin-like 2 (*NUPL2*). ML models play an important role in modern genetic analyses (Libbrecht and Noble, 2015) but considerable variations exist in their application between various medical subspecialties. One of the roles of ML in genomics is to identify the location of specific protein-encoding genes within a given DNA sequence (Mathé et al., 2002). In the field of proteomics, ML has been extensively utilized to predict 3-dimensional folding patterns of proteins. Approaches such as Deep Convolutional Neural Fields (DeepCNF) have been successful in predicting secondary structure configurations (Wang et al., 2016). In proteomics, ML models are also utilized for loop modeling, and protein side-chain prediction (Larranaga et al., 2006).

Imaging biomarker research

Neuroimaging offers unique, non-invasive opportunities to characterize disease-associated structural and functional changes and imaging derived metrics have been repeatedly proposed as candidate biomarkers (Turner et al., 2011; Agosta et al., 2018a; Bede et al., 2018b). The primary role of MRI in current clinical practice is the exclusion of alternative structural, neoplastic and inflammatory pathology in the brain or spinal cord which could manifest in UMN or LMN dysfunction similar to ALS. Diffusion tensor imaging (DTI) has gained a lot of attention as DTI-derived metrics, such as FA, Mean Diffusivity (MD), RD, or Axial Diffusivity (AD) have already been successfully used to identify ALS patients in ML models (RF) (Bede et al., 2017; Querin et al., 2018). The DTI signature of ALS is firmly established thanks to a myriad of imaging studies, and it includes the commissural fibers of the corpus callosum and the bilateral Corticospinal Tract (CST) (Turner et al., 2009; Bede et al., 2014). The latter has been associated to clinical UMN dysfunction, as well as rate of progression in specific sub-regions (Schuster et al., 2016a). White matter degeneration in frontal and temporal regions have been linked to cognitive and behavioral measures (Agosta et al., 2010; Christidi et al., 2017) and specific genotypes (Bede et al., 2013a). While callosal (Filippini et al., 2010; Bede et al., 2013a) and CST (Agosta et al., 2018b) degeneration seems to be a common ALS-associated signature, frontotemporal and cerebellar white matter degeneration seems to be more specific to certain phenotypes (Prell and Grosskreutz, 2013; Bede et al., 2014). From a gray matter perspective, motor cortex atrophy is a hallmark finding irrespective of specific genotypes and phenotypes (Bede

et al., 2012) which is readily captured by cortical thickness or volumetric measures. Other gray matter regions, such as frontal (Lulé et al., 2007), basal ganglia (Bede et al., 2013c, 2018a; Machts et al., 2015), or cerebellar regions (Prell and Grosskreutz, 2013; Batyrbekova et al., 2018) may be more specific to certain patient cohorts. What is important to note, is that considerable white matter degeneration can already be detected around the time of diagnosis which progress relatively little, as opposed to the incremental gray matter findings in the post-symptomatic phase of the disease (Bede and Hardiman, 2017; Menke et al., 2018). The relevance of these observations is that white matter metrics may be particularly suitable for diagnostic models, whereas gray matter metrics in monitoring applications.

3.2.2. Overview of Research in Diagnosis

ML methods have already been extensively tested to aid the diagnosis of ALS (Gordon et al., 2009; Welsh et al., 2013; Sarica et al., 2016; Schuster et al., 2016b; Bede et al., 2017; Ferraro et al., 2017; Fratello et al., 2017; D'hulst et al., 2018; Li et al., 2018; Querin et al., 2018). Diagnostic models are typically developed within a classification framework with limited category labels, such as "healthy" vs. "ALS." Srivastava et al. (2012) implemented a model to discriminate patients within the Spinal Muscular Atrophy (SMA) spectrum. A similar attempt has not been made in ALS yet but could prove very valuable. A number of imaging features have been explored in recent years (Sarica et al., 2016; Schuster et al., 2016b; Bede et al., 2017; Ferraro et al., 2017; Fratello et al., 2017; D'hulst et al., 2018; Querin et al., 2018).

Performance was highest using combined imaging metrics (Bede et al., 2017) outperforming diagnostic models relying solely on clinical features (Li et al., 2018) which typically achieve up to 68% sensitivity and 87% specificity. Current models however are severely limited by small sample sizes and achieve lower true positive rates than the El Escorial's criteria but dramatically improve false negative rates. In general, diagnostic models based on imaging data achieve a sensitivity above 80% which is very encouraging especially given the emergence of larger data sets (Müller et al., 2016). It is crucial to evaluate model performance in comparison to the current gold standard criteria and report both sensitivity (true positive rate) and specificity (true negative rate). Additional metrics seem also necessary such as accuracy and AUC which provides a global indication of the model's performance.

Performance analysis

Welsh et al. (2013),Schuster et al. (2016b),Bede et al. (2017),Ferraro et al. (2017),Fratello et al. (2017),D'hulst et al. (2018), and Querin et al. (2018) only used single-centre imaging data for their model design. Bede et al. (2017) used a canonical discriminant function and achieved an accuracy of 90% (for 90% sensitivity and 90% specificity). Sarica et al. (2016),Ferraro et al. (2017),Fratello et al. (2017), and Querin et al. (2018) used RFs achieving accuracy rates between 77.5 and 86.5%. Schuster et al. (2016b) used a binary logistic regression model and reached 78.4% (90.5% sensitivity and 62.5% specificity). Welsh et al. (2013) and D'hulst et al. (2018) used SVMs reaching an accuracy of 71 and 80%, respectively. A relatively low accuracy of 71% (Welsh et al., 2013) and low specificity of 12.5% (D'hulst et al., 2018) may stem from model overfitting. The complexity of SVM models, class imbalance (D'hulst et al., 2018), data sparsity (Welsh et al., 2013) are some of the factors which may contribute to their relatively poorer performance. Li et al. (2018) used a linear regression model based on clinical data and reached 77.5% accuracy, 68% sensitivity and 87% specificity. Half of the studies (Welsh et al., 2013; Sarica et al., 2016; Bede et al., 2017; D'hulst et al., 2018; Querin et al., 2018) focused on discriminating ALS patients from healthy controls. Four studies (Gordon et al., 2009; Ferraro et al., 2017; Fratello et al., 2017; Li et al., 2018) went further and attempted to identify ALS within a range of neurological diseases including patients with Parkinson's Disease (PD), Kennedy's Disease (KD), PLS, etc. Srivastava et al. (2012) focused on identifying specific SMA phenotypes. Please refer to **Table 2** for an overview of ML papers focusing on the diagnosis of ALS.

Technical analysis

From a methods point of view, all of the above papers overtly present their pre-processing pipeline (Sarica et al., 2016; Schuster et al., 2016b; Bede et al., 2017; Ferraro et al., 2017; Fratello et al., 2017; D'hulst et al., 2018; Querin et al., 2018) and feature selection strategy (Gordon et al., 2009; Srivastava et al., 2012; Welsh et al., 2013; Sarica et al., 2016; Schuster et al., 2016b; Bede et al., 2017; Fratello et al., 2017; Querin et al., 2018). Imaging analyses need to take the effect of age, gender, and education on MRI data into account, as these have a major impact on white and gray matter metrics. Studies control for these demographic factors differently; while age is generally adjusted for (Zhang et al., 2018), the effect of gender (Bede et al., 2013b) and education (Cox et al., 2016) are often overlooked which can affect model development. Judicious feature selection is paramount as model complexity is directly related to the number of features fed into the model. Limiting model complexity, especially in the context of sparse data is crucial to avoid model overfitting. Feature selection is often based, either on group comparisons or a priori imaging or pathological information. Features often include imaging measures of key, disease-associated anatomical regions, such as measures of the motor cortex or pyramidal tracts (Bede et al., 2016). Existing studies use very different validation schemes to test model performance. Cross-validation is the most commonly used (Srivastava et al., 2012; Sarica et al., 2016; Schuster et al., 2016b; Fratello et al., 2017; Querin et al., 2018), followed by holdout validation (Bede et al., 2017; Ferraro et al., 2017) and leave-one-out validation (Welsh et al., 2013; D'hulst et al., 2018). While robust validation schemes are essential, they don't circumvent overfitting especially when limited data are available. "Cross validation" and "leave-one-out" approaches are generally more robust than holdout validation. Special caution should be exercised with regards to validation reports in sparse data situations, where validation schemes have a limited ability to assess model performance. Querin et al. (2018) and Li et al. (2018) both show SFR higher than ten (15 and 12 ,respectively) which comply with minimum SFR recommendations (Raudys, 2001).

TABLE 2 | Research overview: diagnosis.

Key	Dataset(s) origin	Dataset(s) type	Dataset(s) length	Scope	Biomarker(s) type	Pre-processing (if any)	Validation (if any)	Model(s) tested	Performance
Gordon et al., 2009	Eleanor and Lou Gehrig MDA/ALS Research Center	Real-life	34	ALS, UMN, PLS	Clinical	FS	None described	Linear regression	-
Srivastava et al., 2012	Boston Children Hospital	Real-life	46	SMA phenotypes	Clinical, genetic	FS	CV	SVM	AUC (0.928)
Welsh et al., 2013	Michigan MND Clinic	Real-life	63	ALS, healthy	Imaging	FS	LOOV	SVM	AUC:0.7, Acc:71%, Spec:74%, Sens:68.8%
Sarica et al., 2016	Catanzaro Magna Graecia University	Real-life	48	ALS, healthy	Clinical, imaging	SP, FS	CV	RF	Acc:80%
Schuster et al., 2016b	Trinity College Dublin	Real-life	147	ALS, healthy	Imaging	SP, FS	CV	Logistic regression	Acc:78.4%, Sens:90.5%, Spec:62.5%
Bede et al., 2017	Trinity College Dublin	Real-life	150	ALS, healthy	Imaging	SP, FS	HOV	Discriminant function	Acc:90%, Sens:90%, Spec:90%
Ferraro et al., 2017	MND Clinics in Northern Italy	Real-life	265	ALS, UMN, ALS mimics	Imaging	SP	HOV	RF	Acc:87%, Spec:75%, Sens:92%
Fratello et al., 2017	UK PD Brain Bank	Real-life	120	ALS, PD, healthy	Imaging	SP, FS	CV	RF	Acc:80%
D'hulst et al., 2018	University Hospital Leuven and Turino ALS Center	Real-life	370	ALS, healthy	Imaging	SP	LOOV	SVM	Acc:80%, Sens:85%, Spec:12.5%
Li et al., 2018	Australia	Clinical trial	81	ALS, KD, ALS mimics	Clinical	FS	None described	Linear regression	Acc:77.5%, Sens:68%, Spec:87%
Querin et al., 2018	Pitiè Salpêtrière Hospital	Real-life	105	ALS, healthy	Imaging	SP	CV	RF	AUC:0.96, Acc:86.5%, Sens:88%, Spec:85%

CV, Cross Validation; LOOV, Leave One Out Validation; HOV, Hold Out Validation; AUC, Area under the ROC Curve; Acc, Accuracy; Sens, Sensitivity; Spec, Specificity; PD, Parkinson's Disease; FS, Feature Selection; SP, Signal Processing.

3.3. Results in Prognosis

3.3.1. Advances in Biomarker Research

As the precise mechanisms of disease propagation in ALS are largely unknown (Ravits, 2014; Ayers et al., 2015), research has focused on the identification of candidate prognostic biomarkers including potential clinical, biological, imaging, and genetic indicators. Prognostic model performance depends on the feature's ability to capture the disease spread. Optimal prognostic biomarkers should not only discriminate between different ALS phenotypes but categorize individual patients to common disease progression rates (slow vs. fast progressors) (Schuster et al., 2015).

Clinical biomarker research

Several recent studies examined the specific impact of psychosocial factors, cognitive impairment, nutritional status and respiratory compromise, on prognosis. Psychosocial adjustments in ALS may have an under-recognized impact on prognosis (Matuz et al., 2015). The potential effect of mood on disease progression has only been investigated on a relatively small number of samples to date (Johnston et al., 1999).

Biological biomarker research

Recent research suggests that prognostic modeling that does not rely on a priori hypotheses could lead to more accurate prognostic models than does driven by pre-existing hypotheses. For instance, elevations in Creatine Kinase (CK) were linked to LMN involvement and faster disease progression (Rafiq et al., 2016; Goutman, 2017) using the PRO-ACT data (Ong et al., 2017).

Genetic biomarker research

In a clinical setting, genetic testing is often only performed in familial forms of ALS. *C9orf72* repeat expansions account for

40% of hereditary ALS cases and 10% of sporadic ALS cases (Goutman, 2017) and hexanucleotide repeats are associated with specific clinical traits (Byrne et al., 2012). More than 30 genes have been implicated in the pathogenesis of ALS to date and samples are often screened for Angiogenin (*ANG*), Dynactin subunit 1 (*DCTN1*), Fused in sarcoma (*FUS*), Optineurin (*OPTN*), *SOD1*, Transactive Response DNA Binding Protein (*TARDBP*), Ubiquilin (*UBQLN2*), Valosin-Containing Protein (*VCP*) (Chen et al., 2013; Renton et al., 2013; Taylor J. P. et al., 2016), Alsin Rho Guanine Nucleotide Exchange Factor (*ALS2*), Polyphosphoinositide phosphatase (*FIG4*), Probable Helicase Senataxin (*SETX*), Spatacsin (*SPG11*), Vesicle-Associated membrane protein-associated Protein B/C (*VAPB*) (Chen et al., 2013; Renton et al., 2013), Heterogeneous nuclear ribonucleoprotein A1 (*HNRNPA1*), Profilin 1 (*PFN1*), Sequestosome 1 (*SQSTM1*) (Renton et al., 2013; Taylor J. P. et al., 2016), Coiled-coil-helix-coiled-coil-helix domain-containing protein 10 (*CHCHD10*), Matrin 3 (*MATR3*), Serine/Threonine-protein Kinase (*TBK1*) (Taylor J. P. et al., 2016), sigma-1 receptor (*SIGMAR1*), Diamine oxidase (*DAO*) (Chen et al., 2013), Charged multivesicular body protein 2b (*CHMP2B*), Ataxin-2 (*ATXN2*), Neurofilament Heavy (NEFH), Elongator complex protein 3 (*ELP3*) (Renton et al., 2013) as well as Receptor tyrosine-protein kinase (*ERBB4*), Unc-13 homolog A (*UNC13A*), Peripherin (*PRPH*), TATA-binding protein-associated factor 2N (*TAF15*), Spastin (*SPAST*), Lamin-B1 (*LMNB1*), Sterile alpha and TIR motif-containing protein 1 (*SARM1*), *C21orf2*, (never in mitosis gene a)-related kinase 1 (*NEK1*), Granulin Precursor (*GRN*), Microtubule Associated Protein Tau (*MAPT*) and Presenilin 2 (*PSEN2*). IBM Watson software has been successfully utilized to identify other candidate genes; such as *hnRNPU*, *SYNCRIP*, *RBMS3*, *Caprin-1* and *NUPL2* (Bakkar et al., 2017). Genomic research teams have increasingly capitalized on ML methods worldwide, as they can handle copious amounts of data for systematic processing, genomic sequence annotation, DNA pattern recognition, gene expression prediction, and the identification of genomic element combinations (Libbrecht and Noble, 2015).

The benefit of multiparametric datasets

Early machine learning efforts have been hampered by the lack of large data sets in ALS, which is increasingly addressed by the availability of large international repositories, such as those maintained by NISALS (Müller et al., 2016; Neuroimaging Society in ALS, 2018), NEALS (NEALS Consortium, 2018), and PRO-ACT which includes more than 10 000 patient records from 23 clinical trials in total. Similar initiatives had been carried out in other neurological conditions, as part of the Alzheimer's Disease Neuroimaging Initiative (ADNI) (Mueller et al., 2005), the Parkinson's Progression Marker's Initiative (PPMI) (Marek et al., 2011) and Tract HD (Tabrizi et al., 2012). Emerging large data sets, like PRO-ACCT, also serve as validation platforms for previously identified biomarkers. For example, vital capacity was identified as early as 1993 (Schiffman and Belsh, 1993) as a predictor of disease progression and proved relevant in the Prize4Life challenge (Küffner et al., 2014). Other validated biomarkers include creatinine (Atassi et al., 2014; Küffner et al., 2014; Ong et al., 2017), BMI (Atassi et al., 2014; Küffner et al., 2014; Ong et al., 2017), CK (Ong et al., 2017), Alkaline Phosphatase (ALP)(Küffner et al., 2014; Ong et al., 2017), albumin (Ong et al., 2017), total birilubin (Ong et al., 2017), and uric acid (Atassi et al., 2014). Other predictive clinical features such as onset at age, region of onset, and respiratory compromise have long been firmly established (Chio et al., 2009; Creemers et al., 2014).

3.3.2. Overview of Research in Prognosis

While prognostic forecasting has historically been undertaken using traditional statistical approaches in ALS (Ince et al., 2003; Forbes, 2004; Visser et al., 2007; Coon et al., 2011; Atassi et al., 2014; Elamin et al., 2015; Marin et al., 2015; Rong et al., 2015; Tortelli et al., 2015; Wolf et al., 2015; Knibb et al., 2016; Reniers et al., 2017), ML models have an unprecedented potential to identify novel prognostic indicators (Gomeni and Fava, 2013; Hothorn and Jung, 2014; Ko et al., 2014; Beaulieu-Jones and Greene, 2016; Taylor A. A. et al., 2016; Huang et al., 2017; Jahandideh et al., 2017; Ong et al., 2017; Schuster et al., 2017; Seibold et al., 2017; van der Burgh et al., 2017; Bandini et al., 2018; Pfohl et al., 2018; Westeneng et al., 2018). Most prognostic models use clinical features to determine prognosis in ALS but two recent papers enriched their clinical data with imaging measures (Schuster et al., 2017; van der Burgh et al., 2017). Seven studies designed their prediction model around both clinical and biological data, (Hothorn and Jung, 2014; Ko et al., 2014; Beaulieu-Jones and Greene, 2016; Huang et al., 2017; Jahandideh et al., 2017; Ong et al., 2017; Seibold et al., 2017) and nine studies developed their prognostic model based on PRO-ACT data, (Gomeni and Fava, 2013; Hothorn and Jung, 2014; Ko et al., 2014; Beaulieu-Jones and Greene, 2016; Taylor A. A. et al., 2016; Huang et al., 2017; Jahandideh et al., 2017; Ong et al., 2017; Seibold et al., 2017). Prognosis is typically defined either as functional decline or survival and is either approached as a classification problem with predefined categories or as a regression problem with a specific survival or functional thresholds. The most accurate regression approach had a RMSE of 0.52 (with regards to the ALSFRS rate) (Hothorn and Jung, 2014) and one of the most accurate classification method (Ko et al., 2014) reached 66% accuracy, 66% sensitivity, and 65% specificity using a RF. Bandini et al. (2018) achieved 87% accuracy with a SVM model a fairly complex model built on only 64 samples - which puts the model at a high risk of overfitting. For outcome prediction as a regression problem, best results were reached by Pfohl et al. (2018) using a RF. For outcome prediction as a classification problem, best performance was achieved by Westeneng et al. (2018) with 78% accuracy using a multivariate Royston-Parmar model.

Statistical methods

Previous prognostic studies in ALS primarily used traditional statistical approaches, mostly Cox regressions, mixed effect models and Kaplan-Meier estimators. These models have relatively stringent data assumptions which limit model validity and limit data exploration. Nevertheless, they were instrumental in identifying key prognosis indicators in ALS, such as diagnostic

delay (Forbes, 2004; Elamin et al., 2015; Marin et al., 2015; Wolf et al., 2015; Knibb et al., 2016; Reniers et al., 2017), age at symptom onset (Forbes, 2004; Marin et al., 2015; Wolf et al., 2015; Knibb et al., 2016; Reniers et al., 2017), functional disability (Visser et al., 2007; Elamin et al., 2015; Marin et al., 2015; Wolf et al., 2015; Reniers et al., 2017), El Escorial categorization (Forbes, 2004; Marin et al., 2015; Wolf et al., 2015), comorbid FTD or executive dysfunction (Elamin et al., 2015; Wolf et al., 2015; Knibb et al., 2016), site of onset (Forbes, 2004; Elamin et al., 2015), Riluzole therapy (Forbes, 2004; Knibb et al., 2016), vital capacity (Visser et al., 2007), muscle weakness (Visser et al., 2007), involvement of body regions (Visser et al., 2007), gender (Wolf et al., 2015), BMI (Atassi et al., 2014), presence of *C9orf72* mutations (Reniers et al., 2017). Other prognostic studies focused on the macrophage marker Cluster of Differentiation 68 (*CD68*) (Ince et al., 2003), neuropsychological deficits (Coon et al., 2011), creatinine and uric acid levels (Atassi et al., 2014), tongue kinematics (Rong et al., 2015), anatomical spread (Tortelli et al., 2015), and LMN involvement (Reniers et al., 2017). A number of studies have specifically focused on survival (Forbes, 2004; Visser et al., 2007; Coon et al., 2011; Atassi et al., 2014; Elamin et al., 2015; Marin et al., 2015; Tortelli et al., 2015; Wolf et al., 2015; Reniers et al., 2017). Ince et al. (2003) performed an a posteriori analysis of disease progression based on MRI data. Coon et al. (2011) analyzed the impact of language deficits and behavioral impairment on survival. Rong et al. (2015) assessed the implications of early bulbar involvement. To this date, most reliable predictive features are clinical factors, but similar approaches can be extended to biofluid, genetic, and imaging data. Both ML and traditional statistical approaches perform better with multi-modal data. Existing ML studies in ALS show considerable differences in their methodology and validation approaches. Please refer to **Table 3** for an overview of ALS papers focusing on prognostic modeling.

Performance analyses

RF is the most commonly used model in ALS, implemented in eight of the fourteen reviewed studies (Hothorn and Jung, 2014; Ko et al., 2014; Beaulieu-Jones and Greene, 2016; Taylor A. A. et al., 2016; Huang et al., 2017; Jahandideh et al., 2017; Seibold et al., 2017; Pfohl et al., 2018) and it is also one of the best performing methods (Beaulieu-Jones and Greene, 2016; Taylor A. A. et al., 2016; Huang et al., 2017; Pfohl et al., 2018). Boosting, another ensemble method, was tested by Jahandideh et al. (2017) and Ong et al. (2017). The boosting algorithm outperformed the RF model in Jahandideh et al. (2017). NN models were used successfully in two studies: Beaulieu-Jones and Greene (2016) and van der Burgh et al. (2017). Regression models have also been extensively used in ALS, including generalized linear models (Taylor A. A. et al., 2016; Huang et al., 2017; Pfohl et al., 2018), Royston-Parmar models for Westeneng et al. (2018), and non-linear Weibull models (Gomeni and Fava, 2013). Regression models, despite their stringent assumptions, have great potential in clinical applications (Westeneng et al., 2018). Seibold et al. (2017) used an innovative RF approach to establish the impact of Riluzole therapy on functional decline and survival. Out of the ten models built on clinical data, nine were based on PRO-ACT data (Gomeni and Fava, 2013; Hothorn and Jung, 2014; Ko et al., 2014; Beaulieu-Jones and Greene, 2016; Taylor A. A. et al., 2016; Huang et al., 2017; Jahandideh et al., 2017; Ong et al., 2017; Seibold et al., 2017).

Prognosis in ALS is typically either addressed as a classification or a regression problem. In studies using the classification approach, categories are defined based on functional decline (Gomeni and Fava, 2013; Ko et al., 2014; Ong et al., 2017; Westeneng et al., 2018), survival (Schuster et al., 2017; Pfohl et al., 2018), or disease phase (Bandini et al., 2018). Studies using the regression approach predicted survival (Beaulieu-Jones and Greene, 2016; Huang et al., 2017; van der Burgh et al., 2017; Pfohl et al., 2018), Riluzole effect (Seibold et al., 2017), functional decline (Hothorn and Jung, 2014; Taylor A. A. et al., 2016), or respiratory function (Jahandideh et al., 2017). ALSFRS-r is invariably used in these studies, highlighting that it remains the gold standard instrument to monitor disease progression. Most prognostic models rely solely on clinical features,sometimes enriched with biological data. Radiological data are seldom used in these models, and often rely on relatively small datasets; Schuster et al. (2017) included 69 and van der Burgh et al. (2017) 135 subjects. Despite their considerable sample size limitations, these models achieved relatively promising results with accuracy rates above 79%. Unfortunately, as in the case of diagnostic modeling, large datasets of imaging data, especially longitudinal, are still relatively difficult to acquire in single-centre settings.

A variety of metrics have been utilized for model performance evaluation. For classification tasks, these typically include AUC, specificity and sensitivity, accuracy and concordance (C-index), and for regression methods, RMSE, R^2, mean absolute error, and Pearson correlations between real and predicted estimates are usually reported. Approximately half of the reviewed papers used RF to assess variable importance (Hothorn and Jung, 2014; Huang et al., 2017; Jahandideh et al., 2017; Ong et al., 2017; Seibold et al., 2017; Pfohl et al., 2018; Westeneng et al., 2018). Pfohl et al. (2018) carried out correlation analysis and PCA component projection analysis which proved very instructive. Gamma glutamyl-transferase, was identified as a potential prognostic indicator by Ong et al. (2017). Despite the obvious advantages, model testing is only rarely carried out on external data sets (Jahandideh et al., 2017) for which population data should ideally be used (Taylor A. A. et al., 2016). Many referral centres develop models based on local datasets (Schuster et al., 2017; van der Burgh et al., 2017; Pfohl et al., 2018), which are more accessible than population-based data. Population-based data are increasingly available thanks to national (Donaghy et al., 2009; Talman et al., 2016) and regional (Rosenbohm et al., 2017) registries and increasingly thanks to international consortia (Turner et al., 2011; Müller et al., 2016; Westeneng et al., 2018).

The direct comparison of model performances in ALS ML studies is challenging as performance metrics, prediction targets, sample sizes and study designs are hugely divergent. There is little evidence that a specific type of input data, clinical features alone or clinical data enriched with other data types, enhances model performance. This is due to the lack of large

TABLE 3 | Research overview: prognosis with statistical models.

Key	Dataset(s) origin	Dataset(s) type	Dataset(s) length	Scope	Biomarker(s) type	Pre-processing (if any)	Validation (if any)	Model(s) tested
Ince et al., 2003	Newcastle upon Tyne MND clinic	Real-life	81	Progression	Imaging	None described	Not required	Univariate analysis
Forbes, 2004	Scottish ALS-MND Register	Population	1226	Outcome	Clinical	None described	Not required	Cox time dependent regression modeling
Visser et al., 2007	Dutch university hospitals	Real-life	37	Outcome	Clinical, genetic, biological	None described	Not required	Univariate analysis
Coon et al., 2011	Mayo Clinic	Real-life	56	Outcome	Clinical, imaging	None described	Not required	KM analysis
Atassi et al., 2014	PRO-ACT	Clinical trial	8635	Outcome, progression	Clinical, biological	Data cleaning	Not required	Multivariate analysis
Elamin et al., 2015	Irish and Italian (Piemonte) ALS registry	Population	326	Outcome	Clinical, genetic	FS	HOV	Proportional hazards Cox
Marin et al., 2015	FRALim register	Population	322	Outcome	Clinical	None described	Not required	Cox regression (KM)
Rong et al., 2015	-	Clinical trial	66	Progression	Clinical	FS	Not required	Linear Mixed Effect, KM analysis
Tortelli et al., 2015	University of Bari MND Center	Clinical trial	145	Outcome	Clinical	None described	Not required	Bivariate model for correlation
Wolf et al., 2015	Rhineland-Palatinate Register	Population	193	Outcome	Clinical	FS	Not required	Cox proportional hazards
Knibb et al., 2016	South-East England Register	Population	575	Outcome, progression	Clinical	MVR	CV	Cox proportional hazards, ACT
Reniers et al., 2017	University Hospitals Leuven	Real-life	396	Outcome	Clinical	None described	Not required	Univariate and multivariate Cox regression

HOV, Hold Out Validation; CV, Cross Validation; ACT, Accelerated Failure Time; KM, Kaplan Meier; MVR, Missing Value Removal; FS, Feature Selection.

scale databases which routinely store biological samples and imaging data along with clinical observations. It is likely that the incorporation of genetic, biological, and imaging features, will improve prognostic modeling. Some studies candidly discuss their methodological limitations, and model overfitting is the most often cited shortcoming. Data censoring is often mentioned when using PRO-ACT data and selection bias when relying on clinical trial data. Most studies discuss the issues around feature selection and the importance of limiting feature dimension. Model interpretability concerns are sometimes raised when using NN models (van der Burgh et al., 2017). Westeneng et al. (2018) published their findings according to the methodology introduced by Moons et al. (2015) setting an example of performance reporting. Please refer to **Tables 4**, **5** for an overview of ML studies in ALS focusing on prognostic projections.

Data management approaches

Most studies perform some kind of data pre-processing, such as feature selection (Gomeni and Fava, 2013; Ko et al., 2014; Taylor A. A. et al., 2016; Huang et al., 2017; Jahandideh et al., 2017; Schuster et al., 2017; Bandini et al., 2018; Pfohl et al., 2018; Westeneng et al., 2018), signal processing (Schuster et al., 2017; van der Burgh et al., 2017; Bandini et al., 2018), and address missing data (Hothorn and Jung, 2014; Beaulieu-Jones and Greene, 2016; Taylor A. A. et al., 2016; Huang et al., 2017; Jahandideh et al., 2017; Ong et al., 2017; Seibold et al., 2017; Pfohl et al., 2018; Westeneng et al., 2018). Feature importance analysis prior to model design provides important insights before feature selection (Hothorn and Jung, 2014; Taylor A. A. et al., 2016; Huang et al., 2017; Jahandideh et al., 2017; Ong et al., 2017; Seibold et al., 2017; Pfohl et al., 2018). Feature selection

TABLE 4 | Research overview: Prognosis with ML models (1/2).

Key	Dataset(s) origin	Dataset(s) type	Dataset(s) length	Scope	Biomarker(s) type	Pre-processing (if any)	Validation (if any)	Model(s) tested	Performance	Framework
Gomeni and Fava, 2013	PRO-ACT	Clinical trial	338	Progression	Clinical	FS	HOV	non-linear Weibull	AUC:0.96	Classification
Hothorn and Jung, 2014	PRO-ACT	Clinical trial	1822	Progression	Clinical, biological	MVI, VIA	HOV	RF	RMSE:0.52 (ALSFRS rate), PC:40%	Regression
Ko et al., 2014	PRO-ACT	Clinical trial	1822	Progression	Clinical, biological	FS	HOV	RF	Spec:66%, Sens:65%, Acc:66%	Classification
Beaulieu-Jones and Greene, 2016	PRO-ACT	Clinical trial	3398	Outcome	Clinical, biological	MVI	CV	NN, RF, SVM, k-NN, DT, NN with RF (best)	AUC:0.692	Classification
Taylor A. A. et al., 2016	PRO-ACT, Emery ALS Clinic	Clinical trial, real-life	4372	Progression	Clinical	FS, MVR, VIA	HOV	GLM, RF (best)	R^2:58.2%, MC:0.942, ME:-0.627 (ALSFRS score)	Regression
van der Burgh et al., 2017	University Medical Center Utrecht	Real-life	135	Outcome	Clinical, imaging	SP	HOV	NN	Acc:84.4%	Classification
Huang et al., 2017	PRO-ACT	Clinical trial	6565	Outcome	Clinical, biological	FS, MVR, VIA	CV	GP, Lasso, RF (best)	C-ind:0.717	Regression
Jahandideh et al., 2017	PRO-ACT, NEALS	Clinical trial, population	4406	Progression	Clinical, biological	FS, MVI, VIA	CV	RF, XGBoost, GBM (best)	RMSE:0.635 (FVC), R^2:66.9%	Regression
Ong et al., 2017	PRO-ACT	Clinical trial	1568-6355	Progression, outcome	Clinical, biological	MVR, VIA	CV	Boosting	For P: AUC:0.82, Acc:56.5%, Spec:74%, Sens:39%, For O: AUC:0.83, Acc:76.7%, Spec:76.1%, Sens:77.3%	Classification

CV, Cross Validation; HOV, Hold Out Validation; AUC, Area under the ROC Curve; Acc, Accuracy; Sens, Sensitivity; Spec, Specificity; MC,Model Calibration; ME, Mean Error; PC, Pearson's Correlation; DT, Decision Tree; GLM, Generalized Linear Model; k-NN, k-Nearest Neighbors; FS, Feature Selection; MVI, Missing Value Imputation; VIA, Variable Importance Analysis; MVR, Missing Value Removal; P, Progression; O, Outcome; C-ind, Concordance; GP, Gaussian Process; GBM, Gradient Boosting Model; SP, Signal Processing; FVC, Forced Vital Capacity.

is automated when using RF, NN, or boosting models. Missing data management is crucial when dealing with medical data sets as it has a strong impact on data bias and overall model performance. Huang et al. (2017),Seibold et al. (2017),Taylor A. A. et al. (2016), and Ong et al. (2017) discarded data samples with missing features which can introduce further bias in sparse data situations. Mean imputation, which is a simple imputation method, was performed by Jahandideh et al. (2017) and Hothorn and Jung (2014). Simple imputation methods can increase bias in data as these methods assume missing 'completely at random' characteristics which rarely reflect real-life scenarios. Consequently, multiple imputation approaches such as NN approaches (Beaulieu-Jones and Greene, 2016) or MICE (Westeneng et al., 2018) are favored. With few exceptions, Seibold et al. (2017), most studies report their validation framework in detail. Cross-validation schemes are used by some (Beaulieu-Jones and Greene, 2016; Huang et al., 2017; Jahandideh et al., 2017; Ong et al., 2017; Bandini et al., 2018; Pfohl et al., 2018; Westeneng et al., 2018) and hold out validation schemes are implemented by others (Gomeni and Fava, 2013; Hothorn and Jung, 2014; Ko et al., 2014; Taylor A. A. et al., 2016; van der Burgh et al., 2017). Dataset population ranges between 64 and 11 475 samples which explains the considerable methodological differences in pre-processing, data analysis and overall model design. SFR ranges between < 1 (with 135 samples for 2 376 features (van der Burgh et al., 2017)) to close to 1100 (with 6 565 samples for 6 features (Huang et al., 2017)). Small SFRs are mostly due to either data type scarcity (Schuster et al., 2017; van der Burgh et al., 2017; Bandini et al., 2018) or the use of complex models such as NN (Beaulieu-Jones and Greene, 2016).

TABLE 5 | Research overview: Prognosis with ML models (2/2).

Key	Dataset(s) origin	Dataset(s) type	Dataset(s) length	Scope	Biomarker(s) type	Pre-processing (if any)	Validation (if any)	Model(s) tested	Performance	Framework
Schuster et al., 2017	Trinity College Dublin	Real-life	69	Outcome	Clinical, imaging	SP, FS	CV	Logistic regression	Spec:83.34%, Sens:75%, Acc:79.19%	Classification
Seibold et al., 2017	PRO-ACT	Clinical trial	2534-3306	Progression, outcome	Clinical, biological	MVR, VIA	None	RF	Treatment effect on outcome and progression	Regression
Bandini et al., 2018	-	Clinical trial	64	Progression	Clinical	SP, FS	CV	k-NN, SVM (best)	Spec:86.1%, Sens:88.8%, Acc:87%	Classification
Pfohl et al., 2018	Emery ALS Clinic	Real-life	801	Outcome	Clinical	MVI, FS, VIA	CV	GLM, RF (best)	RMSE:547 +/-46 days, R^2:52%, AUC:0.85	Regression, Classification
Westeneng et al., 2018	14 European ALS centers	Real-life	11475	Outcome	Clinical	FS, MVI	CV	MRP	Acc:78%, MC:1.01, AUC:0.86	Classification

CV, Cross Validation; AUC, Area under the ROC Curve; Acc, Accuracy; Sens, Sensitivity; Spec, Specificity; MC, Model Calibration; GLM, Generalized Linear Model; k-NN, k-Nearest Neighbors; MRP, Multivariate Royston-Parmar; FS, Feature Selection; MVI, Missing Value Imputation; VIA, Variable Importance Analysis; MVR, Missing Value Removal;SP, Signal Processing.

Six studies have used less than nine features for model design (Gomeni and Fava, 2013; Hothorn and Jung, 2014; Ko et al., 2014; Huang et al., 2017; Ong et al., 2017; Westeneng et al., 2018) reaching SFRs over 100 samples per feature.

3.4. Advances in Risk Stratification

Accurate patient stratification is not only essential for clinical trial designs but also for individualized patient care (Kiernan, 2018). Current stratification strategies are surprisingly limited and do not utilize patient clustering for pharmaceutical research and medical interventions. Only two drugs have been approved by the FDA to treat ALS to date: Riluzole (Rilutek) and Edavarone (Radicava). While there is some debate if the maximal therapeutic benefit of Riluzole may be in late-stage disease (Dharmadasa et al., 2018; Fang et al., 2018), recent research suggest that Edavarone effect may be superior in the earlier phases of ALS (Goutman, 2017; Kiernan, 2018). It is also noteworthy, that past clinical trials were primarily based on heterogeneous ALS populations. The inconclusive findings of admixed cohorts may not apply to specific patient subgroups (Bozik et al., 2014) or presymptomatic cohorts. Rigorous patient stratification would have an important role in addressing these shortcomings. Unsupervised learning methods, such as the one carried out by Beaulieu-Jones and Greene (2016) using denoised autoencoder and t-distributed Stochastic Neighbor Embedding (t-SNE), provide novel means of monitoring patients. However, as for most unsupervised learning methods, selecting the appropriate number of patient clusters requires extensive empirical testing.

3.4.1. Overview of Stratification Initiatives

Patient stratification in ALS is often explored from a prognostic perspective (Visser et al., 2007; Gomeni and Fava, 2013; Ko et al., 2014; Elamin et al., 2015; Marin et al., 2015; Beaulieu-Jones and Greene, 2016; Ong et al., 2017; van der Burgh et al., 2017; Pfohl et al., 2018; Westeneng et al., 2018) approaching it as a classification problem and patient categories are defined to build the model. Balendra et al. (2014a) analyzed progression patterns using the King's staging system. Clinical stages are potential input variables for stratification, and therapeutic intervention can be tested based on disease subgroups or disease stage.

Patient stratification was performed based on clinical observations alone in seven recent studies (Visser et al., 2007; Balendra et al., 2014a; Ko et al., 2014; Elamin et al., 2015; Burke et al., 2017; van der Burgh et al., 2017; Pfohl et al., 2018). Variables, such as limb involvement (Visser et al., 2007), disease-stage (Balendra et al., 2014a), ALSFRS-r decline (Ko et al., 2014), executive dysfunction (Elamin et al., 2015), behavioral impairment (Burke et al., 2017), and survival (van der Burgh et al., 2017; Pfohl et al., 2018) have been used for patient stratification. Other studies relied on unsupervised techniques to identify patient subgroups. These methods either used model estimation (Gomeni and Fava, 2013; Westeneng et al., 2018), K-means (Ong et al., 2017), a tree-growing algorithm called Recursive Partitioning and Amalgation (Marin et al., 2015) or NNs with a denoising autoencoder (Beaulieu-Jones and Greene, 2016). Clustering was performed either based on clinical features alone (Gomeni and Fava, 2013; Marin et al., 2015; Westeneng et al., 2018) or based on clinical features and biological data (Beaulieu-Jones and Greene, 2016; Ong et al., 2017).

Contrary to supervised learning problems, unsupervised learning methods do not have clear and easily presentable performance metrics. Possible options include the description of inter- and intra-patient subgroup distances and outlier distribution. The optimal number of models (equivalent to cluster number) can be identified using an iterative procedure

for studies based on model estimation (Gomeni and Fava, 2013; Westeneng et al., 2018).

Clustering methods

Patient clustering was performed on various datasets in ALS; clinical trial data (Gomeni and Fava, 2013; Balendra et al., 2014a; Ko et al., 2014; Ong et al., 2017), "real-life data" (Visser et al., 2007; van der Burgh et al., 2017; Pfohl et al., 2018; Westeneng et al., 2018) and population data (Elamin et al., 2015; Marin et al., 2015; Burke et al., 2017). The term "real-life" data is used to samples which derive from local recruitment, typically single-center non-pharmacological studies, where data are acquired prospectively but do not represent entire populations. Access to large patient databases with limited missing data is fundamental to the development of accurate stratification schemes. Recent initiatives such as the Prize4Life challenge (Küffner et al., 2014), the PRO-ACT database and Euro-MOTOR consortium (Rooney et al., 2017; Visser et al., 2018) have proven invaluable resources for research and should be continued and expanded. PRO-ACT's main limitation with regards to patient stratification is its inclusion bias. Working with population data leads to more representative results as clinical trial datasets tend to be associated with considerable bias. The identification of specific patient subgroups is most accurate when the data truly represents an entire patient population.

The maximum number of clusters does not typically exceed five in ALS research; Gomeni and Fava (2013), Ko et al. (2014), Beaulieu-Jones and Greene (2016), Ong et al. (2017), and Pfohl et al. (2018) work with only two patient subgroups, Visser et al. (2007), Elamin et al. (2015), van der Burgh et al. (2017), and Burke et al. (2017) with three patient subgroups, Marin et al. (2015) with four patient subgroups and Balendra et al. (2014a); Westeneng et al. (2018) with five patient subgroups. Depending on the available data, feature type, and data source working with a limited number of clusters may be desirable. This can be particularly challenging in ALS, where a number of phenotypes contribute to clinical heterogeneity. Identifying the correct number of clusters is a common problem in unsupervised learning which can only be solved with *ad-hoc* analyses. Please refer to **Tables 6**, **7** for an overview of studies focusing on risk stratification in ALS.

ALS studies approach patient stratification in strikingly different ways. Visser et al. (2007) proposed an innovative PMA strategy which is based on limb involvement and focuses on symmetrical vs. asymmetrical limb weaknesses. Current ALS phenotyping already considers aspects of limb involvement, but this could be extended to adopt more detailed characterization. Gomeni and Fava (2013) divided patients into slow- and fast-progressing groups based on non-linear Weibull model estimation, which can account for linear, sigmoid or exponential evolutions. Two clusters were retained based on model fitting, as three-cluster attempts proved less conclusive. Balendra et al. (2014a) explored King's stages (Roche et al., 2012) on LiCALS and Mito Target data and demonstrated a viable alternative to ALSFRS-r and traditional patient stratification strategies. Clinical staging is thought to represent pathological stages better than ALSFRS-r. Alternative clinical staging systems, such as MiToS (Chiò et al., 2013a) or Fine'Till 9 (Thakore et al., 2018) could be tested further to assess if they are more sensitive in the earlier or later stages of the disease. Ko et al. (2014) performed an interesting patient classification study based on ALSFRS-r decline but choice of threshold, 0.6 ALSFRS-r point / month was not expounded. Elamin et al. (2015) divided patients into three risk groups based on a scoring system, which was based on site of onset, ALSFRS-r, and executive dysfunction. Marin et al. (2015) identified four groups using an unsupervised ML technique: Recursive partitioning and amalgamation. Membership rules were derived from analyzing ALSFRS-r decline and El Escorial criteria. Beaulieu-Jones and Greene (2016) investigated PRO-ACT survival data using denoising autoencoders, a deep learning model, and used the visualization algorithm t-SNE to visualize how the NN model had divided the subjects according to short vs. long survival. These results are particularly promising as NN models can work well without extensive feature selection. van der Burgh et al. (2017) segregated patients into three classes based on survival times defined by Elamin et al. (2015). Burke et al. (2017) proposed three subgroups for clustering based on executive dysfunction ("non-significant," "mild," and "severe symptoms") using the Beaumont Behavioral Inventory (Elamin et al., 2016), a questionnaire on patient behavior completed by the patient and caregivers. Ong et al. (2017) used unsupervised ML techniques Partitioning Around Medoids and K-Means to identify patient clusters for disease progression and survival. Partitioning Around Medoids and K-Means differ on cluster computing as the former computes the medoid (data point whose average dissimilarity with the other data points is minimal) while the latter computes the average value. Two clusters were optimally suited for both algorithms. Pfohl et al. (2018) used empirically defined survival times based on clinician experience. Westeneng et al. (2018) identified five patient groups after Royston-Parmar model analysis and estimation. Differing patient stratification strategies can be successfully combined as demonstrated by Burke et al. (2017) who analyzed cognitive impairment stratification with regards to King's clinical staging system.

4. DISCUSSION

4.1. Summary of Main Findings

4.1.1. Diagnosis

ML models have been increasingly explored in diagnostic applications in ALS. These models have the potential to supersede the current gold standard diagnostic approach which is based on clinical evaluation and uses the El Escorial criteria. The El Escorial criteria is thought to suffer from low specificity (Goutman, 2017). Recent ML models in ALS have reached comparable sensitivity and specificity values to the El Escorial criteria. The main barriers to model performance stem from limited data availability for training and poor sample to feature ratios. Future strategies should centre on models using multimodal data, and models which discriminate phenotypes

TABLE 6 | Research overview: Patient stratification (1/2).

Key	Dataset(s) origin	Dataset(s) type	Dataset(s) length	Scope	Approach	Clustering feature(s)	Number of clusters found
Visser et al., 2007	Dutch university hospitals	Real-life	37	Progression	Clinical observations	Limb involvement	3
Gomeni and Fava, 2013	ProACT	Clinical trial	338	Progression	Unsupervised (non-linear Weibull model estimation)	Clinical features	2
Balendra et al., 2014a	LiCALS, Mito Target	Clinical trial	725	Progression	Clinical observations	Clinical stages	5
Ko et al., 2014	ProAct	Clinical trial	1822	Progression	Clinical observations	ALSFRS decline rate	2
Elamin et al., 2015	Irish ALS registry, Italy (Piemonte Region)	Population	326	Outcome	Clinical observations	Score based on onset type, ALSFRS rate an executive disfunction	3
Marin et al., 2015	FRALim register	Population	322	Outcome	Unsupervised (RECPAM)	Clinical features	4

RECPAM, Recursive Partitioning and Amalgation.

TABLE 7 | Research overview: Patient stratification (2/2).

Key	Dataset(s) origin	Dataset(s) type	Dataset(s) length	Scope	Approach	Clustering feature(s)	Number of clusters found
Beaulieu-Jones and Greene, 2016	ProAct	Clinical trial	3398	Outcome	Unsupervised learning (DA)	Clinical and biological features	2
van der Burgh et al., 2017	University Medical Center Utrecht	Real-life	135	Outcome	Clinical observations	Survival time based on Elamin2015 categories	3
Burke et al., 2017	Irish ALS Register	Population	383	Progression	Clinical observations	Behavioral impairment based on BBI score	3
Ong et al., 2017	ProAct	Clinical trial	1568-6355	Progression, outcome	Unsupervised (PAM and K-Means)	Clinical and biological features	2x2
Pfohl et al., 2018	Emery ALS Clinic	Real-life	801	Outcome	Clinical observations	Survival time (empirical)	2
Westeneng et al., 2018	14 European ALS centers	Real-life	11475	Outcome	Unsupervised (RP model estimation)	Clinical features	5

DA, Denoising Autoencoders; PAM, Partitioning Around Medoids; RP, Royston-Parmar; BBI, Beaumont Behavioral Inventory.

within the ALS spectrum and distinguish ALS from disease-controls. Optimally, these models should be developed to enable an early, definite, and observer independent diagnosis of ALS.

4.1.2. Prognosis

The development of accurate prognostic models attracts considerable interest, and is fuelled by initiatives like the challenge launched by Prize4Life (Küffner et al., 2014). Prognostic model performance depends heavily on each feature's relevance to disease propagation. Current models rely primarily on clinical findings and laboratory tests which might not be sufficient to predict disease evolution. Despite these challenges, recent models have provided a reasonable gross estimate of death risk (Ong et al., 2017), survival (Schuster et al., 2017; van der Burgh et al., 2017; Westeneng et al., 2018) and progression rates (Ong et al., 2017). The most important constraints of prognostic modeling stem from significant data bias, limited data availability, poor missing data management, and limited sample to feature ratios. Performance reporting should be standardized for model comparisons, reproducibility, and benchmark development. Future studies should include multimodal data, multiple timepoints, include ALS patients with comorbid FTD and appraise disease progression in terms of clinical stages instead

of solely relying on ALSFRS-r. Effective prognostic modeling should also account for disease heterogeneity to provide patients and clinicians with accurate prognostic insights across multiple phenotypes.

4.1.3. Risk Stratification

Novel computerized risk stratification initiatives are urgently required in ALS, as this aspect of ALS research has been relatively ignored to date. Existing studies tend to stratify patients according to rather basic categorization rules, limiting their analyses to a restricted number of clusters and focusing mostly on clinical features. Future research should focus on working with multimodal and longitudinal datasets and analyzing model-derived clustering with commonly used ALS phenotypes. Optimized patient stratification schemes will undoubtedly improve clinical trial design and has the potential to identify clinically relevant ALS subtypes.

5. CONCLUSIONS

ML models have enormous academic and clinical potential in ALS. With the increasing availability of large datasets, multicentre initiatives, high-performance computer platforms, open-source analysis suites, the insights provided by flexible ML models are likely to supersede those gained from conventional statistical approaches. The choice of the ML model need to be carefully tailored to a proposed application based on the characteristics of the available data and the flexibility, assumption and limitation profile of the candidate model. While ALS research to date has overwhelmingly relied on conventional ML approaches, emerging models and neural network architectures have considerable potential to advance the field. Novel models such as "black box" methods however may suffer from similar pitfalls than established algorithms. The meticulous evaluation of data characteristics, appraisal of data bias, missing data, sample to feature ratio is indispensable irrespective of the choice of ML model. Novel models may have outperformed traditional approaches, but data constraints and limitations are often overlooked. Model overfitting is the most commonly encountered shortcoming of recent studies which limits the generalizability of a proposed model. Transparent performance assessment using standardized metrics, robust missing data management and adherence to reporting guidelines are key requirements for future machine learning studies in ALS. Despite the drawbacks of current models and the methodological limitations of recent studies, the momentous advances in the field suggest that ML models will play a pivotal role in ALS research, drug discovery, and individualized patient care.

AUTHOR CONTRIBUTIONS

VG contributed to the design of the study, analyzed the data, and wrote the first draft of the manuscript. VG, GL, PB, FD, J-FP-P, and P-FP contributed to the revision of the manuscript. VG, GL, PB, FD, J-FP-P, P-FP, and GQ read and approved the final version.

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GLOSSARY

ALS : Amyotrophic Lateral Sclerosis
ALSbi : Behaviorally impaired ALS
ALSFRS : ALS Functional Rating Scale
ALSbi : behaviorally impaired ALS
ALSnci : ALS with no cognitive impairment
ALSci : ALS with cognitive impairment
ALSexec : ALS with executive dysfunction
AUC : Area Under the ROC Curve
AD : Axial Diffusivity
CNN : Convolutional Neural Network
CSF : Cerebrospinal fluid
CST : Corticospinal
DeepCNF : Deep Convolutional Neural Fields
DTI : Diffusion Tensor Imaging
FA : Fractional Anisotropy
FTD : Frontotemporal Dementia
GMM : Gaussian Mixture Model
KD : Kennedy's disease
k-NN : k-Nearest Neighbors
LMN : Lower Motor Neurons
MD : Mean Diffusivity
ML : Machine Learning
MND : Motor Neuron Disease
NN : Neural Network
PBP : Progressive Bulbar Palsy
PCA : Principal Component Analysis
PD : Parkinson's Disease
PLS : Primary Lateral Sclerosis
PMA : Progressive Muscular Atrophy
PRO-ACT : Pooled Resource Open-Access ALS Clinical Trials
RBP : RNA-Binding Protein
RD : Radial Diffusivity
RF : Random Forest
RMSE : Root Mean Squared Error
RNN : Recurrent Neural Network
ROC : Receiver Operating Curve
SFR : Sample to Feature Ratio
SMA : Spinal Muscular Atrophy
SVM : Support Vector Machine
t-SNE : t-distributed Stochastic Neighbor Embedding
UMN : Upper Motor Neurons

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Positron Emission Tomography Molecular Imaging Biomarkers for Amyotrophic Lateral Sclerosis

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Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder with limited treatment options. Despite decades of therapeutic development, only two modestly efficacious disease-modifying drugs—riluzole and edaravone—are available to ALS patients. Biomarkers that can facilitate ALS diagnosis, aid in prognosis, and measure drug pharmacodynamics are needed to accelerate therapeutic development for patients with ALS. Positron emission tomography (PET) imaging has promise as a biomarker for ALS because it permits visualization of central nervous system (CNS) pathology in individuals living with ALS. The availability of PET radioligands that target a variety of potential pathophysiological mechanisms—including cerebral metabolism, neuroinflammation, neuronal dysfunction, and oxidative stress—has enabled dynamic interrogation of molecular changes in ALS, in both natural history studies and human clinical trials. PET imaging has potential as a diagnostic biomarker that can establish upper motor neuron (UMN) pathology in ALS patients without overt UMN symptoms, as a prognostic biomarker that might help stratify patients for clinical trials, and as a pharmacodynamic biomarker that measures the biological effect of investigational drugs in the brain and spinal cord. In this Review, we discuss progress made with 30 years of PET imaging studies in ALS and consider future research needed to establish PET imaging biomarkers for ALS therapeutic development.

Keywords: amyotrophic lateral sclerosis, neuroimaging, positron emission tomography, biomarker, diagnostic biomarker, pharmacodynamic biomarker, therapeutic development

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a devastating disorder characterized by degeneration of motor neurons in the brain and spinal cord. It is clinically heterogeneous and shares clinical and pathological features with frontotemporal dementia (FTD). ALS invariably leads to weakness and death; ∼70–80% of ALS patients die within 5 years of symptom onset (1).

Riluzole and edaravone are currently the only disease-modifying treatments for ALS. More efficacious therapy is urgently needed. Fortunately, the recent expansion of knowledge about genetics and pathophysiology of ALS (2) has generated a large pipeline of potential therapeutic agents to be tested in ALS. Biomarkers for ALS are now urgently needed to stratify patients for trial enrollment, to demonstrate biological drug effect, and to guide dose-selection and go-no-go decisions in early phase clinical trials.

Multiple types of biomarkers are being developed for use in ALS (3–5). Electrophysiological biomarkers of the upper motor neurons (UMNs) [transcranial magnetic stimulation (6)] and

lower motor neurons (LMN) [motor unit number index (7) and electrical impedance myography (8)] directly quantify physiology of diseased tissues. Biological fluid-based biomarkers such as phosphorylated neurofilament heavy chain in cerebrospinal fluid (CSF) (9, 10), neurofilament light chain from CSF or serum (10–14), and urine p75 neurotrophin receptor extracellular domain (15) are being evaluated as markers of neuronal degeneration. Neuroimaging biomarkers using magnetic resonance imaging (MRI) or positron emission tomography (PET) techniques can objectively visualize changes associated with the disease processes and help to understand the mechanisms of neurodegeneration *in vivo* (16).

This Review will focus on development of PET molecular imaging biomarkers for ALS. References for this Review were identified by searching PubMed for the terms "amyotrophic lateral sclerosis" or "ALS" or "motor neuron disease" or "MND" AND "PET" or "positron emission tomography." As of October 11, 2018, 222 articles were identified. We excluded articles that were not focused on motor neuron diseases (17), were animal or post-mortem studies (18), were not focused on PET imaging (19), were not dedicated to brain or spinal cord (2), were not written in English (12), were inaccessible (7), studied fewer than 5 ALS or MND cases (20), or were literature reviews or guidelines (21), resulting in 48 papers.

THE DEVELOPMENT OF PET IMAGING IN ALS

PET imaging uses positron-emitting radioisotopes that are incorporated into molecules of interest ("tracers"), which are injected intravenously and enter the central nervous system (CNS). When positrons encounter electrons, they annihilate and emit pairs of gamma rays that travel away from one another at a 180° angle. The detection of gamma ray pairs by the PET camera enables localization of the annihilation event and subsequent three-dimensional reconstruction of radiotracer distribution in the tissue of interest (16). The development of PET tracers that permit visualization of glucose metabolism, cerebral blood flow, neurotransmitter metabolism, neuroreceptor binding, inflammation, and oxidative stress have permitted a deep investigation into the molecular pathophysiology of ALS *in vivo* (**Table 1**).

Glucose Metabolism and Cerebral Blood Flow

The first PET study in ALS, conducted in 1987, used the tracer ^{18}F-fluorodeoxyglucose ([^{18}F]-FDG) to demonstrate that ALS patients with UMN involvement had diffuse cortical hypometabolism compared to healthy controls (18). Subsequent [^{18}F]-FDG PET studies found variable cortical hypometabolism in ALS (22–24). PET studies using radiolabeled carbon dioxide ($C[^{15}O]_2$), which detects alterations in regional cerebral blood flow (40–42), revealed decreased cerebral blood flow to the prefrontal cortex (41–43) and thalamus (41, 43) that correlated with cognitive impairment in ALS. These early PET findings suggested that ALS pathology expanded outside the motor cortex, years before ALS was widely accepted as a disorder on the same spectrum as frontotemporal dementia (FTD).

The 2011 discovery that C9orf72 hexanucleotide repeat expansions cause both ALS and FTD (64–66) motivated new [^{18}F]-FDG PET studies that explored genotype-phenotype correlations and cognition in ALS. One study suggested that ALS patients with C9orf72 expansions had more widespread cortical hypometabolism than sporadic ALS patients (33), though this finding was not replicated (32). Other studies demonstrated frontal and prefrontal hypometabolism in patients with sporadic ALS-FTD compared to ALS patients without FTD (33–35).

In recent years, large cross-sectional [^{18}F]-FDG PET studies have established that sporadic ALS is associated with hypometabolism in the premotor and frontal cortices and hypermetabolism in the brainstem (28, 31, 32). There is now interest in spinal cord imaging: two [^{18}F]-FDG PET studies demonstrated hypermetabolism in the cervical cords of ALS patients (36, 38). These findings suggest potential differences between cortical vs. brainstem and spinal cord metabolism that warrant further exploration.

Neuroinflammation

Neuroinflammation, specifically microglial activation, is a pathological hallmark of ALS (67, 68) and is associated with rate of disease progression (69). The 18 kD translocator protein (TSPO) is highly expressed on activated microglia and astrocytes (70, 71). Radiotracers that bind to TSPO thus can visualize neuroinflammation and gliosis *in vivo*. Indeed, early PET studies of neuroinflammation in ALS used the first-generation TSPO ligands [^{11}C]-PK11195 (44) and [^{18}F]-DPA-714 (21) to demonstrate the presence of widespread glial activation in brains of ALS patients compared to healthy controls.

The second-generation TSPO tracer [^{11}C]-PBR28, which binds TSPO with an 80-fold higher specificity than [^{11}C]-PK11195 (72), has enabled more precise PET evaluation of glial activation. Several [^{11}C]-PBR28 PET studies demonstrated increased tracer uptake isolated to the motor cortices of ALS patients compared with controls (46, 47, 50). Areas of increased uptake correlated positively with Upper Motor Neuron Burden Scale and negatively with ALS Functional Rating Scale-Revised (ALSFRS-R) scores (46, 47, 50). Integrated [^{11}C]-PBR28 PET and MRI scans established that areas of increased uptake co-localize with areas of cortical thinning and reduced fractional anisotropy (47, 50).

[^{11}C]-PBR28 PET studies in patients with primary lateral sclerosis (PLS) found a pattern of glial activation similar to that seen in ALS patients, though tracer uptake was greatest in subcortical white matter in PLS patients and in cortical gray matter in ALS patients (48). The differences between ALS and PLS scans highlight the increased specificity of [^{11}C]-PBR28 tracer and merit further investigation into why such differences in glial activation might exist in these two conditions.

In the largest longitudinal ALS PET study to date, 10 patients underwent [^{11}C]-PBR28 PET scans twice over 6 months. Tracer uptake remained stable despite disease progression, as measured by a 3-point decrease in ALSFRS-R (50). This stability may mirror the pattern of beta-amyloid

TABLE 1 | PET studies in ALS.

References	Tracer	Target(s)	Cross sectional results	Longitudinal results	Clinical correlation
GLUCOSE METABOLISM					
Dalakas et al. (18)	^{18}F-FDG	Glucose metabolism	12 ALS vs. 11 HC: diffuse hypometabolism in cortex and basal ganglia of ALS patients with UMN involvement. Cerebellar metabolism similar between ALS and HC.	4 ALS with 2+ scans; variable changes in metabolism over time	No statistically significant difference in cortical metabolism between ALS patients without UMN signs and HC.
Hatazawa et al. (22)	^{18}F-FDG	Glucose metabolism	12 ALS vs. 11 HC: diffuse hypometabolism, greatest in motor-sensory cortex and putamen. No difference in metabolism in patients without UMN involvement	4 ALS with repeat studies showed reduction in metabolism over time	Cortical hypometabolism associated with disease duration at time of scan.
Ludolph et al. (23)	^{18}F-FDG	Glucose metabolism	18 ALS vs. 12 HC: diffuse hypometabolism in frontal regions not reaching statistical significance	None	Hypometabolism in frontal regions correlates with frontal dysfunction measured by neuropsychologic testing. No correlation between hypometabolism and disease duration at time of scan.
Hoffman et al. (24)	^{18}F-FDG	Glucose metabolism	7 ALS vs. 11 HC: no statistically significant difference when corrected for multiple comparisons	3 ALS with repeated scans after 1 year; no significant reduction in uptake despite clinical progression	Decreased motor strength correlated with hypometabolism in precentral gyri and hypermetabolism in middle frontal gyrus
Garraux et al. (25)	^{18}F-FDG	Glucose metabolism	3 ALS-FTD vs. 46 HC, 10 FTD vs. 46 HC: frontal and anterior temporal hypometabolism	None	No statistically significant differences in cortical hypometabolism between 3 ALS-FTD patients and 10 FTD patients when corrected for multiple comparisons
Jeong et al. (26)	^{18}F-FDG	Glucose metabolism	8 ALS-FTD vs. 11 HC: hypometabolism in bilateral frontal lobes, basal ganglia, thalamus	None	No statistically significant differences in cortical metabolism between 8 ALS-FTD and 29 FTD patients
Renard et al. (19)	^{18}F-FDG	Glucose metabolism	4 ALS-FTD vs. 6 ALS	None	ALS patients with FTD had hypometabolism in dorsolateral prefrontal, medial/lateral premotor cortices, insular cortices, anterior temporal lobes compared to ALS patients without FTD
Boeve et al. (27)	^{18}F-FDG	Glucose metabolism	5 C9 ALS: in 4 of 5, hypometabolism in anterior cingulate, frontal cortices compared to age-segmented normative database	1 ALS with second scan after 2 years showing more prominent cortical hypometabolism	Frontal cortical and anterior cingulate hypometabolism correlated with poor performance on neuropsychological measures of psychomotor speed, word fluency, sustained attention
Cistaro et al. (28)	^{18}F-FDG	Glucose metabolism	32 ALS vs. 22 HC: Hypermetabolism in amygdala, midbrain, pons, cerebellum.	None	13 bulbar onset vs. 19 spinal onset ALS: relative hypometabolism in bilateral frontal cortex, right insula, anterior cingulate, precuneus, interior parietal lobe. Bulbar onset patients with lower neuropsychological scores in verbal fluency
Lai et al. (29)	^{18}F-FDG	Glucose metabolism	10 spinobulbar muscular atrophy vs. 5 HC: hypometabolism in frontal areas	None	None reported
Clark et al. (30)	^{18}F-FDG	Glucose metabolism	9 Primary spastic dysarthria vs. HC: variable degrees of hypometabolism in premotor and motor cortices	None	Hypometabolism in premotor and motor cortices associated with symptom duration >2 years

(Continued)

TABLE 1 | Continued

References	Tracer	Target(s)	Cross sectional results	Longitudinal results	Clinical correlation
Pagani et al. (31)	^{18}F-FDG	Glucose metabolism	195 ALS vs. 40 HC: Hypometabolism in frontal, premotor, occipital cortices. Hypermetabolism in midbrain, temporal pole, hippocampus	None	Bulbar onset ALS patients had more rostral pattern of hypometabolism compared to spinal onset ALS patients. Analysis of Brodmann areas 6, 7, 9-11, 13, 17, 18, 21, 22, 24, 32, 37-40, 47 discriminated ALS from HC scans with 95.4% sensitivity and 82.5% specificity
Van Laere et al. (32)	^{18}F-FDG	Glucose metabolism	59 sALS vs. 20 HC: Hypometabolism in premotor and frontal cortices. Hypermetabolism in hippocampus, amygdala, brainstem, occipital, cerebellum. Similar pattern between 59 sALS, 7 PLS and 11 C9 ALS	None	Severe hypometabolism in frontotemporal regions correlated with shorter survival. Prefrontal hypometabolism is correlated with lower ALSFRS-R scores. Support vector machine analysis discriminated ALS from HC scans with 95.8% sensitivity, 80% specificity; PLS from HC with 57.1% sensitivity, 100% specificity.
Cistaro et al. (33)	^{18}F-FDG	Glucose metabolism	15 C9 ALS vs. 30 sALS: hypometabolism in cingulate, insula, caudate, thalamus, left frontal and superior temporal cortex. Hypermetabolism in midbrain, occipital cortex, globus pallidus, left inferior temporal cortex. 12 sALS-FTD vs. 30 sALS: hypometabolism in orbitofrontal, prefrontal, anterior cingulate, insula. Hypermetabolism in occipital, left precentral/postcentral, superior temporal cortices. 15 C9ALS vs. 12 sALS-FTD: hypometabolism in left temporal cortex.	None	Genotype-phenotype correlation: widespread cortical hypometabolism in C9 ALS more reminiscent of sALS-FTD than sALS, despite lack of FTD diagnosis in C9 patients.
Rajagopalan and Pioro (34)	^{18}F-FDG	Glucose metabolism	18 ALS-FTD vs. 15 HC: hypometabolism in frontotemporal lobes, cingulum, cerebellum, and motor cortex when normalized against pons and whole-brain. Most areas of hypometabolism corresponded with areas of gray matter atrophy.	None	None reported
Canosa et al. (35)	^{18}F-FDG	Glucose metabolism	20 ALS-FTD vs. 150 ALS (94 cognitively normal, 37 with cognitive impairment, 9 with behavioral impairment, 10 with nonspecific impairment): hypometabolism in frontal and prefrontal regions.	None	Continuum of frontal lobe hypometabolism correlates with continuum of cognitive impairment
Van Weehaeghe et al. (17)	^{18}F-FDG	Glucose metabolism	70 ALS (training set), 105 ALS (validation set) vs. 20 HC (used for both training and validation set): hypometabolism in frontal, premotor, inferolateral, parietal cortices. Hypermetabolism in primary visual cortex, cerebellum, upper brainstem, medial temporal cortex. 10 PLS vs. 20HC with similar pattern. Training and validation ALS cohorts had identical hypo- and hyper-metabolism patterns when compared to HC.	None	Frontotemporal hypometabolism predictive of shorter survival. Using volume of interest (VOI)-based discriminant analysis of training set: 88.8% accuracy in classifying ALS or PLS vs. HC in 105 prospective validation cases, if PMA scans excluded. Using voxel-based support vector machine (SVM) approach: 100% accuracy for classifying ALS or PLS vs. HC, if PMA scans excluded.
Marini et al. (36)	^{18}F-FDG	Glucose metabolism	30 ALS vs. 30 HC: hypermetabolism in spinal cord	None	Spinal hypermetabolism (>5th decile) associated with higher mortality rate at 3 years

(Continued)

TABLE 1 | Continued

References	Tracer	Target(s)	Cross sectional results	Longitudinal results	Clinical correlation
Matias-Guiu et al. (20)	^{18}F-FDG ^{18}F-florbetaben	Glucose metabolism, amyloid deposition	18 ALS vs. 24 HC: hypometabolism in frontal area, hypermetabolism in cerebellum. Concurrent use of tracer ^{18}F-florbetaben showed no significant difference in amyloid uptake between ALS and HC.	None	Cognitive impairment associated with decreased frontoparietal metabolism
Buhour et al. (37)	^{18}F-FDG	Glucose metabolism	37 ALS vs. 37 HC: hypometabolism in right paracentral lobule, left inferior parietal gyrus, bilateral thalamus, left superior medial frontal gyrus, cerebellar vermis. Hypermetabolism cerebellar lobules, medial temporal cortex, fusiform cortex.	None	Hypometabolism in hippocampus negatively correlated with changes in memory. Hypometabolism in left fusiform gyrus negatively correlated with theory of mind
Yamashita et al. (38)	^{18}F-FDG ^{11}C-flumazenil	Glucose metabolism, blood flow measured by early flumazenil binding	10 ALS vs. 10 HC: hypermetabolism in spinal cord ipsilateral to weakness at C5 and T1. No difference in flumazenil in spinal cord. Concurrent use of tracer ^{11}C-flumazenil showed no difference in spinal cord uptake between ALS and HC.	None	Cervical hypermetabolism associated with ipsilateral arm weakness
D'Hulst et al. (39)	^{18}F-FDG	Glucose metabolism	ALS (175 training scans from Belgium, 195 validation scans from Italy): minor differences in metabolism between ALS groups across two centers. HC (20 training scans from Belgium, 40 validation scans from Italy): prefrontal hypometabolism in Italian HC compared to Belgian HC cohort. Italian HC scans from patients with lung malignancy (no neurologic disease) who underwent oncologic surveillance PET scans	None	Using SVM analysis of training set, classified ALS or HC from validation set with 95% sensitivity, 12% specificity. Unable to reverse analysis using validation cohort as training cohort and vice versa. Diagnostic algorithm to classify ALS from control scans was unsuccessful when control scans came from patients with non-neurologic illness rather than healthy volunteers
CEREBRAL BLOOD FLOW					
Kew et al. (40)	$C^{15}O_2$	Regional CBF	12 ALS vs. 6 HC: At rest, decreased CBF in sensory and motor cortex, supplementary motor area, parietal regions. With joystick movement task, increased CBF in contralateral motor cortex and adjacent premotor and parietal areas	None	In ALS, poorer verbal fluency associated with decreased CBF in right parahippocampus, bilateral anterior thalamus, right anterior cingulate during joystick movement task No correlation between verbal fluency and resting CBF
Kew et al. (41)	$C^{15}O_2$	Regional CBF	10 ALS vs. 5 HC: decreased CBF during joystick movement task	None	In ALS, poorer verbal fluency associated with decreased CBF in right parahippocampus, anterior thalamus, anterior cingulate during task
Tanaka et al. (42)	$C^{15}O_2$ $^{15}O_2$	Regional CBF	9 ALS vs. 13 HC: non-significant reductions in CBF and oxygen metabolism. 4 ALS with dementia vs. 13 HC: decreased CBF and metabolism in anterior cerebral hemispheres and cerebellum	None	Comparison of CBF between ALS with and without clinical dementia not reported
Abrahams et al. (43)	$C^{15}O_2$	Regional CBF	6 ALS vs. 6 HC: decreased activation (smaller increase in CBF compared to CBF in control condition) during word generation task in right dorsal prefrontal, bilateral inferior parietal lobule, left middle/superior temporal gyri 6 ALS with cognitive impairment vs. 6 HC: decreased activation during word generation task in bilateral dorsolateral prefrontal cortex, medial pre-frontal, premotor, anterior thalamic, insular cortex	None	Poor verbal fluency associated with decreased activation in bilateral prefrontal, premotor, insular cortices, thalamus

(Continued)

TABLE 1 | Continued

References	Tracer	Target(s)	Cross sectional results	Longitudinal results	Clinical correlation
NEUROINFLAMMATION					
Turner et al. (44)	^{11}C-PK11195	TPSO	10 ALS vs. 14 HC: increased uptake in precentral gyri, pons, thalamus, dorsolateral prefrontal cortices	None	Increased uptake correlated with UMN-B. No correlation in ALSFRS-R or disease duration.
Johansson et al. (45)	^{11}C-L-deprenyl-D2	MAO-B—postulated nonspecific measure of astrocytosis	7 ALS vs. 7 HC: increased binding rate in white matter and pons, decreased binding rate in parietal and temporal cortices	2 ALS scans at 8 and 10 months, no change	No statistically significant correlation between binding and clinical characteristics
Corcia et al. (21)	^{18}F-DPA-713	TPSO	10 ALS vs. 8 HC: increased uptake in primary motor, supplementary motor, and temporal cortex. No increased activation in pons of bulbar-onset ALS patients.	None	No correlation between uptake and age, disease duration, or ALSFRS-R
Zurcher et al. (46)	^{11}C-PBR28	TPSO	10 ALS vs. 10 HC: increased uptake in motor cortices and corticospinal tracts	None	Increased uptake correlated negatively with ALSFRS-R, positively with UMN-B score.
Alshikho et al. (47)	^{11}C-PBR28	TSPO	10 ALS vs. 10 HC: increased uptake in left motor cortex correlates with decreased cortical thickness and fractional anisotropy	None	Increased uptake correlated positively with UMN-B score.
Paganoni et al. (48)	^{11}C-PBR28	TSPO	10 PLS vs. 10 HC: increased uptake in anatomically relevant motor regions co-localized with regional gray matter atrophy and decreased subcortical fractional anisotropy	None	No correlation between uptake and UMNB and ALSFRS-R
Albrecht et al. (49)	^{11}C-PBR28	TSPO	10 ALS, 10 HC, 10 low back pain. Occipital cortex may serve as pseudoreference region rather than whole brain for measuring PBR28 uptake.	None	None reported
Alshikho et al. (50)	^{11}C-PBR28	TSPO	53 ALS vs. 21 HC: increased uptake in precentral and paracentral gyri. 11 PLS vs. 21 HC: increased uptake in subcortical white matter of same regions. Increased uptake colocalizes with cortical thinning, reduced frational anisotropy, increased mean diffusivity.	10 scans 6 months apart, no significant change despite decrease in ALSFRS-R by 3 points	Increased uptake in regions of interest correlated positively with UMNB score. Uptake did not change significantly despite clinical decline
Ratai et al. (51)	^{11}C-PBR28	TSPO	40 ALS: PBR28 uptake correlates positively with mI/Cr and negatively with NAA/Cr in precentral gyri.	None	ALSFRS-R score correlated positively with NAA/Cr and negatively with mI/Cr. UMNB score correlated positively with PBR28 uptake and mI/Cr, negatively with NAA/Cr
GABAergic FUNCTION					
Lloyd et al. (52)	^{11}C-flumazenil	GABAa receptor	17 ALS vs. 17 HC: decreased uptake in bilateral prefrontal, parietal, visual association, left premotor/motor cortex.	None	No differences in uptake between ALS patients with or without pseudobulbar affect
Turner et al. (53, 54)	^{11}C-flumazenil	GABAa receptor	24 sALS vs. 24 HC: decreased uptake in premotor, motor, posterior association regions. 10 SOD1 D90A ALS vs. 24 HC: decreased uptake in left frontotemporal junction, anterior cingulate. 2 pre-symptomatic SOD1 D90A—decreased uptake in left frontotemporal junction. 4 PLS vs. HC: relative preservation of anterior and orbitofrontal binding compared to ALS.	None	In sALS, decreased uptake in dominant hemisphere correlated with higher UMN-B score. No correlation between uptake and ALSFRS-R. In SOD1 D90A ALS, uptake correlated positively with ALSFRS-R rather than UMNB.
Wicks et al. (55)	^{11}C-flumazenil	GABAa receptor	12 ALS with cognitive testing	None	Correlation between poorer performance in verbal fluency and reduced binding in right inferior frontal gyrus, superior temporal gyrus, anterior insula.

(Continued)

TABLE 1 | Continued

References	Tracer	Target(s)	Cross sectional results	Longitudinal results	Clinical correlation
					Correlation between poorer confrontation naming and reduced binding in left inferior frontal gyrus/middle frontal gyrus.
Yabe et al. (56)	^{11}C-flumazenil	GABAa receptor	10 ALS with cognitive testing	None	Correlation between writing errors and reduced binding in bilateral anterior cingulate gyrus
SEROTONERGIC FUNCTION					
Turner et al. (57)	^{11}C-WAY100635	5-HT1a receptor	21 ALS vs. 19 HC: marked decreased global cortical binding (21%). Regional decreased binding in frontotemporal regions, cingulate, lateral precentral, parahippocampal, and fusiform gyri	None	Greater decrease in cortical binding in ALS (21%) compared to historical data in depression (12%) and Parkinson's (15%). Trend toward greater reductions in binding in patients with bulbar involvement
Turner et al. (58)	^{11}C-WAY100635	5-HT1a receptor	11 SOD1 D90A ALS vs. 19 HC: decreased global cortical binding (12%), less dramatic when compared with reduction in binding in sporadic ALS vs. HC (21%)	None	Less reduction in cortical binding of D90A ALS compared to sporadic ALS, despite lower ALSFRS-R scores
DOPAMINERGIC FUNCTION					
Takahashi et al. (59)	^{18}F-6-fluorodopa	Levodopa metabolism	16 ALS vs. 13 HC: no difference in mean striatal uptake	None	Negative correlation between 6-fluorodopa uptake and duration of ALS symptoms. No correlation between uptake and severity of symptoms.
Przedborski et al. (60)	^{18}F-6-fluorodopa	Levodopa metabolism	7 SOD1 familial ALS, 7 non-SOD1 familial ALS, 14 HC. 5/14 familial ALS with reduced uptake in nigrostriatal region, more commonly seen in non-SOD1 patients.	None	No correlation between binding and duration of symptoms
Hideyama et al. (61)	^{18}F-6-fluorodopa, ^{11}C-N-methyl-spiperone	Levodopa metabolism and D2/D3 receptor antagonist	5 ALS with clinical parkinsonism: preganglionic and postganglionic striatonigral dopaminergic systems preserved	None	Parkinsonism in ALS patients not correlated with striatonigral dysfunction
Fu et al. (62)	^{18}F-fallypride	D2/D3 receptor antagonist	17 ALS vs. 11 HC: decreased binding in bilateral nucleus accumbens, frontal lobes, superior frontal gyri, left temporal lobe, left angular gyrus. No difference in striatum.	None	None reported
OXIDATIVE STRESS					
Ikawa et al. (63)	^{62}Cu-ATSM	Intracellular reductive state	12 ALS vs. 9 HC: increased uptake in bilateral pre- and post- central gyri and paracentral lobule, right superior parietal lobule.	None	Increased uptake negatively correlated with ALSFRS-R. No correlation between uptake and disease duration

ALS, amyotrophic lateral sclerosis; ALSFRS-R, ALS functional rating scale-Revised; C9, C9orf72 hexanucleotide repeat expansion; CBF, cerebral blood flow; FTD, frontotemporal dementia; HC, healthy control; ml/Cr, myoinositol/creatine ratio; NAA/Cr, N-acetylaspartate/creatine ratio; PMA, primary muscular atrophy; sALS, sporadic ALS; SOD1, superoxide dismutase 1; TSPO, translocator protein; UMN, upper motor neuron; UMN-B, Upper Motor Neuron Burden scale.

brain deposition in Alzheimer's disease, as measured by Pittsburg compound B (PiB) PET imaging: PiB uptake rises in patients developing mild cognitive impairment, then plateaus upon development of Alzheimer's dementia (73). Alternatively, it may reflect a bias toward recruitment of slowly-progressive patients into longitudinal neuroimaging studies. Longitudinal studies with larger sample sizes, rapidly progressing patients, and patients early in the disease course are needed to determine the natural history of glial activation in ALS.

GABAergic Function

Cortical excitability is altered in ALS (6). To evaluate whether loss of GABAergic inhibition contributes to cortical hyperexcitability in ALS, PET studies were conducted using the $GABA_A$ receptor ligand [^{11}C]-Flumazenil. These studies showed widespread reductions in binding in ALS patients compared to controls (52), and found that reduced binding in the frontal lobes (55) and anterior cingulate gyri (56) in ALS patients correlated with poorer performance on language tasks. Additionally, patients with slowly progressive ALS caused by SOD1 D90A

mutations had smaller reductions in binding compared to sporadic ALS patients (53). Taken together, these findings could suggest that loss of GABAergic cortical inhibition is part of ALS pathogenesis, though it is also possible that it reflects generalized cortical neuronal loss rather than specific loss of GABAergic inhibition.

Serotonergic Function

The serotonin 5-hydroxytryptamine (5-HT1a) receptor is expressed widely in the cortex, including on layer III and V pyramidal neurons in the cortex (74). A PET imaging study using the 5-HT1a ligand [^{11}C]-WAY100635 demonstrated decreased tracer binding in the frontotemporal regions, precentral, cingulate, parahippocampal, and fusiform gyri in non-depressed ALS patients compared to healthy controls (57). A follow up study reported smaller reductions in [^{11}C]-WAY100635 uptake in patients with slowly progressive SOD1 D90A genetic ALS compared to sporadic ALS (58). Like the studies using $GABA_A$ ligands, these studies suggest widespread neuronal loss or dysfunction in ALS patients that is less apparent in slowly progressive disease.

Dopaminergic Function

Evidence of extramotor involvement in ALS has raised questions about its overlap with neurodegenerative disorders such as Parkinson's disease. Rare patients with ALS have parkinsonism, and post-mortem evaluation has revealed degeneration of the substantia nigra in ALS (75). To evaluate whether dopaminergic dysfunction plays a role in ALS pathogenesis, several PET studies were conducted using ligands that interrogate levodopa metabolism [[^{18}F]-fluorodopa (59, 61)], bind to dopamine receptors in the striatum [[^{11}C]-N-methylspiperone (61)], and bind to dopamine receptors in the cortex [[^{18}F]-fallypride (62)]. The [^{18}F]-fluorodopa and [^{11}C]-N-methylspiperone studies showed no significant difference in levodopa metabolism or dopamine receptor binding in the striatum of ALS vs. control subjects, even in patients with overt parkinsonism (59, 61). Conversely, the [^{18}F]-fallypride PET study showed decreased dopamine binding in the cortex of ALS patients (62), even though the patients were not noted to have clinical parkinsonism. One interpretation of these studies is that ALS is associated with cortical rather than striatal dopaminergic dysfunction. However, PET studies demonstrating decreased cortical binding of GABAergic, serotonergic, and now dopaminergic ligands in ALS patients argues against dopamine-specific pathogenesis of ALS and supports a generalized cortical neuronal loss or dysfunction in disease.

Oxidative Stress

Oxidative stress is considered one of the pathogenic mechanisms underlying neurodegeneration in ALS (76) and is the proposed target of edaravone, a free radical scavenger recently approved for treatment of ALS (77). The PET ligand [^{62}Cu]-ATSM is a copper-linked small molecule structurally similar to superoxide dismutase (78). It distributes to areas of hypoxia and oxidative stress in PET studies of patients with Parkinson's disease (79) and mitochondrial diseases (80). One [^{62}Cu]-ATSM PET study in ALS showed increased tracer accumulation in the motor cortices, paracentral lobules, and right superior parietal lobule in ALS patients compared to controls (63). Areas of increased uptake negatively correlated with ALSFRS-R score.

Notably, Cu-ATSM was selected as an investigational drug for ALS because human [^{62}Cu]-ATSM PET studies demonstrated effective penetration into the brain. Cu-ATSM's proposed mechanism of action is free radical scavenging and delivery of copper into the CNS (81). Cu-ATSM slowed disease progression in SOD^{G93A} mouse models of ALS (81, 82) and is now entering phase I human clinical trials for ALS (Clinicaltrials.gov NCT02870634).

CHALLENGES AND OPPORTUNITIES IN THE DEVELOPMENT OF MOLECULAR IMAGING BIOMARKERS FOR ALS

The FDA-NIH Biomarker Working Group defines a biomarker as a "characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions" (83). Because PET imaging can localize molecular changes in the brain, it has unique promise for use as a diagnostic, prognostic, and pharmacodynamic biomarker. Its advantages and disadvantages complement other biomarkers being developed for ALS (**Table 2**).

PET Imaging as a Diagnostic Biomarker

Mounting evidence of quantifiable PET imaging differences between ALS and control brains has generated interest in using PET as a diagnostic biomarker for ALS. Indeed, the sensitivity of PET makes it uniquely positioned to detect or confirm UMN dysfunction in suspected ALS patients, which has traditionally been difficult to measure.

Three successive studies recently assessed the diagnostic potential of [^{18}F]-FDG PET in ALS (17, 32, 39). In these studies, the authors used group differences in scans from ALS and control subjects to generate algorithms ("diagnostic algorithms") for classifying individual scans as ALS vs. control. Group-level differences in FDG uptake between ALS and control scans were consistent across time and between two imaging centers. Within one center, the diagnostic algorithm generated from a training cohort achieved high accuracy when classifying scans from a validation cohort (as ALS or control), though accuracy decreased when scans from PLS patients were included in the analysis (17). However, in a multicenter study, the diagnostic algorithm derived from one center's scans (training cohort) achieved 94.8% sensitivity but only 12.5% specificity in classifying scans from a second center (validation cohort) as ALS or control (39). The low specificity was attributed to relative frontal hypometabolism in the validation control scans, compared to training control scans. The validation control scans came from patients with non-neurologic malignancies undergoing surveillance

TABLE 2 | Advantages and disadvantages of potential biomarkers for ALS.

Biomarker type	Advantages	Disadvantages
Neuroimaging biomarkers: Positron emission tomography (PET)	Ability to interrogate disease mechanisms of interest using specific molecular ligands (e.g., energy metabolism, neuroinflammation, neuronal dysfunction, oxidative stress) Dynamic ligand binding enables visualization of treatment effect in central nervous system (CNS) Sensitive to early pathological changes Localizes pathology in CNS	Cost Limited scalability due to expertise and resources required (local cyclotron for production of radioisotopes) Small risk associated with repeated radiation Use may be limited by patient orthopnea
Neuroimaging biomarkers: Magnetic resonance imaging (MRI)	Widely available Advanced techniques permit evaluation of brain activation (functional MRI), white matter tracts (diffusion tensor imaging), and cellular metabolites (magnetic resonance spectroscopy) Free of radiation Localizes pathology in CNS	Large sample sizes required to demonstrate treatment effect limits pharmacodynamic potential (84) Use may be limited by patient orthopnea
Biological fluid-based biomarkers	Scalable Cost-effective Ease of collection Potential for standardization and centralization in core laboratory	Non-localizing
Electrophysiological biomarkers	Directly measures physiology of organs affected by disease Accepted use in diagnosis (electromyography) Sensitive to early pathological changes Good face value for monitoring disease progression	Reliability and reproducibility Sensitive to technical artifacts Potential patient discomfort (electromyography)

brain PET, whereas the training control scans came from healthy volunteers.

These studies highlight the challenges in translating population-level PET data into diagnostic criteria for individual patients. While progress is being made, PET is not yet a valid diagnostic biomarker for ALS. Validation will require longitudinal studies to determine whether prospectively collected scans of patients undergoing evaluation for ALS can distinguish UMN dysfunction before clinical signs emerge. The studies will also need to distinguish motor neuron disease not just from healthy volunteers, but also from disease mimics. If validated as a diagnostic biomarker, PET imaging could shorten the time from ALS symptom onset to diagnosis and facilitate earlier intervention in the neurodegenerative process.

PET Imaging as a Prognostic Biomarker

PET imaging has potential for prognostic use in ALS. Two studies in ALS patients found an association between mortality rate and presence of extensive frontotemporal hypometabolism on [^{18}F]-FDG PET scans (17, 32). Conversely, patients with spinal cord hypermetabolism in the top 20% of one study cohort had a significantly higher mortality rate compared to the rest of the cohort (36). Further longitudinal studies that evaluate whether PET imaging findings can predict the likelihood of future events (such as survival, development of cognitive impairment, or spread of disease from one anatomical region to another) are needed to establish valid prognostic PET biomarkers in ALS.

One intriguing potential use for prognostic PET imaging is in identifying when asymptomatic ALS gene carriers enter a high-risk period for developing clinical disease ("phenoconversion"). Rising levels of serum neurofilament light chain can detect neurodegeneration ∼1 year before phenoconversion in asymptomatic ALS gene mutation carriers (13). To evaluate whether PET imaging can detect also changes that predict phenoconversion, longitudinal [^{11}C]-PBR28 PET studies are being conducted in asymptomatic gene mutation carriers to look for neuroinflammation before disease onset. Prognostic biomarkers of phenoconversion may facilitate development of gene therapy trials designed to prevent ALS, which may be the best opportunity for treating or even curing certain genetic forms of ALS.

PET Imaging as a Pharmacodynamic Biomarker

PET imaging has value as a pharmacodynamic marker in ALS because it can rapidly measure and localize biological activity of investigational agents in the target tissue—the brain. The variety of available PET ligands may enable direct visualization of multiple pharmacologic targets. PET imaging's sensitivity to molecular changes can increase statistical power to detect a drug effect.

[^{11}C]-PBR28 PET is an appealing pharmacodynamic biomarker for ALS clinical trials because binding is dynamic and rapidly responsive to treatment: in Parkinson's disease (85) and traumatic brain injury (86) patients, anti-inflammatory treatment reduced cortical [^{11}C]-PBR28 binding in as little as 4 weeks (85). Additionally, the stability of [^{11}C]-PBR28 uptake in ALS over 6 months of disease progression permits a marked reduction in sample size needed to determine drug effect. A simulated sample size and power calculation using longitudinal [^{11}C]-PBR28 PET data found that 30 participants are needed in a single-arm ALS clinical trial to show a 2% change in [^{11}C]-PBR28 uptake after drug treatment, whereas hundreds of participants are needed to show a 30% reduction in ALSFRS-R slope (50). Currently, four ongoing clinical trials are using [^{11}C]-PBR28 PET as a pharmacodynamic biomarker to assess the biological activity of investigational treatments in ALS

(Clinicaltrials.gov NCT02714036, NCT02469896, NCT03127514, NCT03456882) (87).

In the future, PET imaging using an array of ligands will enable efficient evaluation of multiple pharmacologic targets. Pharmacodynamic data from PET studies may help confirm the biological activity of ALS drugs in the target tissue and inform dose selection based on biological activity. Data derived from these trials will enable deeper understanding of the role of different molecular mechanisms in disease pathogenesis.

CONCLUSIONS AND FUTURE DIRECTIONS

Thirty years of PET imaging has shed light on the pathophysiology of ALS and the expanding boundaries of cortical dysfunction in disease. Because PET imaging can localize molecular changes in the CNS *in vivo*, it has the potential to fill a critical gap in our armamentarium of diagnostic, prognostic, and pharmacodynamic biomarkers for ALS. To realize this potential, major limitations of the research to date will need to be addressed. First, most PET studies in ALS were small. Only 7 published studies enrolled more than 50 ALS patients (17, 31–33, 35, 39, 50), which raises concern for false positive and/or negative findings generated by studies with small sample sizes. Second, minimal longitudinal PET data exists in the ALS literature. A total of 24 ALS patients have had longitudinal PET scans in published studies (18, 22, 24, 27, 45, 50). Third, clinical-radiological correlations reported in the literature are insufficiently characterized and often contradictory. To address these limitations, we must conduct collaborative, multicenter longitudinal studies to collect PET imaging and clinical data in large patient cohorts. Moreover, to ascertain accurate clinical-radiologic correlations, clinical data should be captured by validated instruments that separate motor and cognitive deficits and reliably measure UMN dysfunction.

From a practical standpoint, the widespread use of PET imaging is presently limited by cost, need for expertise and local production of radioactive isotopes. Therefore, PET imaging currently is most useful as a pharmacodynamic biomarker for early clinical trials in ALS. Future multicenter longitudinal studies will allow us to establish the relationship between PET imaging findings and meaningful clinical outcomes, and thus develop and validate the PET imaging biomarkers that can accelerate drug development and advance care for people with ALS.

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SC and NA drafted the manuscript. Both authors made a direct and intellectual contribution to the work and approved it for publication.

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MINI REVIEW
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White Matter Microstructure Breakdown in the Motor Neuron Disease Spectrum: Recent Advances Using Diffusion Magnetic Resonance Imaging

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Motor neuron disease (MND) is a fatal progressive neurodegenerative disorder characterized by the breakdown of the motor system. The clinical spectrum of MND encompasses different phenotypes classified according to the relative involvement of the upper or lower motor neurons (LMN) and the presence of genetic or cognitive alterations, with clear prognostic implications. However, the pathophysiological differences of these phenotypes remain largely unknown. Recently, magnetic resonance imaging (MRI) has been recognized as a helpful *in-vivo* MND biomarker. An increasing number of studies is applying advanced neuroimaging techniques in order to elucidate the pathophysiological processes and to identify quantitative outcomes to be used in clinical trials. Diffusion tensor imaging (DTI) is a non-invasive method to detect white matter alterations involving the upper motor neuron and extra-motor white matter tracts. According to this background, the aim of this review is to highlight the key role of MRI and especially DTI, summarizing cross-sectional and longitudinal results of different approaches applied in MND. Current literature suggests that DTI is a promising tool in order to define anatomical "signatures" of the different phenotypes of MND and to track *in vivo* the progressive spread of pathological proteins aggregates.

Keywords: amyotrophic lateral sclerosis, motor neuron disease, diffusion tensor imaging, fractional anisotropy, network analysis, magnetic resonance imaging, structural connectomics

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INTRODUCTION

Motor neuron disease (MND) is a group of fatal neurodegenerative diseases characterized by progressive damage of the upper motor neurons (UMN) in the cortex and/or lower motor neurons (LMN) in the brainstem and spinal cord. Depending on the relative involvement of UMN and LMN, MND can be classified in a wide range of clinical phenotypes (including amyotrophic lateral sclerosis [ALS], primary lateral sclerosis [PLS], and progressive muscular atrophy [PMA]), characterized by different clinical presentation and progression rate. Advanced brain imaging techniques, such as magnetic resonance imaging (MRI), have been developed over the last decades in order to detect *in vivo* structural and functional brain abnormalities and to monitor

neurodegeneration in the central nervous system of MND patients. Although neurodegeneration primarily affects the gray matter (GM), pathological alterations in the white matter (WM) have also been reported (1), involving not only the corticospinal tract (CST), but also non-motor regions (2).

The present review aims to discuss the current state of the art of MRI within different phenotypes of MND, focusing on WM microstructural alterations, underlining the role of MRI as a tool to understand disease pathophysiology and to provide potential biomarkers for diagnosis and prognostic stratification. Moreover, we also highlight emerging techniques, such as graph analysis, that will likely provide further insights in disease pathogenesis and might help in monitoring disease progression.

DIFFUSION TENSOR IMAGING

Basic Principles

Diffusion tensor imaging (DTI) is the most common MRI technique that allows to investigate WM microstructural changes. DTI is based on the random diffusion of water molecules in the fiber bundles, also known as Brownian motion (3). DTI analysis relies on the concept that, in a spherical volume, the diffusion of water has no preferential direction and spreads equally in three different directions (λ1, λ2, and λ3). Nevertheless, the movement of water molecules within the WM is approximately elliptical, having the greatest movement along axons (axial diffusivity [λ1]) caused by the restriction in the minor axes (radial diffusivity [λ2 and λ3]) imposed by myelin. In order to analyze the diffusion of water molecules, it is possible to define four parameters: (1) fractional anisotropy (FA), which describes how strongly directional is the movement of water molecules within the tissue; (2) radial diffusivity (RD, which is the average of λ2 and λ3); (3) axial diffusivity (AD, or λ1); (4) mean diffusivity (MD, obtained by the average of diffusion in the λ1, λ2, and λ3 axes). While the first three parameters (FA, RD, and AD) describe the spatial variation of water movement, MD reflects the average displacement of water molecules within the volume of interest. Axonal integrity will preserve diffusion parallel to the main fiber direction, resulting in higher FA and lower MD, while damage to the WM will lead to lower FA and higher MD (4). To date, there are several approaches to analyze DTI metrics: regions of interest (ROI) approach, whole-brain voxel-wise methods or tract-based spatial statistics (TBSS). These techniques provide complementary information and are characterized by relative strengths and limitations. The ROI approach is based on the delineation of defined areas or the reconstruction of WM tracts of interest in each subject's native space, in order to extract average DTI metrics to be compared among subjects; although this procedure allows a precise anatomical definition of WM structures and does not involve the coregistration of multiple scan images, it masks local alterations by averaging all voxels within the ROI, usually needs an *a priori* hypothesis and might be influenced by inter-subject anatomical variability (5). The most straightforward approach to assess local DTI alterations is to coregister all subjects' scans and perform statistical tests among groups within each voxel of the whole-brain WM mask; however, whole-brain voxel-wise approaches are sensitive to registration errors (6). To reduce the effects of local misregistrations, TBSS projects all voxels of the DTI image onto the nearest location on a "skeleton" delineating the main WM tracts (7). In addition to these methods, graph theory is one of the most recent approaches to investigate WM changes, building models of structural connectivity in brain disorders based on nodes and edges (8). Current evidence provided by each of these techniques for the study of MND is summarized in the following paragraphs.

The weakness of DTI is the lack of specificity in voxels presenting multiple fiber populations (termed "crossing fibers") (9). In order to overcome this problem, novel data acquisition approaches have been proposed such as high angular resolution diffusion imaging (HARDI), neurite orientation dispersion and density imaging (NODDI) and diffusion spectrum imaging. Although these approaches hold the promise to provide further insights on the pathogenic mechanisms underlying WM degeneration and are likely sensitive to even subtle alterations in several neurodegenerative conditions (10), current evidence in the context of MND is scarce and should be considered preliminary (11, 12).

DTI Signatures in ALS

Several studies have consistently demonstrated decreased FA and increased MD, RD, and AD along the entire CST in ALS patients relative to healthy controls (13–18). Several studies showed specific alterations of DTI metrics only in some parts of the CST: subcortical WM of the precentral gyrus, corona radiata, posterior limb of the internal capsule, cerebral peduncles and pons (19–21). DTI studies have also detected altered metrics in the middle and posterior part of the corpus callosum in ALS patients relative to healthy controls (22, 23). Cervical cord studies also consistently showed DTI alterations in the lateral columns of ALS patients (24–27), which were more severe at more distal cervical segments (25).

Many neuroimaging studies characterized the structural "signatures" in ALS patients with specific underlying genetic mutations. In particular, diffuse WM abnormalities were observed in *C9orf72* repeat expansion carriers (the most common genetic mutation) (28, 29). Particularly, *C9orf72* patients showed an involvement of the CST, whole corpus callosum and superior longitudinal fasciculus compared with healthy controls, in terms of decreased FA and increased MD (29). Only few structural MRI studies were performed in carriers of pathogenic mutations in *SOD1*, showing a relative preservation of brain motor networks compared to sporadic ALS patients (30, 31).

Cross-sectional DTI studies shed light on the pathophysiological processes associated with the development of ALS. However, the definition of biomarkers that could track progressive changes over time has crucial importance. To date, relatively few longitudinal studies focused on DTI changes over time in these patients, due to the difficulties in enrolling enough cases with a rapidly evolving disease who could undergo an appropriate number of follow-up scans. Most of the studies, using a ROI approach or TBSS, showed decreasing values of FA over time in CST, corpus callosum, frontal areas and cerebellum (21, 32–35). One study demonstrated also

that diffusivity increased both in the external and internal capsule (21). Nevertheless, there are also studies showing inconsistent results, probably due to different sample sizes, follow-up intervals and, most importantly, the heterogeneity of MND patients (36–38). The same limitations apply to the few longitudinal studies assessing the evolution of cervical cord DTI alterations (27, 36) that showed diverging results about the entity of cord FA decrease over time. One recent study was performed in ALS patients carrying *C9orf72* mutation, demonstrating the spreading of diffusivity alterations from anterior to posterior WM regions over a 6-month period (39).

Phenotyping the MND Spectrum

DTI measures might also be crucial to distinguish different MND phenotypes. Indeed, DTI metrics were widely used for the identification of "signatures" in PLS. In particular, one study demonstrated that PLS patients showed lower CST FA values relative to healthy controls and ALS patients (40). Degeneration in extra-motor areas has also been found to be similar (41) or even more severe (40) in PLS patients compared to ALS patients. Furthermore, widespread DTI alterations were found to correlate with the severity of cognitive deficits in PLS patients (42). On the other hand, the least extensive microstructural changes were observed in patients with predominant LMN involvement, with diverging results in literature concerning the extent and significance of such damage (43–45). Particularly, a recent two-center study suggested that WM integrity was disrupted along the CST and in frontal and prefrontal regions in patients with predominant LMN disease relative to healthy controls (46). Only patients with predominant LMN involvement and a higher rate of disease progression showed significant WM alterations in the specific ALS-related tract systems (46).

Clinical and Neuropsychological Correlations

Many DTI studies aimed to test the relationship between WM changes and clinical and neuropsychological measures in MND. Decreased FA in the CST related with disease severity and rate of disease progression in ALS, identifying an association between worsening disability and degeneration of WM tracts, both in the brain (21) and the cervical cord (24, 27). These findings support the potential use of connectivity measures as markers of disease progression in ALS. Inconsistencies among different studies have been reported as for the relationship between DTI measures and disease duration in ALS patients, as longer disease duration has been paradoxically associated with both increased FA (47) and increased MD values of the CST (48). These discrepancies may be explained by the different progression rates of the two samples. DTI changes in the CST and corpus callosum, as well as in the cingulum, inferior longitudinal, inferior fronto-occipital, and uncinate fasciculi have been found to correlate with performance at cognitive tests assessing attention and executive functions (49). Additional extensive WM damage to extra-motor frontotemporal tracts has also been shown, underlying variable degree of behavioral and cognitive disturbances in ALS patients (45, 50, 51). Particularly, one study demonstrated that WM abnormalities of the corpus callosum and frontotemporal tracts, including uncinate, cingulum, and superior longitudinal fasciculi, are the best predictor of executive and non-executive deficits and behavioral changes within the MND spectrum (51).

Network-Based Analyses

Network-based analysis of structural connections is a new powerful technique that allows studying the brain of healthy subjects or patients with neurodegenerative disorders. The techniques mentioned so far allow to map WM tracts individually using DTI. Recently, neuroimaging research has moved to the study of the human connectome, which aims to map all the possible pathways of the human brain (52). With such new approach, it is possible to provide information about how networks are embedded and interact in the brain. Using graph analysis and connectomics, brain regions can be depicted as a set of nodes, linked by edges representing structural connections. Maps of structural connectivity are created following the following steps: (1) network nodes are identified applying a selected atlas of GM structures to the brain; (2) following definition of the brain regions, WM tracts are reconstructed using DTI; (3) streamlines of the whole brain touching each couple i and j of the segmented GM nodes are selected; (4) the number of streamlines is calculated for each tract and inserted into a matrix; (5) for each structural connection, the level of microstructural integrity is measured extracting the mean FA, MD, RD, and AD values; (6) finally, all the values are inserted into four different matrices. From the analysis of these matrices, it is possible to provide information concerning the topological organization of network architecture (53). Many studies have examined the global and local graph metrics such as: (1) nodal strength and degree, which provide information regarding the effect of a node in the network; (2) clustering coefficient and local efficiency, which reflect the level of local organization of a network; (3) path length, that is the number of steps needed to connect each pair of nodes; (4) global efficiency, calculated as the inverse of path length, which represents the efficacy of a network to communicate between each pair of nodes; (5) modularity, which gives information regarding segregation of a network, reflecting the level of modular organization (54, 55). To date, modifications of brain topological organization and disruption of structural connectivity have been associated with several neurodegenerative disorders (56–58), including MND.

In a first cross-sectional study, structural brain networks were compared between ALS patients and healthy controls applying network-based statistics (59). ALS patients showed regions with reduced WM connectivity, centered around the primary but also included secondary motor regions (frontal cortex and pallidum). In addition, overall efficiency and clustering coefficient were found to be decreased in ALS patients. A second study studied WM alterations using network analysis, comparing results with those obtained using TBSS (60). The results, consistent with the previous study, showed an impaired motor-frontal-subcortical subnetwork in the ALS patients compared with controls (60). The study also revealed that the results obtained with the network analysis have a strong correspondence with voxel-based approaches (60).

To date, only a few longitudinal studies aimed to investigate the effect of ALS on the brain network over-time. Particularly, one study showed an expanding sub-network of impaired brain connections after six months, with a central role of the primary motor regions (61). The loss of structural connectivity was found to propagate to frontal and parietal regions, supporting the idea that disease spreads along WM connections following a pattern classified into sequential stages (62).

DTI as a Non-invasive *in-vivo* Biomarker of Disease Spreading

Neuropathological studies identified the cytoplasmic inclusions of TDP-43 as the molecular hallmark in up to 98% of ALS cases (63). In the last few years, several studies have speculated that the progressive regional accumulation of TDP-43 aggregates in the brain might be reflected by the consecutive deterioration of WM fiber tracts (61). In light of this, DTI-based approaches have been used to study propagation patterns in the brain of MND patients. A DTI study, using a tract of interest-based staging approach, confirmed the neuropathological progression of ALS in the following order: CST (stage 1); corticorubral and corticopontine tracts (stage 2); corticostriatal pathway (stage 3) and proximal portion of the perforant path (stage 4) (64). Furthermore, the extracted tracts of interest were used to categorize ALS patients into the predefined stages according with their WM damage. Staging categorization at baseline was able to classify 72% of the ALS patients into the different stages. After 6 months, there was an increase in ALS stage in 27% of ALS patients (64). Recent studies applied the *in-vivo* staging approach also to phenotypic variants of ALS. One study aimed to figure out if PLS might be a separate disease or just a slowly progressive variant of ALS (41). Microstructural changes were analyzed using the same approach as "classical" ALS, demonstrating that ALS and PLS patients showed identical alterations in the ALS-related tract systems, considering consequently PLS as phenotypical variant of ALS (41) (**Figure 1**).

The previously considered studies investigated pathology spreading in ALS-related tracts that were selected *a priori*, according with *post-mortem* neuropathological stages. In order to overcome this *a-priori* selection, one study applied network analysis to investigate the underlying pathogenic mechanism of ALS (65). The results showed that regions involved by TDP-43 pathology in early disease stages are highly structurally interconnected in the brain (65). Furthermore, brain regions of subsequent neuropathological stages were found more closely interconnected than regions of more distant stages (65), suggesting that spread of TDP-43 in ALS occurs along axonal pathways (**Figure 2**). The DTI-based *in-vivo* staging of MND patients needs to be confirmed in future longitudinal studies with *post-mortem* confirmation.

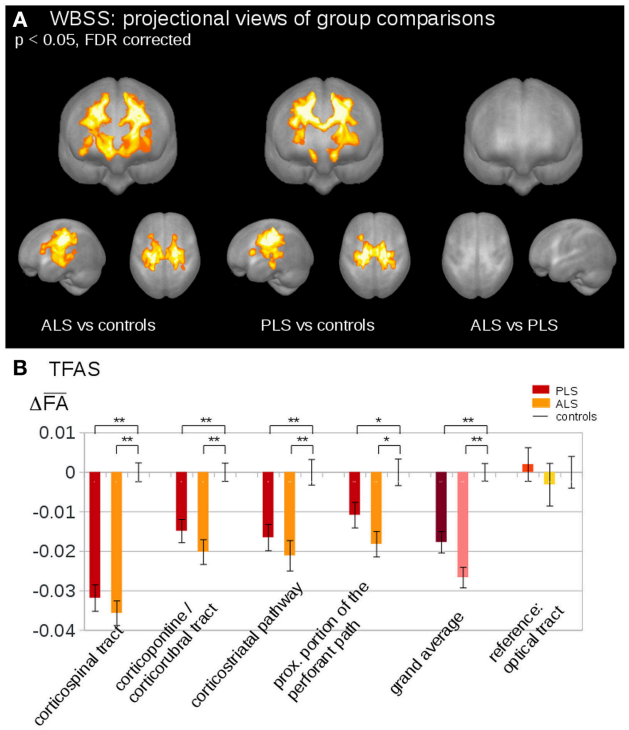

FIGURE 1 | (A) Whole brain-based spatial statistics (WBSS) of fractional anisotropy (FA) maps at the group level for amyotrophic lateral sclerosis (ALS) patients, primary lateral sclerosis (PLS) patients, and controls. WBSS of FA maps demonstrated multiple clusters of regional FA reductions at $p < 0.05$ (corrected for multiple comparisons), projectional views. **(B)** Tractwise fractional anisotropy statistics (TFAS) of FA maps at the group level for ALS patients, PLS patients, and controls. TFAS demonstrated significant regional FA reductions in ALS-related tract systems and in the grand average between ALS patients and controls as well as between PLS patients and controls. No alterations between groups were observed in the reference tract. $^{*}p < 0.05$, $^{**}p < 0.001$. Reproduced with permission from Müller et al. NeuroImage Clinical 2018 (41) (published open-access under a CC BY-NC-ND 4.0 license).

DISCUSSION AND FUTURE DIRECTIONS

In the context of therapeutic trials, it is essential to identify a useful biomarker that might help for diagnosis, stratification and tracking the disease progression within the MND spectrum. In order to provide new drugs that could aid the early treatment of the disease, the identification of such biomarker is a crucial point to be addressed. Within such a framework, MRI has been long recognized as *in-vivo* biomarker and, in the last few years, an increasing number of studies applied advanced neuroimaging techniques in order to understand the underlying mechanisms in MND. Particularly, we highlighted the important role of DTI, as a very useful tool in order to characterize microstructural changes during the progression of the disease, to find "signatures" of the different phenotype of MND and to track *in vivo* the progressive spread of TDP-43 aggregates. In order to detect WM changes of different phenotypes of MND, cross-sectional studies were performed highlighting alterations within specific tracts, especially in the CST as well as in the corpus callosum. In light of the fact that decreased FA and increased MD describe the microstructural damage in MND patients, we support the idea that the most potential promising DTI biomarkers are FA or MD changes in the CST and corpus callosum. Additionally, connectivity

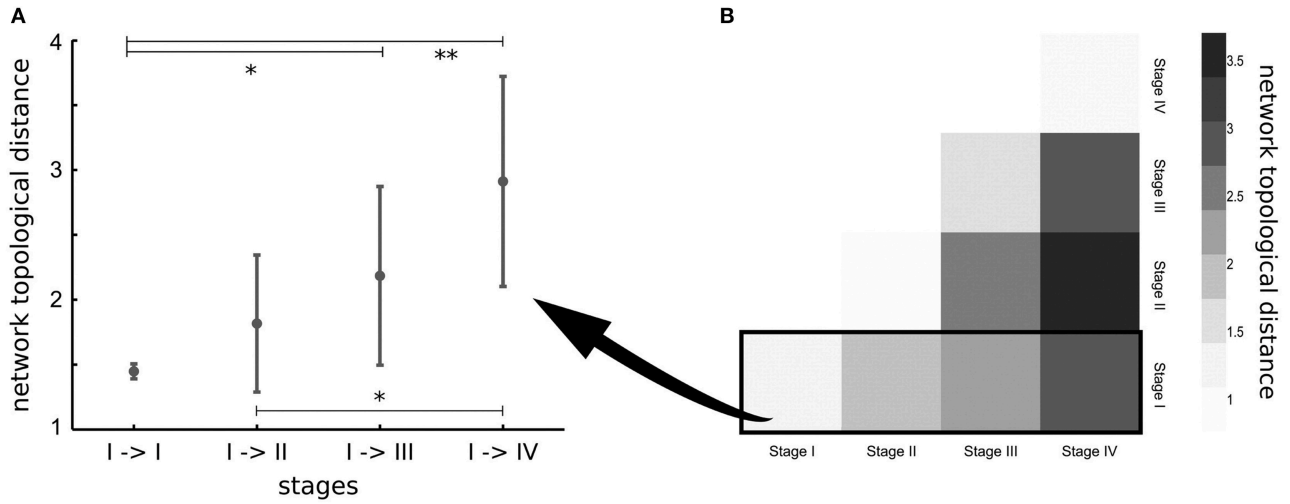

FIGURE 2 | (A) Network topological distance between nodes of stage I, stage I and stage II, stages I, and III and between stages I and IV show a strong ordering effect ($p = 0.002$). Significance of differences in network topological distances between stages is marked as follows: $^{*}p < 0.05$, $^{**}p < 0.005$. **(B)** Matrix of mean network topological distances between all four stages. Reproduced with permission from Schmidt et al. NeuroImage 2016 (65) (published open-access under a CC BY-NC-ND 4.0 license).

measures might potentially be considered as a marker of disease progression. This is because decreased FA and disease severity and rate of disease progression are highly correlated. In the last few years, the focus has shifted towards the analysis of disease progression. Particularly, several longitudinal neuroimaging studies are confirming the recently proposed neuropathological staging model (62), demonstrating an expanding subnetwork of impaired brain connections from the primary motor cortex to frontal and parietal regions. All these findings support the idea that WM tract involvement might be a valid biomarker to assess *in vivo* the spreading of pathological proteins and to track the neurodegeneration process.

In conclusion, DTI analysis has the potential to be a valid technique for use at the individual patient level in the future. However, there is urgent need for more longitudinal studies. The combination of the *in vivo* staging using longitudinal DTI scans with the *post-mortem* classification might be very useful to understand deeply the pathophysiology of the disease and to provide as soon as possible disease-modifying therapies.

AUTHOR CONTRIBUTIONS

SB drafted the first version of the manuscript. All the authors discussed/edited the draft producing the final version of the manuscript.

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The Impact of Cognitive and Behavioral Symptoms on ALS Patients and Their Caregivers

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Previously thought to be a pure motor disease, amyotrophic lateral sclerosis (ALS) is now established as multisystem neurodegenerative disorder that lies on a continuum with frontotemporal dementia (FTD). Cognitive and behavioral symptoms primarily extend to executive function, personality, social conduct, and emotion processing. The assessment and management of cognitive and behavioral symptoms is complicated as they must be differentiated from psychological responses to a terminal diagnosis and progressive physical impairment. This is made more difficult by the limited number of studies investigating how these symptoms specifically affect patients and caregivers well-being. The current review focuses on the impact of cognitive and behavioral symptoms on patient and caregiver well-being and their implications for future research and interventions in ALS. This is an important area of research that could form the basis for more tailored, and potentially more successful, non-pharmacological interventions to improve psychological well-being among patients with ALS and their caregivers.

Keywords: amyotrophic lateral sclerosis, dementia, depression, quality of life, caregiver, burden, adherence, non-pharmacological interventions

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BACKGROUND

Amyotrophic lateral sclerosis (ALS) is a multisystem neurodegenerative disorder which includes a broad spectrum of non-motor symptoms that can dominate the clinical presentation (1, 2). Cognitive and behavioral symptoms include impaired executive function, deficits in social and emotional cognition, apathy, disinhibition, and perseveration similar to that seen in frontotemporal dementia (FTD). Frontotemporal dysfunction of varying severity can affect more than 50% of ALS patients (3), with ∼8–14% meeting full diagnostic criteria for FTD (4–8). As such, early detection and timely management of cognitive and behavioral symptoms is widely acknowledged as an important aspect of contemporary ALS care (9). However, fully assessing cognitive and behavioral symptoms in ALS is made difficult by the fact that these symptoms must be distinguished from psychological reactions to a terminal diagnosis and the progressive physical loss that comes alongside it. Our narrative review focuses on evaluating the impact of cognitive and behavioral symptoms on patient and caregiver well-being and their implications for developing future non-pharmacological interventions in ALS. Gathering this research can help form more appropriate and effective non-pharmacological interventions to improve psychological well-being among patients with ALS and their caregivers.

SEARCH STRATEGY AND SELECTION CRITERIA

For this narrative review references were primarily searched through PubMed. The following terms were systematically searched: "amyotrophic lateral sclerosis"; "motor neuron(e) disease"; "cognitive"; "behavioral"; "depression"; "anxiety"; "quality of life"; "psychological health"; "caregiver"; "carer"; "burden"; "strain"; "stress"; "compliance"; "adherence"; "psychosocial intervention"; "non-pharmacological intervention"; "support"; "manage"; "intervention"; "care"; "caring"; "coping"; "cope"; "frontotemporal dementia." The section on non-pharmacological interventions for cognitive and behavioral symptoms in ALS also used the MEDLINE, EMBASE, PsycINFO, AMED, and CINAHL databases. Searches included papers published in English between May/2013 and July/2018. Research articles relevant to ALS and FTD were included in the review.

PSYCHOLOGICAL SYMPTOMS IN ALS

The psychological impact of ALS has been widely addressed in the literature. Anxiety and depression, particularly depression are often used as clinical markers of psychological morbidity in patients diagnosed with ALS. Self-report measures, particularly the Hospital Anxiety and Depression Scale and Beck's Depression Inventory remain the most widely used measures. Based on the Structured Clinical Interview for the Diagnostic and Statistical Manual of Mental Disorders, the "gold standard" for assessment of depression, the rate of clinical depression ranges between 9 and 12% in ALS (10, 11). Perhaps not surprisingly, self-report measures of depression tend to show more variable rates of depression ranging from 20 to 64% (12–20). Similarly, the prevalence rates of anxiety vary widely, with rates ranging as low as 8% to as high as 88% among patients with ALS (12, 14, 18, 19, 21). The severity of symptoms appear to be predominantly in the mild range. Despite the low rates of clinical depression and anxiety, patients with ALS have been shown to be at increased risk of being diagnosed with depression, anxiety and other neurotic or stress-related disorders following diagnosis (21–25), however this may be attributable to the clinicopathological overlap between ALS and FTD (24).

Management of psychological symptoms is crucial to maintaining quality of life. ALS patients provided with an assistive communication device in the early stages of the disease have been found to experience higher quality of life, particularly in the domains related to psychological and existential well-being (26). Quality of life and depression appear to be largely unrelated to patients' desire for hastened death (27) and end-of-life choices (28). This may be due to satisfactory levels of quality of life typically reported by ALS patients (29, 30). In fact, several studies have shown that caregivers and healthy controls tend to underestimate ALS patients quality of life and psychological well-being (31), possibly reflecting a "disability paradox" (32). However, it should be noted that many quality of life measurements used were not ALS specific.

THE IMPACT OF COGNITIVE AND BEHAVIORAL SYMPTOMS ON ALS PATIENT'S PSYCHOLOGICAL WELL-BEING

To date, there is a paucity of research specifically examining cognitive/behavioral symptoms and patients' psychological well-being. The majority of recent studies on patients' psychological well-being have either excluded patients with cognitive/behavioral symptoms or have not specifically discussed findings in relation to cognitive/behavioral symptoms. This is an important area for future research given emerging findings showing a relationship between depression and cognitive/behavioral changes. Higher levels of depression have been associated with lower cognitive performance on the Edinburgh Cognitive and Behavioral ALS Screen (17), specifically the subtests measuring social cognitive deficits and inhibitory control (12). Findings regarding anxiety and cognitive function are inconsistent, with one recent study finding no relationship (12) and another showing a weak association between anxiety and cognitive performance, perhaps reflecting underlying behavioral changes, namely disinhibition (17). Indeed, the findings available on behavioral and psychological symptoms appear to be more consistent. A large scale study of cognitive and behavioral impairment, and depression showed that patients with behavioral impairment exhibited higher levels of depression and hopelessness (10). This may partly reflect the overlap between depression and behavioral symptoms, namely apathy (33, 34).

THE IMPACT OF COGNITIVE AND BEHAVIORAL SYMPTOMS ON TREATMENT ADHERENCE IN ALS

Adherence to treatment recommendations in ALS can extend survival (e.g., non-invasive ventilation or Riluzole), improve patients' quality of life (35, 36), and likely to reduce caregiver burden. Review articles of cognition and behavioral symptoms in ALS discuss the likely impact of these symptoms on treatment adherence (37–40), however only one study to date has investigated the effect of non-motor symptoms on treatment adherence in ALS (41). Non-adherence to non-invasive positive-pressure ventilation and percutaneous endoscopic gastrostomy recommendations was 75 and 72% respectively for patients with ALS-FTD compared to 38 and 31% those with "motor only" symptoms. Therefore, the presence of a frontotemporal syndrome reduced adherence by half in ALS.

In general, ALS patients are compliant with recommendations made in multidisciplinary clinics (36). Out of a total of 287 recommendations made to 25 patients with ALS, patients complied fully with 59% of the recommendations made by the team. Not surprisingly, recommendations were greatest for physical needs (e.g., medications for symptoms such as spasm, saliva, sleep difficulties and interventions for nutrition and speech) and adherence was also highest for this category of recommendations. Interestingly, while patients with marked

cognitive impairment were excluded in this study, patients with milder cognitive and behavioral symptoms (e.g., executive dysfunction) were included and may help explain why less than half of all recommendations were recalled (40%) and only a small proportion of patients (32%) had retained the written list of recommendations provided after the clinic visit. In total, <5% of the total recommendations were for mental health needs of patients (e.g., anti-depressants) and almost no recommendations (<2% of total recommendations) were made for caregivers (e.g., increase caregiver hours, ALS respite care program, and caregiver training to aid in patient transport).

In general, studies of treatment adherence in ALS have not typically characterized non-motor symptoms in patient cohorts [e.g., physiotherapy exercises (42); respiratory support (43); tolerability of oral vs. tablet Riluzole (44); tolerability of early non-invasive ventilation use (45)] and is an exclusionary criteria in some studies [e.g., aerobic exercise therapy vs. cognitive behavioral therapy (46)]. It is therefore not surprising that motor predictors of treatment adherence are often reported. For example, symptomatic orthopnoea and dyspnoea, nocturnal hypoventilation, and spinal onset of symptoms have been associated with adherence to non-invasive ventilation (47–50). Functional scores (forced volume vital capacity and the revised ALS Functional Rating Scale) have also been identified as independent predictors of adherence to clinical trials and fewer protocol deviations (51).

CAREGIVER BURDEN IN ALS

Several studies have shown that caregiving in ALS affects caregivers' level of distress and quality of life (52). The psychological symptoms experienced by caregivers have a significant impact on caregiver burden (53). Burke et al. (54) demonstrated that caregiver distress explained 39% of the variance in caregiver burden (54). In another study where caregivers were dichotomized into low and high burden groups, there were no differences across groups with respect to motor function (revised ALS Functional Rating Scale), bulbar/spinal onset, or survival time. Significant differences were only found when high and low-burden caregivers were compared on levels of anxiety, depression, distress and quality of life (55). A longitudinal study involving ALS patients with relatively preserved cognition demonstrated that anxiety and depression in caregivers were the best long-term predictors of burden (56).

An interesting study using a mixed methods approach (quantitative and qualitative) to assess burden in 81 informal ALS caregivers, showed that increased psychological distress, hours of care provided, and lower quality of life were the best predictors of caregiver burden, explaining 53% of variance. These caregivers identified difficulties related to four main themes: (a) the caregiving role and tasks associated with management of the condition, (b) psychosocial and emotional impact, (c) limitated time and restricted social life, and (d) significant impact in relationships with others and also identity (the process of "becoming" and "being" a caregiver) (57). Longitudinal studies have also demonstrated that disease severity causes strain and burden in caregivers over time (58, 59).

THE IMPACT OF COGNITIVE AND BEHAVIORAL SYMPTOMS ON CAREGIVER BURDEN

Recent evidence has demonstrated that both cognitive and behavioral symptoms contribute to caregiver burden in ALS (54, 60). A study involving 33 ALS patient-caregiver dyads showed that caregiver burden (Zarit Scale) was associated with executive dysfunction and behavioral changes, such as apathy and disinhibition (54). Similarly, findings from a Chinese study revealed that the degree of frontal dysfunction and behavioral changes (predominantly disinhibition) was significantly associated with caregiver burden (60).

More severe cognitive deficits have also been shown to predict caregiver burden in ALS (61). Conversely, a study involving 84 ALS patient-caregiver dyads found no correlation between caregiver burden (Caregiver Burden Inventory) and cognitive functioning (ALS-Cognitive Behavioral Screen). Only disease progression and behavioral symptoms were correlated with caregiver burden (62). Indeed, the level of burden for caregivers of ALS-FTD patients appears to increase with disease progression compared to a persistently high level of burden among caregivers of FTD patients (63).

Few studies have found specific motor symptoms associated with caregiver burden. One study linked poor motivation and difficulties with everyday skills to higher burden (64), while another study revealed that caregiver burden was predicted by behavioral problems and severity of limb involvement (65).

These findings highlight the support caregivers require from health care professionals and family/friends to not only manage the emotional and physical burden of caregiving (66), but also cognitive and behavioral symptoms that can greatly impact on their caregiving experience.

DISCUSSION

Assessment and management of cognitive and behavioral symptoms forms the larger goal of preservation of quality of life in both ALS patients and caregivers. Timely assessment of cognitive and behavioral symptoms has important prognostic and therapeutic implications. The presence of dysexecutive symptoms is not only likely to impact on patient and caregiver psychological well-being but also decision-making, adherence to life-sustaining interventions, and capacity to engage and benefit from non-pharmacological interventions. Additionally, cognitive and behavioral symptoms may exist before full blown motor symptoms (67) and, therefore, the ability to make informed decisions may be effected early in the disease course. While treatments for symptomatic management in ALS is often most beneficial if initiated early [e.g., (68)] and clinicians value proactive decisions (69), failure to identify cognitive and behavioral symptoms may mean that patients are not fully supported by their health care team and caregivers to undertake informed decision-making that is in accordance with their current personal philosophy and values (70).

The provision of practical support by ALS specialists is known to facilitate adherence in ALS. Increased educational training and adaptation facilitates adherence to non-invasive ventilation (71); telemonitoring decreases emergency room visits and hospital admissions, and follow-up care between clinical visits increase adherence to clinical recommendations (36, 72). Support from other ALS patients through online platforms can also increase treatment adherence such as with medication adherence (73).

It is also essential to recognize the importance of caregivers in the management of ALS from an early stage, informing them about the possibility of burden, offering them health care support, and monitoring their well-being over time (74). Weisser et al. (75) showed that the needs of ALS caregivers were multiple, including practical, social, and psychological needs. A model of coping was subsequently proposed integrating resilience, burden, needs, and rewards (75). An intervention to reduce maladaptive coping strategies has also been found to improve well-being in caregivers of patients with ALS (76). Furthermore, the use of technological approaches (e.g., telemedicine) for ALS patients and their caregivers that live in remote and rural areas which have reduced access to health care services may be especially beneficial (77). Provision of training for health care professionals to help patients and caregivers during the advanced stages of the disease would also ensure that important factors such as fatigue, stress, and ethical challenges related to end-of-life care are adequaly addressed (78).

To the best of our knowledge, there are currently no evidence-based studies which examine interventions to manage the cognitive and behavioral symptoms of ALS patients, though there are a few that examine caregiver burden in response to behavioral symptoms (79). In the absence of such evidence, it is possible to extrapolate findings from intervention studies in non-ALS populations as possible non-pharmacological interventions for cognitive and behavioral symptoms in ALS. Behavioral variant FTD and cognitively impaired ALS show similar cognitive profiles, although cognitive deficits are more severe in patients with behavioral variant FTD in most domains (4). In both disorders, considerable impairment in social cognition, fluency and verbal memory is found, whereas impairment of visual memory and attention is less prominent (3, 4). Due to the similarity of symptoms, studies examining management of behavioral variant FTD may be relevant to ALS, though caution should be used in extrapolating their conclusions.

Environmental management has shown promise in addressing cognitive/behavioral symptoms of FTD patients (80). For example, reducing noise and stimulation, lessening clutter, or simplifying social situations can help patient's better focus on a nominated task or response (80, 81). Removing access to problematic stimuli or modifying public outings to reduce the opportunity for inappropriate interactions are also effective FTD-specific environmental manipulations (80, 81), which could also be implemented when working with ALS patients presenting symptoms of disinhibition or loss of social cognition.

Though the research on behavioral modification in FTD is also limited and consists mainly of case studies and reports, clinicians have typically focused on disinhibition, apathy and compulsive behaviors (80, 82). In a few specific cases, behavioral interventions successfully reduced behaviors that were most distressing for patients' and caregivers', improved the relationship between patients' and caregivers', and helped to prevent the patients from being institutionalized (82, 83). Two case reports used behavior theory techniques including reducing stimuli, introducing new non-verbal cues, and creating reward systems (82, 83). Similarly, Tailored Activities Programs have been shown to reduce agitation in behavioral variant FTD patients (83–85). Support groups for family caregivers have also proved helpful (in person and when using online live streams) and most effective when caregivers are experiencing similar behaviors and challenges with the patient (86, 87).

There are limitations to understanding the impact of cognitive and behavioral changes on patients with ALS and their caregivers. The majority of published studies have been completed in developed countries and thus results cannot be extrapolated to all countries. Also, many of these studies do not take into consideration the various socioeconomic variables attributable to the patient and caregivers daily experience with ALS, such as the individual's wealth or their country's health care system.

Despite the clinical necessity of an intervention to manage cognitive and behavioral symptoms, there has been a lack of research on the topic which further widens the gap between research and practice. Drawing from interventions in dementia, there is a large landscape of possible, untested interventions for cognitive and behavioral symptoms of ALS. Early and comprehensive management of cognitive and behavioral symptoms not only promotes holistic care of patients but would also further enhance caregiver's psychological well-being and likely to reduce the healthcare and societal burden of these symptoms due to poor intervention adherence and avoidable hospitalizations.

AUTHOR CONTRIBUTIONS

JC, SH, PL, and KD wrote the manuscript. EM edited it and had manuscript oversight.

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REVIEW
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Biomarkers of Metabolism in Amyotrophic Lateral Sclerosis

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Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder characterized by the deterioration of motor neurons. However, this complex disease extends beyond the boundaries of the central nervous system, with metabolic alterations being observed at the systemic and cellular level. While the number of studies that assess the role and impact of metabolic perturbations in ALS is rapidly increasing, the use of metabolism biomarkers in ALS remains largely underinvestigated. In this review, we discuss current and potential metabolism biomarkers in the context of ALS. Of those for which data does exist, there is limited insight provided by individual markers, with specificity for disease, and lack of reproducibility and efficacy in informing prognosis being the largest drawbacks. However, given the array of metabolic markers available, the potential exists for a panel of metabolism biomarkers, which may complement other current biomarkers (including neurophysiology, imaging, as well as CSF, blood and urine markers) to overturn these limitations and give rise to new diagnostic and prognostic indicators.

Keywords: amyotrophic lateral sclerosis, ALS, metabolism, biomarker, motor neurone disease

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OVERVIEW

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease caused by the death of motor neurons in the brain and spinal cord. The loss of neuronal input leads to progressive paralysis and patient mortality within 2–5 years from diagnosis (1). ALS likely arises from a combination of genetic susceptibility and environmental exposures (2, 3), although it is recognized that ALS is a complex, multi-system disease (4, 5).

Given the complex and heterogeneous nature of ALS, diagnosis and tracking of prognosis remains difficult. Current diagnostic criteria typically follow tests to rule out other pathological causes of symptoms and include: indicators of upper and lower motor neuron involvement, nerve conduction tests, electromyography and "watchful waiting" (4). As a result, researchers have attempted to utilize a wide range of biomarkers—observable biological measurements that confirm the presence or progression of a change in body status, as a means of diagnosing and following disease progression. While the current range of biomarkers in ALS offer some diagnostic and prognostic benefit, there is a need to identify a biomarker that satisfies the following six attributes: specificity to disease; reproducibility; appearance early in the disease; stability across the diurnal period; independence of dietary status and behavior; and a notable change during disease progression. By meeting these criteria, a biomarker can be used to reliably identify and track disease progression, in a manner that can easily be reproduced in a clinical setting.

Metabolic perturbations occur in ALS patients and in mouse models of the disease; both at the systemic and cellular level (6, 7). Clinically, an increase in resting energy expenditure (REE) and

decline in body mass index (BMI) is linked to worse outcome (8–10), suggesting prognostic potential in metabolic biomarkers. Given that changes in metabolic status are generally reflected in overall body weight, body composition, and tissue/cellular metabolic function, metabolic changes at the anthropometric, tissue and cellular levels may represent appreciable metabolism biomarkers of ALS onset, progression, and/or severity (**Figure 1**). A list of the potential biomarkers of metabolism in ALS, and their quality relative to the aforementioned identifying attributes are summarized in **Table 1**.

ANTHROPOMETRIC BODY MEASURES

Lower premorbid BMI is associated with increased risk for ALS (11–13), and the degree of decline in premorbid BMI predicts ALS risk and survival (14, 15). Lower BMI, or a decline in BMI following diagnosis correlates with worse survival (16, 17), although this association is not always observed (18, 19, 23, 24). Rather, the mortality risk for ALS relative to BMI exists as a U-shaped curve, in which mortality decreases with increasing BMI, until BMI levels indicate premorbid obesity. Thereafter, mortality risk increases again (8, 20). This seemingly complex association could be explained by changes in body composition throughout disease progression.

BMI is often used as an indirect measure of fatness. However, conventional anthropometric measures of BMI and body adiposity index (BAI) do not always accurately reflect changes in fat and/or fat free mass (FFM) in ALS (69). In this regard, fat mass (FM) and FFM at diagnosis are not associated with survival risk (14), yet redistribution of adipose tissue does occur in ALS (29), and visceral fat is correlated with functional status and survival (28). Moreover, serial assessment of body FM indicates that increases in FM are associated with longer survival (14). While a decrease in FFM serves as an independent prognostic factor for shorter survival in ALS (23), we did not identify any studies that document progressive changes in muscle mass as a potential marker of disease progression in ALS. As a hallmark of ALS, however, there is potential to use the loss of FFM as a marker of disease progression. Such measures must consider the technical difficulties associated with assessing FFM in patients who experience significant and progressive disability, while also accounting for whole body and regional changes in FFM, which differ greatly between patients.

Despite BMI and BAI being poor predictors of body composition in ALS, changes in BMI may offer reliable measures for progressive changes in the overall nutritional status of the patient, and by proxy, disease progression. As documented by Kasarskis et al. a progressive decline in body weight is commonly observed in ALS patients in the months prior to death, and this reduction in body weight or BMI likely reflects a state of undernutrition (25). In recent years, lower BMI has been found to be associated with lower ALSFRS-R scores (70), and a loss of body weight (14, 21, 23, 24, 26, 27, 71) and BMI (14, 17, 22, 24) throughout disease course is consistently associated with shorter survival. Not surprisingly, these observations, while serving as markers for disease progression, have resulted in the adoption of interventions aimed at slowing weight loss in ALS (72).

SKELETAL MUSCLE PATHOLOGY

With findings suggesting that FFM is a prognostic factor in ALS (23), analysis of skeletal muscle, the primary component of FFM, may offer insights into tissue-specific metabolism biomarkers. Assessment of cellular metabolic changes in skeletal muscle can be challenging, especially when weighing the clinical benefit against that of an invasive procedure on a patient undergoing significant muscle wasting. Furthermore, heterogeneity in site of disease onset leads to variable muscle pathophysiology between patients (73).

Despite these limitations, creatine kinase, an enzyme that is linked with muscle damage and deterioration, has been studied intensely in ALS. While not strictly a metabolic marker, creatine kinase can be considered as an important modulator of body composition (74). As such, it may indirectly influence systemic metabolic processes. Numerous reports of increased creatine kinase in ALS (36–43), and particularly in limb-onset patients (38, 43), highlight the potential for its use as a marker of disease. However, contradictory observations of associations between creatine kinase and clinical parameters of disease, and disease progression and survival attest to the need for further investigations into determining the utility of creatine kinase as a biomarker in ALS.

MITOCHONDRIAL DYSFUNCTION

In human ALS muscle, mitochondrial defects including dysregulation of respiratory complex I (44), decreased respiratory complex I and IV activity (45, 75), decreased muscle mitochondrial protein expression (75) and upregulation of muscular mitochondrial uncoupling protein 3 (76) indicate that impairments in mitochondrial function could serve as a metabolic marker of ALS. It should be noted, however, that these studies were unable to correlate mitochondrial defects with functional parameters of disease progression, despite studies in animal models reporting a strong relationship between the two (77–79). Therefore, while there is clear evidence of mitochondrial defects in ALS, mitochondrial defects *per se* cannot currently be used as a biomarker due to the difficulty in both easily observing these defects in a clinical setting, and linking such defects to a marker of disease progression and/or survival. Instead, emphasis could be placed on the assessment of the more easily detectable metabolites that drive mitochondrial function.

GLUCOSE METABOLISM

Glucose use in the brain of ALS patients has been evaluated using fluorodeoxyglucose F18 positron emission tomography (F18-PET) (30–33). These studies have identified decreased glucose use in the primary motor cortex of ALS patients, suggesting that this brain region is hypometabolic (32). Other studies have reported a decrease in the use of glucose across other brain regions

TABLE 1 | Classification of potential biomarkers of metabolism in amyotrophic lateral sclerosis (ALS).

Marker	Observation	Utility as a biomarker in ALS						Biomarker Score	References
		Specific to ALS	Reproducible	Pre-diagnostic	Diurnal stability	Independence	Change with progression		
ANTHROPOMETRIC MARKERS									
Body mass index (BMI)	Lower BMI is an indicator of poor prognosis. U-shaped association; lower BMI is associated with increased risk and faster progression whereas BMI in the range of morbid obesity is associated with shorter survival. Degree of premorbid loss of BMI predicts risk of ALS	N	N	Y	Y	N	*Variable*	**2.5**	(8, 11–22)
Body weight	Weight loss correlates with faster disease progression; weight loss suggested as a risk factor for ALS	N	N	N	Y	N	*Variable*	**1.5**	(14, 21, 23–27)
Fat mass	Fat mass at diagnosis is not a determinant of survival. Increased fat mass is correlated with longer survival	N	N	*Insufficient data*	Y	N	Y	**2**	(14, 28)
Fat free mass	Fat free mass at diagnosis is not a determinant of survival. Loss of fat free mass is associated with shorter survival	N	Y	*Insufficient data*	Y	N	Y	**3**	(14, 23)
Fat distribution	Redistribution and increased deposition of fat in muscle	N	*Insufficient data*	*Insufficient data*	Y	N	*Insufficient data*	**1**	(29)
IMAGING MARKERS									
Brain glucose use	Hypometabolism specific to select brain regions; varies between studies	N	*Insufficient data*	*Insufficient data*	*Likely*	*Likely*	Y	**2**	(30–33)
Spinal cord glucose use	Hypermetabolism; changes in glucose metabolism correlates with disease progression	N	*Insufficient data*	*Insufficient data*	*Likely*	*Likely*	*Variable*	**1.5**	(34, 35)
MUSCLE MARKERS									
Creatine kinase	Increased in blood; variability in correlation with disease progression/survival. Greater increase observed in male subjects and limb-onset ALS	N	Y	Y	*Likely*	*Likely*	*Variable*	**3.5**	(36–43)
Mitochondrial function	Decreased activity of complex I and IV. Activity also declines over course of disease	N	*Insufficient data*	*Insufficient data*	Y	Y	*Variable*	**2.5**	(44, 45)
PDK4 levels	Increase in pyruvate dehydrogenase kinase 4 (PDK4) correlated with increased denervation and fuel switch	N	*Insufficient data*	*Insufficient data*	Y	Y	*Likely*	**2.5**	(46)
Glucose	Increased	N	N	*Insufficient data*	N	N	N	**0**	(47)
Sphingolipids	Increased	N	*Insufficient data*	*Insufficient data*	N	N	Y	**1**	(48)
Phosphatidylcholine	Increased	N	*Insufficient data*	*Insufficient data*	N	N	N	**0**	(48)

(Continued)

TABLE 1 | Continued

Marker	Observation	Utility as a biomarker in ALS						Biomarker Score	References
		Specific to ALS	Reproducible	Pre-diagnostic	Diurnal stability	Independence	Change with progression		
Cholesterol + Carriers	Increased	N	*Insufficient data*	*Insufficient data*	N	N	N	**0**	(49)
Lactate	Increased	N	*Insufficient data*	*Insufficient data*	N	N	*Insufficient data*	**0**	(47, 50)
CEREBROSPINAL FLUID (CSF) MARKERS									
Pyruvate	Increased	N	*Insufficient data*	*Insufficient data*	N	N	*Insufficient data*	**0**	(51)
Insulin	Decreased	N	*Insufficient data*	*Insufficient data*	N	N	N	**0**	(52)
Growth hormone	Decreased	N	*Insufficient data*	*Insufficient data*	N	N	N	**0**	(52)
CIRCULATING MARKERS (BLOOD, PLASMA AND SERUM)									
Glucose	Increased (33% of patients achieve World Health Organization (WHO) criteria for impaired glucose tolerance)	N	N	*Insufficient data*	N	N	N	**0**	(53)
Mannose	Increased	N	N	*Insufficient data*	N	N	*Insufficient data*	**0**	(54)
Free fatty acids	Increased	N	N	*Insufficient data*	N	N	N	**0**	(53)
Sphingolipids	Increased	N	N	*Insufficient data*	N	N	N	**0**	(54)
Cholesterol + Carriers	Major variations and contradictory reports mask any specific trend	N	N	*Insufficient data*	N	N	*Variable*	**0.5**	(53, 55–62)
β-hydroxy-butyrate	Increased	N	N	*Insufficient data*	N	N	*Insufficient data*	**0**	(63)
2-hydroxy-butyrate	Increased	N	N	*Insufficient data*	N	N	*Insufficient data*	**0**	(54)
α-ketoglutarate	Increased	N	N	*Insufficient data*	N	N	*Insufficient data*	**0**	(54)
Acetate	Increased	N	N	*Insufficient data*	N	N	*Insufficient data*	**0**	(63)
Adiponectin	Increased	N	N	*Insufficient data*	N	N	N	**0**	(64)

(Continued)

TABLE 1 | Continued

Marker	Observation	Utility as a Biomarker in ALS						Biomarker Score	References
		Specific to ALS	Reproducible	Pre-diagnostic	Diurnal stability	Independence	Change with progression		
Cortisol	Increased	N	N	*Insufficient data*	N	N	N	**0**	(65)
Cortisol (morning peak)	Decreased	N	N	*Insufficient data*	N	N	N	**0**	(65)
Insulin	Decreased	N	N	*Insufficient data*	N	N	N	**0**	(52, 64)
Gastric inhibitory peptide	Decreased	N	N	*Insufficient data*	N	N	N	**0**	(64)
Ghrelin	Decreased	N	N	*Insufficient data*	N	N	N	**0**	(64, 66)
SALIVA MARKERS									
Cortisol (night-time)	Increased	N	*Insufficient data*	*Insufficient data*	N	*Likely*	*Insufficient data*	**0.5**	(67)
Cortisol (Stress-induced)	Decreased	N	*Insufficient data*	*Insufficient data*	N	*Likely*	*Insufficient data*	**0.5**	(67)
Cortisol (circadian rhythm)	Decreased	N	*Insufficient data*	*Insufficient data*	N	*Likely*	*Insufficient data*	**0.5**	(67)
URINE MARKERS									
p75 neurotrophin receptor extracellular domain	Increased	N	*Likely*	*Insufficient data*	Y	Y	Y	**3.5**	(68)

The strength of proposed biomarkers are scored relative to their potential to serve as markers that are specific to ALS, and that conform to the requirements as detailed in text.
Specific to ALS refers to uniqueness of the marker to ALS over other diseases, reproducible refers to whether the indicated change is reproducible across patient cohorts, pre-diagnostic indicates where changes are apparent prior to symptom onset, diurnal stability refers to the consistency of the marker throughout the day, independence indicates the ability of the marker to remain stable regardless of changes in food intake or behavior, change with progression identifies whether the marker changes as disease progresses. For each potential biomarker, a score out of 6 was determined (biomarker score, indicated in bold), where Y (Yes) = 1 point, N (No) = 0 points, Variable = 0.5 points, Likely (supported by animal or statistical modeling studies) = 0.5 points, and Insufficient data = 0 points.

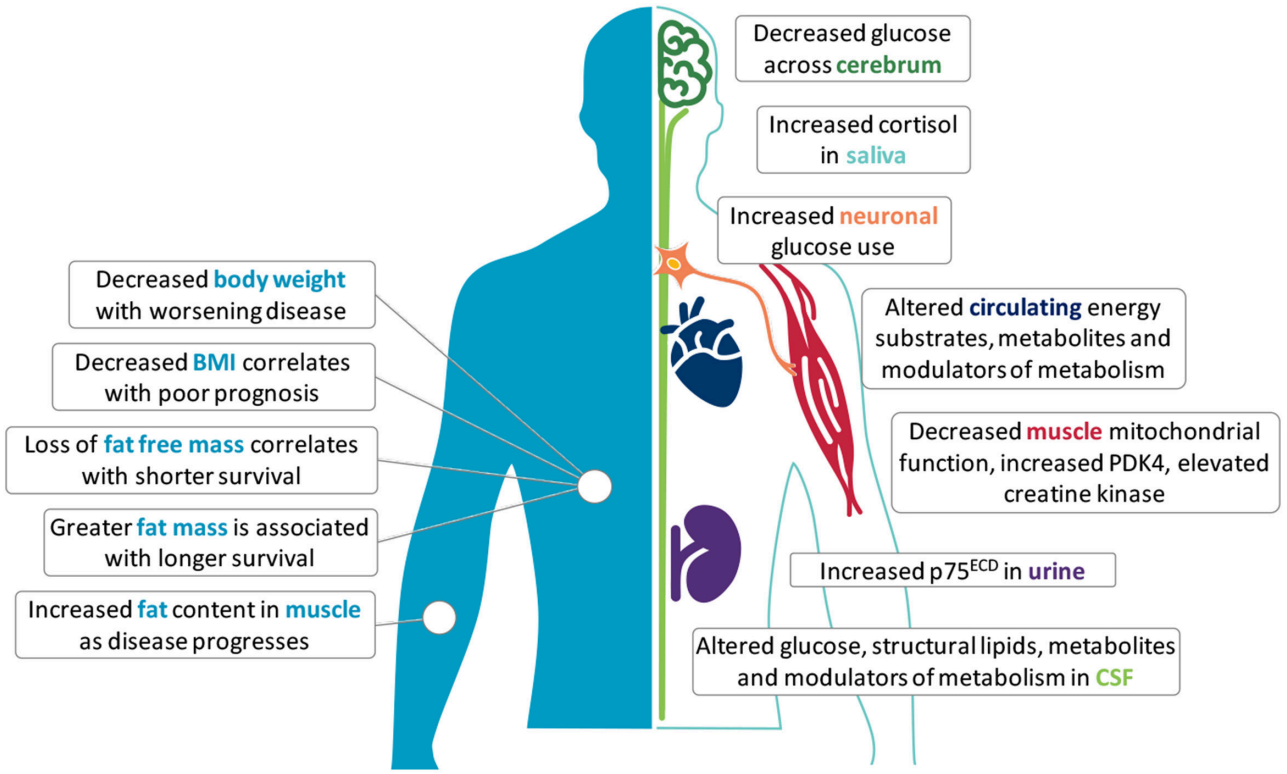

FIGURE 1 | Potential metabolism biomarkers in amyotrophic lateral sclerosis (ALS). Metabolic alterations in ALS offer opportunities to use metabolism biomarkers for the diagnosis, categorization, and tracking of disease. Non-invasive anthropometric measures include body weight, body mass index (BMI), fat free mass, fat mass, and fat distribution. Invasive measures include the use of F18-PET to assess glucose metabolism in the central nervous system, or require the sampling of saliva, blood, cerebrospinal fluid (CSF), muscle tissue, and urine. Although few independent markers are specific, reproducible or able to track disease in ALS, used together with complementary biomarkers (including neurophysiology and imaging), these markers may provide deeper insights into metabolic perturbations that are potentially involved in the onset and progression of disease.

(31, 33); although this may reflect the differences in experimental cohorts. In this regard, Claassen et al. investigated a cohort of patients with primary lateral sclerosis, while the study by Ludolph et al. evaluated ALS patients with both upper and lower motor symptoms. Given that the degree of cerebral hypometabolism in ALS is correlated with the duration of clinically-identified symptoms (30), the ability of the motor cortex to utilize glucose may allow for monitoring of disease progression. However, since brain glucose hypometabolism is not specific to ALS (80), its use as a diagnostic/prognostic marker is limited.

F18-PET has also been used to assess the uptake and utilization of glucose in the cervical spinal cords of ALS patients (34, 35, 81). Overall, observations of spinal cord glucose hypermetabolism (34, 35, 81) is congruent with increased levels of glucose in the CSF of ALS patients (47). In a study by Yamashita et al. glucose hypermetabolism on the ipsilateral side to the patient's symptoms was found to be positively correlated with ALSFRS-R, suggesting that changes in spinal cord glucose metabolism are specific to the affected corticospinal tract and the degree of disease severity (35). By contrast, the study by Marini et al. reported spinal cord glucose hypermetabolism independent of disease duration and functional impairment (34). As such, the degree of glucose use in the spinal cord may present some use for diagnostic testing, but provides limited insights for evaluation of disease progression and prognosis. Indeed, glucose hypermetabolism in the spinal cord extends to other neurological conditions (82, 83), thereby limiting its use as a specific biomarker for ALS. Finally, as the reproducibility of F18-PET in both the brain and spinal cord is low (84), more rigorous testing is required to determine if results are consistent across a heterogeneous ALS population.

Alterations in glucose metabolism in ALS extend beyond the central nervous system (CNS). Glucose tolerance tests conducted by Pradat et al. indicate that ALS patients have a significant increase in blood glucose levels following the provision of a glucose load when compared to age- and sex-matched controls. Within ALS patients, a degree of heterogeneity was observed, with 33% of participants meeting World Health Organization criteria for impaired glucose tolerance (53). Impaired glucose tolerance is in line with reports of insulin resistance in ALS

(85), and could explain observations of increased expression of pyruvate dehydrogenase kinase 4 (PDK4) in skeletal muscle of ALS patients (46). Similarly, mannose, an epimer of glucose that has recently been shown to be a predictor of insulin resistance (86), has been reported to be significantly increased in the plasma of ALS patients (54). While the assessment of glucose tolerance and insulin resistance is relatively straightforward, these tests lack reproducibility and specificity to ALS (87–89). Therefore, although glucose metabolism is altered in ALS, it cannot be used as an independent biomarker for ALS diagnosis and prognosis.

FATTY ACIDS AND KETONES

In patients with ALS, the resting level of circulating free fatty acids (FFAs) is significantly increased (53). While higher levels of FFAs has been linked to impaired glucose tolerance in ALS, it has not been shown to be correlated with any markers of disease progression or severity. Ketones, including β-hydroxy-butyrate (63) and 2-hydroxy-butyrate and α-ketoglutarate (54), which are produced through fatty acid metabolism under fasting conditions, are also significantly increased in ALS. Similar to FFAs, no correlations have been observed between disease status and the expression of ketones. Thus, FFAs and ketones cannot currently be considered as reliable biomarkers for ALS, and the lack of specificity for ALS-centric pathology indicate that they may not present as particularly valuable diagnostic markers individually.

DOWNSTREAM METABOLITES

Metabolites, the downstream indicators of metabolic function, are also impacted in ALS. While not specific to ALS, altered expression of metabolites may offer a potential avenue for biomarker discovery. In line with disease heterogeneity, reported levels of metabolites in the blood and CSF are variable. Notably, the levels of lactate (47, 50) and pyruvate (51) in the CNS are increased, potentially reflecting an increase in metabolic output, or increased release of metabolites into the CSF following neuronal deterioration. Given that mitochondrial dysfunction is observed in ALS, further evaluation of the ratio between these metabolites may hold significant informative value in ALS due to the diagnostic value of this test for mitochondrial disorders (90).

Blood levels of acetate are increased in ALS (63), although this is not readily observed in the CSF (47, 51). Acetate is a key metabolite in the oxidation of fatty acids. As acetate synthesis precedes the formation of citric acid in the Krebs cycle, changes in circulating acetate may occur due to excess production via an increase in fatty acid oxidation, increased release from deteriorating muscle cells, or other disruptions to mitochondrial membrane integrity (e.g., due to the presence of free radicals). Such potential mechanisms align with ALS pathology. As a whole, downstream metabolites hold promise as potential biomarkers, and further work that can interrogate relationships between metabolites and clinical parameters of disease would add merit to their use as metabolic biomarkers of disease.

ENDOCRINE MODULATORS OF METABOLISM

Insulin is an anabolic hormone that has been reported to be decreased in the blood (64) and CSF (52) of ALS patients. By contrast, other studies have reported no significant differences in plasma insulin levels in ALS patients (91, 92). Other anabolic hormones that have been found to be decreased in ALS include growth hormone (in CSF and blood) (52, 92–94) and gastric inhibitory peptide in blood (64). Conversely, hormones that promote catabolism, such as cortisol (65, 67), and adiponectin (64) are increased or dysregulated in saliva and blood of patients with ALS. Furthermore, ghrelin, an important modulator of appetite, is also reduced in the plasma/blood of ALS patients (64, 66). Given that alterations in these hormones are likely to be symbolic of a change in metabolic function/homeostasis, studies that confirm a link between endocrine markers of metabolism and clinical markers of disease offer potential for their development as prognostic biomarkers.

METABOLISM OF STRUCTURAL LIPIDS

While fatty acids and their derivatives serve as energy substrates through mitochondrial respiration, they also play an essential role in maintaining cellular integrity. Phospholipids, particularly phosphatidylcholine, are significantly increased in the CSF of ALS patients (48). Sphingolipids, such as stearoyl sphingomyelin and ceramide, are also increased in patient blood (48, 54). Interestingly, in the study by Blasco et al. predictions of clinical measurements, such as ALSFRS-R, were found to be correlated to CSF sphingomyelins and triglycerides with long-chain fatty acids (48). Such findings are favorable for the development of biomarker assays, but further tests are required to confirm the reliability of predictive models, before use as a prognostic biomarker.

An increase in cholesterol esters has been observed in ALS patient spinal cord (95). However, cholesterol and its carriers prove to be more difficult to characterize, with variable levels of HDL and LDL cholesterol being reported in ALS. In a population-based longitudinal study, a positive association was found between LDL cholesterol and ALS risk (55), however, there was no indication of the impact of LDL on disease progression or mortality. Nonetheless, this could serve as a diagnostic biomarker for ALS risk. Previously, higher levels of cholesterol, LDL, as well as an elevated LDL/HDL ratio in ALS patient blood have been correlated with increased survival (56–58). Conversely, similar increases in total cholesterol, LDL, and HDL cholesterol in ALS patient blood (59, 60) and CSF (49) have not been found to be correlated with disease progression. Furthermore, a small number of studies contradict these findings, reporting that cholesterol, LDL, and HDL levels do not vary between ALS patients and controls (53, 61, 62), although lower levels of serum lipids may correlate with worse respiratory function (61). Based on these contradictory observations, the validity of cholesterol as a biomarker remains uncertain. Further studies that address these disparate data are required.

NOVEL METABOLISM-ASSOCIATED BIOMARKERS

p75 neurotrophin receptor (NTR) belongs to the tumor necrosis factor family of receptors. It is a transmembrane receptor which binds neurotrophins and pro-neurotrophins (96). p75NTR has been implicated in processes of energy expenditure (97), glucose uptake, and insulin sensitivity (98). In ALS, the secretion of the extracellular domain of p75NTR ($p75^{ECD}$) in urine was recently established as a biomarker for disease progression and prognosis (68, 99). Urinary $p75^{ECD}$ increases as disease progresses, and an elevation of urinary $p75^{ECD}$ is observed alongside a decrease in ALSFRS-R scores (68). While it is not clear if increases in urinary $p75^{ECD}$ in ALS match metabolic derangements that accompany disease progression (such as changes in energy metabolism, glucose uptake and insulin sensitivity), the introduction of $p75^{ECD}$ as a fluid biomarker in ALS provides an opportunity for the evaluation and possible co-development of metabolism-associated biomarkers.

CONCLUSION

The complexity and heterogeneity of disease between patients limits the scope for the use of a single reliable biomarker of ALS. Significant changes in metabolism seen in ALS may represent a potential avenue for biomarker development. As documented in this review, a range of markers might be relevant (**Figure 1**). However, as investigations into the cause for metabolic derangements in ALS are ongoing, and little emphasis has been placed on the development of metabolism biomarkers as diagnostic or prognostic indicators, few reliable metabolism biomarkers exist (**Table 1**). Moreover, because metabolic alterations in ALS likely arise from the dysregulation of a number of processes, the utility of biomarkers for assessing early or progressive changes in the metabolic state of ALS patients would necessitate the development of a panel that captures the spectrum of metabolic changes that occur at the systemic and cellular level.

As there is no single biomarker for ALS that sufficiently meets the six major attributes of a biomarker, it is clear that the assessment of biomarkers that cover multiple dimensions of the disease is needed in order to generate a comprehensive view of the state of disease. The complementary assessment of metabolism markers alongside other biomarkers including neurophysiology, imaging, as well as CSF, blood, and urine markers may form a more convincing and reliable diagnostic/prognostic platform, while providing insights into the multifactorial nature of disease.

AUTHOR CONTRIBUTIONS

SEK, TJT, FJS, and STN conducted the literature search and wrote the manuscript. FJS produced all artwork. STN critically revised the manuscript.

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Tracking a Fast-Moving Disease: Longitudinal Markers, Monitoring, and Clinical Trial Endpoints in ALS

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Amyotrophic lateral sclerosis (ALS) encompasses a heterogeneous group of phenotypes with different progression rates, varying degree of extra-motor involvement and divergent progression patterns. The natural history of ALS is increasingly evaluated by large, multi-time point longitudinal studies, many of which now incorporate presymptomatic and post-mortem assessments. These studies not only have the potential to characterize patterns of anatomical propagation, molecular mechanisms of disease spread, but also to identify pragmatic monitoring markers. Sensitive markers of progressive neurodegenerative change are indispensable for clinical trials and individualized patient care. Biofluid markers, neuroimaging indices, electrophysiological markers, rating scales, questionnaires, and other disease-specific instruments have divergent sensitivity profiles. The discussion of candidate monitoring markers in ALS has a dual academic and clinical relevance, and is particularly timely given the increasing number of pharmacological trials. The objective of this paper is to provide a comprehensive and critical review of longitudinal studies in ALS, focusing on the sensitivity profile of established and emerging monitoring markers.

Keywords: motor neuron disease, amyotrophic lateral sclerosis, biomarkers, magnetic resonance imaging, neuroimaging

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a clinically, genetically, and pathologically heterogeneous neurodegenerative condition (1–3). Clinical heterogeneity in ALS is multidimensional owing to variations in upper motor neuron (UMN) and lower motor neuron (LMN) involvement, extra-motor symptoms, age of onset, survival, and progression-rates. Disease heterogeneity hinders biomarker development (3, 4) which in turn impedes the reliable assessment of candidate drugs in clinical trials (1). Current clinical trials recruit relatively heterogeneous cohorts of symptomatic patients, despite the notion that considerable pathological changes can already be detected at the time of diagnosis (5, 6). The considerable variability in progression rates in ALS is another confounding factor in clinical trial designs (1, 7–10). Imaging and electrophysiological markers have been repeatedly proposed as candidate monitoring markers (11, 12), but it is increasingly clear that a panel of several "wet" and "dry" biomarkers may be required to capture subtle changes over short periods of time (13, 14). The objective of this paper is the comprehensive and critical review of longitudinal studies in ALS, focusing on study designs, statistical power, clinical correlations, the sensitivity profile of proposed monitoring markers and their applicability to clinical trials.

METHODS

A formal literature search was performed on PubMed using the core search terms "amyotrophic lateral sclerosis" and "longitudinal" combined with each of the following keywords separately: "staging," "monitoring," "outcomes," "clinical," "clinical trials," "electrophysiology," "neurophysiology," "electromyography," "transcranial magnetic stimulation," "motor unit number estimation," "motor unit number index," "positon emission tomography," "single photon emission computed tomography," "magnetic resonance imaging," "neuroimaging," "imaging," "blood," "urine," "cerebrospinal fluid," "saliva," and "muscle." A supplementary search combined the core search terms with the following keywords: "presymptomatic," "asymptomatic," and "post-mortem." Inclusion criteria included longitudinal studies investigating imaging, neurophysiological, clinical, or biofluid biomarkers in ALS. Animal studies, review papers, opinion pieces, editorials, case reports, and case series were excluded. Only articles written in English and published between January 1980 and August 2018 were reviewed. Based on the above criteria a total of 118 original research papers were selected and reviewed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) recommendations.

Abbreviations: 2D-DIGE, two-dimensional fluorescence difference gel electrophoresis; ABG, arterial blood gas; ACE-3, Addenbrookes Cognitive Examination - Third Edition; ACE-R, Addenbrooke's Cognitive Examination-revised; AD, axial diffusivity; ADM, abductor digiti minimi; ADQ, abductor digiti quinti; AGA, arterial gas analyses; AHB, abductor halluces brevis; ALS, amyotrophic lateral sclerosis; ALSAQ-40, ALS assessment questionnaire; ALS-CBS, ALS Cognitive Behavior Screen; ALS-CFB, ALS computerized frontal battery; ALS-FBI, ALS-Frontal Behavioral Inventory; ALSFRS-r, revised ALS functional rating scale; ALSS, ALS severity scale; ALSSQoL-R, revised ALS-specific Quality of Life questionnaire; APB, abductor pollicis brevis; ARSLA, Association pour la recherche sur la SLA; ATLIS, accurate test of limb isometric strength; BAI, body adiposity index; BMI, body mass index; CALR, Calreticulin; CALSNIC, Canadian ALS Neuroimaging Consortium; CHI3L1, chitinase-3-like protein 1; CHI3L2, chitinase-3-like protein 2; CHIT1, chitotriosidase-1; Cho, Choline; CIDP, chronic inflammatory demyelinating polyneuropathy; CIS20-R, checklist individual strength; CK, creatinine kinase; CLIC1, Chloride intracellular channel protein 1; CMAP, compound muscle action potential; CMCT, central motor conduction time; CMT, Charcot-Marie-Tooth disease; CNS-BFS, Center for Neurologic Study-Bulbar Function Scale; CNS-LS, Center for Neurologic Study-Lability Scale; COWAT, controlled oral word association test; Cr, creatinine; CSF, cerebrospinal fluid; CSP, cortical silence period; CypA, peptidyl-prolyl cis-trans isomerase A; DCMAP, distal compound muscle action potential; D-KEFS, Delis–Kaplan Executive Function System; DTI, diffusion tensor imaging; DWI, diffusion-weighted imaging; ECAS, Edinburgh Cognitive and Behavioral ALS Screen; ECL, electrochemiluminescence; EDB, extensor digitorum brevis; EMG, electromyography; ERp57, protein disulfide-isomerase A3; EURALS, European Registry of ALS Consortium; EUROMOTOR, European multidisciplinary ALS network identification to cure motor neurone degeneration; FA, fractional anisotropy; FAB, frontal assessment battery; FBI, frontal behavioral inventory; FD, fiber density; FEV1, forced expiratory volume; fibs-sw, fibrillation/sharp-waves; FM-ADP, fat mass air displacement plethysmography; FPs, fasciculation potentials; FSS, fatigue severity scale; FUBP1, far upstream element-binding protein 1; FVC, forced vital capacity; GM, gray matter; GSTO1, glutathione S-transferase omega-1; HADS, hospital anxiety and depression scale; HDAC4, histone deacetylase 4; HHD, handheld dynamometry; HSC70, heat shock cognate 71 kDa protein; IL, interleukin; IFN, interferon; IRAK4, Interleukin-1 receptor-associated kinase 4; JPND, EU Joint Programme for Neurodegenerative Disease Research; LGVF, letter guided verbal fluency; LMN, lower motor neuron; MAS, modified Ashworth scale; McDESPOT, multi-component driven equilibrium single pulse observation of T1/T2; MDRS-2, Mattis Dementia Rating scale-Second Edition; MEP, maximal static expiratory mouth pressure; MiND-B, motor neuron disease behavior scale; MIP, maximal inspiratory pressure; MiRNAs, micro-RNAs; MITOS, Milano-Torino staging system; MMSE, mini mental state examination; MMT, manual muscle testing; MND, Motor neuron disease; MoCA, Montreal Cognitive Assessment; MR, magnetic resonance; MRC, Medical Research Council Scale for muscle strength; MRCSS-LL, Medical Research Council sum score; MRI, magnetic resonance imaging; MRS, magnetic resonance spectroscopy; MS, multiple sclerosis; MU, motor unit; MUNE, motor unit number estimation; MUNIX, motor unit number index; MUPs, motor unit potentials; MUSIX, motor unit size index; NAA, N-acetylaspartate; NEALS, Northeast ALS Consortium; NF-L, neurofilament light chain; NI, neurophysiology index; NISALS, Neuroimaging Society in Amyotrophic Lateral Sclerosis; NMR, nuclear magnetic resonance; nUHPLC LC-MS, nano ultra-high performance liquid chromatography tandem mass spectrometry; p75ECD, neurotrophin receptor p75 extracellular domain; PA28a, proteasome activator complex subunit 1; PBA, pseudobulbar affect; PCR, polymerase chain reaction; PDI, protein disulfide-isomerase; PEFT, peak expiratory flow time; PET, positron emission tomography; PGGM, precentral gyruses gray matter; PGRN, progranulin; PLS, primary lateral sclerosis; PMA, progressive muscular atrophy; pNFH, Phosphorylated neurofilament heavy chain; PRDX2, peroxiredoxin-2; PRO-ACT, Pooled Resource Open-Access ALS Clinical Trials; QoL, quality of life; RD, radial diffusivity; RMN, Research Motor Neuron; ROA2, Heterogeneous nuclear ribonucleoproteins A2/B1; RSA, relative surface area; rsfMRI, resting state functional magnetic resonance imaging; SCA, spinocerebellar ataxia; SBMA, spinal and bulbar muscular atrophy; SEIQOL-DW, Schedule for the Evaluation of the Individual Quality of Life-Direct Weighting; SF-36, 36-Item short form health survey; SMA, spinal muscular atrophy; SMUAP, single motor unit action potential; SNIP, sniff nasal inspiratory pressure; SOD1, superoxide dismutase 1; SOP, Standard operating procedure; SPECT, single photon emission computed tomography; SPO2, peripheral capillary oxygen saturation; SVC, slow vital capacity; TA, tibialis anterior; TDP-43, TAR DNA-binding protein 43; TiM, Telehealth in Motor Neuron disease; TMS, transcranial magnetic stimulation; TNF, tumor necrosis factor; TUG, timed up and go test; Tw Pdi, twitch trans-diaphragmatic pressure; TWBC, total white blood cell count; UMN, upper motor neuron; VC, vital capacity; WALS, Western ALS Consortium; WVFI, Written Verbal Fluency Index.

RESULTS

Neuroimaging

The sample size characteristics, study design features, follow-up intervals of longitudinal neuroimaging, neurophysiology, and clinical studies are summarized in **Table 1**. Whilst most longitudinal imaging studies in ALS evaluate cerebral alterations (10), a number of promising spinal studies have now also been published. Spinal imaging has gradually overcome the technical challenges of physiological motion, small cross-sectional dimensions and susceptibility gradients (19, 110–118). The majority of longitudinal studies in ALS are single-center studies eliminating the need for cross-platform MR sequence harmonization and inter-rater reliability tests. Given the low incidence of certain phenotypes such as primary lateral sclerosis (PLS), progressive muscular atrophy (PMA), and spinal and bulbar muscular atrophy (SBMA) however, multisite collaboration is often necessary (119). The infrastructure, funding and governance of such multicenter collaborations are now established via international consortia like the Neuroimaging Society in Amyotrophic Lateral Sclerosis (NISALS) or the Northeast ALS Consortium (NEALS) (16, 23, 120, 121). The need to include disease-controls in addition to healthy controls to describe ALS-specific changes

TABLE 1 | Longitudinal "dry biomarker" studies in ALS: Neuroimaging, Neurophysiology and Clinical Studies.

Author(s) and year of publication	Follow-up interval (months)	Number of patients/Number or controls	Clinical assessment batteries/Functional rating scales	Imaging data	Main study findings
IMAGING STUDIES					
Floeter et al. (15)	6–18	28/28	ALSFRS-R, letter fluency, FBI, MMSE	DWI, structural (T2)	- progression and propagation detected (DTI measures) over 6 months - DTI measures correlated with ALSFRS-R, King's stage and cognitive measures
Kassubek et al. (16)	6	67/31	ALSFRS-R	DTI	- progression detected at group level and 27% of individual patients (DTI measures) - FA correlated with ALSFRS-R
Stampfli et al. (17)	3–6	21/13	ALSFRS-R	T1, DWI	-progression detected (FD values)
Baldaranov et al. (18)	26	6/6	ALSFRS-R	DTI	-progression detected (FA, AD/RD values) and correlated with progression on ALSFRS-R
Bede et al., 2017 (14)	4	32/69	ALSFRS-R	structural, DTI	-progression detected (GM)
de Albuquerque et al. (19)	8	27/27	ALSFRS-R, UMN scale	structural (T1, T2)	- progression detected (AD, MD) - correlation with ALSFRS-R change
Menke et al. (20)	24	16/0	ALSFRS-R, UMN score	T1, DTI, rs-Fmri	- progression detected - correlation with ALSFRS-R decline
Simon et al. (21)	3–6	21/13	ALSFRS-R, MRCSS-LL, MUNE	DTI, structural (T1)	- progression detected (FA values) - correlations with ALSFRS-R change, MUNE, functional disability and strength
Floeter et al. (22)	6	49/28	ALSFRS-R, FBI, MDRS-2, letter fluency, MMSE, D-KEFS	structural (T1)	-progression detected (ventricular volume)
Schulthess et al. (23)	6	135/56	ALSFRS-R	rs-Fmri, DTI	- progression detected (functional connectivity) - correlation with physical disability
McMillan et al. (24)	12	20/25	neuropsychology	structural (T1)	-hypermethylation protective against progression, correlation with protection of some components of neuropsychological assessment
Steinbach et al. (25)	3	16/16	ALSFRS-R, neuropsychology	DTI	-progression detected
Westeneng et al. (26)	5.5	112/60	ALSFRS-R	structural (T1)	- progression detected (volume measures) - correlation with ALSFRS-R
Menke et al. (4)	6	60/36	ALSFRS-R, ACE-R	structural (T1), DTI	-progression detected (GM)
Schuster et al. (27)	3–15	77/60	ALSFRS-R	structural (T1)	-progression detected (cortical thickness)
Stoppel et al. (28)	3	40/42	ALSFRS-R, MRC, neuropsychology	structural, Fmri	- progression detected - correlation with ALSFRS-R and MRC
Verstraete et al. (29)	5.5	24/19	ALSFRS-R	DTI, structural (T1)	- no progression detected - propagation detected
Ignjatovic et al. (30)	6	46/26	ALSFRS-R	structural (T1, T2, FLAIR)	-progression detected (hypointensities in PGGM)
Kwan et al. (31)	1.26–2.08 years	45/19	ALSFRS-R, finger tapping	T1, DTI	-progression detected (cortical thickness, GM volume)
Keil et al. (32)	6	24/24	ALSFRS-R, SF36, FAB, MMSE	DTI, structural (T1, T2)	- progression detected (FA values) - correlations with ALSFRS-R, physical and executive function
Menke et al. (33)	6	24/0	ALSFRS-R	DTI	-progression detected (AD)
Ichikawa et al. (34)	NA	6/NA	NA	NA	-progression detected, correlated to neuropsychology assessment
van der Graaff et al. (35)	NA	48/12	ALSFRS-R, finger tapping	DWI	-progression detected
Zhang et al. (36)	8	17/19	ALSFRS-R	structural (T1), DTI	- progression detected (FA)

(Continued)

TABLE 1 | Continued

Author(s) and year of publication	Follow-up interval (months)	Number of patients/Number or controls	Clinical assessment batteries/Functional rating scales	Imaging data	Main study findings
Agosta et al. (37)	9	16/10	ALSFRS	structural (T1)	- progression detected (GM)
Agosta et al. (38)	9	17/20	ALSFRS	DWI, structural	- progression detected (cord area, cord average FA)
Avants et al. (39)	5.3	4/4	0	structural (T1)	- progression detected (cortical atrophy)
Lule et al. (40)	6	25/15	ALSFRS-R	Fmri, structural (T1)	- progression detected (activity)
Unrath et al. (41)	6	11/0	ALSFRS	MRS, T1	- progression detected (NAA, NAA/Cr+Cho)
Suhy et al. (42)	Every 3 months	28/12	0	MRS, T1, T2	- progression detected (NAA, Cr, Cho)
Block et al. (43)	24	33/20	0	MRS	- progression detected
Irwin et al. (44)		143/0	MMSE, LGVF	structural VBM	- no progression on MRI reported
Kolind et al. (45)	42	30/12	ALSFRS-R, ACE,	mcDESPOT	- progression detected in PLS only
Verstraete et al. (46)	6	45/25	ALSFRS-R	structural (T1)	- no progression reported
Blain et al. (47)	6–12	23/25	ALSFRS-R, ALSS	structural (T2), DWI	- no significant progression detected (DTI measures)
Rule et al. (48)	3–12	45/17	0	MRS, structural (T1, T2)	- no clear pattern of progressive change over time (NAA rations)

Author(s) and year of publication	Follow-up interval (months)	Total number of patients/Total number of controls	Neurophysiology modality	Target muscle	Key study findings
NEUROPHYSIOLOGY STUDIES					
Escorcio-Bezerra et al. (49)	4.3	21/21	MUNIX	tibialis anterior (TA), abductor pollicis brevis (APB) and abductor digiti minimi (ADM) muscles	- progression detected (mean MUNIX)
de Carvalho et al. (50)	3–6	73/37	FPs, MUPs, fibs-sw, jitter- MU physiology	tibialis anterior	- progression detected
Boekestein et al. (51)	8	18/24	MUNIX, HD-MUNE, CMAP, MUSIX	thenar	- progression detected (MUNE, MUNIX)
Cheah et al. (52)	3	37/0	CMAP, axonal excitability	abductor pollicis brevis	- progression detected (CMAP)
Ahn et al. (53)	NA	135/NA	NA	NA	- asymmetric progression (MUNE)
Cheah et al. (54)	3	58/NA	NI, CMAP	abductor digiti minimi and ulnar nerve	- progression detected (NI)
de Carvalho et al. (55)	6	28/0	NI, CMAP, MUNE	abductor digiti minimi muscles	- progression detected (CSP)
Neuwirth et al. (56)	15	7/8	MUNIX, CMAP,	abductor pollicis brevis (APB), abductor digiti minimi (ADM), abductor halluces brevis (AHB), extensor digitorum brevis (EDB)	- progression detected (MUNIX)
Floyd et al. (57)	18	60/33	TMS, CMCT, MEP	abductor digiti minimi (ADM) and tibialis anterior (TA)	-linear progression detected (TMS threshold, CMCT, TMS amplitude corrected)
Gooch et al. (58)	NA	64/NA-1	TMS, MUNE,	NA	-progression detected (MUNE)
Liu et al. (59)	12	112/12	MUNE, CMAP	Abductor pollicis brevis (APB) and abductor digiti quinti (ADQ)	- progression detected (MUNE), correlated to ALSFRS descent
Albrecht et al. (60)	11.5	10/25	MUNE, S-MUAP	extensor digitorum brevis	- progression detected (MUNE)

(Continued)

TABLE 1 | Continued

Author(s) and year of publication	Follow-up interval (months)	Total number of patients/Total number of controls	Neurophysiology modality	Target muscle	Key study findings
Wang et al. (61)	12	20/70	MUNE, SMUP, CMAP, MU loss	thenar	- progression detected - (Thenar MUNE, CMAP)
Chan et al. (62)	24	NA	motor units	thenar	- progression detected
Felice et al. (63)	12	NA	MUNE	thenar	- progression detected (MUNE)
Yuen et al. (64)	6	NA	CMAP, MUNE	abductor digiti minimi	- progression detected (MUNE, fiber density)
Vucic et al. (65)	7–100 days	25/30, 35	cortical and axonal excitability- MEP, CMAP- TMS	abductor pollicus brevis	- aim to determine effect of riluzole
Aggarwal et al. (66)	36	31/57	MUNE	tibialis anterior, abductor pollicis brevis (APB), deltoid, and first dorsal interosseous muscles	- no progression reported
Arasaki et al. (67)	NA	NA	MUNE,	extensor digitorum brevis (EDB)	- no progression reported
de Carvalho et al. (68)	11.6	NA	CMAP, MEP, TMS	NA	- no progression detected
Swash et al. (69)	NA	14/NA	single fiber EMG	NA	- no definite progression detected

Author(s) and year publication	Follow-up interval (months)	Number of patients/Number of controls	Clinical assessment batteries/Functional rating scales	Summary of findings
CLINICAL STUDIES				
ALSFRS-R				
Thakore et al. (70)	NA	3367/0	ALSFRS-R, ALSFRS, bloods-creatinine, uric acid, CK, albumin, sodium bicarbonate, hematocrit, TWBC	- ALSFRS-R progression detected, pre-slope and post-slope have effects on survival
Rooney et al. (71)	NA	407/0	ALSFRS-R	- progression detected in ALSFRS-R subscores
*ACTS trial. (72)	NA	75/NA	ALSFRS	progression detected (ALSFRS-R), associated with motor and pulmonary function
Cognitive and behavior assessments				
Floeter et al. (73)	18	NA	ALSFRS-R, letter fluency, FBI	- progression detected (ALSFRS-R, FBI, letter fluency)
Elamin et al. (74)	NA	186/NA	cognitive testing	- progression detected (cognitive function)
Roberts-South et al. (75)	24	16/12	neuropsychology, language, discourse sampling, perfusion computerized transaxial tomography, pulmonary, clinical	- progression detected (cognitive language deficits)
*Duning et al. (76)	3	10/32	ALSFRS, clinical neuropsychological battery, imaging	- progression detected (DTI)
Poletti et al. (77)	24	168/0	ECAS	- no progression detected, ECAS scores improved over time
Xu et al. (78)	6	108/60	ACE-3, FAB, ECAS executive, MoCA, ALSFRS-R, ALS-FTD-Q, MiND-B	- no progression detected
Gillingham et al. (79)	9	20/36	ALS-CFB, ALSFRS-R	- no progression reported
Mioshi et al. (80)	6	79/53	MiND-B- apathy, disinhibition, stereotypical behavior, ACE-R, ALSFRS-R	- no progression reported
Quality of life assessments				
Jakobsson Larsson et al. (81)	24	36/0	SEIQoL-DW, ALSFRS-R, HADS	- anxiety decreased over time, depression correlated to QOL, QOL remained stable despite physical deterioration

(Continued)

TABLE 1 | Continued

Author(s) and year publication	Follow-up interval (months)	Number of patients/Number of controls	Clinical assessment batteries/Functional rating scales	Summary of findings
BMI and other clinical assessments				
Beck et al. (82)	6	78/39	skin water loss	- progression detected (skin water loss)
Garruto et al. (83)	NA	31/66	bone mass (wrist radiograph)	- progression detected (bone loss)
Ioannides et al. (84)	6	44/29	FM-ADP, BMI, BAI, ALSFRS-R	- BMI and BAI not accurate measures of fat mass in ALS
Peter et al. (85)	3	393/791	BMI, ALSFRS-R	- alterations in body weight present in ALS patients decades before manifestation of symptoms
Nunes et al. (86)	3	37/0	BMI, serum albumin, transferrin, total cholesterol	- no progression reported
Jablecki et al. (87)	NA	NA	clinical scores	- no progression reported
Respiratory and muscle assessments				
Andres et al. (88)	4–21	100/0	ATLIS, ALSFRS, VC	- ATLIS more sensitive to change than ALSFRS and VC
de Bie et al. (89)	12	10/0	RSA, ALSFRS-R, FVC	- progression detected(RSA and ALSFRS-R)
Shellikeri et al. (90)	NA	33/13	kinematic measures of tongue and jaw movement, speaking rate, intelligibility, ALSFRS-R	- progression detected (tongue movement size and speed)
Londral et al. (91)	2–20	19/26	typing activity, ALSFRS-R	- progression detected (typing activity)
Panitz et al. (92)	12	51/0	fatigue severity scale (FSS), CIS20-R-subjective fatigue experience, concentration, motivation, activity, ALSFRS-R, MRC, SVC	- progression detected (FSS, CIS20-R), correlated to ALSFRS-R, and ALSFRS-R progression
Atassi et al. (93)	NA	8635/0	ALSFRS-R, VC	- PRO-ACT database- progression detected (ALSFRS-R and VC)
Watanabe et al. (94)	1.7 years	451/0	ALSFRS-R, MRC, MMT	-progression detected (ALSRS-R)
Leonardis et al. (95)	every 3 months	NA/0	ALSFRS-R, Norris-r, AGA, FVC, MIP, MEP, SNIP	- progression detected (respiratory measures)
Mahajan et al. (96)	NA	362/0	VC	- progression detected (VC)
Pinto et al. (97)	4–6	49/0	Diaphragm amplitude, ALSFRS-R, MIP, FVC, SNIP, SPO2	- progression detected (Diaphragm amplitude, ALSFRS-R, respiratory measures)
Montes et al. (98)	6	31/0	TUG, ALSFRS-R, FVC, MMT	- linear progression detected (TUG) - associated with ALSFRS-R, MMT
Vender et al. (99)	NA	139/0	FVC	- progression detected (FVC)
Wilson et al. (100)	NA	55/NA	respiratory- FVC, FEV1, PEFT	- linear progression detected (PEFT)
Poloni et al. (101)	NA	NA	VC, Motley index, FEV1	- progression detected (respiratory measures)
Andersen et al. (102)	6–59	20/0	respiratory- SVC, cough peak flow, max inspiratory muscle strength, SNIP, max insufflation capacity	- no progression reported
Quaranta et al. (103)	NA	NA	respiratory function	- no progression reported
Proudfoot et al. (104)	24	61/39	eye tracking- anti saccadic, trail making, visual search tasks, ALSFRS-R, ACE-R, UMN, imaging)	- no progression detected
*Lenglet et al. (105)	18	512/0	ALSFRS-R, MMT, SVC	- clinical trial
Yamauchi et al. (106)	Every 6 months	43/30	ALSFRS-R, phrenic nerve conduction study (DCMAP), respiratory function tests (SNIP, FVC), nocturnal pulsed oximetry, MMT	- no progression reported
Mendoza et al. (107)	NA	161/0	MIP, FVC	- no progression reported
Marti-Fabregas et al. (108)	NA	NA	FVC	- no progression detected
Palmowski et al. (109)	NA	NA	electro-oculography	- not well-defined progression

Studies detecting progressive changes are listed first followed by studies not capturing longitudinal changes.
**indicates clinical trial.*

is increasingly recognized (30, 43, 44). With few exceptions (122–124), most ALS imaging studies use 3 Tesla platforms and 7 Tesla systems are more commonly used in post-mortem studies (125, 126). Disease progression has been detected across a range of MR imaging metrics including structural (22, 26), diffusion (16, 18), functional (28, 40), and spectroscopy (41, 42) measures. As the majority of studies have a two-timepoint design, it is often unclear if specific imaging metrics show linear or exponential changes. The few existing multi-timepoint studies suggest that pathological change is not linear (10). The revised ALS functional rating scale (ALSFRS-r) is the most commonly reported clinical measure (16, 18–20), with only few imaging studies reporting associations with staging (15) or neuropsychological performance (15, 24).

Neurophysiology

Most longitudinal neurophysiology studies are single center studies, reducing the risk of inter-rater and inter-center variability (127). As presented in **Table 1**, follow-up interval ranges between 7 days (65) and 3 years (66), and up to 7 follow-up time-points have been included in some studies (57, 60). Surprisingly few studies include disease controls such as peripheral neuropathy (60) or benign fasciculation syndrome (50). Clinical assessments performed in conjunction with neurophysiology typically include ALSFRS-r (51), forced vital capacity (FVC) (55), slow vital capacity (SVC) (56), grip strength (64), pinch strength (58), and manual muscle testing (MMT) (58), however, correlations between neurophysiological measures and clinical assessments are seldom reported. The majority of longitudinal neurophysiological studies focus on upper limb muscles, e.g., abductor pollicis brevis, deltoid, first dorsal interrosseus, extensor digitorum brevis, abductor digiti minimi (51, 52, 55, 60, 61) with relatively few studies evaluating lower limb muscles such as abductor hallicus brevis and tibialis anterior (50, 56, 57, 66). The most commonly reported longitudinal neurophysiological indices include compound muscle action potential (CMAP) (51, 52), single motor unit action potential (SMUAP) (60), MUNE (55, 59), MUNIX (49, 56), neurophysiology index (NI) (54, 55), TMS measures (57, 58), and axonal excitability (52). Progressive neurophysiological changes have been detected by MUNIX (49, 51, 56), MUNE (51, 58, 60), CMAP (52, 61), NI (54), and TMS measures (57) and allowing for study-design limitations, the consensus is that degenerative changes are not linear.

Clinical Biomarkers and Instruments

Robust clinical longitudinal studies in ALS have up to 6 follow-up time points (88, 89, 91), the interval between the assessments can be as short as 3 months (95) and the sample size can be as big as several thousands (70, 93) (**Table 1**). Few multi-timepoint studies include disease controls such as motor neuropathies (91), alternative neuromuscular diseases (78), or neurodegenerative conditions (83). Large, multi-timepoint longitudinal studies invariably suffer from considerable attrition rates, but these are rarely explicitly reported in the manuscript abstracts (10). Detailed genotyping is only available in a minority of longitudinal studies (15, 77, 79, 94). The most widely utilized rating scale in longitudinal studies is the ALSFRS-r (70, 71, 128) which provides a composite score of bulbar, limb and respiratory dysfunction, and is invariably evaluated in clinical trials (72, 105). Quality of life (QoL) in ALS is increasingly evaluated by disease-specific instruments such as the 40-item ALS assessment questionnaire (ALSAQ-40) or the revised ALS-specific Quality of Life questionnaire (ALSSQoL-R) (129–131). A number of symptom-specific instruments are also commonly used such as the Center for Neurologic Study-Bulbar Function Scale (CNS-BFS), a 21-item self-report scale of bulbar function, and the Center for Neurologic Study-Lability Scale (CNS-LS), a 7-item self-report scale of pseudobulbar affect (PBA) (132). Tapping rates, composite reflex scores, The Penn UMN Score (133), the Modified Ashworth scale (MAS) are often used as proxies of UMN degeneration (132).

In clinical trials, muscle strength is often estimated by handheld dynamometry (HHD) (134), manual muscle testing (MMT) (105), scoring systems such as the Medical Research Council (MRC) Scale for muscle strength (135) and some studies also report limb circumference (136). Respiratory function in ALS is typically monitored by sniff nasal inspiratory pressure (SNIP), SVC, or FVC in addition to measures such as early morning arterial blood gas (ABG) and overnight pulse-oximetry (137, 138). Measures of typing ability (91), tongue movements (90), vital capacity (VC) (96), FVC (99), SNIP (97), and diaphragm amplitude (97) all show progressive longitudinal changes. Nutritional markers such as body mass index (BMI) and lipid profile are now established prognostic indicators (139, 140). Cognitive and behavioral domains are routinely assessed thanks to the availability of validated screening instruments such as the Edinburgh Cognitive and Behavioral ALS Screen (ECAS) (141), the Beaumont Behavioral Inventory (BBI) (142) and the ALS Cognitive Behavioral Screen (ALS-CBS) (143). In contrast to the relentlessly progressive motor deficits of ALS, the trajectory of cognitive and behavioral deficits is less clear due to considerable individual variations, genotype-associated profiles (144, 145), differences in assessment strategies and practice-effects (146). Several longitudinal neuropsychology studies do not detect progression (77, 147, 148), progressive behavioral impairment has been noted in the absence of cognitive change (149), and some studies report improved performance as a result of practice effects (77).

Wet Biomarkers

The findings, study design characteristics, and follow-up intervals of longitudinal biofluid studies are summarized in **Table 2**. Phosphorylated neurofilament heavy chain (pNFH), neurofilament light chain (NF-L), progranulin (PGRN), cytokines, TAR DNA-binding protein 43 (TDP-43), cystatin C, creatinine, micro-RNAs (miRNAs), chitotriosidase-1 (CHIT1), chitinase-3-like protein 1 (CHI3L1), chitinase-3-like protein 2 (CHI3L2) have been evaluated in both research studies (152, 153, 157, 158, 162, 164, 168, 171) and clinical trials (150, 156, 157, 160, 161). Markers of iron metabolism and ferroptosis are relatively recent domains of ALS biomarker research (172, 173). Most biofluid studies are either serum (150, 157) or CSF studies (152, 167), but urine (155) and skeletal

TABLE 2 | Longitudinal "wet biomarker" studies in ALS.

Author(s) and year of publication	Follow-up interval (months)	Number of patients / number of controls	Candidate biomarker evaluated	Biofluid	Assessment method used	Summary of conclusion
*Okada et al. (150)	12	57/0	creatinine	serum	NA	- progression detected (creatinine)
Raheja et al. (151)	NA	NA	microRNAs	serum	NA	- progression detected (miR-136-3p, miR-30b-5p, miR-331-3p, miR-496, miR-2110)
Thompson et al. (152)	30	49/52	chitotriosidase (CHIT1), chitinase-3-like protein 1 (CHI3L1), and chitinase-3-like protein 2 (CHI3L2), (phosphorylated neurofilament heavy chain) Pnfh	CSF	nano ultra-high performance liquid chromatography tandem mass spectrometry (nUHPLC LC-MS/MS), ELISA	- progression detected (CHI3L1)
Di Pietro et al. (153)	NA	14/24	micro-RNAs- MIR206, MIR208B, MIR499	skeletal muscle	quantitative real time PCR, Western blot analysis	- progression detected (MIR208B, MIR499, MIR206, HDAC4)
Murdock et al. (154)	Every 6–12 months	119/35	leukocytes	blood	flow cytometry	- progression detected (immune cells), associated with ALSFRS-R
Shepheard et al. (155)	NA	54/45	urinary p75ECD	urine	sandwich ELISA	- progression detected (urinary p75ECD), correlated with ALSFRS-R
van Ejik et al. (156)	NA	1241/0	creatinine	plasma	NA	- progression detected (plasma creatinine), correlated to ALSFRS-R, muscle strength, mortality
Waller et al. (157)	3	22/0	microRNAs, miR-17-5p, miR-223-3p, miR-24	serum	Qiagen miScript-based Qpcr	- progression detected (mir-206, mir-143-3p, mir-374b-5p)
McCombe et al. (158)	27	98/61	pNFH	serum	NA	- progression detected (pNFH)
Lu et al. (159)	36	136/104	neurofilament heavy chain-phosphoform	plasma	ELISA	- progression detected (NfH)
*Levine et al. (160)	6	28/0	tau, pNFH	CSF	ELISA	- progression detected (tau)
*Levine et al. (161)	12	20/0	tau, pNFH	CSF	ELISA	- progression detected (tau)
Wilson et al. (162)	24	44/60	cystatin C	CSF, plasma	quantitative enzyme linked immunosorbent assay (ELISA)	- progression detected (cystatin C)
Gaiani et al. (163)	36	94/82	ALSFRS-R, NFL	CSF	enzyme-linked immunosorbent assay (UmanDiagnostics AB)	- NFL may have role as a biomarker
Lu et al. (164)	48	95/88	CK, ferritin, tumor necrosis factor (TNF)–a, and interleukin (IL)−1b, IL-2, IL-8, IL-12p70, IL-4, IL-5, IL-10, and IL-13, IL-6, IFN-Y	plasma	multiplex electrochemiluminescence immunoassay	- no defined progression
Steinacker et al. (165)	24	125/28	neurofilament light chain (NF-L), progranulin (PGRN), S100	serum, CSF (baseline only)	ELISA, electrochemiluminescence (ECL) immunoassay, ECLIA Elecsys (Roche, Penzberg, Germany)	- no progression reported
Gibson et al. (166)	12	80/0	CK	NA	NA	- no progression detected
Gray et al. (167)	24	41/14	CSF- glucose, lactate, citric acid, ethanol	CSF	H-NMR	- no progression reported
Lu et al. (168)	36	167/78	neurofilament light chain (NFL)	serum, blood, CSF	electrochemiluminescence immunoassay	- no progression detected
Verstraete et al. (169)	NA	219/100	TDP-43	plasma	sandwich ELISA	- no defined progression
Nardo et al. (170)	6	94/64	PRDX2, GSTO1, CLIC1, HSC70, CypA, PDI, ERp57, CALR, PA28a, IRAK4, FUBP1, ROA2, actinNT, TDP-43	blood PBMC	2D-DIGE, mass spectometry	- no progression reported

indicates clinical trial.

muscle-based (153) studies have now also been published. Quantitative enzyme-linked immunosorbent assay (ELISA) is the most commonly used antibody-based technique (13, 174) which can be performed with one antibody (indirect ELISA), or with two antibodies (sandwich ELISA). Increased CSF (13) and serum (175) pNFH detected by ELISA is thought to be a sensitive marker of axonal degeneration in ALS (152, 171, 176, 177). The specificity of this marker however may be inadequate to reliably differentiate ALS from other neurodegenerative conditions (13, 176). Other antibody-based techniques such as Western blot (171) and electrochemiluminescence (ECL) (153, 168) may improve detection sensitivity and reliability (13). Panels of multiple proteins can be evaluated by multiplex immunoassays such as planar or microbead assays (13). Mass spectrometry based methods using chromatin-immunoprecipitation-based surfaces, two-dimensional gel electrophoresis or high-resolution mass spectrometry have identified cystatin-C and transthyretin as candidate biomarkers (178–180). The longest wet biomarker study followed patients for 4 years (164). The majority of studies have at least 2 follow-up timepoints (155, 162, 170) and one study included 13 follow-up timepoints (156, 159). Large multi-center trials include as much as 1,000 participants (156). One of the most striking shortcomings of existing longitudinal studies is that very few included disease controls such as Parkinson's disease cohorts, patients with multifocal motor neuropathy with conduction block, Kennedy's disease, chronic inflammatory demyelinating polyneuropathy (CIDP), cervical or lumbar radiculopathy, Charcot-Marie-Tooth disease (CMT), benign fasciculation, and cramp syndrome etc. (152, 159, 162). Another limitation of many longitudinal studies is the lack of comprehensive genotyping (12) as very few studies report comprehensive screening for ALS-associated mutations (153, 159, 169, 171). Exhaustive clinical profiling, such as medications (152, 164), neuropsychological assessments (171), quality of life indices are rarely reported in longitudinal studies. The majority of studies limit their clinical descriptions to ALSFRS-r, FVC, MRC, and Ashworth scores (153, 161, 162). Serum and plasma biomarkers such as creatinine (150, 156), pNfH (158, 159), and micro-RNAs (157), CSF biomarkers such as CHI3L1 (152), tau (160, 161), and cystatin-C (162), and urinary (155) and skeletal muscle (153) biomarkers are some of the promising tools for detecting disease progression. While no progressive changes have been detected in NFL levels, it is likely to be a useful as a diagnostic biomarker (168, 171).

Studies of Asymptomatic Mutation Carriers

Current clinical trials only recruit symptomatic cases despite accruing evidence that ALS has a long presymptomatic phase (5). Imaging studies of asymptomatic mutation carriers have consistently confirmed disease-specific cerebral and spinal cord changes prior to symptom onset (181–184) indicating that this disease-phase may represent a crucial window for therapeutic or neuroprotective intervention. The majority of presymptomatic studies assess a single time-point, as opposed to the longitudinal tracking of asymptomatic carriers of ALS-causing mutations (15). While the overwhelming majority of presymptomatic studies focus on *C9orf72* hexanucleotide carriers (183, 185–187), no prognostic markers have been validated to predict whether single patients will develop ALS or FTD. Compared to imaging studies, strikingly few presymptomatic neurophysiology studies have been undertaken (66). Studies of asymptomatic ALS-causing mutation carriers have enormous potential for academic research and may pave the way for asymptomatic pharmaceutical trials (5, 181).

DISCUSSION

Clinical trials currently evaluate the efficacy of candidate drugs using the revised ALS functional rating scale (ALSFRS-r), muscle strength assessment tools such as manual muscle testing (MMT), respiratory function indices such as forced vital capacity (FVC), slow vital capacity (SVC) and sniff nasal inspiratory pressure (SNIP), neurophysiological measures and survival (102, 116, 120, 188, 189). These measures however primarily reflect late-stage functional impairment and are not indicative of early stage pathology. Brain and spinal cord imaging has been evaluated as early-stage biomarkers with both diagnostic and monitoring potential (116, 120, 190). The core neuroimaging signature of ALS, irrespective of the disease-stage, includes corticospinal tract (191, 192), corpus callosum (193) and motor cortex degeneration (194). Atrophy in frontotemporal regions has been primarily associated with neuropsychological deficits (195–197) and linked to hexanucleotide repeats in C9orf72 (145, 198). Longitudinal imaging studies are superior to cross-sectional studies as they readily detect dynamic structural and functional changes and may elucidate compensatory processes (10, 14, 23, 28, 40, 120, 199). The emergence of multi-timepoint study designs (14, 20) enable the characterization of anatomical propagation patterns (200) and provide invaluable temporal insights into the disease trajectory of late-stage ALS. Inter-scan intervals as short as 3 months can detect longitudinal changes (14, 18, 120). Many longitudinal studies make use of multiple magnetic resonance (MR) metrics which is particularly useful in establishing an optimal panel of monitoring markers (120). Several longitudinal studies have indicated that white matter degeneration can be detected relatively early in the course of ALS with restricted further progression over time, whereas gray matter pathology shows relentless progression in the symptomatic phase of the disease (4, 14, 120). In addition to structural imaging studies, connectivity-based, metabolic, peripheral nerve, and, whole body muscle imaging have contributed to our understanding of longitudinal changes (20, 201–203).

Needle electromyography and nerve conduction studies play an important clinical role in ruling out alternative conditions and confirming a suspected diagnosis of ALS. Despite variations in local protocols, neurophysiological tests are recognized as objective, reliable and cost-effective tests of neuromuscular dysfunction, and have also been repeatedly proposed as longitudinal markers (55, 204). CMAP is generated by depolarization of muscle fibers through the stimulation of a single nerve, where amplitude reductions are interpreted as

loss of motor axons (205, 206). While CMAP measurements capture longitudinal decline, it is confounded by variations in temperature, limb positioning and electrode placement (56, 207). CMAP-derived measures such as MUNE and MUNIX are now extensively utilized to characterize progressive changes in ALS. MUNE estimates motor neuron numbers, and may detect the rate of motor neuron loss, making it a more reliable method of appraising disease progression than CMAP (208, 209). However, its early-phase sensitivity has been questioned, as its use is limited to distal muscles, and the technique requires considerable training, especially for inter-rater and multi-site comparisons (205, 210). TMS allows the characterization of upper motor neuron dysfunction, and may be particularly useful in detecting progressive changes (57, 205).

Functional rating-scales are often the monitoring instruments of choice in clinical trials (55), as they are easy to administer, cost-effective to utilize and have acceptable inter- and intra-rater reliability profiles (7). The most widely used rating scale in clinical longitudinal studies is the ALSFRS-r. Despite its ease of administration, it has considerable limitations, as it may be disproportionately influenced by LMN dysfunction, does not account for laterality or asymmetry of symptoms, omits cognitive impairment, and may be affected by medications (14, 128, 188, 211).

Proteomics, metabolomics and lipidomics have seen significant advances in ALS research and CSF and serum markers are now used in longitudinal academic and pharmacological studies (172). Potential biomarkers for the detection of disease progression include serum and plasma biomarkers such as creatinine (150, 156), pNfH (158, 159), and micro-RNAs (157), CSF biomarkers such as CHI3L1 (152), tau (160, 161), and cystatin-C (162), and urinary (155) and skeletal muscle (153) biomarkers.

Prediction Analyses

Age at symptom onset (212), BMI (139), bulbar involvement (213), cognitive impairment (214), *C9orf72* genotype status (144), respiratory insufficiency (215), "definite ALS" by the El Escorial criteria (216), and functional disability (217) are the most commonly cited determinants of poor prognosis in ALS. SNIP (218) and less commonly used measures such as twitch trans-diaphragmatic pressure (Tw Pdi) (219) and maximal static expiratory mouth pressure (MEP) were shown to be good predictors of ventilator-free survival (219). A combined panel of several clinical, wet, and dry biomarkers is likely to offer the most accurate prognostic information (115, 120, 216, 217, 220). While cerebral (217, 221, 222) and spinal (115) imaging measures have been repeatedly linked to survival outcomes, these have not been utilized in a clinical setting. Neurophysiological variables, such as phrenic nerve stimulation outcomes (223) and biofluid markers, such as pNFH and NFL (165, 168, 224–226) are also thought to be accurate predictors.

Patient Stratification

Attempts to enroll patients in the early stages of the disease are hampered by the universally long diagnostic delay in ALS (227). Patient stratification in trials is typically based on site of onset (228), instead of other variables which have an established prognostic impact (138, 229). Admixed patient cohorts within a trial may hamper the ability to detect how different phenotypes and genotypes may exhibit a different response to a candidate drug (230–232). The stratification of heterogeneous cohorts is now aided by the development of validated staging systems, such as the King's (233), Milano-Torino (MITOS) (234) or the Fine'til 9 (FT9) (235) staging systems. The King's Staging system is based on the number of body regions affected, and the presence of nutritional or respiratory failure (233). The MITOS staging system is based on the ALSFRS-r, and is particularly sensitive to changes in later stages of the disease (236, 237). However, none of these staging systems account for cognitive or behavioral changes (236). Pathological staging systems suggest a four-stage model of ALS based on anatomical patterns of pTDP-43 load (238, 239). This system has now been validated by *in vivo* neuroimaging studies (240) and signals that accurate pathological staging and patient stratification may be possible based on neuroimaging (199, 240).

International Consortia

Only few ALS centers maintain dedicated biobanking facilities to store and process molecular markers in human biofluid locally. Similarly, relatively few centers are in a position to generate sufficient number of MRI and neurophysiology data sets of rare phenotypes to make meaningful inferences in a single center setting. Brain and tissue banks are also challenging to establish, maintain and fund, despite their invaluable contribution to ALS research (241–243).

Biospecimen samples are also often collected during clinical trials, and discarded after negative outcomes, despite their enormous potential for biomarker discovery (172). One of the most important achievements of biomarker development efforts is the establishment of national and international research consortia such as Association pour la recherche sur la SLA (ARSLA), Neuroimaging Society in ALS (NISALS), Research Motor Neuron (RMN), Canadian ALS Neuroimaging Consortium (CALSNIC), EU Joint Programme for Neurodegenerative Disease Research (JPND), European multidisciplinary ALS network identification to cure motor neurone degeneration (EUROMOTOR) which maintain vital biobanking facilities, registries, data repositories for multicenter data interpretation (121, 244). Clinical trial networks are also increasingly recognized as valuable platforms for multisite data collection and interpretation as they operate with carefully standardized protocols. Consortia such as the European Registry of ALS (EURALS) Consortium, the Western ALS (WALS) Consortium and the Northeast ALS (NEALS) Consortium are other examples (245). NEALS is one of the largest consortia with over 100 member sites from the US, Canada, Mexico, Italy, Lebanon and Australia (246). EURALS coordinates research studies and clinical trials relying on population-based European registries and include centers from Scotland, England, Netherlands, Spain, Ireland, Serbia, Italy, France, and Germany (241, 247, 248). ALS research consortia promote patient-oriented research, maintain biofluid, imaging and DNA banks, and have

the potential to translate scientific advances into pragmatic clinical interventions.

Telehealth

Novel trends in longitudinal data collection include telemedicine-based technologies, wearable sensors and mobile phone applications (230). The continuous collection of data via telephone or telemedicine applications such as the Telehealth in Motor Neuron disease (TiM) system circumvent the inconvenience of patients and caregivers traveling long distances for research appointments (249). Once local data-protection and governance guidelines are complied with, information uploaded from these systems can be made available to healthcare professionals of multidisciplinary teams in real time (249). The feasibility of telehealth for ALS patients via live video-conferencing has also been evaluated (250) and is considered a particularly promising clinical and research platform (249, 250). A number of cognitive-behavioral screening tools have also been adapted for phone administration (251) including modified versions of the ALS Cognitive Behavior Screen (ALS-CBS), the Controlled Oral Word Association Test (COWAT), the Center for Neurologic Study-Lability Scale (CNS-LS) and found to be statistically equivalent to face-to-face assessments (251). Performance on other tests however, such as the telephone versions of the ALS-Frontal Behavioral Inventory (ALS-FBI) caregiver interview and the Written Verbal Fluency Index (WVFI) was not equivalent to clinic-based assessments (251). The continued development of telephone and internet-enabled devices are likely to provide further insights to longitudinal physical, cognitive and behavioral changes (251).

CONCLUSIONS

While clinical indicators of disease progression remain indispensable, neuroimaging, neurophysiology, and biofluid measures are particularly promising, objective, quantitative biomarker candidates. The validation of combined "wet" and "dry" biomarker panels will not only enable the detection of subtle progressive changes in ALS, but allow precision stratification of heterogeneous patient cohorts in clinical trials and improve existing prediction algorithms.

AUTHOR CONTRIBUTIONS

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Pathological Crying and Laughing in Motor Neuron Disease: Pathobiology, Screening, Intervention

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Pathological crying and laughing (PCL) has significant quality-of-life implications in amyotrophic lateral sclerosis (ALS); it can provoke restrictive life-style modifications and lead to social isolation. Despite its high prevalence and quality of life implications, it remains surprisingly understudied. Divergent pathophysiological models have been proposed, centered on corticobulbar tract degeneration, prefrontal cortex pathology, sensory deafferentation, and impaired cerebellar gate-control mechanisms. Quantitative MRI techniques and symptom-specific clinical instruments offer unprecedented opportunities to elucidate the anatomical underpinnings of PCL pathogenesis. Emerging neuroimaging studies of ALS support the role of cortico–pontine–cerebellar network dysfunction in context-inappropriate emotional responses. The characterization of PCL-associated pathophysiological processes is indispensable for the development of effective pharmacological therapies.

Keywords: pathological crying and laughing, pseudobulbar affect, emotional lability, involuntary emotional expression disorder, motor neuron disease, amyotrophic lateral sclerosis, biomarkers, magnetic resonance imaging

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INTRODUCTION

The terms "pathological crying and laughing," "pseudobulbar affect," "emotional lability," and "involuntary emotional expression disorder" are often used interchangeably depending on geographical location and year of publication. Despite the differences in terminology, recurrent episodes of involuntary or exaggerated emotional expression, particularly in the form of crying and laughing, are common in several neurological conditions including, in order of prevalence: motor neuron disease (MND), traumatic brain injury (TBI), multiple sclerosis (MS), stroke, multiple system atrophy-cerebellar type (MSA-C), Alzheimer's disease (AD), and Parkinson's disease (PD) (1–6).

The disorder is particularly common in motor neuron diseases (MND): amyotrophic lateral sclerosis (ALS) and primary lateral sclerosis (PLS). Prevalence estimates vary, but between one quarter and one half of MND patients are thought to be affected (5, 7–11). While there is a paucity of studies comparing PCL prevalence across the spectrum of MND phenotypes, a recent study confirmed the relationship between clinical upper motor neuron dysfunction and PCL prevalence; PCL was most commonly identified in PLS and UMN-predominant patients (39%), followed by typical ALS (29%) and lower motor neuron (LMN) predominant groups (10%). Consistent with this pattern, there was a single case of PCL in a group of 12 patients with progressive

muscular atrophy (PMA) (12). While the manifestations of the episodes may be congruent with the person's contemporary emotional state (10, 13, 14), the magnitude of emotional responses is disproportionate to the emotive stimulus and the social context. Such episodes may cause significant distress, embarrassment, and ultimately may lead to social withdrawal (15).

The primary objective of this review is to systematically appraise PCL-related studies in MND from a dual academic and clinical perspective. We outline established and emerging disease-models based on neuroimaging, neurosurgical and neurophysiological studies. The spectrum of clinical presentations, diagnostic challenges, functional impact, and treatment options are also discussed. We preferentially use the term "pathological crying and laughing" (PCL) in this review, in its broadest sense: encompassing the entire spectrum of presentations. Furthermore, while PCL in ALS is the primary focus of this review, we draw further information from PCL studies in other neurological conditions to discuss unifying, symptom-specific, pathophysiological concepts.

METHODS

A formal literature search was conducted using PubMed/Medline and Embase using the terms "pathological crying and laughing," "pseudobulbar affect," "emotional lability," involuntary emotional expression disorder" separately, and in combination with terms "amyotrophic lateral sclerosis," "motor neuron disease," "pseudobulbar palsy," "clinical trials," "treatment," and "pathology." Only articles in English, published between 1988 and October 2018 were included. A total of 220 articles met these criteria; these were systematically reviewed for information relating to diagnosis, disease-mechanisms, anatomical localization, and treatment options.

Historical Context and Terminology

The abundance of terms used to describe this syndrome epitomizes the tireless efforts to characterize the underpinnings of both physiological and pathological emotional expression. Charles Darwin observed in 1872 that "certain brain-diseases, as hemiplegia, brain-wasting, and senile decay, have a special tendency to induce weeping" (16). Oppenheim (17) used the term "pseudobulbar affect" (PBA), based on the important observation that the disorder commonly occurs in association with motor features of pseudobulbar palsy, a condition resulting from bilateral corticobulbar tract pathology. Although this association is still well-recognized (13, 18), the term PBA may be misleading; new evidence suggests that corticobulbar tract dysfunction alone is neither necessary, nor sufficient to cause PCL (19–23). "Emotional Lability" (EL) was described by Pierre-Marie as early as 1892, a term still commonly used in the literature (24). The term pathological crying and laughing (PCL) was used by Wilson in his influential essay of 1924, in which he introduced his unifying theory of the disorder (25). More recently, the term "involuntary emotional expression disorder" (IEED) has been proposed to encompass the wider range of emotional symptoms which may accompany the disorder (26).

Abbreviations: AD, Alzheimer's disease; ALS, Amyotrophic lateral sclerosis; B.I.D., Bis in die/twice daily; CNS-LS, Center for Neurologic Study-lability scale; DxQ, Dextromethorphan-quinidine; EL, Emotional lability; ELQ, Emotional lability questionnaire; EMA, European Medicines Agency; FDA, US Food and Drug Administration; LMN, Lower motor neuron; MND, Motor neuron disease; MRI, Magnetic resonance imaging; MS, Multiple sclerosis; MSA-C, Multiple system atrophy-cerebellar type; NMDA, N-methyl-D-aspartate; PBA, Pseudobulbar affect; PC, Pathological crying; PCL, Pathological crying and laughing; PD, Parkinson's disease; PLACS, The pathological laughing and crying scale; PLS, Primary lateral sclerosis; PMA, Progressive muscular atrophy; SPECT, Single-photon emission computed tomography; SSRI, Selective serotonin re-uptake inhibitor; TBI, Traumatic brain injury; TCA, Tricyclic anti-depressant; UMN, Upper motor neuron.

Clinical Presentations, Diagnosis and Monitoring

A useful conceptualization of PCL is that symptoms may lie on a spectrum, (27) with infrequent, mood congruent but disproportionate episodes at one end, and frequent, mood incongruent episodes at the other. While there is considerable variability in episode type and character across patients, within individuals episodes tend to be consistent over time in terms of symptom type (i.e., uncontrolled laughing or crying), the context in which the episodes recur, the severity, duration, and the degree of voluntary control that the individual retains over the episodes (26, 28). The wide range of presentations coupled with the lack of unifying terminology, has hampered efforts to establish widely adopted diagnostic criteria. Several criteria have been proposed and later revised, reflecting evolving concepts of the PCL (29). *Poeck's* 1969 criteria (30), focus on episodes that are entirely situation inappropriate or unrelated to the patient's internal emotional state. Recent criteria are less restrictive, encompassing presentations across the entire spectrum (26, 27). Revised criteria proposed by Cummings et al. for "involuntary emotional expression disorder" (IEED) (26) are inclusive of episodes that are either disproportionate to the emotive stimulus or to the individual's internal emotional state. The episodes must represent a distinct change from the patient's emotional reactivity prior to the onset of a neurological disease. These criteria specifically require the exclusion of alternative causes for the symptoms; mood, facial tics, dystonia, or substance effects. More recently, Miller et al. proposed to include supportive features such as the presence of pseudobulbar signs and a proneness to anger (27). The latter reflects accruing observations from several studies (14, 23, 27, 31).

PCL needs to be carefully distinguished from mood disorders through careful clinical evaluation (1, 9, 32). A key difference is that crying in depression or excessive laughter in mania occur in the context of pervasive low or elevated mood, respectively (33). In the case of clinical depression, associated symptoms can also be identified such as anhedonia and insomnia (27, 34). Although some studies report an association between depression and PCL (12), more commonly, no significant association is found (8, 13, 35). The clinical distinction may be particularly challenging in cases where pathological crying co-exists with depressive symptoms (2, 36). Emerging evidence suggests that PCL may respond to selective serotonin reuptake

inhibitors (SSRIs) within days, whereas depression typically only responds to pharmacological intervention after several weeks (37).

Similarly to the multitude of diagnostic criteria proposed for PCL, several screening and symptom severity scoring instruments have been developed. While not all of these have been extensively validated, these tools have been used in both academic research and clinical trials. The pathological laughing and crying scale (PLACS) was validated for use in stroke patients with "PBA" in 1993. The authors used it as an efficacy measure in a small placebo-controlled trial of nortriptyline; treatment was associated with symptom reduction (38). The Center for Neurologic Study-Lability Scale (CNS-LS) was introduced as a self-reported measure of "affective lability" in ALS (39). This short, self-administered questionnaire consists of 7 items; 4 relating to labile laughing and 3 relating to crying. The scale evaluates subjective burden of symptoms over the preceding week, in terms of episode burden and severity. While this scale relies on retrospective patient account, it has been shown to be an accurate indicator of episode frequency (40). The CNS-LS has been adopted as an efficacy measure in several recent clinical trials (41–44). Another assessment tool, the "emotional lability questionnaire" (ELQ) has also been validated in ALS. It extends the period over which symptoms are assessed from one up to four weeks prior to screening, which helps to capture patients who experience less frequent episodes (45, 46). In addition to PCL, it also includes a specific section on abnormal smiling. One of the strengths of the ELQ is that it includes the caregiver's perspective, which helps to identify lack of insight if significantly discordant scores are given by the patient and the carer. Interestingly, in ALS, there is significant agreement between patient and caregiver scores, indicating that patients are keenly aware of PCL symptoms (45). This concordance contrasts with behavioral deficits in ALS-FTD, where patient reports may differ substantially from caregivers reports (47).

Although screening instruments are valuable tools for identifying and monitoring PCL, they don't evaluate the impact of PCL on individual patients. There is evidence, that PCL impacts on the quality-of-life and social functioning of affected individuals (48) and may contribute to carer distress (49). There is a growing effort to understand the individualized experiences of patients with PCL (14, 35, 50, 51).

DISEASE MODELS

Traditional and Revised Hypotheses

The traditional PCL model, proposed by Wilson in 1924 has, until recently been the most widely accepted one (25). Under physiological circumstances, it contends, emotional expression is influenced by both voluntary motor and involuntary emotional centers. These pathways descend onto the medullary "facio-respiratory" centers, which mediate the facial movements and breathing patterns necessary to convey emotion. This model predicts that disruption of the descending voluntary inputs to the brainstem, such as may occur in pseudobulbar palsy, results in disinhibition of involuntary emotional influence on expression. In support of this model, Wilson cited cases of dissociated emotional and voluntary facial expression, such as the observation that some patients with pseudobulbar palsy are unable to make voluntary facial movements but can be observed to smile, laugh or cry in response to emotional stimuli. This phenomenon is usually termed "voluntary facial palsy" (52). The contrary, "mimic palsy" or "emotional facial palsy" is sometimes observed, in which a patient with entirely normal voluntary facial movements, exhibits a complete lack of movement on one side of the face when reacting to emotional stimuli (53).

Parvizi et al. highlighted several limitations of the traditional model (54). Patients with bilateral voluntary facial paralysis, as in Wilson's example do not seem to be excessively prone to PBA, as would be predicted by the "disinhibition" model. Furthermore, patients with severe PBA symptoms are usually able to voluntarily mimic laughing or crying, indicating that involuntary expression can occur alongside intact voluntary control.

The revised model of PCL draws on the increasing appreciation of the role of the cerebellum in cognitive processes (55, 56). This model suggests that the cerebellum plays a key role in gating and modulating emotional output in response to contextual cues from cortical and limbic areas (54). Modulation is believed to be mediated through cortico-ponto-cerebellar pathways. In normal motor control, the cerebellum is known to modulate motor output in response to multiple sensory inputs; disruption of these inputs produces motor modulation deficits, including dysmetria. The involuntary emotional expression resulting from disruption of cortico-ponto-cerebellar emotional circuits has, analogously been termed "affective dysmetria" (57).

Insights From Imaging and Neurophysiological Studies Across Neurological Diseases

Research over the past 30 years has led to a revision of the traditional model of PCL; studies have provided strong evidence to refute a simple causal relationship between PCL and corticobulbar tract dysfunction (58). Instead, a disruption within a widely dispersed network of emotional control appears to underlie the disorder. **Tables 1**, **2** provide an overview of studies highlighting key anatomical regions implicated in the pathogenesis of PCL. An MRI-based lesion study in post-stroke pathological crying classified patients based on symptom severity (70). It found a positive pathoanatomical correlation between lesion size and location and pathological crying severity. Bilateral pontine infarcts were associated with greatest severity, while bilateral anterior hemispheric infarcts were associated with moderate PCL severity.

Among the likely neurotransmitters involved in the physiological expression of emotion, serotonin and glutamate have received particular attention (27, 71, 74). Evidence for dysfunction within these neurotransmitter pathways in PCL comes from the success of serotonergic (31, 38, 75), and anti-glutamatergic drugs (42) in PCL treatment. The selective serotonin re-uptake inhibitors (SSRIs) and tricyclic antidepressants (TCAs), are thought to work by increasing

TABLE 1 | Neuroanatomical regions implicated in PCL circuitry based on clinical observations.

References	Neurological condition	Terminology	*n*	Main study findings	Anatomical localization
CLINICAL CASES					
Parvizi et al. (3)	Cerebellar cyst	PC	1 PC+	Midline cerebellar cyst	Cerebellum (vermis)
Parvizi et al. (59)	MSA-C	PCL	PCL+: 1 PM and 9 clinical MSA-c	Pathological changes confined to cerebellum, basilar pons and olives	cerebellum and brainstem connections
Chattha et al. (60)	PD with STN-DBS	PBA	1 PBA+	Laughter with DBS stimulation of sub-thalamic nuclei	Basal ganglia: STN
Saini et al. (61)	CPM	PBA	1 PBA+	Basilar pons demyelination	CPC tracts: Pons
Martin et al. (62)	Anti-Yo cerebellar degeneration	PBA	1 PBA+	Breast cancer presenting as uncontrollable crying and motor cerebellar syndrome	Cerebellum
McCullagh et al. (20)	ALS	PCL	10 PCL + 8 PCL- 10 HC	Executive dysfunction in PCL+	Pre-motor frontal cortex
Palmieri et al. (45)	ALS	EL	29 EL+	Correlations: (1) bulbar disease and EL (2) bulbar disease and executive impairment. However, no correlation between PCL and cognitive changes	Extra-motor frontal lobe
Olney et al. (13)	ALS	PCL	21 PCL+, 14 PCL–	Laboratory study: PCL+ had impaired regulation of facial expression	Frontal cortex
Hübers et al. (63)	ALS	PCL	10 PCL+, 10 HC	PCL+ more susceptible to mood-incongruent stimuli than controls, PCL associated with emotional lability/suggestibility	Frontal cortex
NEUROSURGICAL CASES					
Krack et al. (64)	PD with STN-DBS	Mirthful laugh	2 PL+	Associated with DBS stimulation of bilateral STN in one patient and right STN in other	Basal ganglia: STN
Okun et al. (65)	PD post-thalamotomy	PBA	1 PBA+	Post-thalamotomy pathological laughing	Basal ganglia
Okun et al. (66)	PD with STN-DBS	PC	1 PC+	Pathological crying with DBS stimulation of left sub-thalamic nucleus	Basal ganglia: STN
Famularo et al. (67)	Cerebellar ependymoma	PL	1 PL+	PC as sole presenting feature of cerebellar vermis tumor abutting the floor of the fourth ventricle	Cerebellum (vermis)
Low et al. (68)	PD with STN-DBS	PC	1 PC+	Pathological crying with DBS stimulation in region of caudal internal capsule (without signs of PBP)	Internal capsule (caudal)
Wolf et al. (69)	PD with STN-DBS	PC	1 PC+	Pathological crying with DBS stimulation of sub-thalamic nuclei	Basal ganglia: STN

CPC, cortico-ponto-cerebellar pathways; CPM, Central Pontine Myelinolysis; Dx, Diagnosis; HC, Healthy control; MSA-C, Multiple system atrophy, cerebellar type; n, sample size; PBP,Pseudobulbar Palsy; PC, Pathological crying; PL, pathological laughing; PM, Post-mortem/Autopsy; SERT, Serotonin transporter; STN-DBS, Subthalamic nucleus, deep brain stimulation; UMN, Upper motor neuron; WM, White matter.

availability of serotonin at synapses in corticolimbic and cerebellar circuits (32).

A SPECT study found reduced brainstem serotonin receptor (SERT) densities in post-stroke PCL (71), providing further evidence of the role of serotonergic transmission in PCL. Serotonergic neurons from the raphe nuclei have widespread projections from the paramedian brainstem to cortical, subcortical, and cerebellar targets (76, 77). Also consistent with the serotonin hypothesis, lesions involving these nuclei and their projections are frequently associated with PCL (70). An MRI study of PBA in MS patients showed an association between symptoms and lesions in key regions: brainstem, bilateral inferior parietal, and medial frontal regions (23).

There is increasing recognition of the role of sensory deafferentation of the cerebellum in PCL (78). Evidence from neurophysiological studies in PCL suggests that the cerebellum may filter emotional output through a "gate-control" mechanism (79). At a cellular level, cerebellar Golgi cells may play a crucial role in gate-control. It has been demonstrated that Golgi cells, when activated from various peripheral inputs, show decreased firing rate, thereby reducing inhibition of granule cells (80). This finding, suggests, that rather than providing "gain-control," Golgi cells may act as a "context-specific gate" on transmission through the mossy fiber–granule cell pathway. A study of PCL in MSA-cerebellar type found a prevalence of 36% in this condition, in which clinically-significant cerebellar dysfunction is apparent (59).This prevalence estimate exceeds those of studies in idiopathic Parkinson's disease (5), suggesting that cerebellar pathology is linked to PCL in Parkinsonian disorders. Several neurological and neurosurgical case-reports have linked PCL to cerebellar pathology, especially in association with vermis pathology (3, 62, 67, 81). These studies support the cerebellar

TABLE 2 | Anatomical conclusions of neuroimaging studies in PCL.

References	Neurological condition	Terminology	*n*	Main study findings	Anatomical localization
Andersen et al. (70)	Stroke	PC	12 PC+	Isolated, bilateral pontine lesions in most severe cases; bilateral basal ganglia lesions in intermediate group; unilateral subcortical lesions in milder	Pons (raphe nuclei), Basal ganglia, Subcortical WM
Murai et al. (71)	Stroke	PC	6 PC+ 9 PC–	Reduced SERT binding ratios in midbrain/pons in PC	Brainstem (raphe nuclei)
Tateno et al. (72)	TBI	PCL	92 PCL+	PLC associated with traumatic frontal lobe lesions, particularly lateral left frontal lobe	Frontal lobes (esp. left lateral)
Ghaffar et al. (23)	MS	PBA	14 PBA+ 14 PBA–	Greater lesion volume in PBA subjects in brainstem; bilateral inferior parietal and medial inferior frontal; right medial superior frontal	Brainstem; Parietal lobes (bilateral inferior); frontal; basal ganglia
Floeter et al. (10)	ALS	PBA	22 PBA+ 25 PBA– 28 HC	PBA+ (vs. PBA-): reduced FA underlying left motor cortex, Increased MD underlying the frontotemporal cortex, the transverse pontine fibers, and MCP. IC pathology in both groups	widespread disruption of CPC tracts in PBA
Wang et al. (21)	Stroke	PCL	56 PCL+, 56 PCL–	PCL associated with pontine infarcts, particularly paramedian lesions	Pons
Christidi et al. (73)	ALS	PCL	28 PCL+ 28 PCL– 25 HC	PCL+ vs. PCL-: Reduced GM volume: left orbitofrontal cortex, frontal operculum, putamen; and bilateral frontal poles. WM pathology: decreased FA in left cingulum bundle, posterior corona radiata	Frontal cortex: left orbitofrontal; and operculum Cingulate WM

CPC, cortico-ponto-cerebellar pathways; CPM, Central Pontine Myelinolysis; Dx, Diagnosis; HC, Healthy control; MSA-C, Multiple system atrophy, cerebellar type; n, sample size; PBP, Pseudobulbar Palsy; PC, Pathological crying; PL, pathological laughing; PM, Post-mortem/Autopsy; SERT, Serotonin transporter; IC, Internal Capsule; STN-DBS, Subthalamic nucleus, deep brain stimulation; UMN, Upper motor neuron; WM, White matter.

gate-control theory of emotional expression, indicating that the disruption to cortico-ponto-cerebellar emotional circuitry may underlie this disorder.

Insights From PCL Studies in Motor Neuron Disease

Corticobulbar tract dysfunction in ALS has been linked to cognitive impairment, and in particular to executive dysfunction (82). A study of PCL in ALS found an association between PCL and poor performance in executive tasks, implicating pre-frontal cortical areas in the disorder (20). Other studies, in contrast have found no such associations (8, 45). Systematic studies of social cognition in patients with PCL are lacking. Despite the conflicting findings, evidence from imaging and neurophysiology studies support the involvement of frontal cortical dysfunction in PCL (73, 79).

Advanced neuroimaging techniques enable the characterization of symptom-specific structural (83, 84), and functional (85, 86) alterations, providing insights into disease mechanisms (87, 88). Given the high prevalence of PCL in MND, it provides unique opportunities to explore PCL-specific network alterations (58). Floeter et al. used MRI diffusion methods to explore the white matter signature of PCL in ALS and PLS (10). Both ALS and PLS patients exhibited considerable white matter pathology in the corticospinal tracts and the corpus callosum. PCL-associated white matter changes were identified in frontotemporal regions, transverse pontine fibers and the middle cerebellar peduncles. A recent multimodal MRI study by Christidi et al. used the CNS-LS to divide a large group of ALS patients into PCL-positive and PCL-negative groups (73). The PCL-positive group showed significant gray and white matter changes compared with the PCL-negative group. The gray matter assessment found reduced volume of left orbitofrontal cortex, operculum, putamen, and of bilateral frontal poles. White matter analyses revealed diffusion abnormalities in the left cingulum, the posterior corona radiata and in the left middle and bilateral inferior cerebellar peduncles. The finding of cerebellar involvement in PCL in ALS again implicates cerebellar dysfunction in the pathophysiology of PCL, across a range of neurological diseases. While it is challenging to detect cerebellar signs clinically in the presence of pyramidal and lower motor neuron degeneration in ALS, imaging studies suggest that cerebellar degeneration is an important feature of ALS pathology, which is likely to contribute to PCL (89–91).

THERAPEUTIC OPTIONS

Antidepressant Medication in the Management of PCL

Divergent pharmacological strategies have been explored in the management of PCL. SSRIs and TCAs are the most frequently used off-label medications (75). Surprisingly, we did

not identify any placebo-controlled trial of any antidepressants for PCL in ALS. In the absence of robust clinical trial data in ALS, evidence from other neurological conditions, most-commonly stroke, is used to guide treatment. There have been positive results in small placebo-controlled trials of SSRIs including citalopram (92), fluoxetine, (31) sertraline (93) and of the TCA nortriptyline (38), in post-stroke PCL. Case-reports and uncontrolled trials reported symptom improvement with amitriptyline (94) and duloxetine (95) in ALS; memantine in AD (96); and mirtazapine in post-stoke PCL (97). A 2010 Cochrane review of treatments for "emotionalism" after stroke concluded that there is "suggestive but not definitive" evidence that antidepressants reduce frequency of symptoms, although it highlighted "several methodological deficiencies" in available studies (98).

Management of PCL in Motor Neuron Disease

In 2010, dextromethorphan/quinidine (Dx/Q) became the first FDA-approved treatment for PCL, following more than a decade of research into the potential benefits of the commonly-used anti-tussive for this indication (99). Dextromethorphan acts as a non-competitive glutamate antagonist on NMDA-receptors and as an agonist on sigma receptors (100). When administered alone, it is rapidly metabolized by first-pass metabolism through the cytochrome P450-2D6 system. The addition of the CYP-2D6 inhibitor, quinidine, dramatically increases the bioavailability of dextromethorphan (101). In 2004, a randomized, double-blinded study compared treatment with Dx/Q (30/30 mg twice daily) with dextromethorphan alone in ALS patients with PBA, defined by a CNS-LS score $\geq$13 (43). The combination not only reduced CNS-LS scores and episode frequency but also led to improvements in quality-of-life measures. Treatment-related side effects including nausea, dizziness, somnolence, and loose stools were relatively common however; about one quarter of patients withdrew from treatment, the majority within 1 week (102). A follow-up study in 2010 assessed whether a lower quinidine dose would reduce adverse effects relative to the earlier trial, while maintaining efficacy (42, 103) The study randomized ALS ($n = 197$) and MS ($n = 129$) patients with PBA to Dx/Q 30/10 mg BID, Dx/Q 20/10 mg BID or placebo BID. Both Dx/Q doses were found to reduce episode frequency, CNS-LS scores and to improve the likelihood of symptom remission compared to placebo. There was a lower discontinuation rate than in the earlier trial. It is interesting to note the considerable placebo response rate across efficacy endpoints in both trials. It must also be pointed-out that CYP450 2D6-poor metabolizers (104) were excluded from the efficacy and safety analyses raising questions about the requirement to screen for this phenotype prior to prescribing (105). Efficacy outcomes were also maintained in a 12-week open label extension study (106). A 52-week open-label study in 553 patients, including 199 patients with ALS, reported no serious drug-related adverse effects (75). However, clinicians must be cognizant of underlying cardiac conditions as quinidine can cause serious QT-interval prolongation (107).

Limitations of Currently Available Treatments

While the emergence of the first FDA-approved drug for PCL is an important advancement, the effective treatment of PCL remains challenging. Unfortunately, there have been no head-to-head trials of Dx/Q and any commonly used antidepressant. This knowledge-gap is particularly problematic given the current cost of Dx/Q, which may be prohibitive. Not only is the price of the approved combination product higher than alternative options, it is dramatically more expensive than the combined cost of its individual components (108). Finally, although Dx/Q was granted approval by the European Medicines Agency (EMA) in 2013, it was subsequently withdrawn by the manufacturer in 2016, on commercial grounds. (109, 110) A 2017 Cochrane review of "symptomatic treatments" in ALS highlighted emotional lability, as a symptom for which there is a "significant gap" in studies regarding the effectiveness of available treatments (111). There is a pressing and unmet need for robust clinical trials of antidepressants in the management of PCL. Finally, there is evidence that ALS patients and carers, lack awareness of the association between PCL and their underlying neurological condition (112), highlighting the importance of enquiring about PCL symptoms in patients with high-risk conditions, such as ALS.

CONCLUSIONS

Pathological crying and laughing is a shared symptom of many neurological conditions across infective, vascular, inflammatory, and neurodegenerative etiologies. Various terminologies have been used to encompass the heterogeneity of symptoms, which vary in severity, emotional congruity, frequency, and degree of control. After centuries of insightful observations, lesion studies and case reports, neuroimaging methods now provide long-awaited *in-vivo* insights into the specific pathophysiological mechanisms underlying the disorder. Patho-anatomical correlations indicate, that irrespective of the pathology (i.e., neurodegeneration, stroke, demyelination, TBI), the disorder occurs due to disruption in circuits involved in the initiation and modulation of emotional output. Key components of the network include sensori-motor cortical regions and their pontine and cerebellar connections. Further research is needed to elucidate the specific role of individual components within the network and their interactions. Effective symptomatic treatments are available; however, further studies are needed to establish individualized treatment strategies for patients experiencing impaired social or occupational functioning.

AUTHOR CONTRIBUTIONS

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Ethical Principles in Patient-Centered Medical Care to Support Quality of Life in Amyotrophic Lateral Sclerosis

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It is one of the primary goals of medical care to secure good quality of life (QoL) while prolonging survival. This is a major challenge in severe medical conditions with a prognosis such as amyotrophic lateral sclerosis (ALS). Further, the definition of QoL and the question whether survival in this severe condition is compatible with a good QoL is a matter of subjective and culture-specific debate. Some people without neurodegenerative conditions believe that physical decline is incompatible with satisfactory QoL. Current data provide extensive evidence that psychosocial adaptation in ALS is possible, indicated by a satisfactory QoL. Thus, there is no fatalistic link of loss of QoL when physical health declines. There are intrinsic and extrinsic factors that have been shown to successfully facilitate and secure QoL in ALS which will be reviewed in the following article following the four ethical principles (1) Beneficence, (2) Non-maleficence, (3) Autonomy and (4) Justice, which are regarded as key elements of patient centered medical care according to Beauchamp and Childress. This is a JPND-funded work to summarize findings of the project NEEDSinALS (www.NEEDSinALS.com) which highlights subjective perspectives and preferences in medical decision making in ALS.

Keywords: ethics, quality of life (QoL), care, amyotrophic lateral sclerosis (ALS), well-being, depression, coping, psychosocial adaptation

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QUALITY OF LIFE IN ALS

Amyotrophic lateral sclerosis (ALS) is among the most devastating neurological conditions: patients lose the ability to speak, to walk and eventually to breathe. On average, patients die within 3 years after symptom onset. If life-sustaining measures such as invasive ventilation are taken, patients may terminate in a locked-in state with a clear mind in a paralyzed body. There is no cure for ALS and care focuses on maintaining functional ability and providing palliative and symptomatic interventions to relieve the burden of symptoms (1). The communication of the diagnosis is a major stressful event for patients, families and caretakers and thus most challenging with regard to medical counseling (2).

There are different ways of how patients cope with these major changes. Quality of life (QoL) is one possible measure of good psychosocial adaptation to disability such as ALS, similarly to depression (3). There have been contradictory reports whether QoL is lost in the course of physical decline (4–6). This discrepancy is partly attributed to selection of patient subgroups (e.g., shortly after diagnosis vs. long-term survivors) and the use of different QoL definitions. QoL is the general well-being of a person and includes physical (individuals' perception of their physical state), psychological (individuals' perception of their cognitive and affective state) and social

dimensions (individuals' perception of the interpersonal relationships and social roles in their life). It is therefore not simply a state of physical integrity (7). QoL is increasingly used to supplement objective clinical or biological measures to evaluate health care provision and interventions in research and clinical trials (8).

There is a debate which QoL measure is truly patient centered. Measures are either based on hedonic concepts focusing on subjective factors and emotional evaluation or eudaimonic concepts with more objective factors of QoL such as physical health or economic status (9). As physical health declines in ALS and mobility becomes heavily restricted, these QoL measures provide evidence for low QoL in ALS simply by the nature of the underlying concept. These clinimetric endpoints are increasingly considered overly reductionistic (10) as they include aspects, which are no longer relevant or are out of range of an immobile patient, e.g., physical activity (11); thus, patients often prefer more subjective scales of QoL as these better capture their emotional state of well-being (**Table 1**). They might as well be regarded as possible outcome measures in clinical trials to determine the subjective benefit of a treatment for a patient. Observations concerning hedonic QoL are often counter-intuitive: simultaneous deterioration of physical integrity and well-being does not necessarily occur (19). Accordingly, ALS patients may experience a surprisingly high subjective QoL and an only moderately increased affective state as compared to healthy subjects (6, 20–27) which can be maintained throughout the course of ALS (27–29). This may even be true in the final state of complete immobility, the locked-in state [LIS; (30, 31)].

The lack of association of severity of illness and subjective QoL has been shown for several diseases and is referred to as the "*well-being-paradox*" (32). Prerequisite for this paradox is a process of psychosocial adaptation to the altered circumstances of severe physical function loss. According to the theory of homeostasis in quality of life, everybody has his/her individual level of well-being which he/she aims to reach which is usually in the range of 70–80% of the maximum QoL (33). Provided that sufficient time (29) and intrinsic (e.g., successful coping) and extrinsic resources (e.g., strong family support) are given, patients may show a process of ongoing change and adaptation of their expectations to the actual circumstances [TOTE model; (34)]. The capability of adaptation is not simply a matter of disease state or general personality traits (21). It can be successfully supported by medical teams through patient centered medical care. The different intrinsic and extrinsic factors in medical care to facilitate QoL in ALS and the individualistic perspective in medical decision making have been evaluated within the JPND-funded project NEEDSinALS (www.NEEDSinALS.com). These factors may be subsumed under the four ethical principles of good medical care according to Beauchamp and Childress (35), namely beneficence, non-maleficence, autonomy and justice (**Figure 1**).

Beneficence

This principle requires that everything should be done in the best interest of the patient. Therapeutic interventions are usually introduced by the physician and their interdisciplinary teams to facilitate QoL in ALS. No cure is available yet, but different therapeutic interventions e.g., non-invasive ventilation (NIV) may be means also to prolong survival (36). Previous studies have provided extensive evidence that ALS patients with NIV have an increased QoL (20, 37, 38). Permanent respiratory insufficiency may lead to disturbed sleep, fatigue and reduced physical fitness, all these symptoms may be relieved by ventilation (39). Thus, ventilation may positively impact QoL and patients with ventilation may show even higher QoL than those without (20). Further, nutritional support is a major element of beneficence in ALS as a loss in BMI is a negative prognostic factor (40). Unfortunately, fear of choking during meals is widely prevalent in patients with bulbar symptoms (41), so many patients fear to eat at all. Thus, introduction of a PEG may be a highly useful approach to improve QoL as it allows for weight control while relieving the patient from the pressure to eat. As patients may nevertheless be able to have oral food intake, the pleasure and sensuality of eating can be maintained which additionally supports QoL. However, in some patients the positive effect of PEG insertion might be outbalanced by "particularly strong feelings of loss of control" (41), highlighting the subjective perspective on patient centered care (8). Other therapeutic interventions may as well-facilitate QoL such as application of botox to stop the debilitating syndrome of "drooling" (sialorrhoea). Also, therapies such as physiotherapy, occupational therapy (ergotherapy) and speech therapy (logopedics) may relieve physical symptoms of pain, muscle tension and stiffness which all finally may help to improve QoL (42).

Apart from therapeutics, there is one major extrinsic factor which may substantially improve QoL which is social support (23). Family is the most frequently named aspect of individual QoL in ALS (26, 29, 43). And, as satisfaction with family was often good among patients, the patient's QoL is also often good (44). For healthy subjects, there are other factors which are important for well-being (finances, career) whereas most ALS show a response shift toward social support in the course (20). Thus, inclusion of family members in clinical counseling and supporting the patient in refocusing on social resources may facilitate QoL.

Apart from these extrinsic factors, QoL in serious illness is highly depending on intrinsic factors, such as resilience which is a general characteristics addressing the capacity to recover quickly from difficulties (45). In this context, there has been evidence in ALS that appraisal of coping potential and mental attitudes may be crucial to adapt (23). A re-set of preferences referred to as response shift (46) may support these inner processes with the ability to see what is still there and what is untouched by the disease [e.g., spiritual well-being; (20)]. Further, reframing, the ability to see the same situation from a different perspective [e.g., instead of looking at what you lose, you pay attention to what is spared such as your emotions, feelings and desires; (20)] can be highly supportive for psychosocial adaptation. Finally, many years of research about adapting to and living with chronic diseases suggest that mindfulness in the sense of accepting the circumstances which cannot be changed without judgement and focus on the present (47) may reduce the negative psychological impact of the illness (48, 49).

TABLE 1 | Examples of most widely used measures of subjective, patient-centered QoL.

Abbreviation	Measure	Procedure	Outcome
MEASURES OF GLOBALE SUBJECTIVE QoL			
ACSA (12)	Anamnestic comparative self-assessment	Culturally independent and well-tolerated measure of general QoL; patient is asked to rate his or her current QoL on a scale from −5 to +5. Minus 5 indicates the worst, plus 5 the best ever experienced QoL. It is thus, a rating within each individual's own framework of QoL	ACSA score between −5 to +5
SEIQoL(-DW) (13)	Schedule for the Evaluation of Quality of Life direct weighting	Overall subjective QoL as judged by the patient through a semi-structured interview. The patients have to (1) name the life areas which are important to their QoL, (2) rate the current level of importance of each area and (3) rate the satisfaction with each of the areas	SEIQoL-Index-Score between 0 and 100%
Ganzini QoL (14)	QoL-single-item question	Single-item question to assess patients self-perceived overall QoL with end-points labeled 1 = "my quality of life is as good as it can be" and 6 = "my quality of life is very bad, horrible."	Score between 1 and 6
Krampe QoL (15)	QoL-single-item question	Single-item question to assess patients self-perceived overall QoL with end-points labeled: "Over the past 7 days, the quality of my life has been": very poor (0)–excellent (10).	Score between 0 and 10
ALSSQoL (16)	ALS-Specific Quality of Life Questionnaire	Fifty item disease-specific questionnaire on 6 domains adressing (1) Negative Emotion; (2) Interaction with People and the Environment; (3) Intimacy; (4) Religiosity; (5) Physical Symptoms; (6) Bulbar Function	Average total QOL score, and 6 domain scores
MEASURES OF GLOBALE SUBJECTIVE QoL COMBINED WITH PHYSICAL QoL			
WHOQOL-BREF (17)	Short version of the World Health Organization Quality of Life (WHOQOL)-Group questionnaire	Twenty-six item non-disease specific questionnaire on Physical, Psychological, Social Relations, Environment within cultural context	Domain scores between 0 and 100
MQoL (18)	The McGill Quality of Life Questionnaire	Subjective QoL according to five subscales: physical function, physical well-being, psychological symptoms, existential well-being and social support	MQoL score as mean of 5 subscales between 0 and 10
	Including MQOL single-item scale (SIS)	Single-item Score (SIS) of the MQoL for overall QoL on a visual analog scale	MQoL SIS score between 0 and 10

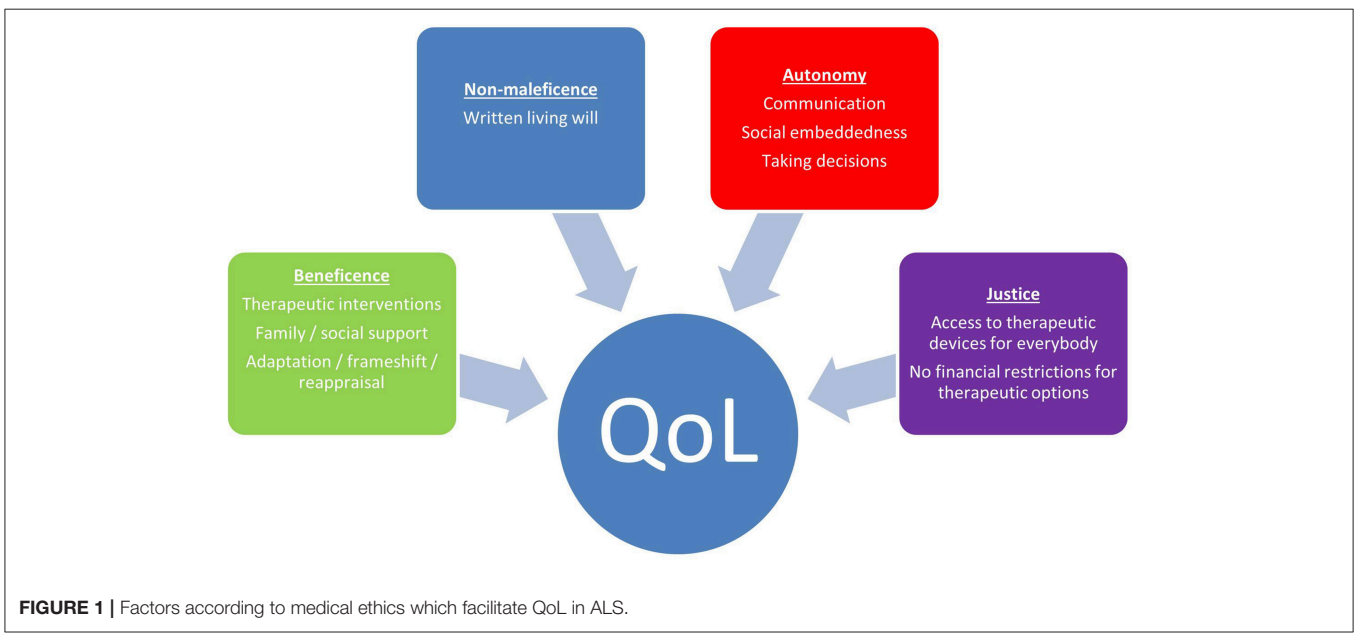

FIGURE 1 | Factors according to medical ethics which facilitate QoL in ALS.

Patients can be encouraged to use these inner resources mentioned above. Psychotherapeutic interventions may help to improve the QoL of patients and may even prolong survival as the psychoemotional state of the patient has impact on QoL (50) and survival time (49). The beneficence of the above mentioned intrinsic factors may as well be addressed in clinical routine by physicians and medical staff to encourage patients to give more room in life for any beneficial intrinsic process.

Beneficence requires the knowledge of the patient's wishes as peer evaluations might not meet the patient's actual needs.

There is evidence for discrepancy between patient's well-being and the perspective on patient's well-being of people without neurodegenerative conditions (27, 29). Peers judgement of patient's well-being is primarily based on personal opinion when they anticipate a low QoL in severely disabled patients. This is true for people without experience in ALS and is even true for caregivers and physicians if they lack experience with ALS (51). Healthy subjects may be blind toward the patient's process of ongoing change and adaptation and instead they may conclude from their personal perspective. The more experienced healthcare professionals are, the more they know about the capacity to adjust and are thus abled to correctly anticipate patient's quality of life, affective state, and wish for hastened death (51).

Thus, the knowledge of and believe in beneficence in ALS is a matter of the physician's experience. As many patients gradually adjust to their situation and also possibly change their therapeutic preferences in the course of the disease (52), beneficence from the patient's perspective is a dynamic construct which needs to be recognized and may be supported by caretakers and medical care teams.

Non-maleficence

Primum non-nocere, refrain from harm is the other side of the coin of beneficence and thus, similar aspects concern maleficence than beneficence. Non-maleficence needs to be considered the moment the diagnosis is communicated. "Breaking the news" is a highly delicate balance between patient's need to be informed which requires veracity and fidelity on the one hand and the right for denial on the other hand which can be a helpful strategy at least shortly after diagnosis (23). Maleficence in the sense of the emotional burden of diagnosis can be reduced by using a thorough approach for breaking the news as it may attenuate negative impact on QoL (53). But also providing sufficient information can prevent maleficence: patients with sudden respiratory insufficiency in an emergency situation who have been informed on all aspects of respiratory support may feel more competent to take the right decision (41).

Advance directives and living wills are crucial to prevent maleficence, e.g., insertion of a tracheostomy in an emergency if the patient does not want to [possibly because he/she is afraid of the burden for others; (52)]. Many therapeutic options secure QoL (e.g., ventilation) but most patients are unable to anticipate this shortly after diagnosis. During the course of ALS, some might dismiss the idea of maleficence of invasive ventilation and might realize the beneficial effect of this therapeutic treatment (29). Therefore, dynamic adjustment to living wills is a key aspect to prevent maleficence.

Preferences regarding therapeutic measures are highly determined by patient's personal values, religious beliefs and cultural background (54).

Cultural differences exist: in Japan, invasive ventilation is more regarded as routine therapeutic treatment than in other countries [33% in Japan; (55)]. In some countries, life prolongation might be regarded as maleficence and more life-shortening treatments are suggested (56). Thus, social context may define what is beneficence and maleficence in the context of cultural norms (54, 57).

Patient's Autonomy

Patient's sense of autonomy is a key issue of quality of life and goes beyond being physically autonomous to perform an action. Autonomy also encompasses the sense of capability to take decisions and the feeling of being an author of one's own action which is a key feature of self-efficacy and thus for QoL (58). Taking decisions also sustains the feeling of social embeddedness disregarding physical disability, e.g., the patient can be included in family decisions and may participate in daily routine if possible (59). This allows the patient to be an active part of daily routine: to participate in decision making, to be asked questions, to express concerns, address fears and anxieties, express wishes, values, desires, and hopes. It is noteworthy that possible minor cognitive deficits in some patients do not interfere with the competency to decide and participate (60).

It is especially challenging to secure patient's autonomy in LIS as there is lack of direct means to communicate in this state. Assisted communication (20, 29) becomes important for individual QoL in the course of ALS, but is not mentioned by patients in early stages of the disease (20). Many patients use letter board for communication which requires considerable effort from a second person to record which item the patient selected from the board. Technical devices may allow for communication but these are time consuming and strenuous to use and also additional assistance is required (59). Thus, knowledge on patient's wishes, desires and thoughts in advanced stages is sparse and there is substantial lack of understanding which factors may impact the dynamics of QoL and affective state in the course of ALS (30). Communication via eye-gaze control is possible, including standardized interviews (61, 62) but the latter are rarely performed. Brain Computer Interfaces (BCI) are promising technologies for communication and interaction (63, 64) but in a subgroup of patients only (65). Other means of BCI-use such as unrestricted access to web browsers of which some are adaptable to home based BCI systems (66) secure new degrees of freedom in severe paralysis (67–70). Some patients already use these techniques in their home environment for communication and painting (71–76) and first evidence support the notion that these techniques positively impact QoL (71, 77). In the future, with major advances in communication technology well-being in ALS might possibly be facilitated. BCIs might also be indirectly used in evaluation and recognition of well-being and emotional state in highly advanced patients (78) such as the amplitude of the N400, a negative deflection of the EEG curve following a meaningful event (79), which was higher in patients with high QoL compared to those with a poor. Thus, the N400 may serve as an objective physiological indicator of individual QoL in non-responsive ALS patients (80). Overall, there is still a long way to go until BCI will be a standard tool for home care for a majority of ALS patients (63, 81). But for patient-centered care, compensation for progressive loss of

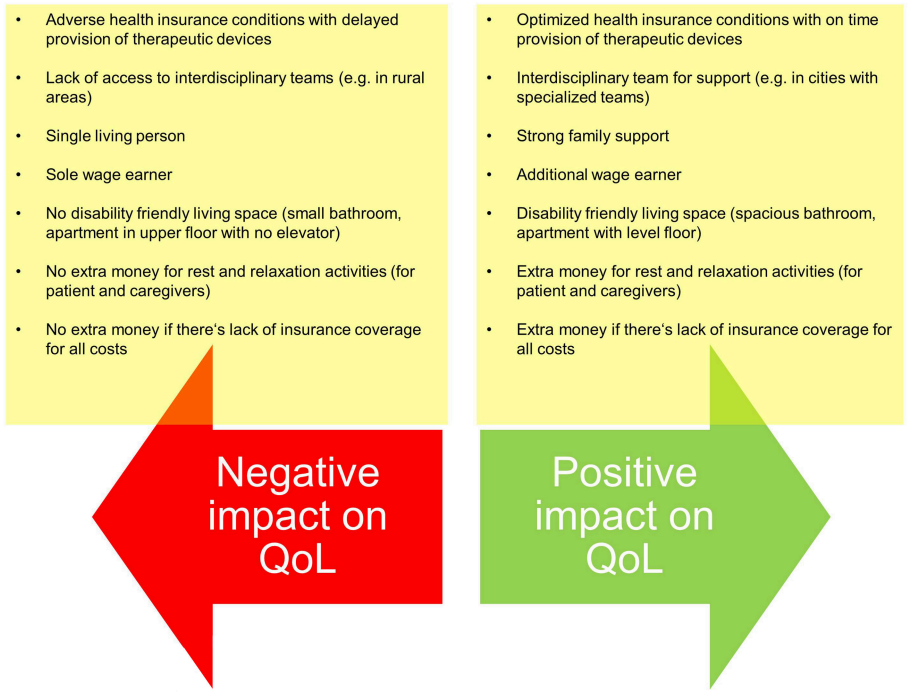

FIGURE 2 | Examples of setting differences which may interfere with justice in medical care despite optimized care provision by the medical team.

verbal speech is mandatory to secure patient's autonomy and QoL (52).

Justice

This ethical principle of care requires that all patients are treated in an equal way without prejudice or social discrimination.. In the sense of justice, patients in similar situations should have access to the same care options. Palliative care intervention improves quality of life in patients and caregivers (82) and medical care may facilitate this positive dynamics by offering this care to every patient. ALS patients expect dignified care (82) but instead, patients are often dissatisfied with health care services (83). Every patient needs to be treated differently according to the actual preferences and needs (54). There is no justice in defining every person by the diagnosis with a nihilistic view of the disease which has to be prevented under all circumstances (48). Instead, to grant justice every patient has to be regarded as an individual with specific needs and the right to be treated the same according to his/her preferences, disregarding mental, societal or financial status.

Further, providing sufficient information according to the patient's needs as outlined above is also a matter of justice. Thus, granting the patient the right for information is similarly a matter of justice as granting the right for not-knowing. In this sense, it is a matter of justice to grant patient's will even if it interferes with the physician's personal and professional opinion.

Finally, justice in medical care is secured in many countries as most healthcare systems secure this kind of justice by providing coverage of (most) costs. Despite that most medical systems are based on a solidary idea allowing for justice, the impact of the disease may vary between patients thus justice in clinical care is not easy to accomplish. There are basic settings which significantly hamper justice in care provision which cannot be changed by the medical team, e.g., there is variance of the paid costs by the insurance companies. Further, in some instances, only basic technical equipment is provided which possibly don't meet the patient's actual needs. Thus, patients have to cover the extra charges for the devices which fully meet their needs. And finally, there are personal settings (e.g., living and working conditions) which may heavily impact patient's life with ALS and which interfere with the principle of justice (**Figure 2**).

CONSEQUENCES AND FUTURE DIRECTIONS

There is evidence that considerate medical care within multidisciplinary teams (84) helps patients to find their own way of coping with the disease to gain or maintain a satisfactory QoL (48). Living with a fatal disease creates a crisis loaded environment and adapting to the disease is a psychological process rendering mandatory a strong support from these specialists' teams (41). Following the ethical principles of medical care as outlined in this text allows for a holistic support of the patient to secure QoL.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

DL, AK, and AL have collected data underlying this review and discussion of data. Text writing was done by DL, thorough revision of the manuscript was performed by all authors.

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MINI REVIEW
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Lipid Biomarkers for Amyotrophic Lateral Sclerosis

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Amyotrophic lateral sclerosis (ALS) is a fatal degenerative disease primarily characterized by the selective loss of upper and lower motor neurons. To date, there is still an unmet need for robust and practical biomarkers that could estimate the risk of the disease and its progression. Based on metabolic modifications observed at the level of the whole body, different classes of lipids have been proposed as potential biomarkers. This review summarizes investigations carried out over the last decade that focused on changes in three major lipid species, namely cholesterol, triglycerides and fatty acids. Despite some contradictory findings, it is becoming increasingly accepted that dyslipidemia, and related aberrant energy homeostasis, must be considered as essential components of the pathological process. Therefore, it is tempting to envisage dietary interventions as a means to counterbalance the metabolic disturbances and ameliorate the patient's quality of life.

Keywords: amyotrophic lateral sclerosis, biomarker, cholesterol, fatty acid, triglyceride

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ALS AND THE NEED FOR BIOMARKERS

Amyotrophic lateral sclerosis (ALS) is a degenerative disease of upper and lower motor neurons mainly characterized by progressive muscle wasting, fasciculations, dysarthria, dysphagia, altered reflexes, and spasticity. It affects about 2 per 100,000 people per year, and usually appears at 40–70 years of age. A significant proportion of cases also presents cognitive or behavioral abnormalities typical of frontotemporal dementia (FTD). The etiology of ALS still remains elusive. About 90% of cases are considered as sporadic. The remaining 10% are inherited mostly in an autosomal dominant manner. Most familial cases can be explained by mutations in four major genes, including *C9ORF72*, *SOD1*, *FUS*, and *TARDBP*. Based on this genetic diversity, multiple pathogenic mechanisms have been implicated in triggering motor neuron degeneration, adding considerable complexity to the understanding of the disease (1).

From a clinical point of view, ALS is easily recognized in its full-blown presentation. However, the diagnostic process may be challenging at very early stages. The diagnosis is based on clinical examination, electrophysiological findings, medical history, and exclusion of confounding disorders. In practice, a correct diagnosis may take as long as 1 year (2). Moreover, disease progression is very heterogeneous. Death may occur between 1 and 5 years after diagnosis, but 20% of patients live longer than 5 years, and 10% survive for more than 10 years (3). Promising biomarkers of diagnosis and prognosis have been proposed based on advanced neurophysiological and neuroimaging techniques. However, many of these practices still lack validation and standardization between clinical centers, and they have been applied only to small cohorts of patients [(4–6), and references therein].

As far as molecular biomarkers are concerned, a great number of molecules have been isolated from patient material, including cerebrospinal fluid, blood and tissues, that recognized and/or monitored ALS with more or less accuracy [(7, 8), and references therein]. To date, changes in the amounts of neurofilament proteins found in cerebrospinal fluid and blood have been postulated as the most promising candidates [(9), and references therein]. However, these and other proposed molecular markers have not reached routine clinical application. Therefore, there is still an incontestable lack of robust and practical biomarkers that could facilitate an earlier diagnosis and improve the prognosis of ALS.

ALTERED ENERGY HOMEOSTASIS AND LIPID METABOLISM IN ALS

Amyotrophic lateral sclerosis was classically attributed to an intrinsic defect of upper and lower motor neurons. Now it is generally accepted that non-neuronal cells surrounding motor neurons, additional neuronal cell types, as well as other cells outside the nervous system participate actively in the pathological process [(10–12), and references therein]. In particular, compelling evidence has emerged over the last decade showing a characteristic imbalance between energy intake and consumption, which is associated with metabolic alterations at the level of the whole body of yet unexplained etiology [(13), and references therein]. Seminal studies revealed that many ALS patients show an increase in energy expenditure, or hypermetabolism, which could account, at least in part, for the decline of their nutritional status (14, 15). It was also recently reported that hypermetabolic patients have a worse prognosis than normometabolic ones (16), which could be related to a detrimental weight loss. In fact, patients that lost more than 5% of body mass at the time of diagnosis had an increased risk of death (17). In addition, a lower body mass index appeared to precede the symptomatic stage of the disease (18). Overall, these studies strongly support that the energy imbalance in ALS could contribute to the rapid deterioration of the patients.

The origin of the hypermetabolism in ALS is currently unknown, although recent studies have pointed to the altered function of hypothalamic neurons involved in the regulation of food intake and energy homeostasis (19, 20). From a therapeutic point of view, pioneering preclinical studies conducted on an ALS mouse model, which carries a mutation in the *Sod1* gene, provided part of the answer to this question. An increase in energy consumption occurs in these mice well-before the onset of the first motor symptoms. This is accompanied by a reduction of adiposity and lower levels of circulating leptin. Most importantly, these studies revealed that sustaining the hypermetabolic rate of ALS mice with a highly-energetic high-fat diet partially protected motor neurons and extended lifespan (21). Likewise, a higher premorbid intake of high-fat food was observed in ALS patients (18). Moreover, a moderate increase in fat mass over the course of the disease was associated with a decreased risk of death, and increasing circulating levels of leptin were positively associated with longer survival (17, 22). Although there is no conclusive evidence of a mechanistic link between the hypermetabolism present in ALS and altered levels of lipids, altogether, these studies suggest that the utilization of lipids as energy substrates could offer benefit, by counteracting an increased metabolic rate and compensating the associated weight loss. In this respect, several pilot studies reported positive effects of highly caloric fat supplements on ALS patients (23, 24). It was also shown that the administration of acetyl-L-carnitine, which supports the transport of fatty acids into mitochondria for being used as energy substrates, retarded the worsening of the patients (25). Recent research has made efforts to identify specific changes in lipid metabolism that could provide clues for future nutritional interventions, as well as serve as robust biomarkers for the disease. This review covers some of the most significant findings published during the last decade.

APOLIPOPROTEIN E AND THE RISK OF ALS

Apolipoprotein E (APOE) is a constituent of lipoprotein particles primarily involved in the transport of triglycerides and their clearance from the bloodstream. It is mainly synthesized in the liver but it is also produced by astrocytes in the brain, where APOE is the most important cholesterol carrier. The human *APOE* gene exists as three major alleles called ε2, ε3, and ε4. The identification of *APOE* ε4 as a risk factor for Alzheimer's disease represented a major breakthrough in the field [(26), and references therein]. On the contrary, most studies on ALS did not observe any association of *APOE* ε4 with an increased risk (27–30), excepted some recent findings (31). Additional reports showed complex interactions between particular *APOE* alleles and other genetic or physiopathological variables. Penco and collaborators identified a combination of seven genetic variants, inluding one affecting *APOE*, that distinguished between ALS patients and control subjects (32). It was also found that individuals who had suffered from head trauma in the adulthood were more prone to have ALS, and this association was stronger in the presence of *APOE* ε4 (33). In contrast, the frequency of *APOE* ε2, which is *a priori* neuroprotective, was higher in ALS patients that had practiced sport regularly (30). *APOE* ε2 also increased the risk of developing FTD in a cohort of patients with ALS (34). It must be noted, however, that the implication of *APOE* in the incidence of this form of dementia is rather controversial. The increase in the probability of having FTD was associated with *APOE* ε2 in some cases, and with *APOE* ε4 in other cases [(35), and references therein].

The influence of *APOE* on the course of ALS has also been contradictory. Initial reports revealed that *APOE* ε4 was associated with earlier age at onset but not with disease duration (27). However, follow-up studies failed to show any relationship between *APOE* ε4 and age at onset or rate of progression, although this allele was more frequent in men with bulbar-onset ALS (28). Parallel investigations did not find any association between the *APOE* genotype and age of onset, site of onset, rate of progression, cognitive impairment or survival (36). Overall, the

implication of *APOE* in the incidence and progression of ALS is therefore not clearly established.

THE INTRIGUING CASE OF CHOLESTEROL

Cholesterol is an essential lipid molecule, which is transported through the bloodstream by several types of lipoprotein particles. In clinical practice, increased levels of total cholesterol or low-density lipoprotein cholesterol (LDL-c), in combination with decreased levels of high-density lipoprotein cholesterol (HDL-c), are indicative of a higher risk of atherosclerotic cardiovascular disease. In the case of ALS, hypercholesterolemia, as detected prior to the onset of motor symptoms, was initially associated with a lower risk (37). However, follow-up studies contradicted these findings. An increase in the premorbid intake of cholesterol was associated with a higher incidence of the disease, as shown after examination of dietary habits obtained from food frequency questionnaires (18). In addition, individuals with increased levels of LDL-c and a higher LDL-c/HDL-c ratio were more prone to develop ALS later (38). Finally, the analysis of GWAS databases revealed that particular alleles predisposing to elevated levels of LDL-c and total cholesterol appeared associated with an increased risk (39).

In many studies, the proportion of hypercholesterolemia individuals or the average contents of total cholesterol and LDL-c were shown to be higher in the ALS population and, in some cases, this increase was noticeable at the time of diagnosis (40–44). In agreement with these findings, a detailed analysis of circulating lipoprotein particles also showed increased levels of LDL-1, which is a LDL subfraction very enriched in cholesterol (44). Other reports, however, did not find clear-cut differences (45–48), or even revealed opposite results (49).

From a prognostic point of view, decreased levels of total cholesterol or LDL-c and a lower LDL-c/HDL-c ratio were associated with a severe respiratory impairment (42, 45). Contrasting with these findings, Delaye and collaborators did not observe any association between several cholesterol parameters and disease progression (44). Yet, most authors agree that hypercholesterolemia, present as elevated levels of total cholesterol and LDL-c or a higher LDL-c/HDL-c ratio, associates with longer survival. This association, however, did not reach significance after adjusting for potential confounding demographic and clinical factors (37, 40, 43, 49–51).

In addition to the biomarker potential of cholesterol *per se*, a few studies have focused on the implication of oxysterols, which are oxidized derivatives mainly involved in maintaining cholesterol homeostasis. Levels of several oxysterol metabolites, including 27-hydroxycholesterol, 24-hydroxycholesterol esters, and 3β,7α-dihydroxycholest-5-en-26-oic acid and other related compounds, were shown to be lower in ALS patients. These changes were detected in blood or cerebrospinal fluid, or both, and they were attributed to a deficit in the metabolism of excess cholesterol, which would result in subsequent toxicity in the brain (46, 52, 53). On the other hand, additional studies reported increased levels of 25-hydroxycholesterol in cerebrospinal fluid and serum of ALS patients. The accumulation of this toxic oxysterol derivative was associated, at least in serum, with a higher rate of disease progression (54).

THE ENERGIZING TRIGLYCERIDES IN ALS

Triglycerides are a primary source of energy for the body but, when accumulated in an excessive manner, they represent an important risk factor for cardiovascular disease. Triglyceride contents should be expected to change in ALS patients according to their characteristic high rate of energy expenditure. In this respect, the proportion of hypertriglyceridemia individuals was more important among ALS patients than in the normal population (55). Hypertriglyceridemia was also found in ALS women (42), and higher triglyceride levels were associated with a better functional status (48). Other reports, however, failed to reproduce these findings (40, 47, 48). Moreover, Blasco and collaborators identified a lipidomic signature in the cerebrospinal fluid of ALS patients, in which certain triglyceride species were found reduced at levels associated with a better prognosis (56). Finally, as in the case of cholesterol, hypertriglyceridemia was associated with longer survival, but this association appeared to have no effect after adjusting for confounding factors (47, 55).

THE ENTRANCE OF FATTY ACIDS ON STAGE

Fatty acids are lipid molecules key for sustaining the structural integrity of cell membranes, providing energy and serving in signaling pathways. They can be mainly transported through the bloodstream attached to a glycerol molecule (that is, in the form of triglycerides) or as non-esterified free fatty acids. The studies relating to the implication of fatty acids as biomarkers for ALS are scarce. Based on food frequency questionnaires, Fitzgerald and collaborators showed that a higher intake of ω3 polyunsaturated fatty acids, which are considered as neuroprotective factors, were associated with a reduced risk of ALS (57). Similar studies did not find the same association but rather reported a higher premorbid intake of *trans*- and saturated fatty acids associated with an increased risk (18). On average, the proportion of polyunsaturated fatty acids in the lipid fraction of clotted blood was decreased in ALS patients while that of monounsaturated fatty acids was concomitantly increased (58). Polyunsaturated fatty acids were also lower in the free fatty acid fraction of plasma (59). Finally, a higher palmitoleic/palmitic fatty acid ratio, indicative of increased adiposity, correlated with a better functional status, and was associated with longer survival (58).

CONCLUSION

Over the last decade, many lipid molecules have been proposed as promising biomarkers for ALS, but none of them has been translated into effective tools in clinical practice. There are several issues of concern that still need to be addressed. On the one hand, the etiology of ALS is multifactorial, and it is likely that the pathological process in subpopulations of patients, with different

genetic and environmental backgrounds, is not the same. In the future, the use of cohorts of well-defined patients should improve statistical robustness. It would also be interesting to compare between patients with ALS and other patients suffering from mimic conditions. On the other hand, lipid changes at the level of the whole body can be affected by a myriad of factors, including genetic, nutritional, physical and pathological factors, which can introduce bias on the results. It is also noteworthy to mention that for those studies that used food frequency questionnaires to estimate food preferences and evaluate eating behavior, they depend, at least in part, on their interpretation probing the patient's perception of food intake, hence lacking sensitivity and objectiveness. Therefore, protocols and measurements need to be standardized between study centers.

Despite some conflicting findings, most studies presented in this review show important alterations of the circulating contents of cholesterol (and related lipoprotein particles), triglycerides and fatty acids, which occur prior to and over the course of ALS. These changes seem to reflect a metabolic environment, which would be appropriate to meet the high energy demands imposed by the increased metabolic rate present in the disease. The understanding of the mechanisms underlying this "low-grade dyslipidemia" is still insufficient but, from a clinical point of view, it leaves open the possibility for therapeutic nutritional intervention. In this respect, recent studies that analyzed the eating behavior of ALS patients revealed marked modifications in their food preferences. In particular, an increase in the intake of saturated fat and meat protein was associated with longer survival (60–62). Moreover, two clinical trials have been initiated, which aim at retarding disease progression by using high-caloric food supplements. The first trial (NCT02306590) is a randomized, parallel-group, double-blind study that compares between placebo and a treatment consisting of a high caloric fatty diet, which is equivalent to an additional intake of 45 g fat per day. The primary objective of this study is to evaluate the impact on survival. The second trial is a randomized, parallel-group, open label study that will determine the effects of a high-protein, high-energy supplement on the functional status of newly diagnosed ALS patients (NCT02152449). The results of these trials as well as the ongoing research on lipid biomarkers and on the understanding of their implication in ALS will certainly pave the way for developing new therapeutic tools.

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The author confirms being the sole contributor of this work and has approved it for publication.

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Spinal Cord Imaging in Amyotrophic Lateral Sclerosis: Historical Concepts—Novel Techniques

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Amyotrophic lateral sclerosis (ALS) is the most common adult onset motor neuron disease with no effective disease modifying therapies at present. Spinal cord degeneration is a hallmark feature of ALS, highlighted in the earliest descriptions of the disease by Lockhart Clarke and Jean-Martin Charcot. The anterior horns and corticospinal tracts are invariably affected in ALS, but up to recently it has been notoriously challenging to detect and characterize spinal pathology *in vivo*. With recent technological advances, spinal imaging now offers unique opportunities to appraise lower motor neuron degeneration, sensory involvement, metabolic alterations, and interneuron pathology in ALS. Quantitative spinal imaging in ALS has now been used in cross-sectional and longitudinal study designs, applied to presymptomatic mutation carriers, and utilized in machine learning applications. Despite its enormous clinical and academic potential, a number of physiological, technological, and methodological challenges limit the routine use of computational spinal imaging in ALS. In this review, we provide a comprehensive overview of emerging spinal cord imaging methods and discuss their advantages, drawbacks, and biomarker potential in clinical applications, clinical trial settings, monitoring, and prognostic roles.

Keywords: ALS (Amyotrophic lateral sclerosis), MRI—magnetic resonance imaging, MND, spinal cord, neuroimaging

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a relentlessly progressive neurodegenerative disorder. Anterior horn pathology and corticospinal tract degeneration has been identified as a core feature of ALS since the earliest descriptions of the condition (1, 2). Despite repeated attempts to detect and characterize spinal cord pathology *in vivo* (3), technological constraints have traditionally precluded reliable quantitative spinal imaging in ALS. Due to the plethora of methodological challenges, such as the small cross-sectional area of the human spinal cord, respiratory, and cardiac movement effects, the overwhelming majority of imaging studies have focused on cerebral alterations in ALS (4).

The diagnosis of ALS is primarily clinical and requires the careful exclusion of ALS-mimics (5). Given the heterogeneity of clinical presentations and the prevalence of atypical phenotypes, diagnostic delay in ALS is not uncommon, and the average period between symptom onset and definite diagnosis is ∼12 months worldwide (6). The median survival from symptom onset ranges from 20 to 48 months (7–9). Progression rates in ALS show considerable variation, and prognosis depends on age at onset, region of onset, co-morbid cognitive impairment, nutritional status, and certain genotypes are associated with faster progression (10–16). Given the considerable clinical, cognitive, and genetic heterogeneity of ALS, there is an unmet need for early diagnostic biomarkers to aid patient stratification into specific phenotypes (17). Clinical trials of ALS continue to rely on survival, functional scores and respiratory measures as outcome measures despite the potential of candidate imaging markers (18).

Magnetic resonance imaging (MRI) not only contributed to the characterization of ALS-associated cerebral changes, it has also contributed important pathophysiological insights, such as the role of inflammation (19), patterns of spread (20, 21), inhibitory dysfunction (22, 23), and network-wise propagation (24, 25). In addition to describing unifying disease-associated signatures, imaging studies of ALS have gradually characterized the features of specific genotypes (26, 27), phenotypes (28, 29), the substrate of cognitive and extra-pyramidal impairments (30), as well as presymptomatic (31) and longitudinal changes (32). Despite the momentous advances however, the overwhelming majority of imaging studies in ALS remain cerebral, overlooking a disease-defining site of ALS pathology; the spinal cord (3).

SPINAL CORD IMAGING

One of the key challenges of spinal cord imaging stems from its elongated dimensions, small cross-sectional area in the axial plane coupled with long sagittal and coronal expansion (33). Furthermore, the cord is surrounded by tissues that have very different magnetic susceptibility profiles and is it subject to both direct (cardiac and respiratory) and fluid-mediated [cerebrospinal fluid (CSF)] movement effects. The main challenges of quantitative spinal cord imaging include (i) partial volume effects, (ii) an inhomogeneous magnetic field environment, and (iii) physiological and patient motion (34).

Abbreviations: 1H-MRS, proton spectroscopy; A-P, anterior-posterior; AD, axial diffusivity; ALS, Amyotrophic lateral sclerosis; ALSFRS-R, revised ALS functional scale; Cho, choline; CNR, contrast-to-noise ratio; Cr, creatine; CSA, cross-sectional area; CSF, cerebrospinal flood; CST, corticospinal tract; DTI, diffusion tensor imaging; FA, fractional anisotropy; fMRI, functional MRI; ihMT, Inhomogeneous magnetization transfer; LMN, lower motor neuron; MD, mean diffusivity; MRI, Magnetic resonance imaging; MRS, Magnetic resonance spectroscopy; MT, Magnetization transfer; MTR, Magnetization transfer ratio; Myo, myo-Inositol; NAA, N-Acetyl Aspartate; NODDI, neurite orientation dispersion and density imaging; RD, radial diffusivity; RL, right-left; SNR, signal-to-noise ratio; TMS, transracial magnetic stimulation; SOD1, superoxide dismutase 1 gene; SOD1+, presymptomatic superoxide dismutase 1 gene.

METHODOLOGICAL CHALLENGES

Partial Volume Effects

Partial volume refers to scenarios where different tissues contribute to the same voxel. In spinal cord imaging this occurs when a voxel is at the CSF/white matter, white matter/gray matter, CSF/vascular, white matter/vascular interfaces. Signals from different tissue densities with different amounts of spins contribute to the total MR signal in these voxels, which results in indistinct tissue-boundaries. Partial volume effects can be reduced by increasing the spatial resolution, but this in turn results in lower signal-to-noise (SNR) and contrast-to-noise ratios (CNR). Magnetic fields strengths of three or seven Tesla compared to conventional 1.5 Tesla platforms (35–38), higher number of phased-array coils with parallel imaging (35, 38, 39), and corrections for physiological motion improves spatial resolution, SNR, and CNR (35, 38, 39).

Physiological and Patient Motion

Due to its proximity to the lungs and the heart, almost the entire spinal cord undergoes repetitive displacement due to respiration, CSF, and cardiac pulsation (40–43). The movement of the human spinal cord linearly increases caudally with distance from the head. The available literature suggest that physiological anterior-posterior (A-P) cord movement (0.60 ± 0.34 mm) exceeds those observed in superior-inferior (SI) (0.4 ± 0.1 mm) and right-left (RL) direction (0.17 ± 0.09 mm) (44, 45). Spinal imaging is also susceptible to movement artifacts from swallowing and patient movements during long MR acquisitions which can create ghosting artifacts (42, 46). By "gating" the acquisition, i.e., synchronizing with the respiratory or cardiac cycles, the effect of periodical movements can be significantly reduced (38, 39, 47). Motion artifacts can also be reduced using "saturation bands" that cover the esophagus, chest, and abdomen, by attenuating signals from moving structures so that it does not corrupt the signal from the spinal cord itself. Velocity compensating gradient sequences and signal averaging across multiple phases of motion can also be applied to minimize motion artifacts. Reducing acquisition time by using fast sequences, i.e., fast-spin-echo, parallel imaging that increases acquisition speed by factors from 1.5 to 3, i.e., SENSitivity Encoding/GeneRalized Autocalibration Partial Parallel Acquisition-type reconstructions, partial Fourier imaging, reducing the size of the phase-encoded direction, and decreasing the k-space matrix size effectively reduce both physiological and subject motion effects (48–53). MRI compatible cervical collars, which minimize involuntary neck movements, may also reduce movement artifacts (46). Co-registration of all data when dealing with multiple series acquisition, e.g., diffusion tensor imaging (DTI) and functional MRI (fMRI), can also be performed to limit the inconsistency in derived maps (54, 55).

Inhomogeneous Magnetic Field Environment

The spinal canal is surrounded by bones, ligaments, disks, arteries, and venous plexi. Its proximity to the esophagus, mediastinum, and the lungs, each containing various amounts of

air, create a challenging scanning environment. Adipose tissue, bone, and air have different magnetic susceptibility profiles, and respiration-induced B0 field fluctuations (43) also contribute to the inhomogeneity of the magnetic field around the spinal cord, resulting in geometric distortions and signal intensity loss (56). To some extent, these artifacts can be counteracted with "shimming." Shimming aims at compensating for field inhomogeneities by creating an auxiliary magnetic field via shim coils (57). While shimming improves overall field homogeneity, it is limited to smooth variations across larger regions and cannot fully compensate for small, and localized field variations, such as those observed at cartilaginous discs between the vertebral bodies. Echo planar imaging sequences, such as DTI, are particularly sensitive to geometric distortions around vertebral disks. In addition to shimming, parallel imaging, and careful slices positioning may reduce magnetic field inhomogeneity, i.e., slices centered in the middle of each vertebral body and perpendicular to the spinal cord (38, 47, 58). The specific geometry of the magnetic field inhomogeneities should be considered in order to correct for its effect (59–61).

SPINAL CORD IMAGING IN ALS

The role of conventional spinal MRI in ALS is to rule of alternative structural, inflammatory or neoplastic pathologies which may result in a combination of upper and lower motor neuron involvement mimicking ALS (62). Compressive myelopathies and radiculopathies are relatively common and early, predominantly lower limb presentations of ALS are sometimes attributed to these radiological findings resulting in laminectomies and other invasive procedures (63, 64). Conventional, clinical spinal sequences are typically only qualitatively interpreted without specific measurements. The majority of clinical spinal scans in ALS are reported as normal, but non-specific signs such as high signal along the corticospinal tracts are occasionally observed on T2-weighted imaging (65–67).

In sharp contrast with clinical sequences, advanced quantitative spinal protocols allow for the detailed and quantitative characterization of spinal gray and white matter integrity (38, 47, 58, 68). These protocols provide high resolution, high SNR, and high CNR images compared to standard clinical sequences. Furthermore, purpose-designed spinal protocols are based on mathematical MR signal modeling (e.g., diffusion-based methods, quantitative magnetization transfer, and MR spectroscopy) and the derived outputs can be quantitatively interpreted to provide accurate, motion-corrected white, and gray matter metrics.

Cord Morphometry

Gross axonal and gray matter loss have traditionally been estimated by measuring spinal cord cross-sectional areas at specific levels and interpreted as a proxy of atrophy in the context of reference normative values (69–72). The "cross-sectional approach" consists of estimating a mean cord cross-sectional area over a representative number of slices at a given vertebral level (70, 71, 73, 74), which can be relatively easily calculated from conventional MR sequences such as T1- or T2-weighted images. A variety of indexes, such as A-P dimension, L-R width, and radial distance can be derived from the cross-sectional area (CSA) approach. These measures reflect on different aspects of pathology, such as global vs. regional, lateral vs. anterior tissue loss, and are often interpreted as predominantly motor or sensory involvement (70, 75). More specific gray and white matter measures can be derived from higher resolution images followed by tissue-type segmentation methods (72, 76, 77). Novel quantitative approaches, such as tensor based morphometry and surface based-morphometry permit a more fine-grained characterization of cord topography and the definition of disease-associated signatures (74, 78). Recent studies demonstrated that spinal cord atrophy, especially gray matter atrophy, correlates with disability and disease progression and may be predictive of respiratory failure and of survival in ALS (58, 70, 72, 73, 79). The main findings of structural spinal cord studies are summarized in **Table 1**.

Diffusion Weighted Imaging

Diffusion weighted imaging (DWI) relies on the evaluation of water diffusion in CNS tissues and is primarily used to characterize white matter integrity (90, 91). DWI-derived metrics, such as axial diffusivity (AD), mean diffusivity (MD), fractional anisotropy (FA), radial diffusivity (RD) enable the quantitative characterization of white matter integrity. Novel high-directional approaches, such as high-angular resolution diffusion imaging (92), q-ball imaging (93), diffusion kurtosis imaging (94), diffusion basis spectrum imaging (DBSI) (95) are particularly well-suited to assess the integrity of crossing-fibers (96, 97). Emerging diffusion techniques such as neurite orientation dispersion and density imaging (NODDI) (98) help to estimate the microstructural attributes of dendrites and axons (99). While in ALS NODDI has been primarily used in cerebral studies in ALS (100, 101), it also has been also piloted in spinal applications (90, 102). Specific DTI indices (AD, RD) have been associated with specific pathological processes, such as axonal (103, 104) vs. myelin-related (105, 106) degeneration, but this interpretation is likely to be simplistic, as DTI measures are affected by axonal density, axonal diameter, myelin thickness and fiber orientation, fiber coherence, and acquisition parameters. DTI has been extensively used to study cerebral changes in ALS and describe phenotype-associated (107), genotype-specific (27), presymptomatic (32), and longitudinal white matter changes in the brain (81). In contrast to the plethora of cerebral DTI studies, relatively few spinal DTI studies have been published in ALS to date (58, 69, 73, 80–82, 85). These have consistently highlighted both motor and sensory tract alterations (**Table 1**).

Magnetization Transfer Imaging

Hydrogen nuclei linked to macromolecules such as the proteins and lipids of the myelin sheet have an extremely short T2 signal. While these macromolecules are not directly detectable by standard MRI sequences, magnetization transfer (MT) imaging enables the characterization of these structures. Macromolecular spins can be saturated using an off-resonance RF pulse, then the magnetization transfer between bound and free pools

TABLE 1 | Quantitative spinal imaging studies in ALS, ALS, amyotrophic lateral sclerosis; ALSFRS-r, the revised ALS functional scale; FA, fractional anisotropy; CSA, cross-sectional area; CST, corticospinal tract; FVC, force vital capacity; ihMT, inhomogeneous magnetization transfer; ihMTR, inhomogeneous magnetization transfer ratio; MD, mean diffusivity; MT, magnetization transfer; MTR, magnetization transfer ratio; MMT, manual muscle testing; SC, spinal cord; SOD1, superoxide dismutase 1 gene.

Author year of publication (references)	Patient cohort *n*	Controls *n*	Spinal imaging technique	Spinal cord region evaluated	Main findings
Valsasina et al. (80)	28 Sporadic ALS	20	CSA/DTI	Cervical spinal cord	Decreased FA and CSA decreased in ALS. Strong correlation between FA and the ALSFRS and moderate correlation between spinal and brain FA
Agosta et al. (81)	17/17 at baseline/follow-up (9 months) Sporadic ALS	20	CSA/DTI	Cervical spinal cord	Longitudinal FA, MD, and CSA changes detected. Brain CST diffusivity measurements are stable over time and do not correlate with cord measures
Nair et al. (82)	14 Sporadic ALS	15	DTI	C2-C6 vertebral levels	Reduced FA and RD in ALS. FA and RD correlate with finger and foot tapping rates. RD correlates with FVC and ALSFRS-R
Carew et al. (31)	23 sporadic ALS, 24 presymptomatic SOD1carriers	29	1H-MRS	C2 vertebral level	Reduced NAA/Cr and NAA/Myo ratios in both SOD1+ and sporadic ALS. Reduced Myo/Cr in SOD1+ subjects but not in sporadic ALS. Reduced NAA/Cho in sporadic ALS but not in SOD1+ subjects
Carew et al. (83)	14 Sporadic ALS	16	1H-MRS	C2 vertebral level	Reduced NAA/Cr and NAA/Myo ratios in ALS. NAA/Myo and NAA/Cho reductions correlate with FVC
Ikeda et al. (84)	19 Sporadic ALS	20	1H-MRS	C2 vertebral level	Reduced NAA/Cr and NAA/Myo ratios in ALS. NAA/Cr and NAA/Myo correlate with ALSFRS and FVC. NAA/Cr, NAA/m-Ins, and m-Ins/Cr are markedly altered in patients with C2 denervation and neurogenic changes
Cohen-Adad et al. (69)	27 sporadic ALS, 2 SOD1-linked familial ALS	21	CSA/DTI/ MT	C2-T2 vertebral levels	Altered DTI and MT metrics in the lateral and dorsal columns. FA correlates with ALSFRS-r. Segmental cord atrophy is associated with disability. FA profile of the cervical cord is suggestive of retrograde CST degeneration i.e., "dying back"
Branco et al. (70)	25 Sporadic ALS	43	CSA	C2 vertebral level	Decreased CSA in ALS. CSA correlates with disease duration, ALSFRS-r, and ALS severity scale
El Mendili et al. (73)	29 at baseline, 14 at follow-up	–	CSA/DTI/ MT	C2-T2 vertebral levels	CSA correlates with MMT. At follow-up, CSA predicts upper limb ALSFSR-R subscores, and FA predicts lower limb disability. CSA and MTR decrease between baseline and follow-up
Wang et al. (85)	24 Sporadic ALS	16	DTI	C2-C4 vertebral levels	CST FA and ADC changes in ALS. No difference in FA or ADC between patients with "definite" and "probable" ALS. No correlations between DTI parameters and modified Norris or ALSFRS-r scores
Iglesias et al. (86)	21 Sporadic ALS	21	DTI	Cervical spinal cord	Abnormal DTI metrics indicate decreased integrity of ascending sensory fibers. Significant correlation between DTI metrics and the depression of the peripheral afferent volley. The combination of SEP and DTI reveals sub-clinical sensory deficits in 85% ALS patients
Rasoanandrianina et al. (58)	10 Sporadic ALS	20	CSA/DTI/MT/ihMT	Cervical spinal cord	Spinal GM and WM atrophy in ALS. GM atrophy correlates with UMN scores. FA and MTR decrease in the CST. Axial diffusivity and ihMT decreased in the CST and dorsal columns. CSA correlates with the ALSFRS-r and spinal ALSFRS-R subscores. DTI and MT/ihMT metrics correlate with disease duration and MRC scores
de Albuquerque et al. (87)	27 at baseline, 27 at follow-up 8 months apart	27	CSA/DTI	C2 vertebral level	Longitudinal reduction in CSA. Cord area reduction correlates with change in ALSFRS-r

(Continued)

TABLE 1 | Continued

Author year of publication (References)	Patient cohort *n*	Controls *n*	Spinal imaging technique	Spinal cord region evaluated	Main findings
Querin et al. (79)	49 sporadic ALS	–	CSA/DTI/MT	C2-T2 vertebral levels	Spinal MRI parameters are more predictive of survival than clinical variables (ALSFRS-R, MMT, and disease duration)
Paquin et al. (72)	27 sporadic, 2 SOD1-linked familial ALS	22	CSA	C3-C6 vertebral levels	Spinal gray matter metrics are more sensitive to discriminate ALS patients from controls than overall cord CSA. Gray matter and spinal cord CSA correlates with ALSFRS-r and MMT arm scores. ALSFRS-r prediction improves when including a combination of gray and white matter CSA
Querin et al. (76)	60 sporadic ALS	45	CSA/DTI/MT	Cervical spinal cord	Random forest classification algorithm leads to good diagnostic performance distinguishing patients with ALS from controls with a sensitivity of 88% and specificity of 85%. The highest discrimination ability was achieved by evaluating RD, followed by FA, and CSA at the C5 spinal level
Piaggio et al. (88)	23 Sporadic ALS	18	CSA	Level of the Foramen magnum	Spinal cord area at the foramen magnum is significantly lower in ALS patients than in control subjects and is significantly correlated to ALSFRS-r. Spinal cord CSA at the foramen magnum correlates with disability in ALS independently of cerebral measures
Grolez et al. (89)	40 at baseline, 40 at follow-up 3 months apart	21	SC volume	Cervical spinal cord	Longitudinal change in cervical spinal cord volume is predictive of slow vital capacity decline and is also associated with survival

can be measured (108). Magnetization transfer occurs by means of cross relaxation processes, such as dipole-dipole interactions and chemical exchange. Magnetization transfer ratio (MTR) is calculated as the percentage difference of MT images with macromolecules signal saturation and one without. MTR enables inferences on myelin content, axonal count, and density as shown by three MS histological studies, and has been used extensively to assess demyelination, remyelination, and degeneration in MS (109–111). Conversely, relatively few studies have used cerebral MT imaging in ALS, and the majority of these focused on corticospinal tract alterations (112–115). Relatively few studies evaluated spinal MT changes in ALS, but they have shown progressive reduction overt time and correlation with muscle weakness (58, 69, 73). The key findings of spinal MT imaging studies in ALS and associated technical challenges are summarized in **Tables 1**, **2**.

Inhomogeneous Magnetization Transfer Imaging

Inhomogeneous magnetization transfer (ihMT) imaging is a novel method (116, 117), which allows the unprecedented characterization of myelin integrity, by isolating key myelin components from the broader macromolecular pool. ihMT shows unparalleled potential to detect and quantify demyelination (118) and may be adapted to spinal applications. ihMT imaging has already been applied to ALS cohorts and demonstrated significant correlation with muscle strength and disability profiles (58).

MR Spectroscopy

Magnetic resonance spectroscopy (MRS) is well-established, non-invasive imaging tool which provides neurochemical insights based on the concentration and relaxation profile of specific metabolites in cerebral and spinal tissues. MRS has been extensively used in cerebral studies of ALS (119), used to assess the therapeutic effect of Riluzole (120, 121), and also used to study brainstem metabolic alterations (122). Cross-sectional and longitudinal (123), single voxel and whole brain multi-voxel studies have both contributed to our understanding of ALS pathophysiology (124). The main targets of proton spectroscopy (1H-MRS) include the following metabolites; N-Acetyl Aspartate (NAA), creatine (Cr), choline (Cho), and myo-Inositol (Myo). These metabolites are typically associated with neuronal integrity/viability (NAA), tissue energy metabolism (Cr), membrane integrity (Cho), and glial function (Myo). (125). Relatively few studies have used 1H-MRS to characterize metabolic changes at the spinal level, and the majority of these studies focused on multiple sclerosis (126, 127) MRS however seems particularly applicable to ALS cohorts, where it promises the characterization of presymptomatic changes and by including both the upper and lower motor components of the motor system, it has led to particularly significant clinico-radiological correlations (31, 83, 84). For the contribution of MRS studies to

TABLE 2 | The advantages and methodological challenges associated with specific spinal imaging techniques.

Imaging technique	Advantages of specific techniques in ALS	Challenges and correction strategies
Diffusion-weighted imaging	Evaluation of specific white matter bundles; motor; and sensory white matter tracts integrity. Availability of multiple derived diffusivity metrics reflecting on various histological aspects of white matter integrity; AD, MD, RD, FA. Emerging high angular resolution diffusion techniques to assess crossing fiber integrity. Derived metrics can be interpreted in comparative, longitudinal, correlation, and machine learning analyses	Motion artifacts: - Gating the acquisition (DWI, CSA, and volume estimation, fMRI, 1H-MRS) - Saturation bands (all modalities) - Velocity compensating gradient sequences (DWI) - Signal averaging across multiple phases of motion (DWI, fMRI, 1H-MRS) - Fast sequences (DWI) - MRI compatible cervical collar (DWI, CSA, and volume estimation, fMRI, 1H-MRS) - Co-registration of all data (DWI, fMRI) - Non-linear co-registration between T1 with and without magnetization transfer saturation pulse (MTR, ihMT) Magnetic field inhomogeneities: - Shimming (all modalities) - Parallel imaging (all modalities) - Corrections for gradients nonlinearity induced geometric distortion (DWI, MT, ihMT, CSA, and volume estimation, fMRI) - Corrections for breathing induced B0 field fluctuations (DWI, fMRI, CSA) Partial volume effect (all modalities) - Higher magnet field strength - Higher number of phased-array coils with parallel imaging - Multi-channel image acquisition - Limiting physiological motion
Magnetization transfer imaging	Evaluation of both white and gray matter integrity. Sensitive detection and measurement of demyelination. Derived metrics can be evaluated at individual and group-level statistical analyses	
Inhomogeneous magnetization transfer imaging	Applicability to both gray and white matter tissue components, superior sensitivity to detect demyelination	
Cross-sectional area and volume estimation	Automated segmentation pipelines enable the estimation of overall cord cross-sectional area and gray and white matter components separately. Gray matter components correlate with clinical and electrophysiological lower motor neuron (LMN) measures, therefore may be regarded an imaging proxy of LMN integrity	
1H-MR spectroscopyd	MRS provides a number of metrics which reflect on focal neuronal integrity (NAA), energy metabolism (Cr), membrane integrity (Cho), and glial function (Myo). MRS readily captures segmental metabolic alterations in symptomatic and presymptomatic ALS cohorts	
Functional MRI	As an emerging technique spinal fMRI has the potential to detect segmental cord activation during motor tasks and at rest	

ALS, amyotrophic lateral sclerosis; ALSFRS-r, revised ALS functional scale; FA, fractional anisotropy; CST, corticospinal tract; FVC, force vital capacity; MD, mean diffusivity; MMT, manual muscle testing.

ALS research and specific methodological considerations please see **Tables 1**, **2**.

Functional MRI

Functional MRI (fMRI) detects local variations in blood oxygenation level-dependent MR signal at rest and during activation paradigms (128). FMRI has been extensively applied to ALS cohorts to describe network changes and assess altered activation patterns when performing motor or cognitive tasks (129–131). Following decades of successful cerebral studies, the first spinal fMRI studies have now been published (55, 132). Emerging spinal cord fMRI studies in healthy controls provide proof of feasibility and the first studies using spinal fMRI in neurological conditions are underway (133).

THE CONTRIBUTION OF SPINAL IMAGING TO ALS RESEARCH

Evidence for Motor Involvement in ALS

Quantitative spinal MRI studies in ALS have consistently detected corticospinal tract and anterior horns degeneration and changes correlated with functional disability (36, 58, 80, 82, 85). Segmental spinal cord atrophy was not only linked to muscle weakness (58, 70, 88), but also to electrophysiological markers such as transracial magnetic stimulation (TMS) and motor evoked potentials (69). Two studies have demonstrated that both white and gray matter atrophy contributes to global cord atrophy in ALS (58, 72), but a recent study indicates that cord atrophy in ALS may be predominantly driven by anterior horn degeneration (72), confirming the role of spinal MRI as a putative LMN marker. DTI and MTR indices of the corticospinal tract (CST) correlated with TMS facilitation motor thresholds, a functional parameter that reflects pyramidal tract integrity.

Longitudinal Spinal Cord Changes in ALS

In contrast to the plethora of longitudinal cerebral studies in ALS (21), relatively few longitudinal spinal studies are available to demonstrate that spinal MRI metrics can track subtle progressive changes over time (73, 81, 87, 89). These longitudinal studies captured decreasing CST MTR and progressive cord atrophy (73, 87) While some longitudinal studies also captured progressive DTI alterations (81), other studies did not (73). Some studies suggest that CSA estimates may be more reliable markers of longitudinal cord pathology than MTR or DTI metrics (73, 87). Progressive cord atrophy not only mirrors clinical progression, but early cervical cord atrophy is thought to predict respiratory dysfunction in ALS (89, 134). Furthermore, spinal MRI metrics may be superior predictive indicators of survival than clinical measures (79). Given the scarcity of longitudinal spinal imaging studies in ALS, it remains to be established which imaging metrics capture early ALS-associated changes, therefore may be used in diagnostic applications, and which metrics can track changes in the later stages making them suitable as monitoring markers.

Evidence for Sensory Involvement in ALS

Several spinal MRI studies (58, 69) have captured dorsal column degeneration using DTI, MT, and ihMT imaging, and one study demonstrated progressive sensory tract degeneration over time (135). Dorsal column pathology can be detected relatively soon after symptoms onset, which suggests that sensory involvement is a core and relatively early feature of ALS. Combined spinal DTI and neurophysiology studies have also confirmed considerable sensory pathway degeneration in ALS patients without sensory symptoms (86). The combined MRI-neurophysiology approach revealed sub-clinical sensory deficits in 85% of ALS patients. These findings suggest that sensory dysfunction may have been underestimated by previous studies and that sensory afferent pathways may be affected early in the course of ALS and are important facets of ALS pathogenesis (69, 86). In contrast to longitudinal cerebral studies (4, 32), longitudinal spinal studies suggest that dorsal column metrics (73), and CST DTI indices (87) may be relatively constant (135).

Evidence for Spinal Metabolic Alterations in ALS

1H-MRS studies in ALS have shown reduced NAA/Cr and NAA/Myo ratios at the C2 vertebral level (31, 83, 84). One spinal MRS study captured reduced NAA/Myo and NAA/Cr ratios in presymptomatic superoxide dismutase 1 gene (SOD1+) carriers (31). In addition to group-level differences in symptomatic and presymptomatic ALS cohorts, NAA/Myo and NAA/Cho reductions correlate with force vital capacity (FVC) and revised ALS functional scale (ALSFRS-r) and inversely correlated to the rates of decline (31, 83, 84).

FUTURE DIRECTIONS

Existing spinal studies in ALS indicate that it is possible to detect disease-specific imaging signatures at a group level, and emerging machine-learning studies (76) have demonstrated that it may be possible to classify individual scans into "ALS" and "Healthy" groups. Despite the pioneering studies however, it is clear that spinal imaging lags behind cerebral imaging. Cerebral imaging has shown that phenotype and genotype specific patterns can be detected, multi-time point longitudinal studies have shown divergent rates of gray and white matter degeneration, studies have been validated by post mortem examination and robust multi-site studies have also been published (136). It is likely that improved coil designs with higher number of phased-array elements, new generation scanners with higher gradients optimized for advanced diffusion-weighted imaging, ultra-high filed platforms with superior spatial resolution, and SNR, spinal imaging will contribute unprecedented insights in ALS. It is conceivable that spinal imaging will contribute to the longstanding debate about dying back and dying forward, and ALS being a primarily spinal vs. cerebral disease. Spinal imaging provides a unique opportunity to appraise both lower and upper motor neuron degeneration. It is also likely that imaging sequences currently primarily used in cerebral imaging in ALS such as resting state fMRI, task-based fMRI, quantitative susceptibility weighted imaging, presymptomatic imaging, texture analyses, and post mortem imaging will filter down to spinal applications. Data-sharing initiatives, cross-platform harmonization, inclusion of upper motor neuron (UMN) and lower motor neuron (LMN) predominant ALS cohorts, correlations with advanced neurophysiological techniques are trends of ALS imaging which is likely to be adopted in spinal studies. One of the key ambitions of multiparametric spinal imaging is to overcome the methodological challenges of thoracic and lumbar imaging.

CONCLUSIONS

The momentous advances in spinal imaging in ALS suggest the spinal metrics may soon be used as validated diagnostic, monitoring, and prognostic markers, contributing both to individualized patient care and pharmacological trials.

AUTHOR CONTRIBUTIONS

ME, GQ, PB, and P-FP contributed equally to the conceptualization, drafting, and revision of the manuscript.

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Split-Hand Syndrome in Amyotrophic Lateral Sclerosis: Differences in Dysfunction of the FDI and ADM Spinal Motoneurons

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The F-wave test allows for the non-invasive assessment of spinal motoneuron excitability. We investigated the difference in spinal motoneuron dysfunction between the first dorsal interosseous (FDI) and abductor digit minimi (ADM) muscles by investigating F-waves and to assess the contribution of spinal mechanisms to split-hand syndrome in patients with amyotrophic lateral sclerosis (ALS). Sixty-five consecutive ALS patients and twenty age- and gender-matched healthy controls (HCs) were enrolled. Motor nerve conduction studies and F-waves were performed bilaterally on median and ulnar nerves in all subjects. HCs revealed prominently longer F-wave latencies, lower chronodispersion, mean F-wave amplitude, and mean and maximal F/M amplitude ratio ($P < 0.001$) in the FDI compared to the ADM. However, no significant differences in almost all F-wave parameters between the FDI and ADM were observed in ALS patients with affected hands except the minimal and mean F-wave latency. These data suggest that excitability is greatly changed in the spinal motoneurons innervating the FDI. Furthermore, the mean F-wave amplitude ($r = 0.454$, $P = 0.002$) of the FDI was significantly correlated with the FDI/ADM CMAP amplitude ratio in ALS patients with affected hands but not of the ADM. Our findings suggested that the dysfunction of spinal motoneurons between the FDI and ADM was different in ALS, and spinal motoneuron dysfunction was associated with development of the split-hand phenomenon.

Keywords: amyotrophic lateral sclerosis, F-wave, motor neuron, split-hand, first dorsal interosseous muscle, motor neuron disease

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a rapidly progressive neurodegenerative disorder involving both upper and lower motor neurons (LMNs) and is often characterized by muscle weakness and atrophy, especially the small hand muscles. Dissociated atrophy of intrinsic hand muscles as an early and specific clinical feature of ALS, termed the split-hand sign, refers to preferential weakness and wasting of the abductor pollicis brevis (APB) and first dorsal interosseous (FDI) muscles with relative sparing of the abductor digit minimi (ADM) (Kuwabara et al., 1999, 2008; Wilbourn, 2000; Eisen and Kuwabara, 2012; Eisen et al., 2017). Menon et al. (2014b) reported that the split-hand sign was often evident in 62% of patients at the time of visiting and in 95% at follow-up.

Although the thenar complex muscles (APB/FDI) and hypothenar muscles (ADM) constituting the split-hand are innervated through the same spinal segments (C8 and T1), the FDI and ADM, which are differentially affected, share ulnar innervation (Weber et al., 2000; Kuwabara et al., 2008; Eisen and Kuwabara, 2012). Corticomotoneuronal input and spinal/peripheral mechanisms have been suggested to be involved (Weber et al., 2000; Bae et al., 2009; Eisen and Kuwabara, 2012; Shibuya et al., 2013; Menon et al., 2014b; Eisen et al., 2017), and cortical dysfunction is considered as the likely pathophysiological mechanism underlying the split-hand phenomenon, while axonal dysfunction may appear as a downstream process (Menon et al., 2014a,b). The spinal mechanisms underlying the development of split-hand in ALS remain controversial. For example, Cengiz et al. (2018) reported no significant difference in cutaneous silent period measurements between the ADM and FDI, suggesting no role of spinal cord excitability changes in split-hand syndrome. However, Wilbourn (2000) reported the finding of split-hand in ALS in 1992, as well as in other diseases with only LMN dysfunction, and suggested that the lesion responsible for the ALS split-hand was at the level of the cervical anterior horn cell (Schelhaas et al., 2003). Further, Fang et al. (2016) found differences between the dysfunction of spinal motoneurons innervating the APB and the ADM in ALS. Thus, in the present study, we examined the hypothesis that spinal mechanisms contribute to split-hand in ALS.

The F-wave is a late response that reflects antidromic activation of motoneurons. Previous studies have shown that F-waves were not only used to assess changes in the excitability of spinal motoneurons (Espiritu et al., 2003; Lin and Floeter, 2004; Argyriou et al., 2006) but also as a probe to determine the activity of the motor cortex (Mercuri et al., 1996; Rivner, 2008). A direct comparison of the F-wave variables of the FDI and ADM innervated by the same nerve and the same spinal segments may provide more valuable information on the excitability changes of the spinal motoneuron pool and shed light on the complex mechanisms of split-hands. To date, the characteristics of multiple F-wave variables in the FDI have not been assessed in healthy subjects and ALS in previous studies. Therefore, the aims of this study were to (1) identify the characteristics of F-waves of the FDI, and (2) to ascertain the difference in spinal motoneuron dysfunction between the FDI and ADM in patients with ALS and HCs and to clarify the spinal pathophysiology of split-hand.

MATERIALS AND METHODS

Subjects

Sixty-five consecutive patients diagnosed as having definite, probable and laboratory-supported probable sporadic ALS according to the revised El Escorial criteria were included in this study. All patients were recruited at the department of neurology in Peking Union Medical College Hospital between December 2017 and November 2018. Patients with ALS complicated by diabetic neuropathy, alcohol abuse, carpal tunnel syndrome, cervical myelopathy, and other neurological disorders were excluded. Control data were obtained from 20 age- and gender-matched healthy volunteers, whose nerve condition studies were normal. In each patient the muscle strength was assessed using the Medical Research Council (MRC) score, and a total MRC score was calculated for the following muscle groups assessed bilaterally: shoulder abduction, elbow flexion, elbow extension, wrist dorsiflexion, finger abduction, thumb abduction, hip flexion, knee extension, and ankle dorsiflexion (Menon et al., 2014b). The maximum possible total MRC score was 90. The clinical status of each patient was evaluated with the ALS Functional Rating Scale-Revised (ALSFRS-R) and upper motor neuron (UMN) score, as previous studies described (Cedarbaum et al., 1999; Grapperon et al., 2014). Two groups were established from the ALS patients, an affected hand group with wasting and weakness in the intrinsic hand muscles, where the data from the more affected hands were analyzed (45 patients), and an unaffected hand group, where the data for bilateral hands were analyzed in this group (20 patients). The hand was considered to be unaffected if the intrinsic hand muscles contained APB, FDI and ADM of normal strength; no wasting or weakness; and the nerve conduction studies (NCSs) were within normal limits. The hands of the healthy controls (HCs) were analyzed bilaterally. To estimate the influence of UMN involvement in the split-hand phenomenon, two subgroups were formed from ALS patients in the affected hand group, designated as the P group (pyramidal signs) and the NP group (no pyramidal signs). A more conservative but robust criterion for UMN lesion was used in the present study, requiring both increased tendon reflexes and positive Hoffman's sign in defining the presence of pyramidal lesion in the arm (de Carvalho et al., 2002). The study was approved by the Peking Union Medical College Hospital Clinical Research Ethics Committee (Beijing, China), and all participants provided signed informed consent.

Nerve Conduction Studies

All patients underwent routine NCSs and electromyography (EMG) using an EMG machine (Medtronic-Dantec Electronics, Skovlunde, Denmark). A peak-to-peak amplitude of maximal compound muscle action potential (CMAP) was elicited by using supramaximal (120%) surface stimulation of the median and ulnar nerves at the wrist and recorded from the APB, FDI and ADM muscles according to previously described standard methods (Stimulus duration: 0.1 ms; Filter setting: 20 Hz–10 kHz Gain: 200 μV/division; Sweep speed: 5 ms/division). Specifically, for FDI recording, the active electrode (G1) was placed on its belly and the reference electrode (G2) at the medial aspect of the proximal interphalangeal joint of the index finger (Kuwabara et al., 2008). The distance between the cathode and active (G1) recording electrodes for ADM muscles was 6.5 cm, while the distance between the cathode and active (G1) electrode for the FDI muscle was 8–10 cm. There was no evidence of conduction block or M response temporal dispersion in ALS patients. The skin temperature was maintained above 32°C. The following parameters were obtained: distal motor latency (DML), motor conduction velocity (MCV), CMAP amplitude (peak-to-peak), and the FDI/ADM CMAP amplitude ratio.

F-Wave Studies

The F-waves of ulnar nerves were recorded with surface electrodes attached to the skin over the FDI and ADM muscles,

the same position as in motor nerve conduction studies (de Carvalho et al., 2002; Kim, 2011). One hundred consecutive supramaximal (120%) percutaneous stimuli were delivered to the ulnar nerve at the wrist at a frequency of 1 Hz with the cathode proximal to the anode (Filters setting: 20 Hz–3 kHz; amplifier gain: 200 μV/division). A peak-to-peak deflection from baseline of at least 40 μV was accepted as an F-wave (Peioglou-Harmoussi et al., 1985). The following F-wave variables were measured in the FDI and ADM: the minimum, mean and maximum latency corrected according to the subject's height (FLmin/H, FLmax/H, FLmean/H) (ms/m); chronodispersion; persistence; mean and maximum F-wave amplitude (peak-to-peak); mean and maximum F/M amplitude ratio (average or maximum peak-to-peak amplitude of F-waves expressed as a percentage of maximum distal CMAP amplitude); and the number of repeater F-waves. The repeater F-waves were identified as having the same shape, latency, and amplitude, and were calculated by the following indices as described by Chroni et al. (Chroni et al., 2012): index repeating neuron (index RN) (number of repeating neuron/ number of traces with different F-wave shapes in a series of 100 stimuli × 100), and index repeater F-waves (index Freps) (total number of F-wave repeaters/total number of traces with F-waves in the same nerve × 100). Due to the nature of the applied F-wave technique, which requires recording of a significant number of F-waves, we only examined the FDI and ADM muscles with strength of MRC of 2 or higher. And nerves without F-waves or the CAMP amplitude ≤ 2.0 mV were excluded from our analysis.

Statistical Analysis

All analyses were performed using SPSS for windows version 24.0 (SPSS, IBM, Chicago, IL, United States). Normality was checked by the Shapiro–Wilk test. Normally distributed data are expressed as the mean ± SD and were compared using one-way ANOVA and the Student-Newman-Keuls (SNK) test. Mean values of measured variables between the FDI and ADM within the same group were compared using Student's *t*-test. Non-normally distributed data are expressed as the medians (IQR) and were compared using the Kruskal–Wallis *H*-test. Once the null hypothesis was rejected, pairwise comparisons of the groups were tested using the Mann–Whitney *U*-test and Bonferroni correction with a significance level of $P < 0.017$. The relationship between the F-wave parameters and FDI/ADM CAMP amplitude ratio was assessed using Pearson's correlation and Spearman's rank correlation test. For comparison of the frequency distribution of categorical variables (gender and disease onset), the χ^2 test was used. The level of statistical significance was established at $P < 0.05$.

RESULTS

The clinical profiles of the ALS patients and HCs are presented in **Table 1**. Among the ALS groups, all patients studied herein had a clinically predominant LMN syndrome and none had a pure UMN syndrome. The total MRC scores were higher in the unaffected hand group than the affected hand group. Disease duration, UMN score and ALSFRS-R were not significantly different between the affected hand and unaffected hand groups. The age at examination, gender ratio, and height were comparable between patients and controls.

Table 2 summarizes the overall comparisons between motor conduction values obtained from the ulnar nerves (FDI and ADM) of both patients with ALS and HCs. In HCs, the mean CMAP amplitude in FDI was greater than that in ADM, and the mean FDI/ADM CMAP amplitude ratio was calculated as 1.38. A significant reduction of FDI/ADM CMAP amplitude ratio (0.9 ± 0.3) was observed in the affected hand group compared with HCs, confirming that the split-hand phenomenon was evident in the present ALS patients (Kuwabara et al., 2008).

The results of F-wave variables are displayed in **Table 3**. When the FDI and ADM were compared in HCs, the FDI showed

TABLE 1 | Clinical profile of participants.

Parameters	Affected hand (A, *n* = 45)	Unaffected hand (B, *n* = 20)	HCs (C, *n* = 20)	*P*-value		
				A vs. C	B vs. C	A vs. B
Age (year)	53.42 ± 8.82 (34–66)	51.20 ± 9.71 (35–69)	52.4 ± 9.13 (39–73)	>0.05	>0.05	> 0.05
Gender (male:female)	25:20	7:13	11:9	>0.05	>0.05	> 0.05
Height (cm)	165.84 ± 8.57	163.85 ± 7.56	166.3 ± 8.18	>0.05	>0.05	> 0.05
Disease duration (months)	14.73 ± 8.89 (3–45)	11.70 ± 6.97 (4–27)	NA	NA	NA	0.158
Disease onset (bulbar: upper limbs: lower limbs)	9:28:8	9:3:8	NA	NA	NA	0.002
Total MRC scores	73.44 ± 10.27 (43–88)	83.35 ± 6.78 (67–90)	NA	NA	NA	< 0.001
UMN scores	39.24 ± 14.03 (4–64)	32.10 ± 12.87 (5–54)	NA	NA	NA	0.057
ALSFRS-R	40.44 ± 4.18 (28–47)	42.30 ± 2.76 (36–46)	NA	NA	NA	0.099

HCs, healthy controls; MRC, Medical Research Council; UMN, upper motor neuron; ALSFRS-R, amyotrophic lateral sclerosis functional rating scale-revised; NA, not applicable.

TABLE 2 | Results of nerve conduction studies and split-hand.

Parameters	Affected hand (A, *n* = 45)	Unaffected hand (B, *n* = 40)	HCs (C, *n* = 40)	*P*-value		
				A vs. C	B vs. C	A vs. B
DML (ms)						
FDI	3.60 ± 0.40	3.45 ± 0.38	3.42 ± 0.32	**0.005**	0.519	0.089
ADM	2.47 ± 0.45	2.20 ± 0.26	2.19 ± 0.25	**0.001**	0.784	**0.002**
CMAP amplitude (mV)						
FDI	7.05 ± 4.31	17.91 ± 4.73	17.78 ± 3.77	**< 0.001**	0.825	**< 0.001**
ADM	7.49 ± 3.41	13.31 ± 2.80	14.62 ± 2.85	**< 0.001**	0.027	**< 0.001**
FDI/ADM CMAP amplitude ratio	0.90 ± 0.30	1.42 ± 0.28	1.38 ± 0.21	**< 0.001**	0.593	**< 0.001**
MCV (m/s)						
FDI	56.99 ± 4.53	60.11 ± 1.94	60.18 ± 1.45	**< 0.001**	0.159	**< 0.001**
ADM	55.89 ± 3.66	60.12 ± 1.22	60.20 ± 1.46	**< 0.001**	0.102	**< 0.001**

DML, distal motor latency; FDI, first dorsal interosseous; ADM, abductor digit minimi; CMAP, compound muscle action potential; MCV, motor conduction velocity; HCs, healthy controls. All data are expressed as the mean ± SD. Values with significant differences printed in bold characters.

TABLE 3 | Results of F-wave variables in the ALS patients and the healthy controls.

Parameters	Affected hand (A, *n* = 45)	Unaffected hand (B, *n* = 40)	HCs (C, *n* = 40)	*P*-value		
				A vs. C	B vs. C	A vs. B
Minimal F latency (ms/m)						
FDI	16.37 ± 0.95**	15.84 ± 0.71**	15.13 ± 0.46**	**< 0.001**	**< 0.001**	0.022
ADM	15.62 ± 0.92**	14.93 ± 0.79**	14.50 ± 0.47**	**< 0.001**	**0.016**	**< 0.001**
Maximal F latency (ms/m)						
FDI	18.93 ± 1.78	17.70 ± 0.92*	16.74 ± 0.57**	**< 0.001**	**< 0.001**	**< 0.001**
ADM	18.59 ± 1.70	16.99 ± 0.89*	15.13 ± 0.46**	**< 0.001**	**< 0.001**	**< 0.001**
Mean F latency (ms/m)						
FDI	17.41 ± 1.15*	16.53 ± 0.72**	15.82 ± 0.54**	**< 0.001**	**< 0.001**	**< 0.001**
ADM	16.80 ± 1.03*	15.81 ± 0.84**	15.17 ± 0.52**	**< 0.001**	**< 0.001**	**< 0.001**
F-wave chronodispersion (ms)						
FDI	4.22 ± 2.27	3.04 ± 0.95*	2.49 ± 0.55*	**< 0.001**	**0.001**	**0.004**
ADM	4.91 ± 2.31	3.34 ± 0.70*	2.88 ± 0.66*	**< 0.001**	**0.003**	**< 0.001**
F-wave persistence (%)						
FDI	62 (41)	96.5 (9.25)*	100 (0.75)	**< 0.001**	**< 0.001**	**< 0.001**
ADM	71 (54.5)	99 (1)*	100 (0)	**< 0.001**	**0.002**	**< 0.001**
Mean F-wave amplitude (μV)						
FDI	269 (216.5)	178.5 (127.25)*	174.5 (90.75)**	**0.002**	0.648	**0.015**
ADM	266 (199.5)	257.5 (124)*	264.5 (126.5)**	> 0.05	> 0.05	> 0.05
Mean F/M amplitude ratio (%)						
FDI	3.99 (5.96)	1.04 (0.6)**	1.03 (0.57)**	**< 0.001**	0.950	**< 0.001**
ADM	3.76 (3.47)	2.13 (1.23)**	1.85 (0.80)**	**< 0.001**	0.258	**< 0.001**
Maximal F/M amplitude ratio (%)						
FDI	11.97 (9.72)	3.37 (3.28)*	2.80 (1.82)**	**< 0.001**	0.020	**< 0.001**
ADM	10.01 (11.51)	5.86 (4.09)*	4.33 (2.52)**	**< 0.001**	0.017	**< 0.001**
Index RN (%)						
FDI	16.67 (23.07)	1.62 (3.23)**	0 (0)	**< 0.001**	**< 0.001**	**< 0.001**
ADM	18.18 (29.81)	0 (1.79)**	0 (0)	**< 0.001**	0.017	**< 0.001**
Index Freps (%)						
FDI	55.81 (51.57)	4.12 (13.33)**	0 (0)	**< 0.001**	**< 0.001**	**< 0.001**
ADM	50 (59.12)	0 (3.77)**	0 (0)	**< 0.001**	0.017	**< 0.001**

*HCs, healthy controls; FDI, first dorsal interosseous; ADM, abductor digit minimi. Normally distributed data are expressed as the mean ± SD, and non-normally distributed data are expressed as the medians (IQR). Values with significant differences printed in bold characters. **P <0.001, *P <0.05, between the FDI and the ADM in each group. For comparisons of F-wave variables among affected hand group, unaffected hand and healthy control group, Bonferroni correction with a significance level of P < 0.017.*

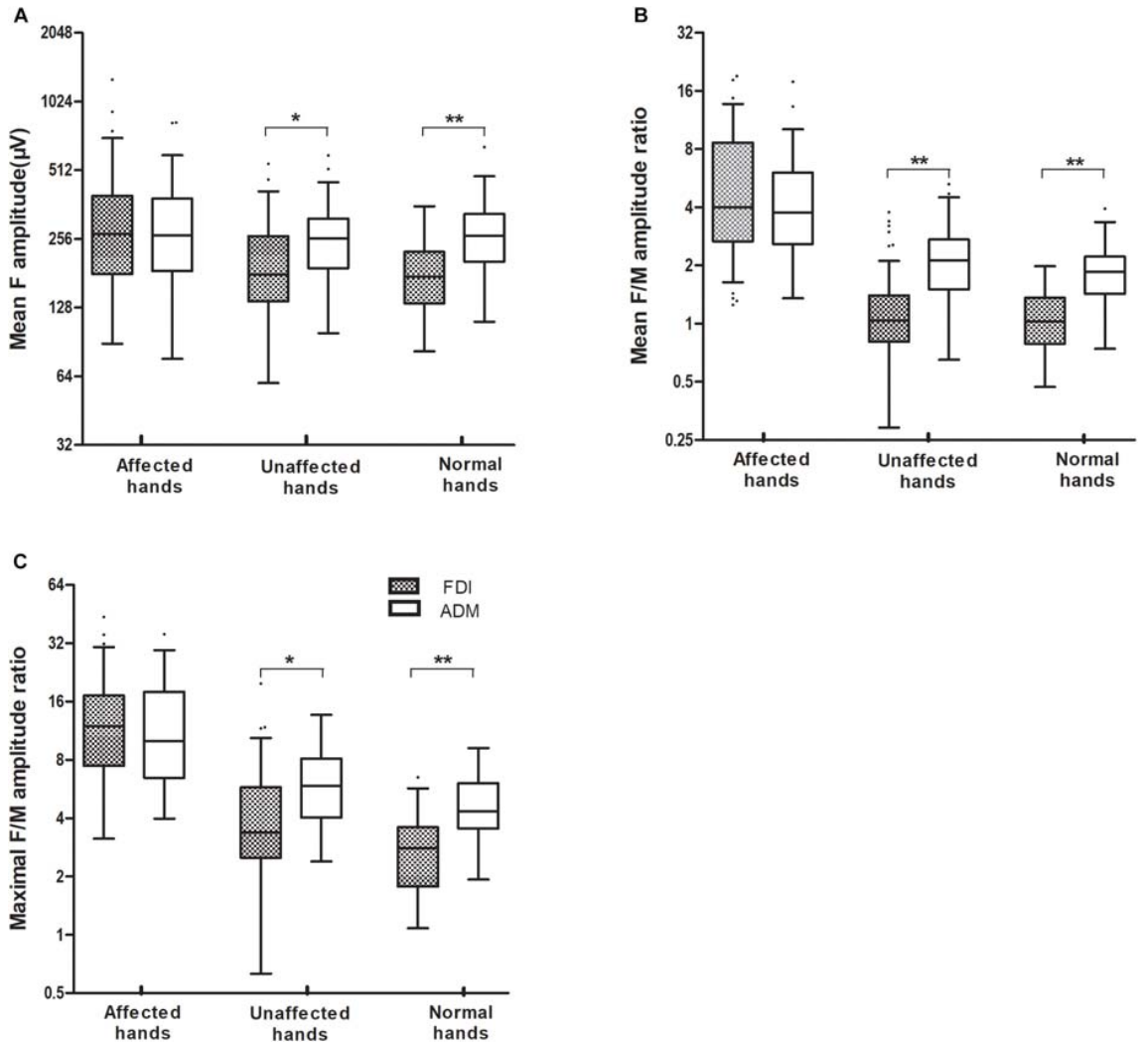

FIGURE 1 | The mean F-wave amplitude **(A)**, and the mean and maximal F/M amplitude ratios **(B,C)**, recorded over the FDI and ADM muscles were significantly increased in ALS patients with affected hands compared with those with unaffected hands. When the FDI and ADM were compared in HCs and the unaffected hand group, the FDI showed a noticeably lower mean F-wave amplitude, and a lower mean and maximal F/M amplitude ratios, than for the ADM. By contrast, there were no differences in F-wave measurements between the FDI and ADM in the affected hand group. $^{**}P < 0.001$; $^{*}P < 0.05$. The numerical data on the Y-axis were logarithmically transformed (Log2).

noticeably longer F-wave latencies and lower chronodispersion, mean F-wave amplitude, and mean and maximal F/M amplitude ratios than the ADM. This trend was similar in the unaffected hand group (**Figures 1A–C**). In contrast, no differences between the FDI and ADM for F-wave measurements were observed in the affected hand group except the FLmin/H and FLmean/H. Concerning the F-wave variables in the unaffected hand group, the F-wave latencies, persistence, chronodispersion of the FDI and ADM and index RN, and index Freps of the FDI were significantly changed compared to controls, along with the relative normal mean F-wave amplitude, mean and maximal F/M amplitude ratios of the FDI and ADM and index RN and index Freps of the FDI (column B vs. C). **Table 4** shows the comparison of F-wave parameters in ALS patients with pronounced split-hands between subgroups of those with (P) and without (NP) pyramidal signs. No difference was observed between the P and NP groups. Additionally, the difference between the FDI and ADM was not significant in both groups.

The results of the correlation analysis conducted between the F-wave parameters and FDI/ADM CMAP amplitude ratio displayed in **Supplementary Table S1**. Combining these parameters, it was evident that the F-wave amplitude ($r = 0.454$, $P = 0.002$) of the FDI was significantly correlated with the FDI/ADM CMAP amplitude ratio in the affected hand group, but not with the ADM. There was no significant correlation between other F-wave variables in both the ADM and FDI and the FDI/ADM CMAP amplitude ratio in the affected hand group of ALS patients. No significant relation was observed between the F-wave variables and FDI/ADM CMAP amplitude ratios in the unaffected hand group and HCs.

TABLE 4 | Comparison of F-wave parameters in ALS patients with split-hands between subgroups of those with (P) and without (NP) pyramidal signs.

Parameters	P group (n = 21)	NP group (n = 24)	P-value
F-wave persistence (%)			
FDI	62 (40)	59 (42.75)	0.637
ADM	84 (50)	52.5 (60)	0.255
Mean F-wave amplitude (μV)			
FDI	219 (180)	310.5 (286)	0.481
ADM	209 (162)	326.5 (181.25)	0.062
Mean F/M amplitude ratio (%)			
FDI	3.47 (3.01)	5.03 (7.39)	0.387
ADM	2.89 (1.74)	4.26 (3.41)	0.055
Maximal F/M amplitude ratio (%)			
FDI	11 (8.97)	15.2 (18.11)	0.106
ADM	9.09 (7.65)	13.47 (12.10)	0.116

FDI, first dorsal interosseous; ADM, abductor digit minimi; P, with pyramidal signs; NP, without pyramidal signs; Non-normally distributed data are expressed as the medians (IQR).

DISCUSSION

Results Related to the Changes in ALS

Our NCSs variables of the FDI and ADM and F-wave values of the ADM in HCs and patients with ALS showed a close resemblance to the previous findings (Peioglou-Harmoussi et al., 1985; Kuwabara et al., 2008; Buschbacher et al., 2015; Fang et al., 2016). In the present study, ALS patients with an unaffected hand did not show significant changes in DML, CMAP amplitude, or MCV recorded over the FDI and ADM in contrast with HCs. While a significantly decreased CMAP amplitude was associated with increased DML and slowed MCV in both the FDI and ADM were observed in our patients with affected hands. These findings are compatible with the chronic denervation/reinnervation process, and are associated with the pathophysiological changes in ALS (de Carvalho et al., 2002; Argyriou et al., 2006).

We used F-waves as an indicator of dysfunction of spinal motoneurons. The F-wave amplitudes are related to the excitability of spinal motoneurons and axonal compensatory reinnervation (Argyriou et al., 2006; Hachisuka et al., 2015). Specifically, the F/M amplitude ratio was used as a quantified index of the proportion of the motoneuron pool, as this measure is minimally influenced by muscle wasting (Argyriou et al., 2006). In ALS patients, the mean F-wave amplitude, and mean and maximal F/M amplitude ratios, were increased in the FDI and ADM. Similar findings were reported and inferred that both anterior horn cell hyperexcitability (Argyriou et al., 2006) and the formation of large post-reinnervation motor units due to LMN dysfunction (Drory et al., 2001) are important factors. In the present study, ALS patients also showed reduced F-wave persistence and increased repeater F-waves. Similar changes were reported in post-polio syndrome (PPS) (Hachisuka et al., 2015). Both F-wave persistence and repeater-F waves are influenced by the number of functional LMNs and motoneuron excitability. A low F-wave persistence indicates loss of function of LMNs and decreased excitability of the motoneuron pool (de Carvalho et al., 2002; Argyriou et al., 2006; Rivner, 2008). With respect to the mechanism of production of repeater F-waves in PPS, it was proposed that loss of motoneurons or decreased excitability of some anterior horn cells caused the remaining anterior horn cells with increased excitability to produce more frequent repeated backfiring (Hachisuka et al., 2015). This underlying pathophysiology in PPS may also explain the increased repeater F-waves in ALS (Chroni et al., 2012; Hachisuka et al., 2015). In addition, the F-wave latencies and chronodispersion were markedly prolonged in our ALS group. However, the F-wave latencies commonly thought to be influenced by height and preferential loss of fast-conduction neurons and the chronodispersion represent the conduction velocity of the motor neurons recruited, which are valuable markers of the conduction properties of motor axons (Fisher, 1998; Espiritu et al., 2003; Rivner, 2008). Their prolongation may be related to axonal degeneration, demyelination secondary to proximal axonal swellings or loss of fast conducting fibers (Argyriou et al., 2006; Hachisuka et al., 2015). As such, analyzing F-waves, especially the amplitude, mean and maximal F/M amplitude ratios, persistence, and the repeater F-waves, may provide an indicator of changes in spinal motoneuron pool excitability (Espiritu et al., 2003; Lin and Floeter, 2004). Intriguingly, we note that F-wave latencies and chronodispersion prolongation, persistence decline and repeater F-waves increase progressively early in patients with unaffected hands, suggesting early dysfunction of motor axons and LMNs in ALS and that subtle subclinical alterations may be reliably assessed by F-wave test.

Results Related to Split-Hand Syndrome

Our study shows a significantly decreased FDI/ADM CMAP amplitude ratio (<0.9) in ALS patients, and this finding reflects the split-hand phenomenon in ALS (Kuwabara et al., 2008; Menon et al., 2013) and presents evidence of LMN involvement. Further, there was a higher CMAP amplitude of the FDI and a lower amplitude of F-waves compared with the ADM in HCs, which has not been previously reported. The reduction in F-wave amplitude is often caused by damage to the LMNs and decreased motor neuron excitability (Taniguchi et al., 2008). The lower amplitude of F-waves, and the lower mean and maximal F/M amplitude ratios in the FDI compared with the ADM of HCs, is likely caused by physiological differences in the excitability of their motoneuron pools, and may relate to central impulses in favor of inhibition in the FDI (Menon et al., 2014c). Similar physiological differences, including lower mean F-wave amplitude, and lower mean and maximal F/M amplitude ratios, in the FDI, compared with the ADM, were also observed in ALS patients with unaffected hands. In addition, compared with the ADM, lower F-wave persistence and increased repeater F-waves were observed in the FDI in the unaffected hands group. These findings suggest a greater degree of spinal motoneuron hypoexcitability and loss of function of LMNs in the FDI. However, patterns of F-wave measurements changes in this study, especially those parameters detecting excitability of the spinal motoneuron pool (F-wave persistence, amplitudes, mean and maximal F/M amplitude ratio and repeater F-waves), were similar in FDI and ADM in ALS patients with affected

hands. The absence of differences in F-wave variables between the FDI and ADM in ALS may imply a significantly enhanced excitability of spinal motoneurons innervating the FDI. We also found a significant correlation between F-wave amplitude in the FDI with the FDI/ADM CMAP amplitude ratio, but not with the ADM, suggesting that the different changes in spinal motoneuron excitability between the FDI and ADM were associated with development of the split-hand phenomenon in ALS.

At the segment spinal motoneuron level, the excitability of the motoneuron pool may be affected by the excitatory and inhibitory central nervous system (Mastaglia and Carroll, 1985; de Carvalho et al., 2002). To further clarify the impact of UMN activity drive on the excitability of the anterior horn cells in ALS patients with split-hand, we examined the F-wave parameters in our subgroup, including persistence, amplitude, and the F/M amplitude ratio, which presumptively are influenced by the corticospinal tract and cortical activity (Lin and Floeter, 2004; Rivner, 2008; Hara et al., 2010). However, we found no differences in F-waves between the P and the NP subgroups or between the FDI and ADM subgroups. The differences in segmental motoneuron excitability were not closely correlated to UMN involvement in our study. Thus, we suspect that the pathophysiology of the split-hand may also have spinal mechanisms.

Our study has some limitations. This was exploratory research with a small sample size. Thus, more patients and follow-up studies are required to confirm our findings on spinal motoneuron excitability associated with split-hand syndrome in ALS. Because of the stimulation of the ulnar at the wrist, the distance from the stimulus site to the target muscle is considerably longer for the FDI than for the ADM. The comparison of F-wave latencies in the FDI and ADM may be of less value. Owing to the predominant involvement of LMN in ALS, signs of pyramidal lesions may be difficult to detect. Moreover, we defined pyramidal lesions in the upper limbs requiring both increased tendon reflexes in the arm and Hoffman's sign, which may lack sensitivity. So, subclinical or possible involvement of UMN cannot be excluded in the NP group, and because of the relative small sample size, the results of F-wave parameters between the P and NP subgroup need to be verified in a larger population of ALS patients. Moreover, F-waves do not allow for accurate measurement of changes in UMN excitability influenced on spinal motoneurons, and a reliable method is needed for further studies. Combining transcranial magnetic stimulation (TMS) with the F-wave test investigates the UMN involvement and spinal motoneuron excitability at the same time and on the same patient groups could elucidate the pathophysiological basis of the split-hand in ALS.

In summary, the present study draws attention to a particular pattern of F-wave abnormalities in the FDI and ADM. Spinal motoneurons innervating the FDI have physiologically greater inhibitory modulation than the ADM, and in ALS, the enhanced excitability is more prominent in spinal motoneurons innervating the FDI that is consistent with the split-hand sign. Although cortical mechanisms could also be involved, we propose that spinal motoneurons dysfunction is associated with the development of the split-hand syndrome.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Peking Union Medical College Hospital Clinical Research Ethics Committee (Beijing, China) with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Peking Union Medical College Hospital Clinical Research Ethics Committee (Beijing, China).

AUTHOR CONTRIBUTIONS

Z-LW, ML, and LC designed the experiments and/or interpreted the data. Z-LW and QD performed the experiments and analyzed the data. ML, SL, and KZ contributed to reagents, materials, and analysis tools. Z-LW and LC drafted the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2019.00371/full#supplementary-material

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MINI REVIEW
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Magnetic Resonance Spectroscopy in ALS

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Proton magnetic resonance spectroscopy (MRS) provides a means of measuring cerebral metabolites relevant to neurodegeneration *in vivo*. In amyotrophic lateral sclerosis (ALS), neurochemical changes reflecting neuronal loss or dysfunction (decreased N-actylaspartate [NAA]) is most significant in the motor cortex and corticospinal tracts. Other neurochemical changes observed include increased myo-inositol (mIns), a putative marker of gliosis. MRS confirmation of involvement of non-motor regions such as the frontal lobes, thalamus, basal ganglia, and cingulum are consistent with the multi-system facet of motor neuron disease with ALS being part of a MND-FTD spectrum. MRS-derived markers exhibit an encouraging discriminatory ability to identify patients from healthy controls, however more data is needed to determine its ability to assist with the diagnosis in early stages when upper motor neuron signs are limited, and in distinguishing from disease mimics. Longitudinal change of NAA and mIns do not appear to be reliable in monitoring disease progression. Technological advances in hardware and high field scanning are increasing the number of accessible metabolites available for interrogation.

Keywords: biomarker, magnetic resonance spectroscopy, neuroimaging, amyotrophic lateral sclerosis, neurodegeneration

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BACKGROUND AND TECHNICAL CONSIDERATIONS

Magnetic resonance imaging has emerged as a promising tool to provide a biomarker in neurological and psychiatric disorders. Routine structural MRI is not helpful in this regard in ALS as signal intensity and gross volume changes in T1 and T2 weighted images is not apparent in the vast majority of cases (1). Advanced imaging and post-processing methods are necessary to reveal pathology that is not evident to the naked eye. Numerous studies have demonstrated the potential of MRS in research and clinical care in brain disorders, including ALS. Results have been consistent amongst investigators using different methods to quantify key metabolites such as NAA, and renewed interest along with advancing technology are leading to studies probing previously inaccessible chemicals such as Gama-aminobutyric acid (GABA).

With routine structural MRI, the abundance and microenvironment of protons is quantified resulting in essentially images of the distribution of water since it is the most abundant proton-rich molecule. The most basic MRS experiment quantifies instead protons in molecules other than water. The experiment is usually a measurement from a defined volume (rather than the whole brain), and produces a spectrum rather than an image. Different peaks in the spectrum arise from different protons and their microenvironment. The positioning along the x-axis of peaks is dependent on the spin frequency of the protons contributing to the peak, with the area under the

peak dependent on the number of protons. Small shifts in frequency can occur due to magnetic field perturbations arising from nearby molecules, leading to a change the shape of a peak (singlets, doublets, triplets, etc.). The frequency of a peak and its splitting structure are key elements used in the identification of the metabolite from where the peak arises.

Images can be produced from metabolites such as NAA, however these are of much lower resolution than structural MRI (which is essentially MRS of water) because of the very low concentrations of such molecules. The lower concentration of the target metabolites also means that MRS scans are comparatively longer than routine structural imaging. Rather than a structural evaluation, MRS is a means of quantifying neurochemistry in the brain of low abundance metabolites. MR spectra can be obtained using other nuclei, including phosphorus, fluorine, carbon, and sodium. These typically require alternate hardware (e.g., specific RF coils) to that typically available with clinical and clinical research systems used for proton MRS.

Metabolites

The metabolites that are visible and quantifiable is dependent on a number of factors, and requires a sufficient concentration typically in the range of micromoles/gm. Spectral resolution and SNR must be sufficient to accurately identify and quantify individual peaks, and this is determined by many factors including B0 field strength and homogeneity, acquisition sequence (PRESS, STEAM, MEGA-PRESS, etc.), and TE, amongst others. Higher field strengths and lower TE in general give access to more metabolites.

There are a number of metabolites detectable using contemporary methods that have relevance in neurological disease (**Figure 1**). N-acetylaspartate (NAA), along with a small contribution from N-acetylaspartylglutamate, is localized only in neurons and their processes, and thus NAA serves as a marker of neuronal integrity. The total creatine peak arises from metabolites (creatine plus phosphocreatine) involved in energy metabolism. Total choline (choline, phosphorylcholine, glycerophosphorylcholine) is a marker of membrane turnover. Increased levels are reported with cell proliferation, both neuronal and glial.

Beyond NAA, there are a number of metabolites that can be measured which are of particular relevance to neurodegeneration in ALS. Myo-inositol (mIns) has a preferential distribution in glial cells, and is as such a putative glial marker. Glutamate is the primary CNS excitatory neurotransmitter. It is difficult to separate using routine MRS techniques from glutamine, and is thus may be expressed as "Glx." GABA is the primary inhibitory neurotransmitter in the brain. Glutathione functions as an antioxidant. Glutamate, GABA, and glutathione can be measured at ultrahigh field (7 T), or high field (3 T) using advanced spectral editing methods.

Acquisition

MRS can be performed using the same hardware systems as for structural imaging. The lowest field strength advised, and indeed what many papers to date report experiments from, are studies at 1.5 T. The benefits of high field imaging include access to more metabolites, shorter acquisition times, and higher spatial resolution. The former comes from increased SNR and increased chemical shift dispersion. The benefit is particularly relevant to detecting metabolites that have very low concentration or a complex resonance peak structure such as glutamate and GABA.

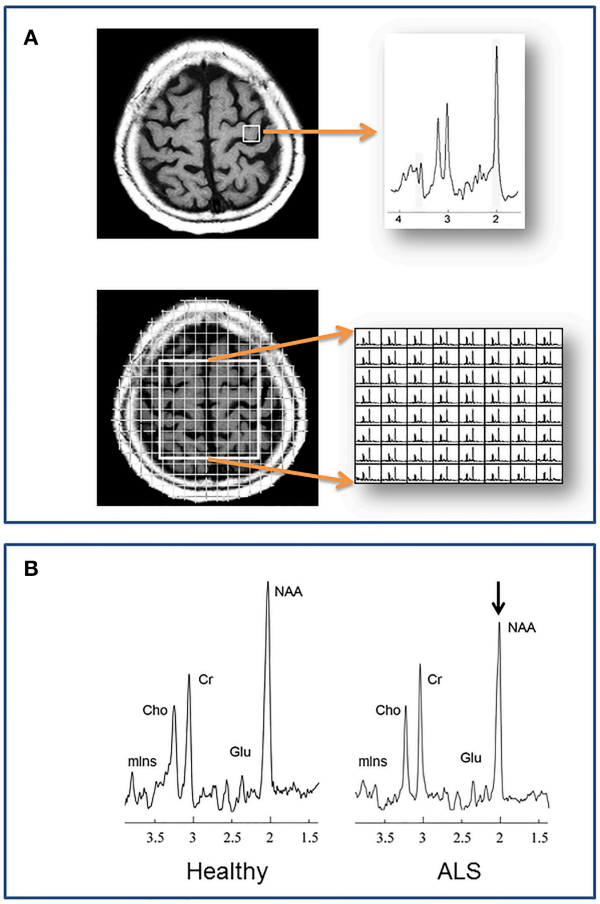

FIGURE 1 | (A) Localization methods. Neurochemical data are acquired from specified volumes during a single MRS scan. A single spectrum is recorded in single voxel spectroscopy (SVS), such as from the left precentral gyrus in the example at top. With magnetic resonance spectroscopic imaging (MRSI) multiple spectra are acquired, such as from a 2 dimensional plane centered over the central sulcus in the example at bottom. **(B)** A representative spectrum from the motor cortex of a healthy individual compared to one from a patient with ALS. N-acetylaspartate is reduced in ALS, reflecting reduced neuronal integrity. Cho, choline; Cr, creatine; Glu, glutamate; mIns, myo-inositol; NAA, N-acetylaspartate.

Unlike whole-brain structural imaging the location from where a spectrum is acquired usually must be pre-defined. Traditional localization schemes to define where spectra are acquired, include single voxel spectroscopy (SVS) and multivoxel spectroscopic imaging (MRSI) (**Figure 1**). In the former, a single spectrum is acquired from a discrete volume of interest (VOI), such as the motor cortex, internal capsule, etc. With MRSI, individual spectra are acquired from multiple regions within a 2-dimensional slab or a 3-dimensional volume. These volumes are positioned at the time of scanning, and the acquired spectra

within the volume are selected after processing. Critical steps during data acquisition, but beyond the scope of this review, are water and lipid suppression, and shimming to minimize local field inhomogeneity.

Data Post-processing and Quantification

Post-processing of data includes a number of steps (e.g., residual water suppression, Fourier transformation, phase correction), with ultimately the production of spectral peaks. These are baseline corrected and fitted. The area under a fitted peak correlates with the number of protons contributing to the signal and thus metabolite density. Processing and quantification is available on MRI consoles, or with stand-alone software such as LCModel (2).

It is paramount to be aware that a metabolite resonance reflects its contributing protons throughout the voxel being sampled, including all cell and tissue types (neurons, glia, gray matter, white matter) and compartments (intracellular, extracellular). The derivation of absolute concentrations (i.e., mmol/L) requires additional MR experiments and processes to correct, for example, partial volume effects, coil loading, field inhomogeneity, and relaxation effects with a potential concern to data reliability. Resonance signals are thus often reported as a ratio to a reference metabolite, such as Cr or Cho (NAA/Cr); this inherently performs the aforementioned correction, however it requires the assumption that the reference metabolite is stable in the disease under question. Normalization with a water signal is used by some as an alternative and obviates the issue of whether Cr or Cho are unchanged, though comes with its own issues.

Recent Advances

High and Ultrahigh Field Imaging, and "New Metabolites"

Within the last decade, research and clinical MR systems have transitioned from a low field of 1.5 T to a high field of 3 T. Studies at the latter are becoming common place, with studies at the ultrahigh field of 7 T emerging.

The benefits of high field imaging include access to more metabolites, shorter acquisition times, and higher spatial resolution. The former comes from increased SNR and increased chemical shift dispersion; this is particularly relevant to metabolites that have very low concentration or complex resonance peak structure such as glutamate, GABA, and mIns. Higher field systems are accompanied by a number of challenges that require attention for successful spectroscopy experiments: greater main (B0) and applied RF (B1) field inhomogeneity and chemical shift mis-registration, altered T1 and T2 relaxation times, greater safety concerns, and higher purchase and operating costs (3).

3D MRSI and Automated Quantification

Single voxel spectroscopy and MRSI constrain the acquisition of data from small and discrete regions (volume of interest). These spatial restrictions are necessary, in part, for optimization of field homogeneity. Thus, MRS scans demand an additional level of knowledge, expertise, and experience from the MR technologist required for accurate positioning of the VOI. Larger sampling of the brain can be done with multislice MRSI (4–6), or 3D MRSI (7), however these further increase acquisition times. Echo-planar spectroscopic imaging (EPSI) has been an exciting development as it permits high resolution volumetric (whole brain) spectroscopic imaging in a single acquisition within a clinically acceptable timeframe (8). It has been applied in ALS to study the neurochemistry of the CST in its 3-dimensional extent (9, 10), and of multiple spatially discrete areas (11, 12).

RESULTS

At the time of writing, a general survey reveals there have been just over 60 papers published describing human proton MRS experiments in ALS, with inclusion of ∼1,400 patients with ALS or related motor neuron disease (primary lateral sclerosis, progressive muscular atrophy). The majority of papers have interrogated neurochemistry of the motor system, namely the primary motor cortex and corticospinal tract. Published works also report findings in "extra-motor" regions including the prefrontal cortex, subcortical gray, brainstem, and spinal cord. Longitudinal MRS studies are few, as they are with other imaging modalities. With few exceptions, studies published since 2011 have been done at high field (3 T) or ultrahigh field (7 T).

Participants in studies have consisted of patients meeting El Escorial Criteria for ALS with combined upper and lower motor neuron signs. The number of MND participants in each study range from 7 to 169, with many studies having 10–30. Some have included subjects with no UMN signs (PMA) (6, 11, 13–16) generally showing the expected correlation of more normal NAA in such subjects. All studies have been conducted at a single center, except for a prospective multicenter study conducted at 4 sites in the Canadian ALS Neuroimaging Consortium [ClinicalTrials.gov # NCT02405182 and in press (Neurology: Clinical Practice)].

Cross-Sectional

Motor Cortex

The regional focus of most studies has been on the motor cortex or CST. NAA ratios to Cr, Cho, or Cr+Cho are reduced in the precentral gyrus (4–6, 12, 13, 15, 17–39). A decline in absolute quantities of NAA (14, 16, 21, 22, 31, 40–43) corroborate these observations of reduced ratios of NAA. A gradient effect can be observed when spectra are acquired from the motor cortex and regions immediately surrounding it, such that less prominent reductions are present in the postcentral gyrus and premotor areas compared to the precentral gyrus (13, 25).

Corticospinal Tract

The corticospinal tract has been interrogated using various methods. One group found reduced NAA/Cr+Cho) in the centrum semioval (CSO) and internal capsule combined, but not individually in these two regions (4). In part contrary to this, a study using a coronal MRSI method in the plane of the CST found reduced NAA/Cr in the precentral gyrus and corona radiata, but normal levels in the internal capsule and cerebral peduncle (44). Another found reduced NAA/Cr in both the motor cortex and

IC (32). NAA of the entire CST was found to be reduced using a whole-brain 3D spectroscopic acquisition protocol (9, 10).

Extra-Motor Regions

The presence of frontotemporal lobar degeneration (FTLD) is supported by reduced NAA indices in various frontal regions including the dorsolateral (11, 23) and mesial prefrontal (19, 45) cortices. Mesial prefrontal cortex neurochemistry is abnormal in patients who for the most part are not cognitively impaired, suggesting MRS may be more sensitive to detecting FTLD than clinical measures (45). "Extra-motor" degeneration was similarly demonstrated in the mid-cingulate gyrus (34), thalamus (34, 46), and basal ganglia (46). As expected, NAA is normal in the parietal and occipital lobes (5, 11, 23, 25, 26, 33, 40) and cerebellum (14).

Brainstem

Reductions in NAA indices are described by most (21, 43, 47, 48) but not all studies (33) that have examined the brainstem.

Spinal Cord

MRS of the upper cervical spinal cord revealed substantially reduced NAA ratios 25–40% in patients with ALS (49, 50). Notably, a single voxel was used enclosing the breadth of the cord. Thus, the spectrum included contributions from both white matter tracts and the anterior horn and other cells in the gray matter. One group extended their methods to investigate neurochemical changes in asymptomatic SOD1+ individuals (51). They found comparably reduced NAA/Cr and NAA/mIns in asymptomatic (39.7% and 18.0%) and patients with ALS (41.2% and 24.0%) compared to healthy controls, inferring the presence of neurochemical changes early in the disease and even before symptoms or signs are present.

Other Metabolites

Reflective of astrogliosis, mIns is increased in the motor cortex (29, 40, 43, 48, 52, 53). The NAA/mIns ratio may be a more robust marker of degeneration as it reflects the combined pathology of decreased neuronal integrity and gliosis with the individual metabolite levels becoming abnormal in opposite directions in the motor cortex (16, 29, 48) and mesial prefrontal cortex (45).

Given one of the putative pathophysiological mechanisms is excitotoxicity, one may have expected Glu to be increased. However, results have been conflicting for the motor cortex where it (or Glx) were normal (16, 21, 43, 52), increased (32), or decreased (40). Studies at 7 T where its quantification may be more precise were conflicting with levels in the motor cortex normal (48) or increased (53). Glx was increased in the medulla (54) along with a negative correlation with the ALSFRS bulbar subscore. Later studies of the pons revealed normal pontine Glu or Glx (43, 48). MRS measurements of the inhibitory neurotransmitter GABA in the motor cortex have been reported to be reduced using the MEGA-PRESS technique at 3T (43, 55), but normal using a STEAM sequence at 7T (53). As discussed above MRS measurements will largely reflect the intracellular metabolic rather than synaptic neurotransmitter pool; as such, reductions may simply be the result of neuronal loss.

Initial findings of decreased glutathione in the primary motor cortex (35) which would have been supportive of a role for oxidative stress in the pathogenesis of ALS were not replicated by subsequent studies at 3 T or 7 T (48, 53).

Diagnostic Accuracy

A number of studies have assessed the discriminatory power of NAA and its ratios in the motor cortex to separate ALS patients from healthy controls. Sensitivity ranges from 53 to 100%, specificity ranges from 37 to 100%, with the average amongst the studies ∼80% for both. MRS improves the accuracy when combined DTI assessment of the corticospinal tract (56, 57) or of signal change on structural imaging (36, 56).

Longitudinal

A number of studies suggest a decline in NAA indices over varying intervals; interpretation of these reports is difficult due to small numbers of patients (5, 19, 22, 52, 58, 59).

In a more rigorous design, longitudinal change in absolute NAA and its ratio to Cr and Cho were measured every 3 months out to 1 year. Changes were seen in the motor cortex and outside the motor cortex over 3 and 9 months, respectively depending on the El Escorial designation, but overall did not follow a consistent pattern (27). In a treatment trial of growth hormone, the placebo arm of 20 patients did not have any change in motor cortex NAA/(Cho+Cr) at 0, 6, or 12 months (60).

In a larger study of 43 patients, 30 had at least one follow up scan on a 3 month interval, demonstrating a non-significant ($p = 0.06$) decline in motor cortex NAA/Cr (6).

Recently, longitudinal neurochemical observations were made at 7 T at 6 and 12 months. Motor cortex NAA/mIns declined and pontine Glx increased. In a sub group analysis, this pattern of neurochemical change was not present in those whose upper limb and bulbar function did not deteriorate over time (61).

Correlations

The presence of correlations with an imaging finding provides a degree of biological validity to the imaging metric. Not surprisingly, NAA indices are more reduced in patients with a greater severity of UMN findings on neurological examination (13–15, 20, 28, 31, 40, 52), however this is not always the case (48). As a measure of UMN function, finger tapping has the advantage of being objective and providing a continuous measure. Correlations with tapping have been reported in a number (4, 6, 16, 18, 39), but not all, (44) studies. A few studies have also noted a correlation with the El Escorial criteria (15, 27, 48). Reports are conflicting with respect to associations with disease duration, progression rate, or disability as quantified by ALSFRS-R. With respect to the latter this is not surprising given that disability is largely driven by muscular weakness which in turn is dependent considerably on LMN status.

The evaluation of neurochemical associations with cognitive or behavioral impairment is limited in ALS. As would be expected dorsolateral prefrontal cortex NAA/Cr correlates with cognitive measures of executive function, including verbal fluency (11) and the Wisconsin Card Sorting Test (23). However, mesial prefrontal cortex NAA/mIns did not correlate with the Addenbrook Cognitive Examination or verbal fluency (45); this may have been due to the localization of the voxel (mesial rather than

dorsolateral) or that the ACE may not be an optimal cognitive screening measure in ALS (62).

The marked clinical heterogeneity of patients with ALS makes prognostication a difficult task, yet this would be extremely helpful in clinic for counseling patients and to assist as an enrichment strategy in clinical trials. MRS was the first neuroimaging modality to reveal an association of cerebral degeneration with survival. Reduced motor cortex NAA/Cho was the strongest predictor of shorter survival, followed by older age and shorter symptom duration (30).

MONITORING TREATMENT

There have been several studies evaluating treatment effects using MRS. The commencement of riluzole, an antiglutamatergic agent, is accompanied by an increase in NAA/Cr in the motor cortex observed at 1 day (63) and 3 weeks (58) after its initiation. Increases in NAA/Cr suggest the existence of a population of metabolically dysfunctional neurons amenable to treatment. This supposition is supported by the observation of maintained NAA/Cr levels in ALS patients in contrast to a decline in NAA/Cr in healthy controls who received creatine supplementation (24). Changes in NAA/Cr were not observed with gabapentin (25), intrathecal BDNF (26), or minocycline (64). Preliminary observations have also been made on the Glx signal with creatine supplementation (24, 65). In contrast to the studies discussed thus far, there have been reports from studies that have performed sub-analyses on patients comparing those who are taking riluzole to those who are riluzole-naïve (43, 48); these have had varying results.

CONCLUSIONS AND FUTURE DIRECTIONS

What has MRS delivered in the field of ALS thus far, and what is needed?

Cross sectional changes reflecting cerebral neuronal impairment (abnormal NAA indices) are consistently present, and with reasonable accuracy in discriminating patients from controls in group analysis. However, with regards to diagnostic utility, a biomarker of cerebral degeneration will be most helpful in the clinic for patients presenting with LMN signs but insufficient UMN signs; MRS data (as for much of the neuroimaging field) is lacking for such patients. In the more immediate future, MRS should be able to play a part in addressing phenotypic heterogeneity, as associations have been demonstrated with various behavioral measures. Future studies addressing diagnostic potential and heterogeneity would benefit from larger sample sizes, deep phenotyping, inclusion of disease mimics, incorporation of other imaging modalities (e.g., DTI), and incorporation of biofluids for correlative and validation analyses. Of note, there is very little known of the association of cerebral neurochemicals with cognitive impairment in ALS.

There is sensitivity to measuring longitudinal change in metabolites that appears best observed with time intervals of at least 3 months. However, there is considerable variability, which currently prohibits its use as a biomarker of disease progression. The experience of MRS to date of assessing response to therapy has been largely proof of principle. Progress in this area has been hampered in part by the lack of robust disease modifying therapies upon which to frame spectroscopy experiments. Inclusion of MRS in phase II clinical trials may provide opportunities validating metabolites as measures of disease progression, target engagement, or therapeutic response.

The feasibility for MRS to be applied for clinical and routinely for research applications, especially for multicenter efforts and to allow inter-study comparison of results, will require refinement, optimization, and standardization of acquisition and processing protocols, in parallel with greater user expertise. Reference to general (66) and ALS-specific guidelines (67) are starting points for such an endeavor. The advent of whole brain MRSI combined with automated quantification is a significant advancement that could facilitate the modality's uptake to more research labs and eventually clinics.

Advances in technology (higher fields, new sequences) are already permitting the quantification of previously undetectable disease-relevant metabolites and of anatomical regions previously inaccessible (spinal cord). This will continue to provide opportunities for exploring biological insights *in vivo* and for evaluating novel disease markers that may meet the desperate need of a biomarker in ALS.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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A Systematic Review of Suggested Molecular Strata, Biomarkers and Their Tissue Sources in ALS

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Amyotrophic lateral sclerosis (ALS), also known as motor neuron disease, is an incurable neurodegenerative condition, characterized by the loss of upper and lower motor neurons. It affects 1–1.8/100,000 individuals worldwide, and the number of cases is projected to increase as the population ages. Thus, there is an urgent need to identify both therapeutic targets and disease-specific biomarkers–biomarkers that would be useful to diagnose and stratify patients into different sub-groups for therapeutic strategies, as well as biomarkers to follow the efficacy of any treatment tested during clinical trials. There is a lack of knowledge about pathogenesis and many hypotheses. Numerous "omics" studies have been conducted on ALS in the past decade to identify a disease-signature in tissues and circulating biomarkers. The first goal of the present review was to group the molecular pathways that have been implicated in monogenic forms of ALS, to enable the description of patient strata corresponding to each pathway grouping. This strategy allowed us to suggest 14 strata, each potentially targetable by different pharmacological strategies. The second goal of this review was to identify diagnostic/prognostic biomarker candidates consistently observed across the literature. For this purpose, we explore previous biomarker-relevant "omics" studies of ALS and summarize their findings, focusing on potential circulating biomarker candidates. We systematically review 118 papers on biomarkers published during the last decade. Several candidate markers were consistently shared across the results of different studies in either cerebrospinal fluid (CSF) or blood (leukocyte or serum/plasma). Although these candidates still need to be validated in a systematic manner, we suggest the use of combinations of biomarkers that would likely reflect the "health status" of different tissues, including motor neuron health (e.g., pNFH and NF-L, cystatin C, Transthyretin), inflammation status (e.g., MCP-1, miR451), muscle health (miR-338-3p, miR-206) and metabolism (homocysteine, glutamate, cholesterol). In light of these studies and because ALS is increasingly perceived as a multi-system disease, the identification of a panel of biomarkers that accurately reflect features of pathology is a priority, not only for diagnostic purposes but also for prognostic or predictive applications.

Keywords: circulating biomarkers, ALS, patients stratification, multi-system biomarkers, motor neuron disease

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurological disorder with an adult onset around 54–67 years old (1). Its clinical hallmark is the degeneration of both upper and lower motor neurons (2, 3), leading to progressive muscle atrophy and weakness, and ultimately to paralysis. Death, often resulting from swallowing problems and respiratory failure (4, 5), generally occurs within 2–4 years from disease onset (6–8), although 5–10% of ALS patients survive over 10 years (7). ALS has a median incidence of about 2.8 cases per 100,000 persons per year and a median prevalence about 5.4 cases per 100,000 persons for a median age at 61.8 ± 3.8 years (1). The incidence and prevalence thus increases with age and reaches a cumulative lifetime risk of 1 in 400 after 80 years old (9, 10). Due to the projected aging of the global population, ALS cases are expected to increase by 69% in the next 25 years (11), underlining the urgent need to identify causes, biomarkers and therapeutic targets for ALS.

The causes of ALS are largely unknown, with ∼90% of cases being sporadic (sALS) while only ∼10% are familial ALS (fALS) (12). Intensive research since the 1990's has aimed to unravel the mechanisms involved in motor neuron degeneration. These studies suggest that ALS is a complex disease driven by a combination of several systemic parameters. To date, up to 30 genes (**Figure 1**) are described as monogenic causes of ALS, with the most frequent being C9orf72, SOD1, FUS, and TARDBP/TDP43 (13–15). In motor neurons, these identified mutations are functionally associated with an alteration of electrophysiological properties (16), accumulation of stress marks (17) and sensitivity to stress (18) (**Figure 2**). However, these monogenic forms

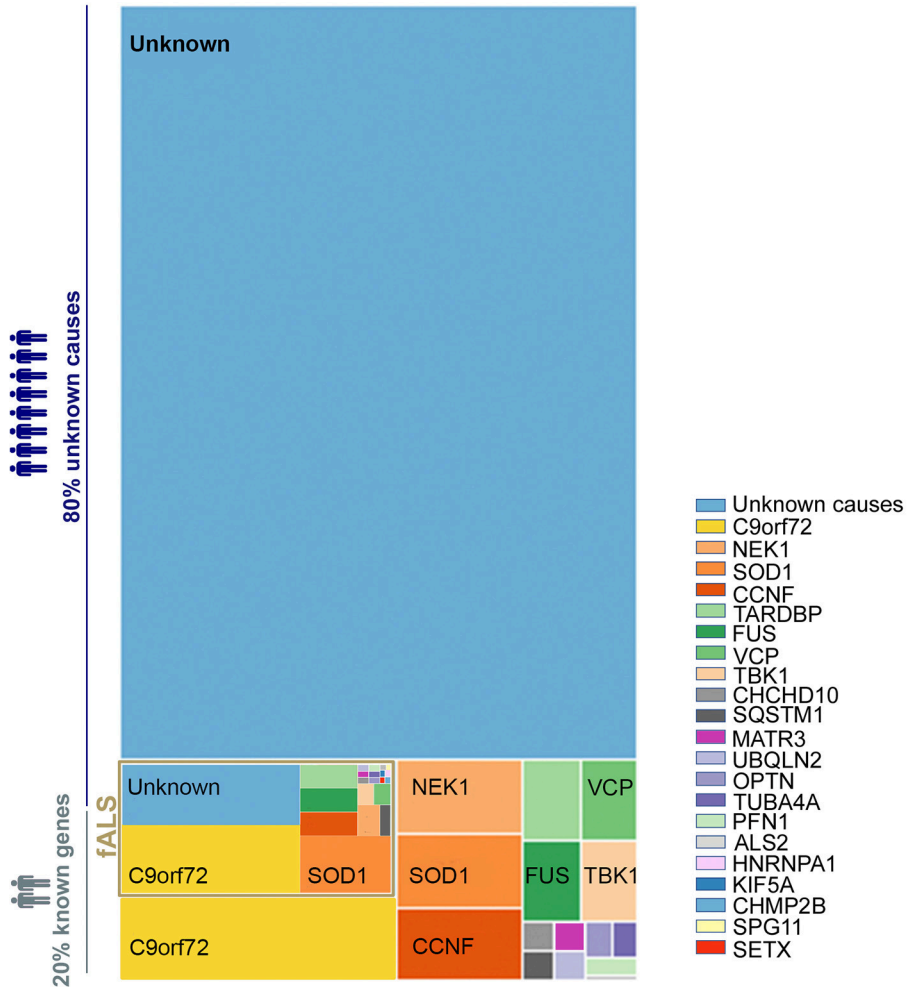

FIGURE 1 | Distribution of genetic basis among the ALS population. A treemap representation of the proportion of ALS patients carrying known causative mutation. The full rectangle represents 100% of all ALS cases. The fALS are highlighted in gold with a frequency adjusted to represent 7.5% of the total (as fALS is estimated at 5–10% of all ALS cases). The two light blue blocks represent those with no known ALS-associated gene mutation among sporadic and familial cases. Cases with known mutations are represented in the other blocks, broken down by affected gene. The color code for each gene is preserved between familial and sporadic cases. The size of each block is proportional to the percentage of ALS associated to the considered genes–proportions given in Volk et al. (13). Overall, some 80% of ALS cases (sALS and fALS combined) are not explained by a known mutation.

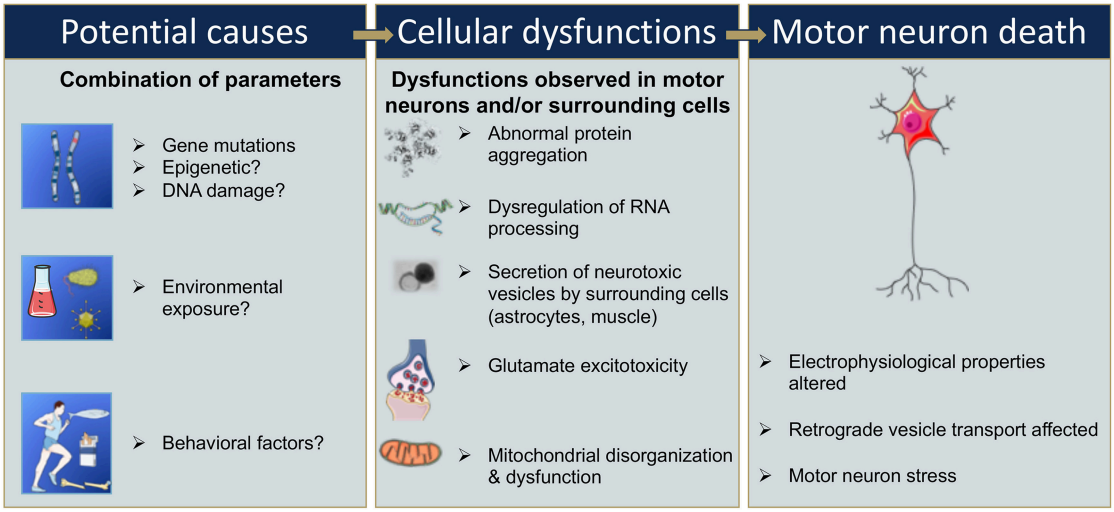

FIGURE 2 | Sequential events that could be involved in motor neuron death in ALS. Gene mutations, epigenetic changes, or DNA damage that occur either spontaneously or due to environmental risk factors such as exposure to toxins or infectious agents, or behavioral factors, have all been proposed as potentially leading to cellular dysfunction (9, 13, 14, 20–23). Cellular dysfunction could include abnormal protein aggregations, alteration of RNA processing, secretion of neurotoxic vesicles by surrounding cells such as astrocyte, muscle cells, glutamate excitotoxicity, and mitochondrial disorganization and dysfunction leading to oxidative stress (24–30). These cellular dysfunctions may take place in motor neurons and/or surrounding cells and, combined or alone, could lead to an alteration of the electrophysiological properties of the motor neuron, and/or to an induction of secretion of neurotoxic elements by surrounding cells, in either case ultimately leading to motor neuron death (16–18).

explain only 15% of sporadic cases and 66% of familial cases (12) (**Figure 1**).

Furthermore, the penetrance of these disease-associated mutations is quite variable and can increase with age (12, 19). The variability in penetrance as well as the lack of identification of a single associated gene mutations in 85% of sALS suggests that some ALS cases have a multigenic component, and/or involve epigenetic modification, and/or result from DNA damage, environmental risk factors, or viral infections (9, 14, 20–23) (**Figure 2**). In these cases, it is likely a combination of these factors that leads to cellular dysfunction such as glutamate-mediated excitotoxicity (24), abnormal protein aggregation (25), mitochondrial disorganization and dysfunction (26, 27) contributing to the oxidative stress (28–30) (**Figure 2**). Adding to the complexity of ALS, several studies suggest that not only the motor neurons are affected but also the surrounding cells, and that these cells participate in the propagation and burden of the disease. For instance, activated microglia cells release superoxide and nitric oxide metabolites, elements that are toxic to neuronal cells (31). Astrocytes can also participate in the propagation of neurotoxic elements (32, 33) such as SOD1 aggregates (34–36), and a failure of astrocytes to remove extracellular glutamate may mediate excitotoxicity (37–39). Ultimately, the intracellular dysfunction of the motor neuron combined with aberrant secretion of neurotoxic elements of surrounding cells leads to motor neuron stress, aberrant electrophysiological properties, and consequently to motor neuron death (**Figure 2**).

In the absence of a reliable diagnostic test for ALS, diagnosis is based on clinical and electrophysiological criteria such as evidence for progressive involvement of both upper and lower motor neurons and exclusion of diseases mimicking ALS as set out in the Revised El Escorial Criteria (REEC), Airlie House criteria (AHC) and Awaji criteria (2, 40). The process of diagnosis can be lengthy and there is a typical diagnostic delay of 9-15 months from onset to diagnostic confirmation (41). Considering that the average survival from onset is 2–4 years (6–8) and that efficacy of Riluzole is improved by early treatment (42), there is an urgent need to improve diagnostic speed and accuracy for ALS. One way of achieving this is the identification of biomarkers specific to ALS pathology, to enable the development a reliable fast diagnostic test. As well as diagnostics, it is also important to identify prognostic biomarkers that can be used to monitor the status of the pathology–various candidates may serve both these purposes. The identification of ALS biomarkers will contribute to a better understanding of the disease pathogenesis, and permit targeted drug development and patient stratification for more efficient clinical trials, assuming that different sub-cohorts of ALS patients respond differently to treatments. Biomarker discovery can be achieved by examining the "omics" contents of ALS patient tissues.

The present review has two aims: (1) to identify pathways commonly affected in genetic forms of ALS, and stratify the patients accordingly, and (2) to explore previous genomic, transcriptomic, proteomic, metabolomic and miRNomic studies of ALS published during the last decade, and summarize the findings, highlighting potential biomarker candidates for ALS disease management and treatment.

Genetic Markers for ALS Patient Stratification

The first gene identified to be associated with ALS was SOD1 in 1993 (43). Since then 29 new genes have been identified (13–15),

representing the most frequent genetic mutations included in current diagnostic processes (13, 44) (**Figure 1**). These 30 genes offer crucial clues in understanding the pathogenesis of ALS—some of the gene products interact with each other (14)—and enable the identification of diverse cellular pathways that are disrupted in ALS patients (**Table 1**). Even if most ALS cases are sporadic, the pathways disrupted in familial cases may also be affected in sporadic cases, as both sALS and fALS can share common molecular signatures or functional biological effects such as FUS or TDP43 protein aggregations or accumulation of stress granules formation (45), disruption in RNA processing (46), or disruption of autophagy and mitochondrial functions (47). When sorting the genes associated to ALS according to their primary cellular functions, several categories of dominantly affected pathway can be highlighted, such as (1) mitochondrial metabolism and turnover, (2) axonal transport and the cytoskeleton, (3) autophagy and proteostasis, (4) endosomal and vesicular trafficking, (5) DNA repair, and (6) ribostasis/RNA alteration/Nucleocytoplasmic transport—with most of the genes being involved in multiple pathways. It may be possible to group patients into strata depending on which combination of pathways is dysregulated, and to recruit patients accordingly for translational research and clinical trials. We have cautiously assigned each causal gene to one of 14 strata, depending on the profile of its affected pathways (**Table 1**). These groupings represent our effort to summarize current understanding and are not intended to be definitive—indeed, it will be important to modify and update them on an ongoing basis with improvements in the knowledge of protein function and the impact of mutations. Although these 14 strata are directly applicable to only 20% of total ALS cases (**Figure 1**), future work may determine whether (and which of) these molecular signatures are implicated in the remaining cases.

The Search for Circulating Biomarkers

The identification of circulating markers associated with ALS pathology would be important tools to provide early disease diagnosis and to track progression or treatment. There has been a concerted focus aimed at identifying such biomarkers in different body fluids over the past 20 years. In **Table S1**, we summarized 76 studies that investigated proteins, miRs, mRNAs, and metabolites as potential biomarkers in cerebrospinal fluid (CSF) or blood (blood cells, serum or plasma). To date, little has been done investigating urine-based biomarkers, and thus urine biomarker analyses are not reported in the current review. CSF is the most frequently used sample source, and several studies (**Table S1**) report a consistent decrease in protein levels of transthyretin—involved in neurogenesis, nerve repair and axonal growth (171)—and cystatin c—an endogenous cysteine protease inhibitor that can protect motor neurons against neurotoxicity by stimulating autophagy and inhibition of cathepsin B (172). In addition, CSF cystatin C protein levels positively correlated with the survival of ALS patients and could be thus potentially used as a prognostic biomarker (173). However, both transthyretin and cystatin C decreases are not specific to ALS patients and a similar pattern is observed in other neurodegenerative diseases (173) such as Alzheimer's (171), suggesting that the protein levels of both transthyretin and cystatin C level are a common signature for neuron vulnerabilities and neurodegeneration. The protein levels of neurofilament light chain (NF-L) and the phosphorylated form of neurofilament heavy chain (pNFH) were also consistently found to be increased in the CSF of ALS patients across multiple studies (**Table S1**), with a high level of either NF-L or pNFH predicting a shorter life expectancy (174–178). NF-L and pNFH are markers for axonal damage (179). In this context, similarly to M-creatine kinase for myofiber fragility in muscular dystrophy (180), NF-L and pNFH thus directly reflect the health of the neurons –the cells specifically impacted by ALS.

Combining NF-L and pNFH with other markers that reflect the "health status" of other tissues such as glial cells, skeletal muscle, or inflammatory response, may represent a useful addition, as ALS is now perceived as a multisystemic disease. Such a multi-marker approach may represent a useful complement to a panel of biomarkers to test the efficacy of drugs in clinical trials. In this respect, miR-451—an inhibitor of microglial cell activation (181)—was consistently decreased in leukocytes of ALS patients (**Table S1**), while the pro-inflammatory MCP-1, secreted by the glial cells and neurons (182), was found to be increased in both serum and plasma (**Table S1**). Both miR-451 and MCP-1 could thus potentially inform the status of inflammatory cell recruitment and activation (181, 182). In addition, miR-206, which is essential for skeletal muscle growth and regeneration (183), as well as miR-338-3p, a regulator of neuromuscular junctions (184), are consistently upregulated in leukocytes—with miR-206 also consistently reported to be upregulated in serum and plasma samples across multiple studies (**Table S1**). In this context, miR-206 and miR-338-3p could be clinically useful candidate biomarkers of the health status of skeletal muscle (185).

Regarding circulating mRNAs, no obvious consistent candidates have been identified yet across previous studies (**Table S1**). With regard to analyses of circulating metabolite candidates, huge variation is observed between studies, though there was a general tendency for upregulation of specific metabolites in serum and plasma (**Table S1**), which is consistent with the hypermetabolism observed in some ALS patients (186). For instance, creatine, which is linked to cell energy metabolism, was consistently increased in CSF and plasma across studies (**Table S1**). Pyruvate and glucose were also found to be increased in CSF and plasma of ALS patients (**Table S1**), potentially reflecting a dysregulation of glycolytic metabolism as observed in SOD1-G93A motor neurons (187), and in some ALS cases (188, 189). This upregulation of glycolysis correlates with a shorter survival time and thus could be used as a prognostic biomarker (188, 189). Similarly, the upregulation of cholesterol and LDL observed in CSF and plasma across studies (**Table S1**) could also reflect a global dysregulation of lipid metabolism in ALS patients (190, 191). Other neurotoxic metabolites, such as homocysteine, were consistently increased in all body fluids (**Table S1**). Altogether, these data suggest a global dysregulation of the energy metabolism in ALS patients.

Other types of molecules could be investigated as biomarkers in ALS, such as long non-coding RNA (lncRNA), which can act in cis to either silence or enhance the expression of proximal genes

TABLE 1 | Summary of the 30 genes presently known to have monogenic association with ALS, and their primary functions.

		Pathways affected											
Gene name, full name	**References**	**Mitochondria**	**Axonal transport**	**Cytoskeleton**	**Autophagy**	**Proteostasis**	**Endosomal trafficking**	**Vesicular trafficking**	**DNA repair**	**Ribostasis**	**Global RNA alteration**	**Nucleocytoplasmic transport**	**Suggestion of possible Strata**
SOD1, Cu,Zn-xuperoxide dismutase-1	(48–52)	x			x	x							1
OPTN, Optineurin	(53–58)	x			x								
CHCHD10, Coiled-coil-helix-coiled-coil-helix domain containing 10	(59–63)	x				x							
NEK1, NIMA related kinase 1	(64–71)	x		x					x				2
KIF5A, kinesin family member 5A	(15,72,73)			x									3
NEFH, Neurofilament heavy subunit	(74–80)		x	x									
TUBA4A, Tubulin alpha 4a	(81–86)		x	x									
DCTN1, Dynactin subunit 1	(87–90)		x					x					4
PFN1 , Profilin 1	(55,91–94)		x	x		x							5
ELP3, Elongator protein 3	(95–98)			x						x	x		6
C9orf72, Chromosome 9 open reading frame 72	(18,99–106)				x	x	x				x	x	7
CHMP2B, Charged multivesicular body protein 2B	(50,107–114)				x	x	x						8
VCP, Valosin-containing protein	(50,55,115–121)				x	x	x						
FIG4, Phosphoinositide 5-phosphatase	(18,50,122–124)				x		x	x					
VAPB, Vesicle-associated membrane protein B	(55,125–129)					x		x					
UBQLN2, Ubiquilin 2	(55,130–134)				x	x							9
TBK1 , TANK binding kinase 1	(55,58,64,135)				x	x							
SQSTM1, Sequestosome 1	(50,55,136,137)				x								
CCNF, Cyclin F	(55,138–140)					x							
TARDBP, TAR DNA binding protein	(45,50,138,141,142)					x				x	x		10
hnRNPA1, Heterogeneous nuclear ribonucleoprotein A1	(138,143,144)					x				x	x		
hnRNPA2B1, Heterogeneous nuclear ribonucleoprotein A2/B1	(144–148)					x				x	x		
ALS2, Alsin	(65,149–151)						x						11
SPG11, Spatacsin vesicle trafficking associated	(152,153)							x					
C21orf2, Cilia and flagella associated protein 410	(154)								x				12
SETX, Senataxin	(155–158)								x	x	x		13
FUS, Fused in sarcoma	(55,138,159–162)								x	x	x		
ATXN2, Ataxin 2	(45,138,163,164)									x	x		14
ANG, Angiogenin	(165–168)									x			
MATR3, Matrin 3	(138,169,170)										x		

The list of genes is taken from Volk et al. (13) and Chia et al (14). The references given in the second column indicate papers providing experimental evidence of the primary pathways (or molecular functions) affected in cell and animal models harboring the respective mutation. These pathways are given in columns 3-13–it should be noted that our understanding of the implicated pathways may change in future as more is known regarding the effects of mutations. We grouped together pathways commonly affected across genetic forms of ALS, and we suggest 14 potential strata based on the profiles of affected pathways. These groupings represent our effort to summarize current understanding and are not intended to be definitive–indeed, it will be important to modify and update them on an ongoing basis as the knowledge of protein loss and gain of function improves.

TABLE 2 | Circulating biomarker candidates consistently observed and confirmed across studies.

Biomarker categories	Biomarker name	CSF	Serum	Plasma	BMC/ Leukocytes	References
Proteins	Transthyretin	↓↓				(203,204)
	Cystatin C	↓↓↓↓↓				(173,175,203–205
	A peptic fragment of the neurosecretory protein VGF	↓		↓		(205,206)
	C-reactive protein (CRP)	↑	↑			(204,207)
	Neurofilament heavy chain phosphorylated pNFH	↑↑↑↑	↑	↑		(174–178)
	Neurofilament light chain (NF-L)	↑↑				(174,208)
	Chitotriosidase	↑↑				(175,179)
	MCP1- alpha		↑	↑		(209,210)
	TDP43				↑↑	(211,212)
miRs	miR-451				↓↓	(184,213)
	miR-338-3p				↑↑	(184,214)
	miR-206		↑↑↑	↑	↑	(169,202,215–217
	miR-133b		↑↑			(217,218)
Metabolites	Pyruvate	↑		↑		(219,220)
	Ascorbate	↑↑				(219,221)
	Glutamine	↓	↓	↓		(222–224)
	Aspartate	↑		↑		(225,226)
	Serine	↑		↑		(224,225)
	Glucose	↑↑				(227,228)
	Creatine	↑		↑↑		(220,224,228)
	Creatinine	↓		↓↓		(220,224,228)
	α-hydroxybutyrate	↑		↑↑		(220,224,228)
	Cholesterol	↑		↑↑		(190,224,229)
	Homocysteine	↑	↑	↑↑		(230–232)
	Glutamate		↑	↑↑		(223,226,233)

*This table is a summary of the detailed **Table S1**. Data are organized per category of molecule investigated. In each category, the source material is indicated as follows: Gold = CSF, Light blue = Serum, Dark Blue = Plasma, Gray = Blood cells. ↑ = Concentration increased in ALS patients compared to controls, ↓ = Concentration decreased in ALS patients compared to controls. The number of arrows indicates the number of papers describing the increase or decrease of the biomarker considered, in a given tissue. The papers describing these changes are referenced in the last column.*

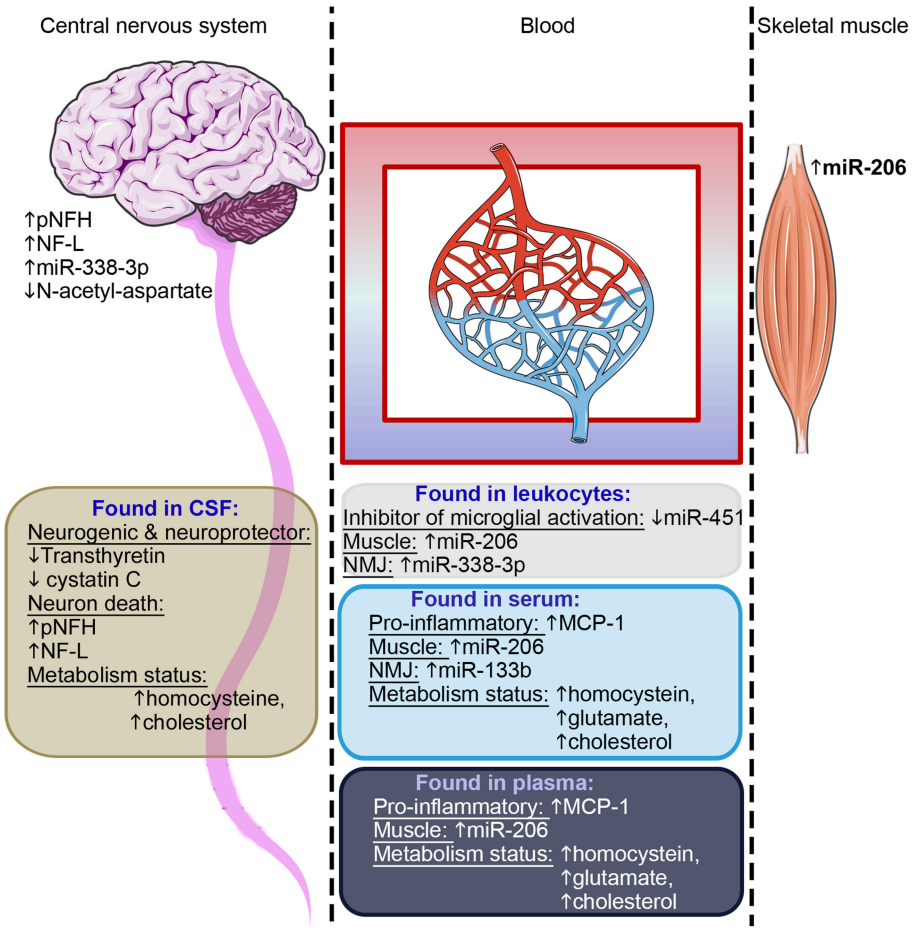

FIGURE 3 | Summary of candidate biomarkers consistently found across studies. Candidates observed in CSF are highlighted in brown, in leukocytes in gray, in serum light blue and in plasma dark blue. These candidate biomarkers reflect the motor neuron health, the inflammatory status, skeletal muscle health, and metabolism status–as indicated in each text block. Some of these candidates were found in postmortem central nervous tissue or on muscle biopsies. NMJ, neuromuscular junction.

(192) and which are known to have a key role in normal neuronal development, as well as in development and progression of neurodegenerative diseases [see (193) for review]. The lncRNA have also been detected in body fluids and have been suggested as potential diagnostic and/or prognostic biomarkers in, but not only, lung cancer (194), triple negative breast cancer (195) and cardiovascular diseases (196). In this context, lncRNA could be investigated as new biomarker candidates for neurodegenerative diseases (193), including ALS.

EXPLORING POTENTIAL ALS SIGNATURES IN TISSUE

Studying changes at the molecular level of specific tissues affected in ALS should improve our understanding of the disease mechanisms and multi-systemic impact.

Postmortem brain or spinal cord have been widely investigated. Accumulation of pNF-H and NF-L in brain tissue (**Table S2**) positively correlate with the accumulation of these markers in CSF (**Table S1**), and may be reflective of motor neuron breakdown (179). Similarly, miR-146a and miR-338-3p, both increased in spinal cord (**Table S2**), are also detected at a greater level in circulating blood cells of ALS patients (**Table S1**). These two miRNAs are involved in the regulation of the inflammatory response (197) and the neuromuscular junction (184, 198). In addition, miR-206, a skeletal muscle growth regulator (183), is increased in ALS muscles across studies [**Table S1**, 2 studies show significant increases (199, 200), the third study only shows a tendency toward an increase in levels (201)]. Together these data reinforce the suggestion that these candidate biomarkers may have utility in determining the status of motor neurons, inflammatory cells and muscle in ALS at different stages of the disease.

When looking at the proteomic and transcriptomic signature of ALS tissues, most observations have not been reproduced across studies. This lack of repeatability could be attributed to numerous factors, such as: different study populations; different types of control subject; different sample sources; different stages of the disease; and the use of different methodological strategies (**Table S2**).

However, when looking at the different pathways affected in nervous or muscle tissues, we can identify dominant signatures. For instance, skeletal muscle exhibits a dysregulation of pathways involved in muscle atrophy/growth, cytoskeletal maintenance and metabolism, while the central nervous system exhibits inflammatory and excitotoxicity features accompanied by disruptions in axonal transport, cell death, autophagy, metabolism, and RNA processing (**Table S2**). Concordantly, the systematic decrease of N-acetyl-aspartate observed *in vivo* by magnetic resonance spectrometry in the central nervous system across studies reflects (**Table S2**) neuron degeneration. These markers likely capture most strongly the endpoints of ALS disease, including degeneration processes in motor neuron death, and muscle denervation and atrophy, and it will be important for future studies to identify biomarkers that track early features of the disease.

CONCLUSION

The number of monogenic forms, combined with potential multisystemic contributions to ALS pathology, render it difficult first to unravel physiopathological events, and then to understand which of these events could be pharmacologically targeted. However, by taking a wide-angle view of the pathways affected in different monogenic forms of the disease, it is possible to discern patient strata, with each stratum potentially representing a separate target for therapeutic intervention. Such a strategy is directly applicable to monogenic forms of ALS—known in ∼20% of current ALS cases—and future work may discover the extent to which each of these potential targets are transferrable to the 80% of cases in which causal links (genetic or otherwise) have not been identified. Identifying biomarkers to diagnose ALS patients and predict their progression (prognostic biomarkers) may also lead to the identification of patient strata in these non-causally linked forms of ALS.

Identifying such biomarkers in ALS is a significant challenge as it involves the assessment, not only of motor neuron health status, but also that of other cell types affected in ALS such as astrocytes, microglia, skeletal muscle and inflammatory cells. In this review, we collated across a large number of recently published studies on ALS biomarkers covering several different cell and tissue types (76 studies on body fluids and 42 studies on tissues), and identified only a relatively few candidates that are consistently identified as potential biomarkers across multiple independent studies. These candidate biomarkers are predominantly reflective of motor neuron health, the inflammatory status, and skeletal muscle health (**Figure 3**). As ALS is increasingly recognized as a multi-systemic disease, it is thus important to track the progression or the recovery of these multiple tissues during clinical trials. In addition, some of these candidates have been confirmed in murine models, e.g., miR-206 in SOD1-G93A mice reflects disease progression in the murine model (202), making them interesting candidates for assessment in pre-clinical studies. As a multi-systemic disease, it is likely that a panel of biomarkers will be needed to fully capture features of ALS pathology.

Considering the different source tissues and the potential implication of each of these in the pathology, our capacity to detect them in accessible fluids, and also the desire to have biomarkers that are confirmed in multiple studies, we would suggest that a useful approach to obtain an overall picture of disease progress in any given patient, may be to combine biomarker candidate molecules from across those listed in **Table 2**. For example, of biomarkers confirmed in multiple studies, we could suggest a panel of Cystatin C, pNFH and NF-L, all reflecting neuronal survival, MCP1 as a pro-inflammatory marker, the MiRs 206 and 133b reflecting muscle origin and neuromuscular junction, respectively, and some indicators of dysregulated metabolism such as homocysteine, glutamate, or cholesterol. Such a panel (or a variation of it with similarly diverse properties in terms of tissue origin), would be useful to assess the overall "health status" of different tissues. However, all of the biomarkers so far proposed require further validation, as would any specific combination of them.

The development of a heterogeneous multi-biomarker panel—likely including robust new biomarkers and the biomarkers cited in this report—could be seen as a priority, not only for diagnostic purposes but also for prognostic or predictive applications.

AUTHOR CONTRIBUTIONS

UV, VM, and MS collated the data from the literature, and wrote the paper. WD and SD organized the data, wrote the paper. AB, WD, and SD edited the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2019.00400/full#supplementary-material

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BRIEF RESEARCH REPORT
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Increased Interleukin-6 Levels in the Astrocyte-Derived Exosomes of Sporadic Amyotrophic Lateral Sclerosis Patients

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Neuroinflammation plays an important role in amyotrophic lateral sclerosis (ALS) pathogenesis. However, it is difficult to evaluate inflammation of the central nervous system (CNS) or the relationship between neuroinflammation and disease progression in ALS patients. Recent advances in the field of exosomes and CNS-derived exosomes extraction technology provide the possibility of measuring the inflammatory status in the CNS without brain biopsy. In this pilot study, we extracted astrocyte-derived exosomes from the plasma of sporadic ALS patients and age-, sex-matched healthy controls and determined Interleukin-6 (IL-6) levels by an enzyme-linked immunosorbent assay (ELISA). The IL-6 levels in astrocyte-derived exosomes were increased in sALS patients and positively associated with the rate of disease progression. However, the association between IL-6 levels and disease progression rate was limited to patients whose disease duration were less than 12 months. These data suggest an increased inflammatory cascade in the CNS of sALS patients. Our pilot study demonstrates that CNS-derived exosomes could be useful to reveal neuroinflammation of the CNS in ALS patients.

Keywords: amyotrophic lateral sclerosis, astrocytes, exosomes, disease progress, interleukin-6

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a rare, progressive neurodegenerative disease that affects upper and lower motor neurons and leads to fatal paralysis (Brown and Al-Chalabi, 2017). Ultimately, most ALS patients die within 3–5 years after disease onset due to respiratory failure. Approximately 90–95% of ALS cases are the sporadic type (sALS), and the remaining cases are the familial type (fALS). To date, more than 20 genes that cause fALS and sALS have been identified (Brown and Al-Chalabi, 2017). Scientific advances in genetic studies in the ALS field have improved our understanding of ALS pathogenesis. However, the exact etiology and pathogenesis of ALS are still unknown. As a result, there is no effective treatment for the disease. Riluzole and edaravone are the only two approved drugs for the treatment of ALS, and they solely delay disease progression for several months (Kumar et al., 2016; Rothstein, 2017).

Numerous intrinsic and extrinsic factors are involved in ALS motor neuron degeneration. One possible factor involved in motor neuron degeneration in ALS is neuroinflammation. Accumulating evidence indicates that ALS patients have chronic inflammation, as demonstrated by activated microglia and astrocytes, as well as infiltration of peripheral monocytes and lymphocytes into

the CNS (Zhao et al., 2013; Liu and Wang, 2017). Increased serum/plasma and CSF levels of some cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), IL-8, and interferon-beta (IFN-β), have been detected in ALS patients when compared to controls (Ono et al., 2001; Mitchell et al., 2009; Fiala et al., 2010; Mitchell et al., 2010; Italiani et al., 2014; Ehrhart et al., 2015; Liu et al., 2015; Hu et al., 2017). Beyond demonstrating ongoing inflammatory processes in ALS patients, these inflammatory biomarkers could also be used as diagnostic and prognostic biomarkers for clinical use because they have been reported to distinguish ALS from healthy or disease controls (Vu and Bowser, 2017; Gonzalez-Garza et al., 2018) and to predict the disease prognosis (Su et al., 2013; Liu et al., 2015). Activated microglia and astrocytes in the CNS play a vital role in the neuroinflammation process in ALS patients; however, the determination of the inflammatory biomarkers in serum/plasma and CSF only indirectly reflects the status of the CNS. Recently, scientific advances in the field of exosomes and CNS-derived exosome extraction technology have provided the possibility of measuring the inflammatory status in the CNS without brain biopsy.

Exosomes are approximately 30–100 nm extracellular vesicles with lipid bilayer membranes that are secreted by almost all types of cells, including neurons, microglia and astrocytes (Raposo and Stoorvogel, 2013; Yanez-Mo et al., 2015). Exosomes contain proteins, lipids and RNA and transfer them between cells. Therefore, exosomes play an important role in intercellular communication. Moreover, different cell types can secrete exosomes with different biomarkers, which could help to identify the exosome source (Beninson and Fleshner, 2014). Due to their specific characteristics, exosomes have attracted large amounts of attention in various studies ranging from mechanistic analyses to clinical research (Jarmalaviciute and Pivoriunas, 2016; Goh et al., 2017). In addition, exosomes can cross the blood-brain barrier (BBB) from both directions. As a result, CNS-derived exosomes can be detected in the blood and may help to reveal the pathophysiology of brain diseases without the use of brain biopsy and CSF analysis (Mustapic et al., 2017). In recent studies, several strategies to extract CNS-derived exosomes from peripheral blood have been reported (Mustapic et al., 2017; Kuwano et al., 2018). However, CNS-derived exosome-based studies focusing on ALS have not been previously reported.

Based on the above information, we hypothesized that inflammatory biomarkers in astrocyte-derived exosomes (ADEs) may increase and may be associated with clinical features in ALS patients. In this pilot study, we extracted ADEs from the plasma of sporadic ALS patients and age-, sex-matched healthy controls to determine the IL-6 levels in ADEs and, ultimately, we detected increased IL-6 levels in ADEs of sALS patients, which were positively associated with the rate of disease progression.

PARTICIPANTS AND METHODS

Participants

This study was approved by the Ethics Committee of the Perking University Third Hospital, Beijing, China. All ALS patients and age-, sex-matched healthy control individuals signed the informed consent before peripheral blood samples were drawn. Patients and controls were recruited from the Department of Neurology of Perking University Third Hospital. Clinically definite and probable sALS patients were diagnosed based on the EI Escorial revised criteria (Brooks et al., 2000) and further evaluated by the revised ALS functional rating scale (ALSFRS-R) (Cedarbaum et al., 1999). The rate of disease progression (ΔFS) was calculated as follows: ΔFS = (48 -ALSFRS-R at "time of diagnosis")/duration from onset to diagnosis (month) (Kimura et al., 2006).

Plasma Sampling in ALS Patients and Controls

Samples containing two milliliters of peripheral blood from ALS patients and healthy control individuals were collected into EDTA tubes. To extract plasma, blood samples were centrifuged at 1500 *g* for 10 min to remove blood cells. Then, the supernatant was subjected to another centrifugation at 2500 *g* for 20 min to remove the platelets and cell debris. Finally, the plasma was stored at −80°C until use.

Extraction of ADEs From Plasma

The method to extract the ADEs from plasma was modified from a previously published article (Mustapic et al., 2017). Briefly, 0.25 ml plasma was incubated with 0.2 μl thromboplastin (System Biosciences, Mountain View, CA, United States) for 5 min. Then, 298 μl calcium- and magnesium-free Dulbecco's Balanced Salt Solution (DBS^{-2}) was added with protease inhibitor cocktail (Roche, Indianapolis, IN) and phosphatase inhibitor cocktail (Thermo Fisher Scientific), followed by centrifugation at 10,000 rpm for 5 min at 4°C. The supernatants were harvested, followed by addition of 126 μl per tube of ExoQuick (System Biosciences, Mountain View, CA, United States). After a second centrifugation at 1500 g for 30 min at 4°C, total exosomes were harvested by removing the supernatant. To enrich ADEs, total exosomes were resuspended in 250 μl of ddH_2O with protease inhibitor cocktail and phosphatase inhibitor cocktail and incubated for at least 120 min at 4°C. Then, 1.5 μg biotinylated mouse anti-human glutamine aspartate transporter (ACSA-1) antibody (Miltenyi Biotec, Auburn, CA, United States) in 50 ml of 3% bovine serum albumin (BSA; 1:3.33 dilution of Blocker BSA 10% solution in DBS^{-2}; Thermo Fisher Scientific) was added per tube and mixed for 60 min at room temperature, followed by the addition of 10 μl streptavidin-agarose Ultralink resin (Thermo Fisher Scientific) in 40 ml 3% BSA and incubation with mixing for another 20 min at room temperature. After centrifugation at 400 *g* for 10 min at 4°C, the supernatant was removed, and each pellet was suspended in 200 μl cold 0.1 M glycine-HCl (pH = 3.0) by gentle mixing for 10 s and centrifugation at 4,500 *g* for 5 min. The supernatants were then harvested, and 25 μl of 3% BSA and 15 μl of 1 M Tris–HCl (pH = 8.0) were added. Finally, 260 μl mammalian protein extraction reagent (M-PER, Thermo Fisher Scientific) was added, and the solution was mixed. The resultant 0.5 ml lysates of ADEs were stored at −80°C. Evidence for enrichment of exosomes

from neural sources in plasma has been demonstrated previously (Mustapic et al., 2017).

Measurement of IL-6 Levels in ADEs and Plasma

Astrocyte-derived exosome proteins were quantified using a single-plex high-sensitivity and high-dynamic-range ELISA for IL-6 (Rockville, MD, United States Cat# K151AKC) (Chaturvedi et al., 2015) and by using enzyme-linked immunosorbent assay (ELISA) kits for the tetra-spanning exosome marker CD81 (Cusabio Technology, Wuhan, China), according to the suppliers' directions. The mean value for all determinations of CD81 in each assay group was set at 1.00, and the relative values for each sample were used to normalize their recovery. The plasma IL-6 levels in both groups were also measured. The protein levels were measured by board-certified laboratory technicians who were blinded to the clinical information.

To ensure the specificity of the tests, negative control groups were set up in this study. In the negative control group one, the biotinylated anti-ACSA-1 antibody was replaced with 3% BSA. In the negative control group two, the total exosomes solution resuspended from ExoQuick pellet was replaced by ddH2O.

Statistical Analyses

Data are presented as numbers, means and standard deviations, or medians (interquartile range, IQR) as appropriate. Normal distributions of datasets were assessed by the Shapiro–Wilks test. Unpaired Student *t*-tests, χ^2 test or one-way ANOVA, followed by Tukey analysis, were used to examine differences between groups. Pearson's correlation was used for statistical correlation analysis. The differences between groups were considered significant if the *p*-value was less than 0.05 (two-tailed). All statistical analyses and graphs were performed using GraphPad Prism 6 (GraphPad Software Inc., San Diego, United States).

RESULTS

In this pilot study, 40 ALS patients and 39 healthy controls were recruited. The detailed clinical information for these two groups are summarized in **Table 1**. The ALS patients and controls were comparable, as there was no difference in age or sex ratio between the two groups. Of the 40 ALS patients, 12 were bulbar onset and 28 were limber onset; 10 ALS cases were diagnosed as definite, and the remainder were probable. The median delay of diagnosis for all patients was 9.23 months. The mean ALSFRS-R score for the patients was 39.83 ± 1.08, and the median disease progression rate was 0.56. The extracted ADEs were validated by western blot. The result showed that the ADEs were positive for CD63, but negative for calnexin (**Supplementary Figure S1**). The ADEs were also verified by transmission electron microscope (**Supplementary Figure S2**). In the CD81 and IL-6 test, the negative control group one and two were all at background levels. The CD81-normalized levels of IL-6 in ADEs were significantly higher in ALS patients (40.40 ± 2.11 pg/ml) than in controls (22.45 ± 1.90 pg/ml) (**Figure 1A**). However, among 40 ALS patients and 39 healthy controls, the IL-6 was detectable only in 12 controls and 15 ALS patients. There was no difference in detection rate between the two groups. The plasma IL-6 levels ranged from 0.13 to 4.58 pg/mL in controls and 0.39 to 15.69 pg/ml in ALS patients (**Supplementary Figure S3A**). There was no difference in plasma IL-6 levels between controls and ALS patients (p = 0.3614) and there was no correlation between IL-6 levels in plasma and ADEs (r = 0.3384, p = 0.2173 for ALS group; r = −0.2657, p = 0.4038 for control group; **Supplementary Figures S3B,C**).

The ALS patients were further divided into subgroups according to the following: onset site: bulbar onset (ALS-B) or limber onset (ALS-L); diagnosis level: definite (ALS-D) or probable (ALS-P); and disease duration: less than 12 months (ALS < 12) or greater than or equal to 12 months (ALS ≥ 12). As shown in **Figures 1B–D**, compared with the control group, the levels of IL-6 in ADEs were increased in all ALS subgroups. However, there was no difference between the ALS subgroups.

The correlations of the levels of IL-6 in ADEs with clinical parameters are shown in **Figure 2**. The IL-6 levels correlated positively with the disease progression rate (r = 0.4696, p = 0.002). However, IL-6 levels in the ADEs of ALS patients did not correlate with total ALSFRS-R scores (r = −0.2021, p = 0.2110), diagnosis delay (r = −0.1735, p = 0.2845) or patient age (r = −0.1087, p = 0.5560). In controls, IL-6 levels also did not correlate with age (data not shown). When the patients were separated into two groups according disease duration (ALS < 12 m or ALS ≥ 12 m), a positive correlation between IL-6 levels and disease progression was only verified in the ALS < 12 m group (r = 0.6605, p = 0.015) (**Figure 3A**) but not in the ALS ≥ 12 m group (r = 0.3510, p = 0.1291) (**Figure 3B**).

DISCUSSION

The present study demonstrated that the levels of IL-6 in ADEs of sALS patients were increased and positively associated with the rate of disease progression, especially in patients at an earlier disease stage. These data suggest that the inflammatory cascade is augmented in the CNS of sALS patients. Analysis of CNS-derived exosomes in peripheral blood has recently attracted immense attention. Numerous studies have demonstrated that CNS-derived exosomes could be helpful to understand the

TABLE 1 | Characteristics of ALS patients and healthy controls.

	Control	ALS	*p*
Cases (male/female)	39 (25/14)	40 (26/14)	1
Age (mean ± SE)	55.74 ± 1.32	54.35 ± 2.02	0.57
Onset site: bulbar/limb	NA	12/28	NA
Diagnosis delay (months)	NA	9.23 (9.68)	NA
Definite/probable	NA	10/30	NA
ALSFRS-R	NA	39.83 ± 1.08	NA
ΔFS	NA	0.56 (0.71)	NA
IL-6 (pg/ml)	22.45 ± 1.90	40.40 ± 2.11	<0.001

Diagnosis delay, interval from the initial symptoms to diagnosis; ALSFRS-R, revised amyotrophic lateral sclerosis functional rating scale; ΔFS, disease progression rate.

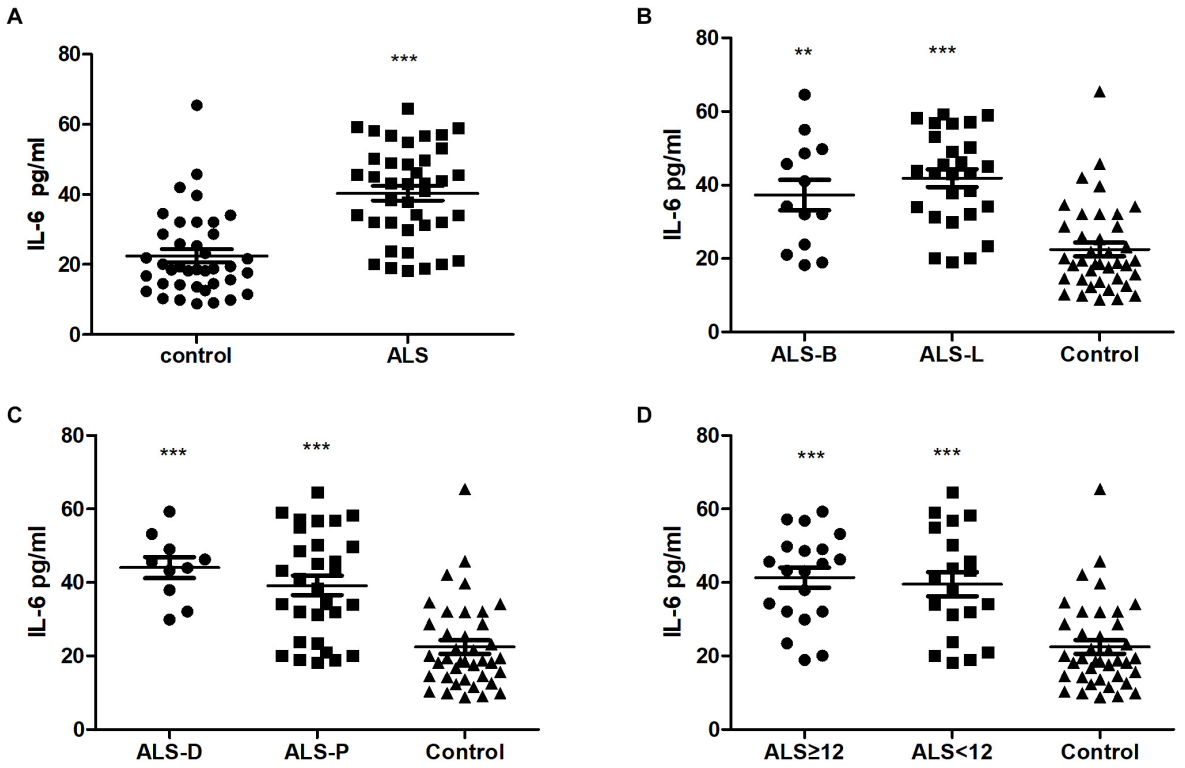

FIGURE 1 | Comparison IL-6 levels in ADEs from plasma of ALS and healthy subjects. Panels **(A–D)** show the levels of IL-6 in ADEs of **(A)** ALS patients and controls; **(B)** ALS patients with bulbar (ALS-B) or limb onset (ALS-L) and controls; **(C)** definite ALS (ALS-D) or probable ALS (ALS-P) and controls; **(D)** ALS duration ≥ 12 months (ALS ≥ 12) or <12 months (ALS < 12) and controls. **, *** indicate $p < 0.01$ and $p < 0.001$, respectively, compared with controls.

pathophysiology of brain disease and the identification of biomarkers (Abner et al., 2016; Winston et al., 2016; Goetzl et al., 2018; Ohmichi et al., 2018). However, to our knowledge, no studies have been reported on CNS-derived exosomes in ALS patients. Therefore, our pilot study is the first to demonstrate that CNS-derived exosomes could be useful to reveal the pathophysiology of CNS in ALS patients.

Several inflammatory biomarkers have been found to be linked to ALS. As a well-known cytokine, IL-6 has been extensively investigated in neurodegenerative disorders and associated with ALS in numerous studies (Sekizawa et al., 1998; Ehrhart et al., 2015; Lu et al., 2016; Blasco et al., 2017; Hu et al., 2017). However, the results are not consistent across all studies (Moreau et al., 2005; Tanaka et al., 2006). In addition, one study reported an increase in IL-6 levels at the late stage of disease (Lu et al., 2016), whereas another study reported that the levels of IL-6 were high at disease onset followed by a subsequent decline (Ehrhart et al., 2015). The plasma IL-6 levels were also measured in this study. However, the IL-6 was detectable in only 12 controls and 15 ALS patients and undetectable in most of the samples. Among the 12 controls and 15 ALS patients, the IL-6 levels were highly variable and no difference has been found between the two groups. The highly variable plasma IL-6 levels in our study and the contradictory results from previous studies indicate that the peripheral IL-6 levels may be influenced by complex factors. A recent study showed that the levels of IL-6 in blood could be influenced by aging and respiratory dysfunction in ALS (Pronto-Laborinho et al., 2019). Thus, determining the IL-6 levels in blood may not be a good way. CNS-derived exosomes could directly reflect the situation in the CNS, and peripheral factors might have little effect on cytokines in CNS-derived exosomes. Therefore, the measurement of IL-6 levels in CNS-derived exosomes, compared with blood or CSF, may be better to illuminate the actual role of IL-6 in ALS. Astrocytes have been reported play an important role in the pathogenesis of ALS, and the predominant CNS source of IL-6 is the activated astrocyte (Van Wagoner and Benveniste, 1999). Hence, in this pilot study, we chose to measure IL-6 levels in ADEs. Compared with the plasma IL-6 levels, the IL-6 levels in ADEs were relatively high and stable and the IL-6 levels in the ADEs didn't correlate with age. Moreover, it was supposed that there may be connection between IL-6 levels in plasma and ADEs. However, no correlation had been found between two groups. All these results indicate that CNS-derived exosomes may be a promising object to help find biomarkers for ALS.

The important findings of our study were that IL-6 levels in ADEs increased in sALS patients and were positively associated with the rate of disease progression. These data suggest that the IL-6 in ADEs may be a candidate biomarker for ALS. However, neuroinflammation is a common phenomenon in almost all neurological disease. Therefore, it is believed that the IL-6 levels in ADEs probably increase in other neurological conditions. Actually, it has been reported that the IL-6 levels in ADEs

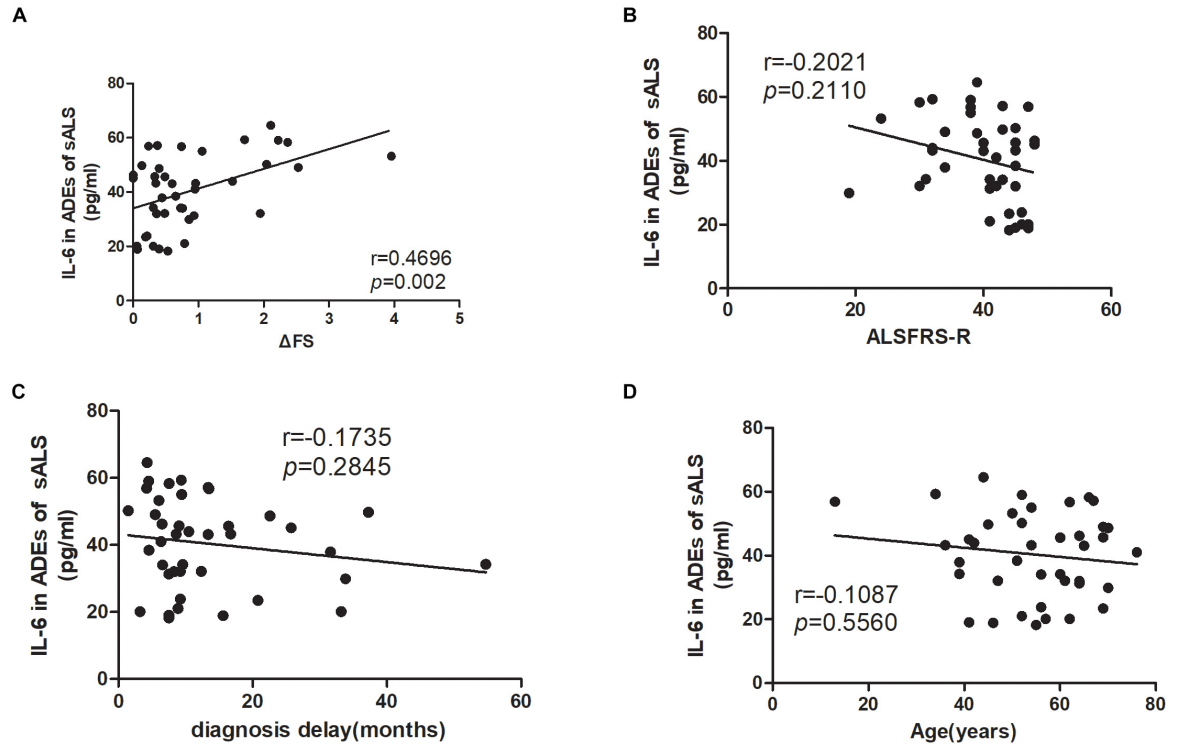

FIGURE 2 | Correlations between IL-6 levels in ADEs of ALS patients with the disease progression rate, ALSFRS-R score, diagnosis delay and patient age. **(A)** shows that the IL-6 levels in ADEs of ALS patients positively correlate with the disease progression rate. However, the IL-6 levels in ADEs of ALS patients do not correlate with the ALSFRS-R score **(B)**, diagnosis delay **(C),** and patient age **(D)**.

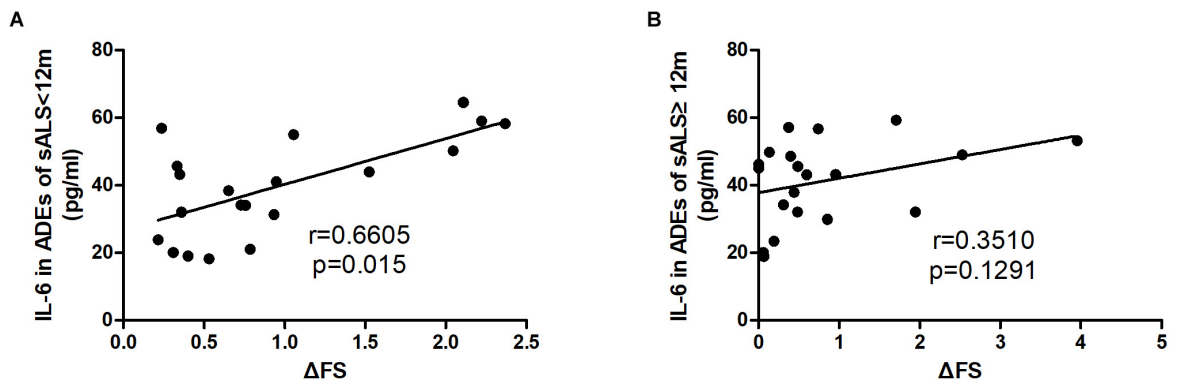

FIGURE 3 | Correlations between IL-6 levels in ADEs of ALS subgroups with the disease progression rate. ALS patients were divided into two groups according to the disease duration. **(A)** The IL-6 levels in ADEs of the ALS < 12 group positively correlate with the disease progression rate. However, the Il-6 levels in ADEs of the ALS12 group do not correlate with the disease progression rate **(B)**.

increased in AD patients (Goetzl et al., 2018). Thus, the IL-6 levels in ADEs may not be suitable to help discriminate ALS from other neurological diseases. According to our study, measuring the IL-6 levels in ADEs may be helpful to reflect the neuroinflammation status and predict disease progression.

We could not determine the precise role of IL-6 in ALS patients because of its complex physiological functions. Increased IL-6 secretion could be a neuroprotective reaction against CNS damage or a pro-inflammatory agent (Spooren et al., 2011). However, most views consider IL-6 as a pro-inflammatory cytokine in ALS patients. The anti-IL-6 antibody, tocilizumab, has been proposed as a therapeutic drug for ALS (Fiala et al., 2013). Therefore, we speculate that the increase in IL-6 observed in this study was harmful to ALS patients. Our further analyses revealed that the levels of IL-6 did not differ between ALS subgroups, and the correlation between IL-6 and the rate of disease progression was only observed during the initial 12 months. These results indicated that IL-6 produced by

astrocytes might be more important during the early stage of disease. However, our sample size was limited, and the results should therefore be confirmed in further studies.

CONCLUSION

The present study demonstrated that the levels of IL-6 in ADEs of ALS patients were increased and positively associated with the rate of disease progression, especially in patients at an earlier disease stage. Our pilot study is the first to demonstrate that CNS-derived exosomes could be useful to reveal the pathophysiology of CNS in ALS patients.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

This study was approved by the Ethics Committee of the Perking University Third Hospital, Beijing, China. All ALS patients and age-, sex-matched healthy control individuals signed the informed consent before peripheral blood samples were drawn.

AUTHOR CONTRIBUTIONS

DF conceived the study, provided the financial support, and responsible for the project management. DF and YC designed the study, responsible for preparing and revising the manuscript, and had key roles in the study. KX and LC took part in the design of the study and in sample collection, and undertook data checking.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2019.00574/full#supplementary-material

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Biological Significance of microRNA Biomarkers in ALS—Innocent Bystanders or Disease Culprits?

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MicroRNAs (miRNAs) represent potential biomarkers for neurodegenerative disorders including amyotrophic lateral sclerosis (ALS). However, whether expression changes of individual miRNAs are simply an indication of cellular dysfunction and degeneration, or actually promote functional changes in target gene expression relevant to disease pathogenesis, is unclear. Here we used bioinformatics to test the hypothesis that ALS-associated miRNAs exert their effects through targeting genes implicated in disease etiology. We documented deregulated miRNAs identified in studies of ALS patients, noting variations in participants, tissue samples, miRNA detection or quantification methods used, and functional or bioinformatic assessments (if performed). Despite lack of experimental standardization, overlap of many deregulated miRNAs between studies was noted; however, direction of reported expression changes did not always concur. The use of *in silico* predictions of target genes for the most commonly deregulated miRNAs, cross-referenced to a selection of previously identified ALS genes, did not support our hypothesis. Specifically, although deregulated miRNAs were predicted to commonly target ALS genes, random miRNAs gave similar predictions. To further investigate biological patterns in the deregulated miRNAs, we grouped them by tissue source in which they were identified, indicating that for a core of frequently detected miRNAs, blood/plasma/serum may be useful for future profiling experiments. We conclude that *in silico* predictions of gene targets of deregulated ALS miRNAs, at least using currently available algorithms, are unlikely to be sufficient in informing disease pathomechanisms. We advocate experimental functional testing of candidate miRNAs and their predicted targets, propose miRNAs to prioritise, and suggest a concerted move towards protocol standardization for biomarker identification.

Keywords: microRNA, amyotrophic lateral sclerosis, ALS, MND, biomarker, ALS genes, bioinformatics, neurodegeneration

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INTRODUCTION

MicroRNAs (miRNAs) are small non-coding RNAs, typically 20–22 nucleotides (nt) long, which act as post-transcriptional regulators of gene expression (1). MiRNA seed sequences provide specificity for the 3′ untranslated region (UTR) of target mRNA, leading to mRNA degradation or translational inhibition (2). Around a third of human gene products are regulated by miRNAs (3), being present in both intracellular and extracellular environments and in almost all biological fluids (4, 5). Extracellularly, miRNAs are detected within membrane vesicles and also freely, forming complexes with other macromolecules (6).

Amyotrophic lateral sclerosis (ALS) is characterized by the progressive loss of upper and lower motor neurons in the spinal cord, cerebral cortex, and brainstem, resulting in muscle weakness and wasting (7, 8). Life expectancy is 2–5 years after onset (9). Approximately 5% of ALS patients develop frontotemporal dementia (FTD) and the ALS-FTD spectrum is hereafter referred to as ALS (10). Around 90% of ALS cases are sporadic (sALS) and 10% are familial (fALS), being associated with inherited mutations. Multiple genes have been linked with ALS (11). Interestingly, some ALS-associated genes, including *TARDBP* and *FUS,* encode RNA-binding proteins which are involved in miRNA processing (12), and indirectly implicate miRNAs in ALS pathophysiology. However in addition to RNA metabolism, ALS-associated genes show diverse functions, with roles in intracellular transport, proteostasis, axonal outgrowth, and glutamatergic signaling (7).

MiRNAs are unusually well-preserved in a range of biological samples, including blood plasma, serum, and cerebrospinal fluid, and are measurable with greater sensitivity and stability than proteins (5, 13). As a result, the last decade has seen a drive to identify specific miRNA biomarkers for ALS, in order to potentiate more rapid and accurate diagnosis, disease stratification and monitoring. Numerous studies have demonstrated deregulation of miRNAs in ALS patients, most aiming to identify clinically-relevant biomarkers.

Relevant to the ALS context, CSF miRNAs are potentially good representatives of central nervous system (CNS) disorders, since a blood-CSF barrier would prevent CNS miRNA dilution in the wider circulation (14). However, it is possible for miRNAs to transfer across this barrier, such that blood miRNAs may provide a window on nervous system dysfunction (15). Although the functional significance of circulating miRNAs is less clear, it has been demonstrated that cells can transfer functional miRNAs between one another in an exosome-mediated manner (16). It has been proposed cells can select the miRNAs to be released (17), although cells also shed material when degenerating. Thus, extracellular vesicles (EVs) may reflect the cells of origin, and some of these circulating miRNAs potentially mirror ALS pathophysiology.

Despite considerable efforts, no specific, robust diagnostic molecular biomarker set has been identified for ALS (18). Recently, Dardiotis et al. (19) reviewed the results of 24 studies, from 2010 to 2017, documenting miRNAs reported in ALS biomarker studies, aiming to clarify those most appropriate for future evaluation. In this same Frontiers issue, Joilin et al. (20) review recent attempts to define a "biomarker-relevant" signature of miRNAs, discussing their great potential and the challenges once the field moves toward clinical validation. However, beyond the key importance of biomarker identification, most studies so far do not attempt systematic bioinformatic or experimental functional interpretation of transcripts targeted by ALS-relevant miRNAs. Consequently, whether changes in miRNA expression simply reflect cellular dysfunction and degeneration, or are active participants in the functional changes of target genes relevant to disease pathogenesis, is unknown.

Here, we also focus on miRNA profiling studies comparing expression levels of miRNAs from ALS patients and controls, over the 2013–2018 period. Our approach aims to evaluate various strategies that can be used to analyse these deregulated miRNAs: *number of reported studies for a given miRNA*, *predicted functional targets,* and *tissue distribution* (i.e., where detected). We document the overlap between miRNAs reported as deregulated in these studies; and for these miRNAs, propose a series of *in silico* methods to identify those predicted to target known ALS genes, evaluating current limitations of such predictions in informing disease pathogenesis. Finally, we consider the source of patient tissue samples used for miRNA profiling, highlighting overlap of given miRNAs and revealing the importance of sample analyzed.

RESULTS AND DISCUSSION

Literature Analysis

To define relevant studies we performed a PubMed literature search with the MeSH terms "microRNA" AND "amyotrophic lateral sclerosis" from 1/1/2013-31/12/2018. We identified 27 peer-reviewed studies fulfilling our selection criteria, which specifically included those recording and comparing levels of multiple miRNAs directly from ALS patients and controls (**Table S1**). Of these studies, 15 were previously considered by Dardiotis and colleagues, whilst Joilin et al. (20) in this same issue considered 11 of the studies presented here.

Detailed observation noted a large degree of variation between the studies, from sample source (serum, plasma, whole blood, CSF, spinal cord, muscle etc.), numbers and clinical characteristics of patient participants (both sALS and fALS) and controls (healthy and other diseases), to the methods used for sample preparation, miRNA profiling and analysis. Additionally, we identified the need for reporting specific arms of mature miRNAs, since in ambiguous cases we could only assume the dominant strand as that reported/detected (miRBase release 22.1: 2018).

In those few studies that investigate functional implications potentially derived from miRNA changes, a wide variety of bioinformatic approaches were used to identify possible mRNA targets of deregulated miRNAs, including different versions of TargetScan, Pictar, miRanda, DIANA-Tarbase, and miRtarbase. Further attempts to identify those gene/signaling networks targeted, built on protein-protein interaction (PPI) networks, gene ontology and pathway analysis, generating a variety of outcomes (21, 22).

Most Commonly Deregulated microRNAs

As a first approach to select potentially pathologically relevant miRNAs, we ranked them according to the number of times they were reported as deregulated in different studies. In the 27 miRNA profiling studies, a total of 559 miRNAs were shown as deregulated. Among these, nine miRNAs were reported six or more times, compared to 38 reported in five or more studies, directing the threshold selected for our analysis. Those nine most frequently reported ($\geq$6) are shown in **Table 1A**. Since any miRNA deregulation could have deleterious effects on gene targets, initial selection did not discriminate between up- vs. down-regulation. Indeed, for many miRNAs, the reported

direction of deregulation was inconsistent between studies, which may be accounted for by differences in the analytical protocol and/or miRNA profiling technique.

Of the most frequently reported miRNAs, hsa-miR-133a-3p was found deregulated in 9/27 studies. The high ranking of hsa-miR-133a-3p may be explained by the fact it is a known myomiR, enriched in muscle tissue (24) and several of the analyzed studies focused on expression levels of myomiRs alone, potentially introducing tissue bias (25–29) (**Table S1**). However despite its myomiR label, hsa-miR-133a-3p has also been suggested as motor neuron enriched (30).

Frequently Deregulated microRNAs and Target Prediction of ALS Genes

To connect biomarker reporting and potential functional relevance we have outlined an *in silico* method to determine whether these commonly reported miRNAs preferentially target selected known ALS-associated genes (http://alsod.iop.kcl.ac.uk/ [last updated 2015], an ALS bioinformatics repository online database) (31). The 37 ALS genes considered were (in alphabetical order):

ALS2, ANG, ARHGEF28, ATXN2, C9orf72, CHCHD10, CHGB, CHMP2B, CRYM, DAO, DCTN1, ERBB4, FIG4, FUS, GLE1, LUM, MATR3, NEFH, OPTN, PARK7, PFN1, PLEKHG5, SETX, SIGMAR1, SOD1, SPG11, SQSTM1, SS18L1, SYNE, TAF15, TARDBP, TBK1, TRPM7, TUBA4A, UBQLN2, VAPB, and *VCP*.

Although not updated since 2015, this database provides information regarding the ALS patients harboring mutations in these genes, such as patient numbers (fALS and sALS), gender and mean onset age as well as site of disease (bulbar/limb). Additionally, all ALS genes reviewed by Kirby et al. (10) except *hnRNPA1* are included in this list. For the prediction analysis we used DIANA-microT-CDS v5.0 (32, 33). As reviewed by Riffo-Campos et al. (34), the DIANA-microT attempts to apply a more balanced predictive approach, displaying TargetScan, and miRanda comparisons in its analysis.

From the *in silico* analysis, 8/9 most frequently deregulated miRNAs were predicted to target at least one of these ALS genes (**Table 1A**), with hsa-miR-9-5p, predicted to target 4/37 of the genes. There appeared to be no obvious relationship between the total number of ALS genes the individual miRNAs were predicted to target and the number of studies reporting these miRNAs as deregulated. The most frequently predicted ALS targeted genes were *ARHGEF28*, *CHMP2B,* and *MATR3* (2/9 miRNAs). The total count of predicted ALS target genes for the combined nine miRNAs was 18, and overall, 15/37 ALS genes were predicted as targets of at least one of the nine miRNAs. Whilst this approach highlights the potential of *in silico* predictive methods, evaluation of comparable analyses with less frequently reported deregulated miRNAs is also merited.

Other Deregulated microRNAs and Target Prediction of ALS Genes

To determine if the most commonly identified ALS miRNAs are the most relevant, the same analysis must be performed with an identical number (nine) of randomly selected miRNAs, which although reported to be deregulated in the 27 ALS studies, appeared in fewer than six reports. Randomization was achieved by selecting from all deregulated miRNAs, without duplicates, using a Microsoft Excel randomization function. The results of this preliminary analysis are shown in **Table 1B**. Of the nine miRNAs, deregulation was reported in between 1 and 5 (of 27) studies. 7/9 of these randomly selected miRNAs were predicted to target at least one of the 37 ALS genes. Further, one of the miRNAs, hsa-miR-766-3p (deregulated in 2/27 studies) was predicted to target 4/37 ALS genes. The most frequently predicted ALS targeted gene was *ERBB4* (4/9 miRNAs). The total count of predicted ALS target genes for the combined nine miRNAs was 16, comparable to that of the nine most commonly deregulated miRNAs (18, **Table 1A**). Overall, 10/37 ALS genes were predicted as targets of at least one of the nine miRNAs. Although this second analysis could indicate all deregulated miRNAs are equally important in their capacity to potentially target ALS relevant genes, a further *in silico* step requires comparison with a group of miRNAs not deregulated in biomarker studies.

Analysis of Randomly Selected and Non-deregulated microRNAs

To investigate if predicted gene targets for deregulated ALS miRNAs reflected an enrichment compared to non-deregulated miRNAs, we performed an example test with nine randomly selected mature miRNAs not reported as deregulated in any of the 27 studies and performed the same analysis (**Table 1C**). We selected from all *Homo sapien* mature miRNA sequences recorded on miRBase release 22.1: October 2018 (35). One of the random miRNAs, hsa-miR-603-3p, was predicted to target 6/37 of the ALS genes, including *ERBB4.* The major ALS gene, *TARDBP,* was predicted most frequently (3/9 miRNAs).

Compared to the nine most frequently reported (**Table 1A**) or not frequently reported deregulated miRNAs (**Table 1B**), this random miRNA selection gave a total count of 13 predicted ALS target genes (**Table 1C**), representing only 8/37 of the ALS genes. Notably, 5/9 of these random miRNAs were predicted to target at least one of the 37 ALS genes. Further selections of different sets of nine random miRNAs showed similar results (not shown).

The systematic approach outlined above would potentially allow the use of statistical analysis (i.e., binomial test) to indicate whether deregulated miRNAs from ALS patient studies more frequently target ALS genes (at least based on *in silico* predictions) than randomly selected miRNAs, but we suggest greater numbers of miRNAs would need to be considered. This analysis would not be trivial and is outside the scope of this article, which seeks to propose a workflow. Further, consideration of additional (to the 37 used here) ALS genes is likely merited, again expanding the complexity of the analysis. Current ALS genes also relate to different signaling networks, and more specific gene pathways may be required for target enrichment. In summary, we have defined a systematic *in silico* analysis that should be extended in the future to investigate functional links between deregulated miRNAs and ALS pathological processes.

TABLE 1 | The ALS genes predicted by DIANA-microT-CDS v5.0 to be targets of (A) the nine most frequently reported miRNAs from the studies, (B) nine deregulated miRNAs randomly selected from all ALS studies, and (C) nine randomly selected miRNAs not reported to be deregulated in the ALS studies.

	miRNAs	Number of Studies deregulated (out of 27)	Direction of deregulation (up, down or both)	ALS genes predicted by DIANA-T-CDS						
(A) Most Frequently Reported miRNAs	hsa-miR-133a-3p	9	Both	*TUBA4A*	*VAPB*					**MiRNA hits (8/9)** **Genes (15/37)** **Total (18)**
	hsa-let-7a-5p	7	Both	*ARHGEF28*						
	hsa-miR-127-3p	6	Both							
	hsa-miR-155-5p	6	Both	*TBK1*	***UBQLN2***					
	hsa-miR-206-3p	6	Both	*ATXN2*	***MATR3***					
	hsa-miR-26a-5p	6	Both	*ARHGEF28*	*ERBB4*	*MATR3*				
	hsa-miR-455-3p	6	Both	*TARDBP*						
	hsa-miR-9-5p	6	Both	***CHMP2B***	*CRYM*	*NEFH*	***TRPM7***			
	hsa-miR-124-3p	6	Both	***CHMP2B***	*SIGMAR1*	*SQSTM1*				
(B) Random miRNAs from ALS studies	hsa-let-7b-5p	5	Down	*ARHGEF28*						**MiRNA hits (7/9)** **Genes (10/37)** **Total (16)**
	hsa-let-7c-5p	4	Down	*ARHGEF28*						
	hsa-miR-204-3p	1	Down	*ERBB4*	*VAPB*					
	hsa-miR-766-3p	2	Both	***DAO***	*DCTN1*	*SIGMAR1*	*VAPB*			
	hsa-miR-212-3p	2	Down	*C9orf72*	*ERBB4*	*FIG4*				
	hsa-miR-329-3p	2	Down							
	hsa-miR-876-3p	1	Down	*ERBB4*	*MATR3*	*TUBA4A*				
	hsa-miR-302a-5p	1	Down	***ARHGEF28***	*ERBB4*					
	hsa-miR-154-5p	3	Down							
(C) Random miRNAs absent from ALS studies	hsa-miR-3168-5p			*TARDPB*						**MiRNA hits (5/9)** **Genes (8/37)** **Total (13)**
	hsa-miR-875-5p									
	hsa-miR-611-5p									
	hsa-miR-603-3p			*ATXN2*	*ERBB4*	*MATR3*	*OPTN*	*TARDBP*	***VAPB***	
	hsa-miR-500b-5p			*DCTN1*	*ERBB4*					
	hsa-miR-325-5p									
	hsa-miR-764-5p									
	hsa-miR-665-3p			*DCTN1*						
	hsa-miR-4277-5p			*DAO*	*TARDPB*	*VAPB*				

Note that we report mature miRNAs; where specific miRNAs were not reported we considered dominant strands as reported on miRBase release 22.1: October 2018. For (A), all miRNAs deregulated in at least six studies were considered. For (B) nine miRNAs were randomly selected from those reported by <6 of the 27 studies. Further analysis regarding strand specificity was also performed on this group and on the nine miRNAs not found deregulated in the studies (C). MiRNAs in bold have confirmed interactions with the target experimentally, using miRTarBase v7.0 (23).

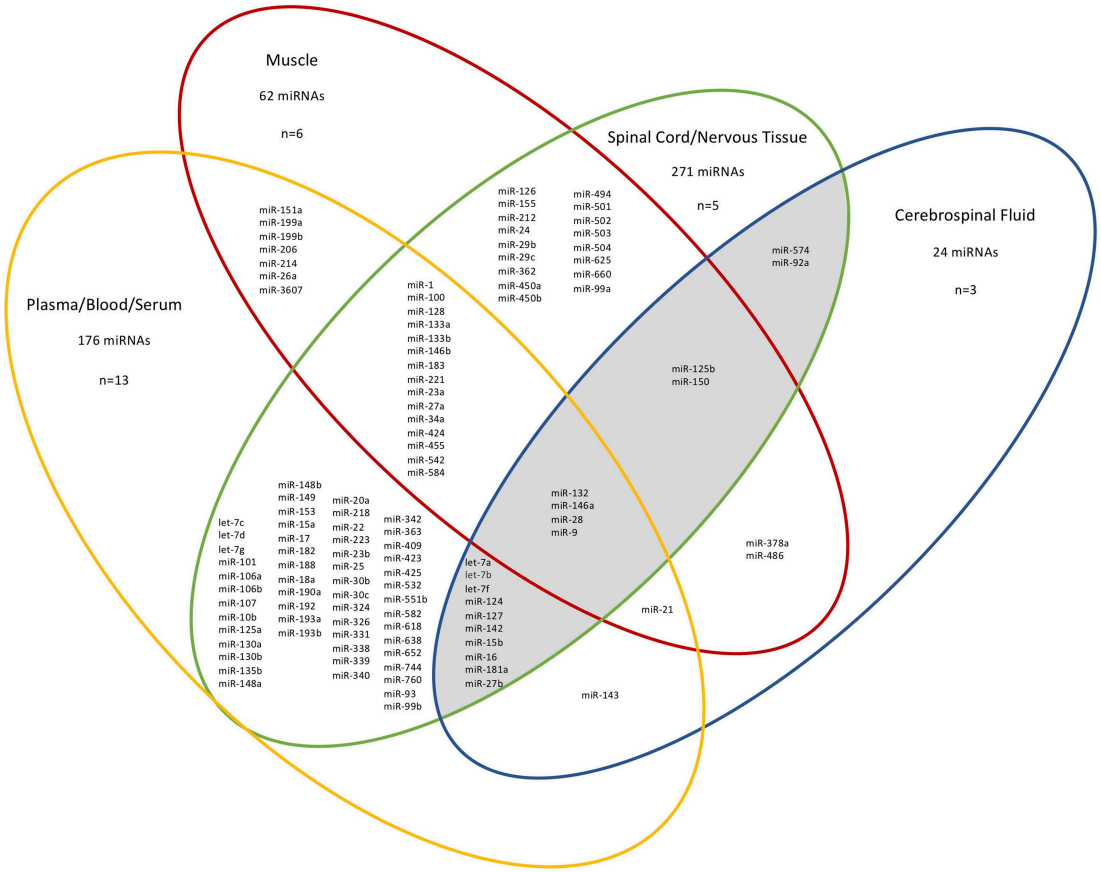

FIGURE 1 | Different tissue sources and overlap of miRNAs identified from ALS patients in 26/27 studies. n=the number of papers examined in each compartment. For simplicity, specific miRNA arms are not shown. The CSF-spinal cord/nervous tissue overlap is shaded. The miRNAs deregulated between patient neuromuscular junction and control blood and deregulated miRNAs from sALS patient fibroblasts are not included since they do not belong in any of the distinct source group compartments used here (21, 36). The total miRNAs present in each group are given. MiRNAs within a single compartment are not shown.

Tissue Distribution of Deregulated miRNAs

Next, we considered the different tissue sources of the ALS-relevant miRNAs identified in 26/27 studies ($n = 410$ unique miRNAs, for exclusions see **Figure 1** legend), and grouped the miRNAs based on four "compartments": CSF, spinal cord/nervous tissue, muscle, and plasma/blood/serum. **Figure 1** shows the overlap between studies after mapping the deregulated miRNAs to the compartment (tissue) they were determined in. 265/410 deregulated miRNAs were present in at least two different tissues. We noted considerable correspondence between miRNAs extracted from plasma/blood/serum with those from CSF (total of 16/24 miRNAs within the CSF group), supporting the notion that blood miRNAs can provide a window into CSF changes (15). Since ALS is a neurodegenerative disorder, those miRNAs deregulated in patient CSF and spinal cord/nervous tissue were of particular interest and these 18 miRNAs are shown in a shaded region in **Figure 1**. Of these 18 miRNAs, two are exclusive to only these two sources (hsa-miR-92a-5p/3p and hsa-miR-574-5p/3p). Notably, of the nine most frequently reported miRNAs found to be deregulated in the 27 studies (**Table 1A**), four overlapped with these 18 miRNAs.

Most Frequently Deregulated miRNAs Within the CSF-Spinal Cord/Nervous Tissue Overlap

As noted above, four miRNAs in the CSF-spinal cord/nervous tissue overlap are also amongst the most frequently reported deregulated miRNAs (**Table 1A**; hsa-miR-124-3p, hsa-miR-127-3p, hsa-let-7a-5p, and hsa-miR-9-5p). The latter is discussed in the section MicroRNAs Present in all Tissue Sources.

Hsa-miR-124-3p was reported down-regulated in ALS patients in five studies (21, 37–40), with only one study finding it upregulated (41). Despite the caveats to the predictive approach highlighted above, it is notable that the predicted targets of this miRNA are *CHMP2B, SQSTM1,* and *SIGMAR1*. Hsa-miR-124-3p has been shown to be deregulated in the spinal cord and brainstem of *SOD1* transgenic mice and has been linked to astrocyte differentiation and neurogenesis in the mouse brain (42, 43). Further, hsa-miR-124-3p is found to be expressed almost exclusively in the brain and spinal cord (44).

Hsa-miR-127-3p was not predicted to target any of the ALS genes and was found almost consistently down-regulated in ALS patients (22, 37, 39, 45, 46) with only one study

reporting its upregulation (41). Whilst little is reported in relation to ALS, hsa-miR-127-3p has been found deregulated in FTD patients compared to control groups and Alzheimer's disease patients (47). This result is consistent with hsa-miR-127-3p predominantly being expressed in brain tissue (44).

Hsa-let-7a-5p, most highly expressed in the cerebellum, is predicted to target *ARHGEF28* (**Table 1A**) (44). Let-7a-5p has been found downregulated in the plasma of Parkinson's disease patients compared to healthy controls, showing it may not be useful as an ALS specific biomarker (48).

MicroRNAs Unique to the CSF-Spinal Cord/Nervous Tissue Overlap

The two miRNAs unique to the CSF-spinal cord/nervous tissue group are hsa-miR-92a-5p/3p and hsa-miR-574-5p/3p. Hsa-miR-92a-5p/3p's predicted targets are *CHCHD10, TARDBP, PLEKHG5,* and *NEFH* and hsa-miR-574-5p/3p's are *VAPB* and *SIGMAR1*. According to a miRNA tissue atlas, both miRNAs show neither specific tissue specificity nor ubiquitous expression (44). Despite this, deregulation of these miRNAs in ALS could be tissue specific.

MicroRNAs Present in all Tissue Sources

Hsa-miR-132-5p/3p, hsa-miR-146a-5p/3p, hsa-miR-28-5p/3p, and hsa-miR-9-5p/3p were deregulated in all tissue samples (**Figure 1**) and are all predicted to target at least one ALS gene. Hsa-miR-132-3p has been implicated in a range of neurodegenerative disorders including multiple sclerosis, Parkinson's disease and Alzheimer's disease, demonstrating wider relevance beyond ALS (49). This is consistent with the miRNA tissue atlas, where it is primarily expressed in the brain (44). Downregulation of miR-146a-5p in cortical aberrant astrocytes has been implicated in motor neuron degeneration in ALS, whereas its upregulation has been implicated in motor neuron loss in spinal muscular atrophy (50, 51). No links between miR-28-5p/3p and ALS have yet been made, consistent with it being predicted to target just one ALS-associated gene (*SETX*). Mutations in *TARDBP* have been reported to cause deregulation of miR-9-5p and miR-9-5p/3p has been shown to be upregulated in mutant *SOD1* mice (52, 53). MiR-9-5p has been implicated in axon extension and branching *via* targeting of *Map1b* (54). It is also predominantly expressed in the brain and spinal cord (44).

CONCLUSIONS

We have shown that miRNAs found deregulated in published studies investigating ALS patients have limited overlap, likely due to the wide variation in tissue extraction and miRNA detection methods. Future emphasis should therefore be on standardizing tissue extraction and miRNA profiling methods.

However, we identified nine miRNAs repeatedly reported as deregulated in the 27 studies. Despite these miRNAs being commonly predicted to target ALS-associated genes, the randomly selected miRNAs not found deregulated in ALS patients, showed similar predictions. Therefore, our *in silico* analysis provided no clear correlation between deregulated miRNAs and the collection of ALS-linked genes analyzed. This indicates that whilst the ability to predict thousands of candidate genes with *in silico* methods remains informative, they should be used with caution and in combination with other methods, of which experimental functional testing is recommended. Although limitations of the bioinformatics approach may explain our observations, the currently identified ALS-associated genes may offer a limited view on the pathological pathways altered during disease progression. It is thus tempting to suggest *in silico* analyses are currently underpowered. In the future it would be interesting to perform this bioinformatics approach using ALS genes grouped by their relation to specific functional pathways, for example proteostasis or RNA metabolism.

We have additionally shown the source can influence the miRNAs detected, since only four deregulated miRNAs appeared in all tissue sources analyzed. Importantly, we have shown the four miRNAs reported most frequently deregulated appear in CSF, spinal cord/nervous tissue and blood/plasma/serum. This suggests miRNAs may indeed "travel" between CSF and blood, the latter potentially providing a clinically accessible source which may mirror ALS pathology in the CNS. We therefore propose four miRNAs—hsa-miR-124-3p, hsa-miR-127-3p, hsa-let-7a-5p, and hsa-miR-9-5p—as good candidates for further study and suggest blood, serum or plasma as a clinically accessible source.

Overall we have demonstrated the need for a multifaceted approach, utilizing patient data, bioinformatics, but most critically, experimental follow-up, to resolve the true biological significance of these implicated miRNAs and determine the real disease culprits of ALS.

Note Added After Submission

Whilst this manuscript was under review, Yao et al. (55) identified *SQSTM1* as a target of miR-124-3p. Notably our analysis had indicated that hsa-miR-124-3p, present in the CSF-spinal cord/nervous tissue overlap (**Figure 1**), was one of the most frequently reported deregulated miRNAs (6/27 studies) and was predicted to target *SQSTM1* (**Table 1A**).

AUTHOR CONTRIBUTIONS

SF wrote and edited the manuscript. RL, FD-B, and RM-R critically reviewed and edited the manuscript.

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The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2019.00578/full#supplementary-material

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MINI REVIEW
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Unbiased MRI Analyses Identify Micropathologic Differences Between Upper Motor Neuron-Predominant ALS Phenotypes

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Amyotrophic lateral sclerosis (ALS) is an incurable and progressively fatal neurodegenerative disease that manifests with distinct clinical phenotypes, which are seen in neuroimaging, and clinical studies. T2- and proton density (PD)-weighted magnetic resonance imaging (MRI) displays hyperintense signal along the corticospinal tract (CST) in some ALS patients with upper motor neuron (UMN)-predominant signs. These patients tend to be younger and have significantly faster disease progression. We hypothesize that such ALS patients with CST hyperintensity (ALS-CST+) comprise a clinical subtype distinct from other ALS subtypes, namely patients with UMN-predominant ALS *without* CST hyperintensity, classic ALS, and ALS with frontotemporal dementia (FTD). Novel approaches such as fractal dimension analysis on conventional MRI (cMRI) and advanced MR techniques such as diffusion tensor imaging (DTI) reveal significant differences between ALS-CST+ and the aforementioned ALS subtypes. Our unbiased neuroimaging studies demonstrate that the ALS-CST+ group, which can be initially identified by T2-, PD-, and FLAIR-weighted cMRI, is distinctive and distinguishable from other ALS subtypes with possible differences in disease pathogenesis.

Keywords: MRI, UMN-predominant ALS, corticospinal tract hyperintensity, diffusion tensor imaging, micropathologic differences, fractal dimension, ALS phenotypes

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INTRODUCTION

Etiology and site of origin of amyotrophic lateral sclerosis (ALS) within the central nervous system (CNS) are unknown (Mitsumoto et al., 1998). ALS diagnosis is based on motor neuron degeneration in both the CNS and peripheral nervous system (PNS), which include the upper motor neuron (UMN) and lower motor neuron (LMN), respectively. However, whether ALS begins in the CNS (Eisen et al., 1992) or PNS (Chou and Norris, 1993) is debated. Even if we consider a CNS origin, precisely where degeneration begins along the UMN pathway is unknown, as it can be

anywhere along its rostrocaudal extent. If pathology originates in the corticomotoneuron within the cerebral cortex it would be considered a "neuronopathy"; if it originates somewhere along the axon in motor tracts (e.g., corticospinal and corticobulbar) within the subcortical white matter or spinal cord, it would be considered an "axonopathy." If ALS is an axonopathy, degeneration would begin distal to the neuronal cell body, and proceed retrogradely to affect it later; if it is a neuronopathy, the neuronal cell body would be affected first with subsequent loss of the entire axon because of wallerian degeneration.

In previous studies, we have evaluated brain MRI changes in patients with ALS based on their clinical phenotype and extent of UMN or cognitive impairment, including in those with UMN-predominant ALS, classic ALS [expressing relatively equal amounts of UMN and lower motor neuron (LMN) dysfunction], or ALS with frontotemporal dementia (ALS-FTD). Although an ALS diagnosis relies on the clinical presence of both UMN and LMN signs, a proportion of patients with ALS present with evidence of only UMN abnormalities and develop LMN signs later. A hyperintense signal is visible along both corticospinal tracts (CST's) on conventional T2-, proton density (PD)-, and FLAIR- weighted MRI in some patients with predominant or exclusive UMN signs (Mitsumoto et al., 1998), while others do not (Matte and Pioro, 2010), even though both patient groups have comparable degrees of clinical UMN dysfunction. A review of the literature revealed 17–67% (median 40%) of ALS patients with CST hyperintensity (Pioro, 2006), while a preliminary analysis at the Cleveland Clinic found this change in ∼30% of ALS patients (Matte and Pioro, 2010). Although the precise cause of CST hyperintensities is unknown, an early radiologic-histopathologic study showed demyelination and wallerian degeneration in fibers of the tract (Yagishita et al., 1994). Even though ALS is primarily a motor neuron disorder, previous studies (Abrahams et al., 1996, 2005; Chang et al., 2005; Mezzapesa et al., 2007; Sage et al., 2007) have demonstrated involvement of extramotor regions subserving cognition and behavior, especially in ALS patients with dementia. Unlike Alzheimer's dementia, cognitive impairment in ALS patients with dementia predominantly affects frontotemporal regions of the brain and is termed frontotemporal dementia (FTD).

Unlike LMN abnormalities, which can be identified by routinely used electromyography (EMG), even if such signs are subclinical, objectively identifying UMN abnormalities (Brooks et al., 2000) is more challenging. Techniques applied to assess the latter such as transcranial magnetic stimulation and proton magnetic resonance spectroscopy are more labor intensive, and primarily used in research settings (Kaufmann et al., 2004). The neurologic examination remains the gold standard for detecting UMN abnormalities, but this is relatively subjective and dependent on the skill and acumen of the clinician. If in contrast, LMN changes like muscle atrophy, hypotonia, and hyporeflexia are very prominent, coexistent UMN signs can be masked, making diagnosis of ALS very difficult. Therefore, we evaluated conventional neuroimaging techniques used during routine clinical evaluation to provide non-invasive objective measures of UMN involvement.

The focus of this review is to summarize our previously published findings of how non-biased conventional MRI sequences acquired at 1.5T have identified differences between ALS patients with specific clinical phenotypes. Our goal was to demonstrate the utility of widely accessible routine clinical MRI in revealing unique macropathologic differences *in vivo* between such ALS patient groups and possibly to gain insights into disease pathogenesis and progression.

PATIENT DATA CONSIDERED

Groups of individuals evaluated by conventional clinical T2/PD/FLAIR-weighted MRI included: (1) UMN-predominant ALS patients *with* CST hyperintensity (ALS-CST+), (2) UMN-predominant ALS patients *without* CST hyperintensity (ALS-CST−), (3) patients with classic ALS (ALS-Cl), and (4) ALS patients with frontotemporal dementia (ALS-FTD), and (5) neurological controls. UMN-predominant ALS patients were defined as those with LMN signs that were either absent, or if present, were restricted to only one neuraxial level (bulbar, cervical, or lumbosacral) at time of MRI. UMN-predominant patients with CST hyperintensity were those in whom hyperintense signal was observed along the CST bilaterally in T2-, FLAIR-, and especially PD-weighted images. Patients with ALS-FTD displayed cognitive or behavioral impairment during clinical evaluation, as assessed by EP Pioro, including disturbances of language, executive function and impulse control. Such patients underwent bedside evaluation, including MoCA testing, extensive formal neuropsychometric testing by an experienced neuropsychologist, and usually both.

MRI STUDIES

After identifying CST hyperintensity on T2-, FLAIR-, and PD-weighted sequences in several ALS patients with UMN-predominant phenotype, we were puzzled when we observed other patients, relatively indistinguishable at initial clinical evaluation, who did *not* have CST hyperintensity. Since diffusion tensor imaging (DTI) could provide more insight with its diverse metrics (which are based on diffusion of water molecules), we studied the DTI metrics: fractional anisotropy (FA), axial diffusivity (AD), radial diffusivity (RD), and mean diffusivity (MD) along the CST in ALS patients of ALS-CST+ and ALS-CST− groups, compared to neurologic controls (Rajagopalan et al., 2011). Four levels along the rostrocaudal extent of the CST (identified by diffusion tensor tractographic reconstruction) in the white matter (WM) were examined: (1) subjacent to primary motor cortex (subPMC), (2) centrum semiovale at top of lateral ventricle (CSoLV), (3) posterior limb of internal capsule (IC), and (4) cerebral peduncle (CP), as shown in **Figure 1**. This allowed us to determine in our UMN-predominant ALS patients the level(s) where abnormalities in DTI metrics occur along the CST. Furthermore, it enabled us to determine whether quantitative differences exist corresponding to the qualitative presence or absence of CST hyperintensity.

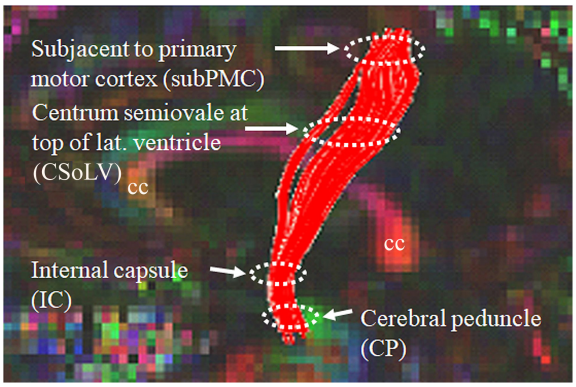

FIGURE 1 | Sagittal view of control subject brain with superimposed FA color map showing tractography-derived virtual CST fibers between subjacent to primary motor cortex (subPMC) rostrally and cerebral peduncle (CP) caudally. DTI metrics are also obtained at two intervening CST levels, including centrum semiovale at top of lateral ventricle (CSoLV) and posterior limb of the internal capsule (IC). cc corpus callosum.

Volumetric Studies in ALS-CST+ and ALS-CST− Patients

Our gray matter voxel based morphometry study (Rajagopalan and Pioro, 2014) revealed no significant difference in gray matter (GM) volume between ALS-CST+ and ALS-CST− groups in any brain region. Also, our brain parenchymal fraction (which includes GM and WM volume) study (Rajagopalan and Pioro, 2015) failed to reveal any significant difference in brain parenchymal fraction values between ALS-CST+ and ALS-CST− groups.

DTI Metrics Distinguish Between ALS-CST+ and Other ALS Patient Groups

Fractional anisotropy values were reduced in both ALS-CST+ and ALS-CST− groups when compared to controls. On the other hand, the AD and RD metrics showed significant differences at the internal capsule level only between controls and the ALS-CST+ group but not in the ALS-CST− group. It is in the posterior limb of the IC that hyperintensity is usually reported in the ALS literature (Yagishita et al., 1994; Ellis et al., 1999; Toosy et al., 2003; Pioro, 2006). Considering that AD and RD metrics reflect axonal and myelin integrity (Beaulieu, 2009), their abnormality in the ALS-CST+ group but not the ALS-CST− group suggests micropathologic differences along the CST. These results suggest that ALS patients with CST hyperintensity probably have different underling pathology from those who do not, which could arise from differing pathogenic mechanisms.

We further investigated whether neuroimaging, and specifically DTI metrics along the CST could objectively differentiate the ALS-CST+ group from the other ALS subtypes and neurologic controls (Rajagopalan et al., 2013b). In this study, we found that FA and AD values were lowest in the ALS-CST+ group when compared to controls and also when compared to the other ALS groups at rostral CST levels. When considering the CST separately in each hemisphere, significant FA differences were observed between controls and both ALS-CST+, and ALS-CST− groups (**Figure 2**). These findings, as well as significant differences in AD values between controls and patients in the ALS-CST+ group (but not between neurologic controls and those in ALS-CST− group) suggest differing micropathologies in the subcortical axons of the various ALS patient groups (**Figure 3**). Furthermore, AD, MD, and RD measures were significantly different between the ALS groups, distinguishing these values at IC and CSoLV levels of CST between patients in ALS-CST+ and ALS-FTD groups (**Figures 3–5**).

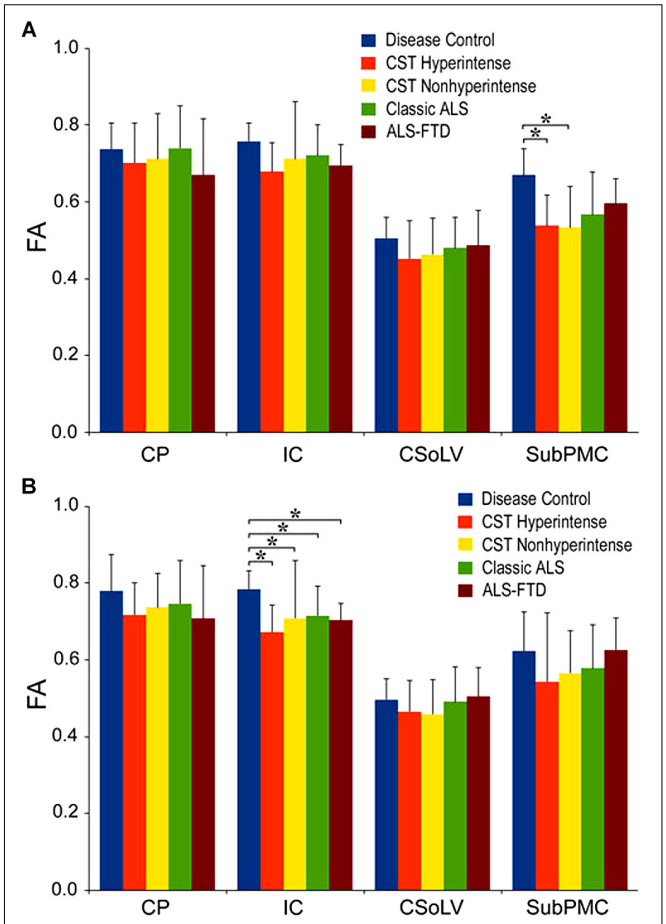

FIGURE 2 | Fractional anisotropy (FA) values at four CST levels in left **(A)** and right **(B)** hemispheres of ALS patients compared to controls showing significant differences as $^*P < 0.05$. CP, cerebral peduncle; IC, posterior limb of internal capsule; CSoLV, centrum semiovale at top of lateral ventricle; subPMC, subjacent to primary motor cortex. Reproduced with permission from Springer Nature.

Diffusion Tensor Tractography Reveals Motor Fiber-Specific Truncation

The above studies demonstrated distinct pathological changes in regions of interest (ROIs) along the CST in the ALS-CST+

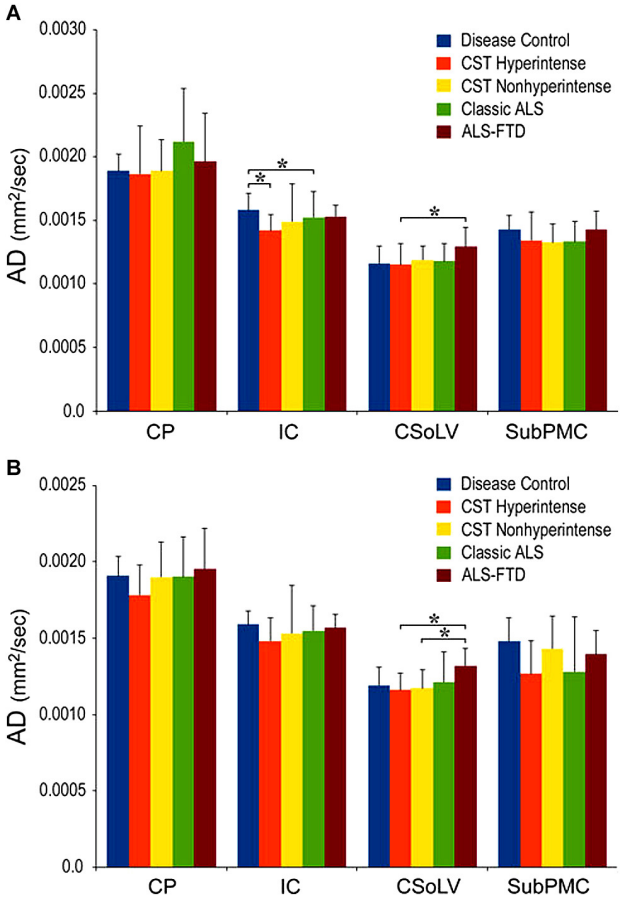

FIGURE 3 | AD values at four CST levels in left **(A)** and right **(B)** hemispheres of ALS patients compared to controls showing significant differences as $^*P < 0.05$. CP, cerebral peduncle; IC, posterior limb of internal capsule; CSoLV, centrum semiovale at top of lateral ventricle; subPMC, subjacent to primary motor cortex. Reproduced with permission from Springer Nature.

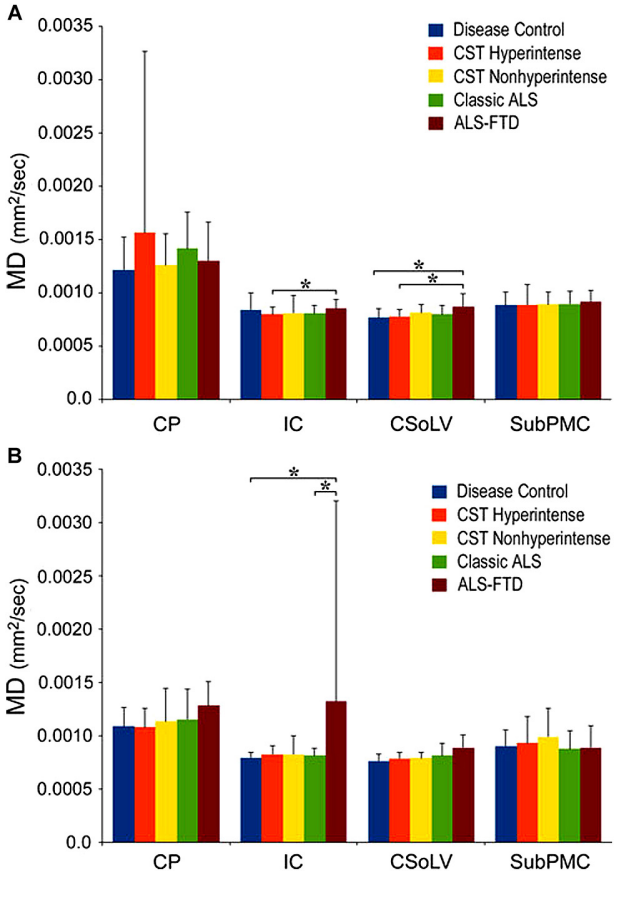

FIGURE 4 | Mean diffusivity (MD) values at four CST levels in left **(A)** and right **(B)** hemispheres of ALS patients compared to controls showing significant differences as $^*P < 0.05$. CP, cerebral peduncle; IC, posterior limb of internal capsule; CSoLV, centrum semiovale at top of lateral ventricle; subPMC, subjacent to primary motor cortex. Reproduced with permission from Springer Nature.

group compared to other ALS groups and neurologic controls. However, the ROI approach is limited because of operator bias where voxels are placed, and evaluation of the CST only where voxels are placed, rather than along its entire length. In order to more accurately and objectively identify areas along the tract's entire length, we used diffusion tensor tractography (DTT) to reconstruct a "virtual" CST (Rajagopalan and Pioro, 2017). DTT identified virtual CST fibers between the CP and just beneath (Sub) the primary motor cortex (PMC) in ALS-CST+ patients, ALS-CST− patients, and neurologic controls. Surprisingly, we observed partial *absence* of virtual CST fibers in both groups of ALS patients but not in any controls. Specifically, these fibers were absent ("truncated") at the SubPMC level, which is between the PMC and CSoLV levels in several patients of both ALS-CST+ and ALS-CST− groups, as shown from a representative patient in **Figure 6**. Of note, no truncation was observed in any of the neurologic control subjects. CST truncation occurred primarily in ALS-CST+ patients (9 of 21, 42.8%) and less frequently in ALS-CST− patients (4 of 24, 16.6%; $P = 0.049$). Further, the frequency of virtual CST truncation was significantly ($P = 0.018$) higher in all ALS patients (both ALS-CST+ and ALS-CST− groups combined) than in the control group. To determine if this truncation was specific to descending motor fibers, we identified virtual non-motor fiber tracts connecting the primary sensory cortex (PSC) and subcortical white matter. Because most of these sensory fibers are afferents to the PSC, they should generally be unaffected by corticomotoneuron degeneration. In fact, truncation of such virtual non-motor (sensory) tracts occurred in only one subject from each of the ALS patient groups: 1 of 21 (4.7%) in ALS-CST+, and 1 of 24 (4.1%) in ALS-CST− groups. Our DTT findings of subcortical truncation of essentially only virtual motor (and not sensory) fibers, as shown from a representative patient in **Figure 6**, suggest microanatomic specificity of the underlying pathophysiologic process. This is in keeping with the notion that the sensory system remains relatively unaffected in ALS with the minority of patients reporting sensory symptoms.

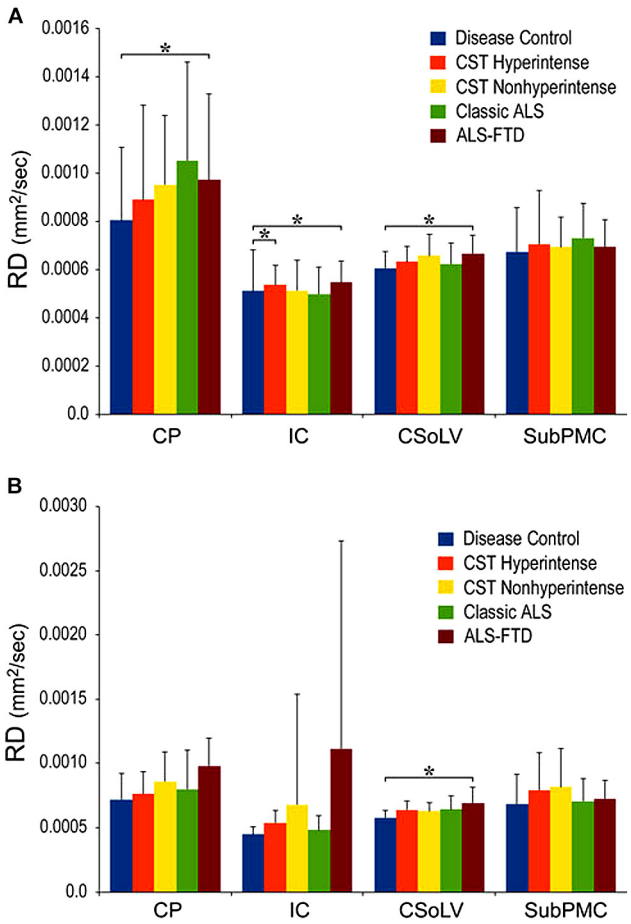

FIGURE 5 | Radial diffusivity (RD) values at four CST levels in right **(A)** and left **(B)** hemispheres of ALS patients compared to controls showing significant differences as $*P < 0.05$. CP, cerebral peduncle; IC, posterior limb of internal capsule; CSoLV, centrum semiovale at top of lateral ventricle; subPMC, subjacent to primary motor cortex. Reproduced with permission from Springer Nature.

Importantly, the truncation of motor fibers more frequently in one ALS phenotype than another, no truncation in neurologic controls, and differential involvement of motor but not sensory fibers all suggest that virtual CST truncation is and disease- and fiber-type specific. Therefore, these results further support unique pathologies along the CST in these two UMN-predominant ALS patient groups.

Disease Progression Rates Differ in Patients of ALS-CST+ and ALS-CST− Groups

Differences in the DTT findings between the two groups are supplemented by clinical observations of significantly shorter duration of symptoms prior to MRI in the ALS-CST+ group (9.6 ± 5.5 months, mean ± SD) compared to ALS-CST− group (36.4 ± 44.2 months, $P < 0.001$), as previously reported (Rajagopalan and Pioro, 2017; **Table 1**). The shorter disease duration in patients of the ALS-CST+ group compared to those of the ALS-CST− group translated into much faster disease progression in the former patients, even though both groups had essentially identical motor function scores of the revised ALS functional rating scale (ALSFRS-R) at time of MRI (34.6 ± 7.8, mean ± SD, vs. 34.1 ± 8.1, respectively). The monthly decline in ALSFRS-R (ΔFS) was three time higher in the ALS-CST+ group (1.38 ± 1.64, mean ± SD) compared to the ALS-CST− group (0.46 ± 0.43; $P = 0.001$), indicating a significantly faster decline of motor function in the former group of patients. Of note, duration of disease in the ALS-CST− group averaged 3 years prior to MRI (36.4 ± 44.2 months, mean ± SD), reflecting their slow progression, and was over 48 months in a one-third of them. This suggests that some of these slowest progressing patients without CST hyperintensity may have, in fact, represented a group with primary lateral sclerosis (PLS) (Gordon et al., 2006).

Fractal Dimension Analyses Reveal ALS Group Differences in White Matter Complexity

At a microscopic level, ALS pathology includes axonal swelling with neurofilament accumulation, dendritic attenuation, and wallerian degeneration of axons (Cluskey and Ramsden, 2001). Evidence of such micropathology, including axon degeneration and demyelination can be detected at a macroscopic level *in vivo* by certain MRI techniques (Metwalli et al., 2010). Neuronal degeneration with resultant loss of

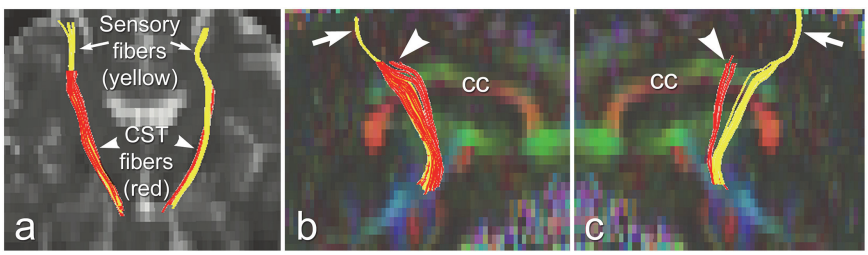

FIGURE 6 | Truncated virtual CST fibers arising from primary motor cortex (red, arrowheads) are contrasted with intact sensory fibers projecting to/from primary sensory cortex (yellow, arrows) in an UMN-predominant ALS patient with faster disease progression rate. Virtual tracts are projected on a coronal b0 image **(a)**, and on sagittal images of left **(b)**, and right **(c)** hemispheres. cc, corpus callosum. Reproduced with permission from Elsevier.

TABLE 1 | Clinical parameters of ALS patients.

Clinical measure/ALS subgroups	ALS-CST+ Mean ± SD	ALS-CST− Mean ± SD	*P*
n	21	24	NS
Age	52.3 ± 11.4	59.5 ± 12.1	< 0.05
Symptom duration prior to MRI (months)	9.6 ± 5.5	36.4 ± 44.2	< 0.001
ALSFRS-R score	34.6 ± 7.8	34.1 ± 8.1	NS
Disease progression rate	1.38 ± 1.64	0.46 ± 0.43	0.001

Key: SD, standard deviation. ALS-CST+, ALS patients with predominant upper motor neuron (UMN) signs and hyperintense signal along the corticospinal tract (CST) on conventional proton density (PD) and T2-weighted images and no clinical dementia. ALS-CST−, ALS patients with predominant UMN signs without CST hyperintensity and no clinical dementia (ALS-CST−). ALSFRS-R, ALS functional rating score-revised. NS, not significant.

dendrites and axons has been shown to reduce complexity of subcortical WM structure. Therefore, measuring WM structural complexity may reveal the effects of neuronal degeneration occurring in ALS.

Fractals are geometry objects that are self-similar at different scales, and were first proposed by Mandelbrot. The fractal dimension (FD) is a non-integer number that characterizes the morphometric variability of a complex and irregular shape. FD analysis can quantitatively measure the internal shape complexity of brain WM from MRI by characterizing multifractal behavior of different textures instead of using only pixel intensity values (Liu et al., 2003). Higher FD values reflect more WM complexity, as would be expected in healthy states, whereas lower values result with aging, and when WM becomes diseased more amorphous. In a study of patients with multiple sclerosis, reduced brain WM FD values were proposed to represent a more amorphous tissue state resulting from inflammation, decreased myelin content, and increased water content (Esteban et al., 2007).

We used FD analysis to evaluate WM structural degeneration in each of the four ALS patient groups: ALS-CST+, ALS-CST−, ALS-Cl, and ALS-FTD. In this study (Rajagopalan et al., 2013a), we estimated three quantitative measureable WM features using FD shape representations, including WM skeleton, GM/WM surface structure, and WM general structure. The skeleton captures the central line of the WM structure, which preserves the topological and geometric information of the WM, and represents its interior structure complexity. The surface structure comprised of voxels at the gray matter (GM)-WM interface, represents the shape of gyral and sulcal convolutions over the cortical surface. Finally, FD of general structure incorporates all WM voxels, including those at the GM/WM interface and skeleton in segmented images, and thereby represents brain volume. Because FD measures of skeleton, surface structure, and general structure represent different components of WM, they provided novel information about ALS-induced changes in brain WM structure and shape. General structure and skeleton FD values were significantly different between ALS-CST+ and ALS-FTD groups. Whole brain skeleton (P = 0.001) and general structure (P = 0.02) were significantly higher in ALS-CST+ patients compared to ALS-FTD patients, as shown in **Figure 7**. Although not significant, whole brain skeleton FD values in ALS-CST+ group patients trended higher than those in ALS-CST− (P = 0.10) and ALS-Cl (P = 0.10) groups. However, neurologic controls and ALS patients revealed no significant differences in FD values. These results indicate that shape complexity in the ALS-CST+ patient group was significantly greater than in the ALS-FTD group, and trended higher than in the other two patient subtypes. Although the significance of this higher FD in ALS-CST+ patients is unclear, it is likely related to differences in integrity of axons, myelin, and other changes within the neuropil, including inflammatory processes.

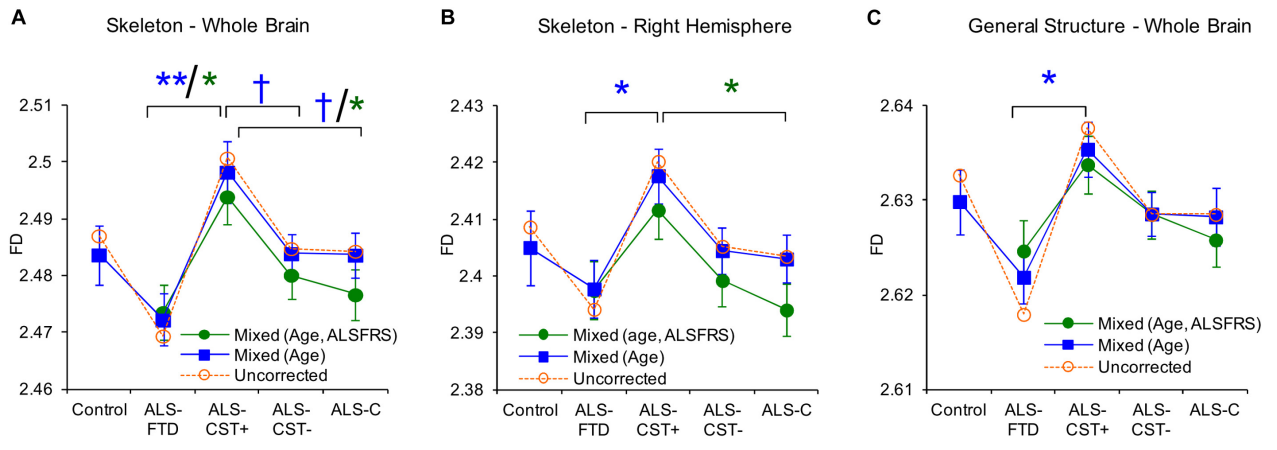

FIGURE 7 | Between group comparisons show significant differences in fractal dimension values of skeleton-whole brain **(A)**, skeleton right hemisphere **(B)**, and general structure whole brain **(C)**. Uncorrected means are represented as dashed lines and corrected means (mixed model) and standard error of the mean are shown as solid lines. Data for mixed models with gender, age as covariates are shown in blue or with gender, age, and ALSFRS-R as covariates are in green. Corrected mean comparisons between groups are performed using the Tukey multiple comparison method. $^{\dagger}p < 0.1$,$^{*}p < 0.05$, $^{**}p < 0.001$. Reproduced with permission from *PlosOne*.

Clinical Differences Between Patients in ALS-CST+ and Other Groups

Patients in the ALS-CST+ group were younger when compared to those in the ALS-CST− group ($P < 0.05$) (**Table 1**). In contrast, patients with ALS-FTD were significantly older than ALS-CST+ patients and neurologic controls. ALS-CST+ group revealed significantly shorter symptom duration compared to those in ALS-CST− ($p < 0.001$) and ALS-FTD groups ($p < 0.05$), indicating earlier neurologic evaluation after symptom onset. Also, disease progression rate was significantly faster in ALS-CST+ patients than in ALS-CST−, ALS-Cl, and ALS-FTD groups, as had been observed in a preliminary study of another group of ALS patients (Matte and Pioro, 2010). The revised ALSFRS-R score, which is a validated measure of physical function in ALS (Cedarbaum et al., 1999), was significantly lower (worse) in ALS-FTD patients compared to the ALS-Cl patients but essentially identical in ALS-CST+ and ALS-CST− patients at time of MRI.

CONCLUSION

The aforementioned brain MRI studies uniformly revealed objective differences in patients with the various ALS subtypes: UMN-predominant ALS with CST hyperintensity (ALS-CST+), UMN-predominant ALS without CST hyperintensity (ALS-CST−), classic ALS (ALS-Cl), and ALS with FTD (ALS-FTD). Specifically, patients in the ALS-CST+ group show distinctive and distinguishable changes from the others, including patients in the ALS-CST− group, which appear phenotypically similar, at least in relation to extent of UMN dysfunction. Coupled with the patients' distinct clinical characteristics, these neuroimaging abnormalities strongly suggest that CST hyperintensity, as revealed by conventional MRI (cMRI) T2/PD, and FLAIR sequences used during routine clinical evaluation, is not artefactual or non-specific but identifies a unique ALS patient group. *We hypothesize that ALS-CST+ patients comprise a distinct phenotype from ALS-CST−, ALS-Cl, and ALS-FTD with unique micropathology of the CST and potentially important differences in ALS pathogenesis.* Prescreening ALS patients for the presence of CST hyperintensity may be useful when enrolling or stratifying into clinical trials.

AUTHOR CONTRIBUTIONS

VR and EPP conceptualized the idea for the manuscript. VR wrote the manuscript. EPP made substantive revisions to the manuscript.

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frontiers in Neurology

MINI REVIEW
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Post-polio Syndrome: More Than Just a Lower Motor Neuron Disease

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Post-polio syndrome (PPS) is a neurological condition that affects polio survivors decades after their initial infection. Despite its high prevalence, the etiology of PPS remains elusive, mechanisms of progression are poorly understood, and the condition is notoriously under-researched. While motor dysfunction is a hallmark feature of the condition, generalized fatigue, sleep disturbance, decreased endurance, neuropsychological deficits, sensory symptoms, and chronic pain are also often reported and have considerable quality of life implications in PPS. The non-motor aspects of PPS are particularly challenging to evaluate, quantify, and treat. Generalized fatigue is one of the most distressing symptoms of PPS and is likely to be multifactorial due to weight-gain, respiratory compromise, poor sleep, and polypharmacy. No validated diagnostic, monitoring, or prognostic markers have been developed in PPS to date and the mainstay of therapy centers on symptomatic relief and individualized rehabilitation strategies such as energy conservation and muscle strengthening exercise regimes. Despite a number of large clinical trials in PPS, no effective disease-modifying pharmacological treatments are currently available.

Keywords: postpolio syndrome, PPS, polio, poliomyelitis, neuroimaging, biomarker, clinical trials, motor neuron disease

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INTRODUCTION

Poliomyelitis was one of the most acutely debilitating infections of the twentieth century that affected millions in the 1940 and 1950s and more recently in India during an outbreak in 1988 (1). Following the introduction of the polio vaccine in the mid-1950s and early 1960s, there has been a dramatic decline in the number of new polio cases and it is estimated to be 99% eradicated today. Despite the enormous progress in the eradication of the polio virus, 15–20 million people across the world still suffer from the sequelae of the infection (2). A large proportion of polio survivors has been presenting with a constellation of new neurological symptoms that has been described as Post-Polio Syndrome (PPS). The description of PPS is attributed to Jean-Martin Charcot in 1875 but was only widely recognized by the medical community in the early 1980s (3). PPS is characterized by new neurological deficits after a long period of neurological stability, typically at least 15 years after the initial polio infection. PPS may manifest as new, persistent, and progressive muscle weakness, atrophy, limb fatigability, myalgia, arthralgia, and dysphagia, but also as generalized fatigue, which typically has a considerable impact on the patients' quality of life. The estimates of the percentage of polio patients affected by PPS are inconsistent, varying between 20 and 85% (4, 5) depending on the diagnostic criteria applied (2). As a result, despite the rarity of acute polio infection in the modern world, PPS is likely to persist for the next few decades. Despite

its prevalence, post-polio syndrome remains surprisingly under-researched and poorly characterized. The purpose of this review is to provide a comprehensive overview of the aetiological, genetic, diagnostic, prognostic factors, and treatment modalities in PPS while highlighting key gaps that require further research.

METHODS

A literature search was performed on PubMed using the search term "post-polio syndrome," "postpolio syndrome" or "post-polio syndrome" alone and in combination with "epidemiology," "pathophysiology," "clinical features," "fatigue," "neurophysiology," "brain imaging," "electromyography," "inflammation," "diagnosis," "management," "clinical trial," "longitudinal," "cross-sectional," "case report," "autopsy," and "post mortem." Only articles written in English and published between January 1980 and May 2019 were selected for literature review. Identified publications were categorized into "academic" papers discussing pathophysiology, genetic susceptibility, biology, and "clinical" papers focusing on diagnostic criteria, management, rehabilitation, and clinical trials.

Abbreviations: 101-PNR, 101- point numeric rating; 10MWT, 10-meter walk test; 2MWT, 2-minute walk test; 6MWT, 6-minute walk test; ALS, Amyotrophic lateral sclerosis; BDI, Beck depression inventory; BiPAP, Bilevel positive airway pressure; CAS, cytokine analysis study; CBT, Cognitive behavioral therapy; CK, Creatine kinase; CMAP, Compound muscle action potential; CMV, Controlled mechanical ventilation; CSE, Clinical study extension; CSF, Cerebrospinal fluid; CSF-MC, cerebrospinal fluid mononuclear cells; ELISA, Enzyme-linked immunosorbent assay; EMG, Electromyography; ESS, Epworth sleepiness scale; FIS, Fatigue impact scale; FSS, Fatigue severity scale; FVC, forced vital capacity; HDsEMG, High density surface electromyography; HHD, hand-held dynamometry; IASP, International Association for the Study of Pain; IBM-FRS, Inclusion body myositis functional rating scale; IPAP, inspiratory positive airway pressure; KAFO, Knee ankle foot orthosis; LIC, lung insufflation capacity; LVR, Lung volume recruitment; MAF, Multidimensional assessment of fatigue; MD, Myotonic dystrophy; MEP, Maximal expiratory pressure; MFI-20, Multidimensional functional inventory; MFM scale, Motor function measurement scale; MIP, Maximal inspiratory pressure; MMPI, Minnesota multiphasic personality inventory; MRC, Medical Research Council Scale for muscle strength; MRI, Magnetic resonance imaging; MRS, Magnetic resonance spectroscopy; MUAP, Motor unit action potential; MV, Minute ventilation; MVA, Maximal voluntary activation; MVC, Maximal voluntary contraction; MVIC, Maximal isometric voluntary contraction; NHP, Nottingham health profile; NIPPV, Nasal intermittent positive pressure ventilation; NIV, Non-invasive ventilation; OSA, Obstructive sleep apnea; PASE, Physical activity of the elderly; PBMC, peripheral blood mononuclear cells; PCF, unassisted peak cough flow; PFS, Piper fatigue scale; PFT, Pulmonary function test; PLMS, Periodic limb movements of sleep; PPL, Polio problem list; PPS, Post-polio syndrome; PV, Polio virus; qMRI, quantitative magnetic resonance imaging; QMT, Quantitative motor test; rCT, randomized controlled trial; RDBPC, Randomized double-blind placebo controlled; REE, resting energy expenditure; RLS, Restless leg syndrome; RNA, Ribonucleic acid; RQ, respiratory quotient; RR, respiratory rate; RT-PCR, Reverse transcription polymerase chain reaction; rTMS, Repetitive transcranial magnetic stimulation; S-SFEMG, Single fiber electromyography stimulation; SF-36, 36-item short form survey; SFEMG, Single fiber electromyography; SFQ, Short fatigue questionnaire; SIP, Sickness impact profile; SIPP, Self-reported impairments in persons with late effects of polio; *SMN* gene, Survival motor neuron gene; SNIP, Sniff nasal inspiratory pressure; SSS test, Sit-stand-sit test; tDCS, Transcranial direct current stimulation; TQNE, Turf's quantitative neuromuscular examination; TUG test, Timed-Up-and-Go test; UW-SES, University of Washington self-efficacy scale; VAS, Visual analog scale; VAS-F, Visual analog scale for fatigue; VCO_2, carbon dioxide production; VO_2, oxygen consumption; WBV, Whole body vibration; WHOQOL-BREF, World Health Organization quality of life abbreviated scale.

RESULTS

Pathophysiology

During the acute poliomyelitis infection, 95% of those infected remain asymptomatic or only suffer flu-like symptoms while the remaining 5% succumb to the paralytic form of the disease. Acute poliomyelitis is typically spinal, affecting the limbs and respiratory musculature, but bulbar manifestations affecting speech and swallow are also well-documented. Polioenterovirus type 1 is the main cause of meningeal, spinal cord and brain inflammation as it can cross the blood-brain barrier independently from poliovirus receptors (6, 7). Ensuing anterior horn degeneration, and apoptosis post infection has been widely recognized as the hallmark feature of paralytic poliomyelitis. Following the acute phase, axonal sprouting takes place reinnervating the muscle of the affected regions (8, 9). Motor units gradually become abnormally enlarged, up to 7-fold their original size (10) rendering them metabolically unsustainable (11). This process can take up to three decades from the acute infection to the development of PPS symptoms (12). The concomitant denervation-reinnervation process is evidenced by electromyography (EMG) findings (13–17) and muscle histology showing small angulated fibers (18, 19) and muscle fiber type-grouping (15). Metabolic stress (11, 20), overuse (21, 22), physiological aging (20, 23), and persistent inflammation (24) are also thought to contribute to gradual motor unit failure. Motor units loss has been consistently correlated to functional decline in longitudinal studies (13, 14, 25, 26). Overuse of functioning muscle units is thought to induce detrimental structural alterations (27, 28). Cellular adaptation in the muscles, such as fiber alteration from type II (fast) to type I (slow) (28), changes in contractile properties (29–31), and muscle hypertrophy (9) are likely to contribute to muscular fatigue and myalgia in PPS. The persistence or reactivation of polio virus in polio survivors has also been suggested with conflicting reports. Two research studies (7, 32) have identified polio-virus (PV) genomic sequences in the CSF and peripheral leucocytes as well as high serum IgM anti-PV antibody titres, which were absent in stable polio survivors and in other neurodegenerative groups (33). Other studies however could not confirm these findings (34). An inflammatory or autoimmune basis to post-polio syndrome has also been proposed. This hypothesis originates from post mortem observations of inflammatory changes in the spinal cord of PPS patients (35, 36). The role of inflammation is also supported by *in vivo* evidence. Increased serum and CSF levels of pro-inflammatory cytokines and peptides such as TNF-α, IFN-γ were repeatedly observed in PPS (37–39). Furthermore, TNF-α and IFN-γ levels respond to IVIg therapy in PPS, and remain unchanged in controls (37, 38, 40). However, no correlations have been detected between symptom severity (38), rate of decline (37), and pro-inflammatory peptide levels. Skeletal muscle biopsies also exhibit inflammatory changes and increased expression of prostaglandin E2 synthetic pathway enzymes (41). Relatively limited evidence exists to support the autoimmune basis of PPS. One study identified high titres of PV antibodies concurrently with high levels of regulatory T cells (42), while another study (43) found normal levels of immune

complexes in PPS patients. No specific anti-muscle or anti-neuronal autoantibodies have been associated with PPS (44). A genetic predisposition for PPS has also been investigated, but no conclusive risk profile has been identified to date. *SMN* gene deletion (45, 46) associated with spinal muscular atrophy (SMA) was not reported in PPS, but Fc-gamma receptor IIIA polymorphisms may play a role in the predisposition to PPS (47).

Neuropathology and Neuroimaging

Post-mortem studies are conflicting with regards to cerebral involvement in post-polio syndrome. Post-mortem studies (48) from 50 to 70 years ago suggest that polio virus preferentially affects the reticular formation, posterior hypothalamus, thalamus, putamen, caudate, locus co-eruleus, and substantia nigra which may account for the late-onset fatigue and attention deficit (49–52). Interestingly, cortical involvement is relatively selective and preferentially involves the precentral gyrus and pre-motor areas. A more recent case report (53) and a retrospective analysis of formalin-fixed central nervous system (CNS) tissue of a small cohort of patients (33) arrived at a different conclusion. They identified no cerebral involvement at all, but selective spinal cord pathology affecting the anterior roots with dorsal root sparing. These studies detected enterovirus RNA in spinal cord only. There have also been rare reports of polio patients developing ALS with characteristic histopathological findings (54, 55). Compared to other motor neuron diseases (56), there is a striking paucity of brain (57) and spinal cord imaging studies in PPS (58). Magnetic resonance imaging (MRI) has been used to evaluate volumetric changes (59) and to correlate anatomical changes to post mortem findings (48). The main focus of existing brain imaging studies in PPS was to explore the substrate of fatigue. Multiple hyperintensities were identified in the reticular formation, putamen and medial lemniscus in the majority of PPS patients (48) which is consistent with previous post mortem studies (49–52). A large study of 118 participants compared the brain volume profile of 42 PPS patients, 49 multiple sclerosis patients and 27 controls, and no statistically significant volume reductions were identified in PPS (59). No association was identified between fatigue and brain volumes. The majority of existing studies are cross-sectional which provide limited insights into progressive longitudinal alterations (60). There is an ongoing longitudinal, case-control study to characterize spinal cord alterations in PPS (61).

Diagnosis

Post-polio syndrome is a clinical diagnosis, supported by electrophysiological findings and possible mimics need to be reassuringly ruled out. An extensive work-up including laboratory tests, imaging studies, cerebrospinal fluid sampling, detailed electrophysiological evaluation, and muscle biopsies may be required to exclude alternative diagnoses. The diagnostic criteria for PPS was first proposed by Halstead in 1991 (62) and evolved over time to the current March of Dimes diagnostic criteria (63, 64) which include:

1. Prior paralytic poliomyelitis with evidence of motor neuron loss, as confirmed by history of the acute paralytic illness, signs of residual weakness and muscle atrophy on examination, or signs of denervation on EMG.
2. A period of partial or complete functional recovery after acute paralytic poliomyelitis, followed by an interval (usually 15 years or more) of stable neuromuscular function.
3. Gradual onset (rarely abrupt) progressive and persistent new muscle weakness or abnormal muscle fatigability (decreased endurance), with or without generalized fatigue, muscle atrophy, or muscle and joint pain. Onset may at times follow trauma, surgery, or a period of inactivity. Less commonly, bulbar dysfunction or respiratory weakness occurs.
4. Symptoms that persist for at least a year.
5. Exclusion of alternative neuromuscular, medical, and orthopedic problems as causes of symptoms.

PCR amplification of poliovirus RNA in the CSF is indicative of prior history of poliomyelitis (6, 7, 32) and the presence of pro-inflammatory cytokines may also be detected (39, 65). Proteomic CSF markers such as gelsolin, hemopexin, peptidylglycine alpha-amidating monooxygenase, glutathione synthetase, and kallikrein 6 have been proposed as diagnostic markers but supporting evidence from larger studies is lacking (4). On muscle biopsy, hypertrophic muscle fibers type I (66, 67), indicative of compensatory reinnervation and small angulated fibers, indicative of active denervation (19) may be observed. CSF sampling and muscle biopsy also allows the exclusion of other neuromuscular mimics. People with PPS typically undergo detailed spinal imaging to rule out alternative structural, neoplastic, compressive, or inflammatory spinal etiologies which could manifest in lower motor neuron dysfunction (58, 68–70). Electromyography (EMG) is an invaluable tool to assess suspected post-polio cases, as it allows the confirmation of a prior history of poliomyelitis while excluding differential diagnoses (71). A variety of EMG techniques have been used in post-polio research studies including single fiber EMG (SFEMG), high density surface EMG (HDsEMG) (72), and macro-EMG. Ongoing denervation can be detected on conventional EMG by the presence of fibrillation and fasciculation potentials and increased jitter on SFEMG in newly weakened muscles (73). Needle EMG can also readily detect sub-clinically affected muscles in PPS (74). EMG measures correlate well with muscle strength and endurance (75, 76). While EMG provides important insights, EMG measures don't differ significantly between those with PPS and stable polio (77) and thus EMG is not regarded as an electrodiagnostic tool to confirm PPS (73). PPS is therefore a clinical diagnosis supported by laboratory tests.

The Spectrum of Clinical Manifestations

Post-polio patients characteristically experience new onset muscle weakness, decreased endurance, muscle atrophy, myalgia, and fasciculations (78). Additional symptoms often include generalized fatigue, cold intolerance, dysarthria, dysphagia, and respiratory compromise (79, 80). New symptoms typically occur in previously affected areas but sub-clinically affected body regions can also get affected (74). Ambulatory difficulties often necessitate assistive devices, and may lead to increased fall

risk (81). PPS is also associated with a wide range of non-motor symptoms. Frank sensory deficits may be detected and paraesthesias are often reported by PPS patients. Changes in sensory evoked potentials have been linked to cord atrophy on MRI (82). There have been consistent reports of cognitive deficits (83) in PPS including word finding difficulties (84), poor concentration, limited attention, memory impairment (85), and mood disturbances (86). The non-motor aspects of PPS are often under evaluated despite their considerable quality of life implications (87). Due to the combination of motor disability (88) and non-motor symptoms, many patients engage less in social activities (89) which may lead to social isolation. Generalized fatigue is one of the most distressing sequelae of PPS which is likely to be multifactorial due to muscle unit pathology, weight-gain, respiratory compromise, polypharmacy, and poor sleep (**Figure 1**). The identification of the key "fatigue-factors" in individual patients is indispensable for the effective pharmacological and non-pharmacological management of fatigue. Fatigue is thought to exhibit circadian variations throughout the day (90). Sleep disorders such as restless leg syndrome (RLS) (87, 91–94), sleep related breathing disturbances (95), obstructive sleep apnoea (OSA) (96), excessive daytime somnolence (EDS), and periodic limb movement in sleep (PLMS) (97) are not only often reported in PPS but they are likely to play an important role in the pathogenesis of fatigue in PPS (98, 99). Fatigue is thought to be more severe in PPS with RLS, and correlate to the severity of RLS (87). The simultaneous onset of RLS and PPS symptoms (91) and the positive response to pramipexole in an uncontrolled trial by Kumru et al. (93) have been interpreted as a pathophysiological link between RLS and PPS (98). The putative link between RLS and neuroimmunological alterations (100, 101) may also suggest shared pathophysiological processes between PPS and RLS (99). Furthermore, a higher incidence of cauda equina syndrome (102) and renal impairment (103) has also been reported in PPS but the association between these syndromes remains to be elucidated.

Progression, Assessment, and Monitoring

The majority of longitudinal studies (14, 25, 104–107) detect progressive muscle weakness, which contributes to deteriorating gait performance (107) and declining mobility (105). Quantifying the rate of decline in PPS is challenging and no reliable functional predictors have been validated. Male gender is thought to be a negative prognostic indicator (108), but PPS is more common in females (12). Most PPS patients who participated in research studies have lived with PPS for over 13 years suggesting that PPS is a relatively slowly progressive condition. There have also been however sporadic reports of rapidly progressive and life-threatening forms of PPS (109), which raises the question of occasional misdiagnoses or a link between PPS and amyotrophic lateral sclerosis (ALS) (54). The severity of PPS-associate disability is typically evaluated clinically but a number of rating scales and questionnaires have been developed and validated for both clinical and research use. In addition to mobility and dexterity, these instruments evaluate the non-motor aspects of the condition such as fatigue, pain, sleeping disturbances, and mood (110). Clinical tests used to assess motor disability include the 6-min walking test (6MWT) (111) at self-preferred speed, the 2-min walking test (2MWT) at maximal speed (112), Timed-Up-and-Go test (TUG) (113), 10 meters walking test (10MWT), Sit-Stand-Sit test (SSS) (114). Muscle strength is typically appraised by manual muscle testing using the MRC scale, or more objectively using a dynamometer during maximal isokinetic and isometric voluntary contraction. Endurance is measured using isometric contraction peak torque, isometric endurance, tension time index (TTI) or recovery of torque after endurance test (76). Quantitative muscle mass assessment can be performed using ultrasound parameters such as muscle echo intensity and muscle thickness which are non-invasive tools for disease monitoring (115). The most commonly used instruments to assess non-motor domains include the Fatigue Severity Scale (FSS) (116), Fatigue Impact Scale (FIS), Piper Fatigue Scale (PFS), Short Fatigue Questionnaire (SFQ), Nottingham Health Profile (NHP), Physical activity scale for the elderly (PASE) (117), Polio Problem List (PPL), Visual analog scale (VAS) (118), Multidimensional Fatigue Inventory (MFI-20) (119), World Health Organization quality of life abbreviated scale (WHOQOL-BREF) (120), University of Washington Self-Efficacy Scale (UW-SES) (121), Sickness Impact Profile (SIP), 36-item Short Form Health Survey (SF-36) (112). Sleep disturbances (97) and respiratory function can be formally assessed through polysomnography and pulmonary function tests (PFT) (122, 123). RLS is typically diagnosed clinically (124) and most commonly evaluated using the validated international RLS rating scale (IRLS) (87, 93, 125). Maximal inspiratory and expiratory pressures (MIP and MEP), sniff nasal inspiratory pressure (SNIP) (126), and arterial blood gases are validate markers of respiratory function in PPS.

Non-pharmacological Interventions

The effective management of the heterogeneous symptoms of PPS requires individualized care in a multidisciplinary setting (127). Expert input from physiotherapists, occupational therapists, speech and language therapists, respiratory physicians, podiatrists, psychologists, dieticians, pain specialists, social workers, nurse specialists, and orthotists are needed to meet the multifaceted care and support needs of PPS patients (128). Individualized lifestyle modifications and energy conservation strategies are indispensable in the effective management of PPS (129). PPS-specific training regimens alternating active intervals and rest have been developed to improve cardiorespiratory fitness, conserve energy during routine activities, and maintain independence (130). Isokinetic, isometric, resistance, and endurance training are thought to improve muscle strength and endurance without further muscle unit degeneration (131–140). Combining aerobic and flexibility training is also thought to improve QoL. Supervised training is advised in those with significant disability (141). Training in a warm environment may have longer lasting effects than training in colder temperatures (142). Patients with arthralgia may benefit from dynamic water exercises (143) as well as exercising in a group setting (144). Deconditioning of the cardiorespiratory system (145) may limit the effectiveness of aerobic training in PPS (146), therefore

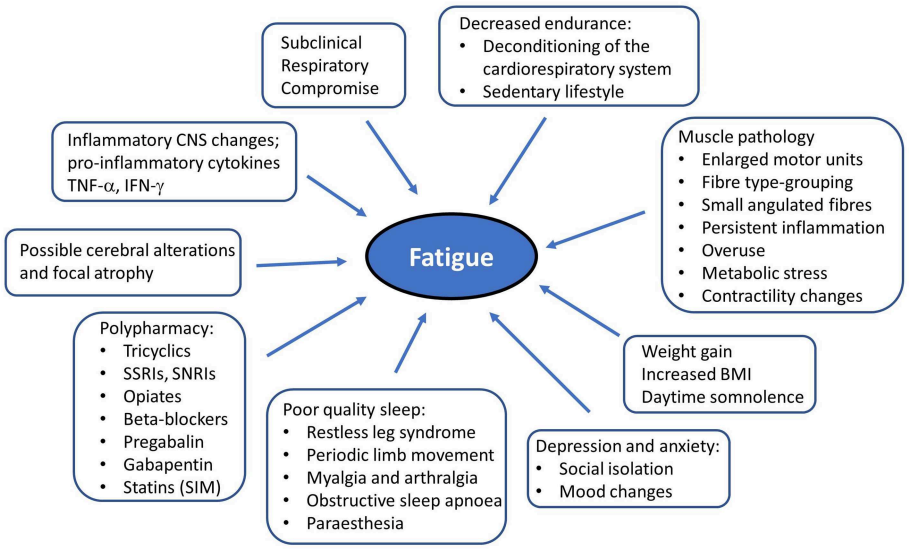

FIGURE 1 | Putative factors in the etiology of generalized fatigue in post-polio syndrome. RLS, Restless leg syndrome; PLMS, periodic limb movement in sleep; CNS, Central nervous system.

aerobic regimens must be carefully tailored to individual fitness levels (147). While some studies show improved endurance following mid- to high-intensity aerobic exercises (139, 140), a recent study (148) highlights that high-intensity aerobic exercise may not be beneficial in PPS patients with fatigue. Due to the heterogeneity of disability profiles in PPS, individualized training regimes and exercises that don't rely on anti-gravity strength are particularly important (148–150). Home-based arm ergometry for example is a well-tolerated and safe form of aerobic exercise (149, 150). Whole body vibration (WBV) has been proposed as an alternative to exercise in PPS (151) and improved mobility was reported in a small study (152), but no improvement was noted in muscle strength or gait performance (153). Orthoses are commonly prescribed for PPS patients to improve mobility and reduce pain. New powered-type Knee Ankle Foot Orthosis (KAFOs) offer limited benefits on gait symmetry or walking speeds but were shown to improve base support, swing time, stance-phase, and knee flexion during swing phase (154). The emergence of novel, light-weight materials such as carbon fiber (155) and the biomechanical analysis of individual walking patterns have helped to optimize orthosis-design for patients. The use of MIG3 Bioceramics fabrics for example had beneficial effects on pain and periodic limb movement (156). Other lifestyle modification such as weight loss, smoking cessation, increased physical activity, and modification to daily activities have all been beneficial to patients with PPS (22). There are sporadic reports that anodal transcranial direct current stimulation (tDCS) of premotor regions (157), repetitive transcranial magnetic stimulation (rTMS) of the left prefrontal cortex (158) and static magnetic fields (159) may ameliorate fatigue, improve sleep, reduce pain, and even improve motor functions in PPS, but these studies have not been replicated. PPS patients with bulbar involvement require expert phonatory and swallowing assessments by a speech-and-language therapist (160) and careful follow-up. Instrumental modalities such as ultrasonography and videofluoroscopy (161) and clinical instruments (162) can be used to detect progressive bulbar dysfunction and appraise the risk aspiration. Compensatory swallowing techniques, dietician input for food consistency alterations, individualized speech therapy, and laryngeal muscle training may be helpful in PPS patients with bulbar involvement (163). PPS patients who suffer from respiratory compromise and sleep related breathing disorders benefit from lung volume recruitment (LVR) (164) and non-invasive ventilation (NIV) such as Bi-PAP (165) or nasal intermittent positive-pressure ventilators (NIPPV) (166). Invasive ventilatory support with a tracheostomy is seldom required in PPS (167).

Addressing the non-physical aspects of PPS; mitigating psychological responses, emotional reactions, frustration, and fear of falling are equally important aspects of multidisciplinary care (168). Despite its positive effects on self-esteem (169), cognitive behavioral therapy (CBT) is not superior to standard multidisciplinary care in the treatment of fatigue (170–172). Psychotherapy is primarily aimed at reducing anxiety, improving depressive symptoms (173), alleviating pain (174, 175), and enhancing subjective well-being (176). Hope-oriented psychotherapy and encouraging participation in work (177) promote resilience in polio survivors and is associated with improved social functioning (178), satisfaction with social roles, improved quality of life, and superior mental health (179). Peer-support groups are also instrumental in buffering the impact of a functional impairment on psychosocial well-being (180). Furthermore, a reduction of physical demands at work and ergonomic adaptations at the workplace not only help PPS patients to maintain their occupational activities but enjoy their work (181). Rehabilitation nurses also play an important role in

TABLE 1 | Pharmaceutical and non-pharmaceutical clinical trials in post-polio syndrome; study characteristics and key outcomes.

References	Study Design/selection criteria of PPS patients	Number of follow-up time points	Follow-up interval (months)	Number of participants receiving drug/placebo	Assessment tools used	Key study findings
PREDNISONE						
Dinsmore et al. (183)	RDBPC/U	3	3	7/7	MRC scale, MVIC using electronic strain gauge tensiometer, fatigue on a 0–3 scale	- Short-lived improvement in muscle strength - No improvement in fatigue - Not recommended
AMANTADINE						
Stein et al. (184)	RDBPC/S (fatigue)	2	2	10/13	FSS, VAS-F, MMPI, BDI, somatization scale, reaction time evaluation	- Not superior to placebo for fatigue
PYRIDOSTIGMINE						
Trojan et al. (185)	RDBPC/S(fatigue/muscle weakness)	6	at 6 weeks, 10 weeks, and 6 months	43/42	SF-36, modified TQNE, MVIC by electronic strain gauge, Hare Fatigue Symptom Scale, FSS, IGF-1 serum levels	- Very weak muscles became slightly stronger - IGF-1 increased in compliant patients - No clear benefits on QoL, muscle strength, and fatigue
Horemans et al. (186)	RDBPC/S (fatigue and muscle weakness)	5	0.75	31/31	NHP, FSS, 2MWT at comfortable pace, time to walk 75 m at fastest speed, ambulatory activity monitor, MVC by chair dynamometer, MVA by interpolated stimulation; muscle fatigability by sEMG during 30 s sustained isometric contraction at 40% of MVC, NMJ defects by jitter on S-SFEMG	- No significant effects on fatigue - Significant effects on walking distance - Little effects on walking duration, muscle strength, MVA - Limited benefits in physical performance
MODAFINIL						
Chan et al. (187)	RDBPC cross-over/S (fatigue)	12	0.25	7/7 Cross-over 7/7	PFS, ESS, aural digit spans, reaction time	- Not effective in fatigue
Vasconcelos et al. (188)	RDBPC cross-over/ S (fatigue)	2	1.5	18/18 Cross-over 18/15	FSS, VAS-F, FIS; SF-36	- Not superior to placebo in fatigue and QoL improvement
CO-ENZYME Q10						
Skough et al. (189)	Parallel RDBPC/S(ability to perform resistance training)	2	3	7/7	Sit-stand-sit test (SSS); Timed up and go (TUG) test, 6MWT, dynamometer, bloods for CK, LD	- No change in CK or LD - No additional effects of the Co-enzyme Q10 supplementation during resistance training
Peel et al. (190)	Parallel RDBPC/S (fatigue)	2	2	54/49	MAF (revised Piper Fatigue Scale), FSS	- Not effective in fatigue
LAMOTRIGINE						
On et al. (191)	RDBPC/S (ambulatory with lower limb involvement only)	3	0.5	15/15	VAS, NHP, FSS	- Superior to placebo for pain, fatigue, and QoL as detected in VAS, NHP, FSS

(Continued)

TABLE 1 | Continued

References	Study Design/selection criteria of PPS patients	Number of follow-up time points	Follow-up interval (months)	Number of participants receiving drug/placebo	Assessment tools used	Key study findings
INTRAVENOUS IMMUNOGLOBULIN (IVIg)						
Gonzalez et al. (38)	Controlled open-label/U	2	1.5-2	16PPS; 26OND/0	CSF for CSF-MC, PB for PBMC, real-time quantitative RT-PCR for relative quantitation of mRNA	- Significant decrease of CSF-MC expression of TNF-α and IFN-γ not seen in PBMC expression of cytokines
Kaponides et al. (192)	Uncontrolled open-label/S (ambulatory, BMI < 28)	3	at 2 and 6 months	14/0	Dynamic dynamometer, 6MWT, SF-36	- No significant effect on muscle strength and physical performance
Gonzalez et al. (193)	RDBPC/U	2	3	67/68	Dynamometer, SF-36, 6MWT, TUG, PASE, sway, sleep quality, VAS, MFI-20	- Positive changes in muscle strength, physical activity, and those with significant pain - No change on QoL, fatigue sleep quality, "better" limb muscles or mild pain
Farbu et al. (40)	RDBPC/U	5	3	10/10	MAF (revised Piper Fatigue Scale), FSS, CSF, and PB for expression of cytokines (TNF-α, IFN-γ, IL-6, IL-1β, IFN-β, IL-10) using ELISA	- Positive effects on pain after 3 months - No effects on muscle strength and fatigue - TNF-α increased in CSF
Werhagen et al. (194)	Uncontrolled open-label/S (pain)	2	6	45/0	Neurological examination, sensory testing, soft tissue palpation, and joint assessment, VAS, pain classified according to IASP	- Better results on pain in younger, those with more pronounced paresis, had acute polio <10 yo
Östlund et al. (195)	Uncontrolled open-label/S(fatigue, muscle weakness)	2	6	113/0	SF-36, PASE, VAS	- Likely responders include those with pain intensity above VAS of 20 mm, younger than 65 yo, and paresis in lower extremities
Gonzalez et al. (65)	RDBPC and controlled quantitative cytokine study/U	2	12	CSE: 20/21 CAS: 20/30	CSE: SF-36, 6MWT, VAS CAS: CSF and PB for cytokines (TNF, IL-23, IFN-γ, TGF-β, IL-10, IL-13) using RT-PCR	- Improvement in QoL but not in pain and walking ability compared to placebo - Decline in CSF IFN-γ and IL-23, TNF, and increase in IL-10 and IL-13 - No changes in PB cytokine levels
Bertolasi et al. (196)	RDBPC/U	3	2	24/26	SF-36, MRC scale, dynamometer, 6MWT, VAS, 101-PNR, FSS	- Improvement in QoL; mental activity subscale - No effects on gait, muscle strength, fatigue, and pain
L-CITRULLINE						
Schmidt et al. (197)	RDBPC/U	5	6	15/15	6MWT, MFM scale, qMRI, MRS, bloods for muscle necrosis (CK), oxidative stress (8OHDG, 4-HNE), nitrosative stress(nitrotyrosine, cGMP), mitochondrial-related	- Ongoing clinical trial

(Continued)

TABLE 1 | Continued

References	Study Design/selection criteria of PPS patients	Number of follow-up time points	Follow-up interval (months)	Number of participants receiving drug/placebo	Assessment tools used	Key study findings
					genes (*Citratsynthase, Cytochrome C oxidase subunit 1, Succinate dehydrogenase subunit A)*, QMT using HHD, SIPP,IBM-FRS,WHOQOL-BREF	
RESPIRATORY SUPPORT						
Kaminska et al. (164)	Feasibility/S(restrictive respiratory defects)	2	3	7ALS, 7PPS, 5MD	SF-36, SIP, standard spirometry (FVC, FVC% predicted, LIC, LIC-FVC difference, PCF, MIP, MEP)	- LVR Feasible - Encouraging effects on respiratory mechanics - LIC increased
Gillis-Haegerstrand et al. (165)	Randomized comparative/S(using VCV)	2	30 min	8	BP, oxygen saturation, ABG, indirect calorimetry (SaO_2, VO_2, VCO_2, REE, RQ, RR, IPAP)	- BiPAP PSV decreases oxygen cost of breathing in PPS with respiratory failure without decreasing ventilation efficiency. - Significant $PaCO_2$ decrease using this ventilation modality. - Maintains adequate ventilation in PPS patient with resp. failure
Barle et al. (167)	Comparative /S (nocturnal invasive CMV)	7	30 min	9	BP, oxygen saturation, ABG, indirect calorimetry (SaO_2, VO_2, VCO_2, REE, RQ, MV,RR, IPAP)	- Invasive BiPAP reduces oxygen cost of breathing in long-standing tracheotomized PPS compared to CMV.
EXERCISE PROGRAM						
Murray et al. (149)	Assessor blinded rCT/U	2	2 months	26/29	6-MAT, PASIPD, 6MWT, FSS, SF-MPQ-2, QMA, exercise log	- Home-based ergometry is a well-tolerated form of aerobic exercise - No improvement of physical fitness, fatigue, activity - Slight decrease in BP in interventional group
PRAMIPEXOLE						
Kumru et al. (93)	Uncontrolled open label/U	3	At 0, 2 months and 6 months	16/0	RLS severity scale	- Significant decrease of RLS severity detected on RLS rating scale - Maintenance of improvement of RLS with pramipexole at 6 months follow-up

rCT, randomized controlled trial; S, selected (i.e., fatigued); U, unselected; RDBPC, Randomized double-blind placebo controlled.

the setting of realistic health goals, encouraging resiliency, and providing emotional support (182).

Pharmacological Trials

Several randomized controlled clinical trials (RCT) were conducted in PPS (**Table 1**). High-dose prednisone (183), amantadine (184), and modafinil (187, 188) showed no superiority to placebo in the management of fatigue. Prednisone therapy, showed a short-lived improvement in muscular strength but no meaningful functional improvement (183). The evidence for the benefit of pyridostigmine therapy remains conflicting. Some studies (185) identified no benefit on muscle function while others reported a slight improvement in walking performance (186). Co-enzyme Q10 supplements are thought to have no effect on muscle strength, endurance or fatigue in PPS (189, 190). A small RCT of lamotrigine, demonstrated improvements in VAS, NHP, and FSS suggesting that it may be beneficial to treat pain and fatigue and improve quality of life (191). Given the inflammatory and autoimmune hypothesis of PPS pathogenesis, intravenous immunoglobulin has been extensively investigated for its potential therapeutic effects. Its benefit with regards to pain, muscle strength, physical functioning, and quality of life is inconsistent. Improved pain control and overall vitality (192, 196) seem to be the main benefit of intravenous immunoglobulin (IVIg) treatment. Two small uncontrolled trials (38, 194) and two larger RCTs (40, 65) arrived to similar conclusions with regards to pain control and improvement in serum and CSF inflammatory markers. The main indicators for response to IVIg include severe pain, fatigue, <65 years of age, and paresis mainly affecting the lower extremities (194, 195, 198). Studies are somewhat conflicting on its effect on muscle strength (65, 193). These findings however encourage further large RCTs to establish the target PPS cohort for IVIg treatment, treatment intervals, and dose optimisation. A single-center, double-blind RCT trial of L-citrulline (197) is currently underway to investigate its effect on muscle metabolism and function. It is at clinical phase IIa and has proven to be of beneficial in muscular dystrophies in improving endurance in both aerobic and anaerobic exercise. The symptomatic management of non-motor symptoms in PPS also has considerable quality of life benefits. Restless leg syndrome in PPS often responds to dopamine agonists such as pramipexole (93, 199). The use of analgesics and antidepressants such as amitryptiline, duloxetine, and codeine may decrease physical discomfort and improve mood but need careful monitoring as they may worsen fatigue and lead to poor concentration. Adverse reactions to certain anesthetic agents are well-documented in PPS. Post-anesthesia fatigue, somnolence, and weakness are well-recognized, and fatal outcomes due to respiratory arrest have also been reported (200, 201). The diagnosis of PPS needs to be carefully discussed with the anaesthesiologists, so the appropriate muscle relaxants and anesthetics can be used, and patients should be advised of the possibility of a prolonged post-operative phase (202).

CONCLUSIONS

Despite being one of the most devastating neurodegenerative conditions in the world, surprisingly limited research is undertaken in post-polio syndrome. Its pathogenesis remains elusive, no sensitive diagnostic tools have been developed, and validated prognostic and monitoring markers are lacking. Non-motor symptoms of PPS have considerable quality of life implications and are notoriously challenging to manage. The etiology of fatigue in PPS is yet to be elucidated and successful individualized management strategies are needed to maintain mobility, independence, and patient autonomy. There is striking a paucity of neuroimaging studies in PPS that could provide anatomical insights into the substrate of extra-motor symptoms. Ultimately, the characterization of PPS-associated pathology may help research efforts in other motor neuron diseases.

AUTHOR CONTRIBUTIONS

The manuscript was drafted by SL and PB. The manuscript was edited, adjusted, and reviewed for intellectual content by RC, EF, DM, and OH.

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MINI REVIEW
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Past and Future of Neurotrophic Growth Factors Therapies in ALS: From Single Neurotrophic Growth Factor to Stem Cells and Human Platelet Lysates

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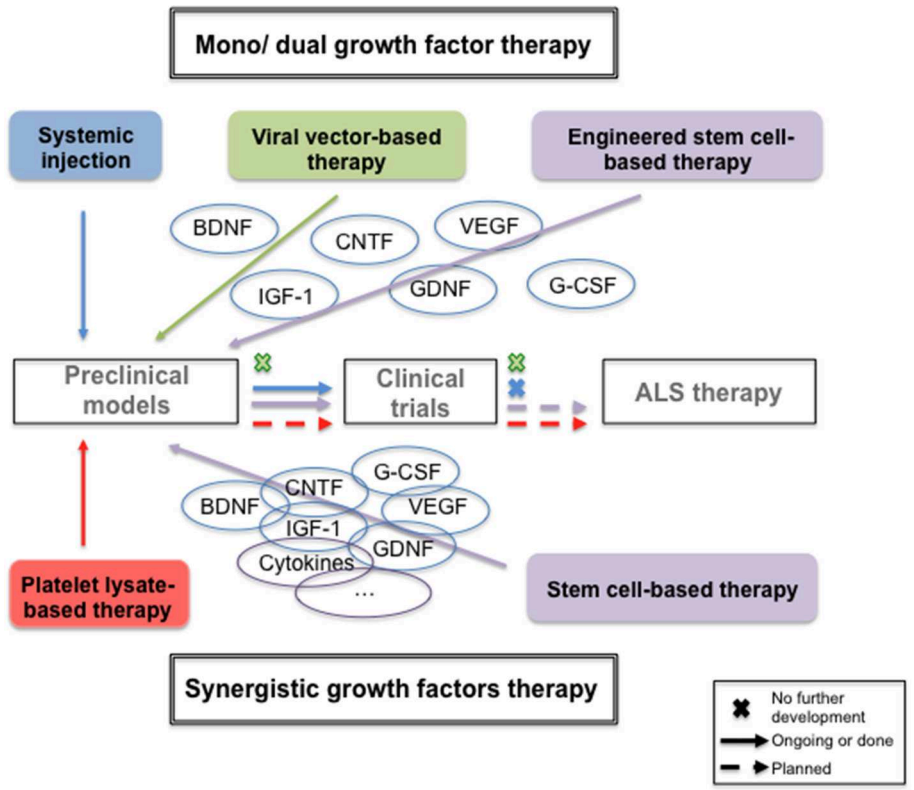
Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that typically results in death within 3–5 years after diagnosis. To date, there is no curative treatment and therefore an urgent unmet need of neuroprotective and/or neurorestorative treatments. Due to their spectrum of capacities in the central nervous system—e.g., development, plasticity, maintenance, neurogenesis—neurotrophic growth factors (NTF) have been exploited for therapeutic strategies in ALS for decades. In this review we present the initial strategy of using single NTF by different routes of administration to the use of stem cells transplantation to express a multiple NTFs-rich secretome to finally focus on a new biotherapy based on the human platelet lysates, the natural healing system containing a mix of pleitropic NTF and having immunomodulatory function. This review highlights that this latter treatment may be crucial to power the neuroprotection and/or neurorestoration therapy requested in this devastating disease.

Keywords: Amyotrophic lateral sclerosis, growth factors, therapeutic, stem cell, human platelet lysate

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease affecting the upper and lower motor neurons in the cerebral cortex, brainstem and spinal cord that lead to a progressive, irreversible muscle paralysis, and swallowing and respiratory dysfunctions. Death eventually occurs 3–5 years after diagnosis (1). The majority of ALS cases (90%) are sporadic with unknown cause (2). To date, there is no curative treatment in ALS. Therefore, the development of new and effective treatment is highly urgent. Among the different approaches, the delivery of neurotrophic factors (NTFs) is explored since the 90's because NTFs are necessary to regulate several physiological processes such as neuronal differentiation and survival, axonal outgrowth and synapses maintenance (3–5), proliferation and differentiation of stem cells in the nervous system (6–9). Therefore, these trophic factors represent a promising therapeutic strategy to treat neurodegenerative diseases (10) such as ALS.



Graphical Abstract | From single to synergistic neurotrophic growth factors therapies.

PRECLINICAL EVIDENCE OF NEUROTROPHICS GROWTH FACTORS ABILITIES TO TREAT AMYOTROPHIC LATERAL SCLEROSIS (TABLE 1)

Recombinant NTFs Delivery by Injection

Some trophic factors have been demonstrated to promote cell survival and be protective in both *in vitro* and *in vivo* models of neuronal degeneration: Ciliary Neurotrophic Factor (CNTF), Brain-derived Neurotrophic Factor (BDNF), Glial-Derived Neurotrophic Factor (GDNF), Insulin-like Growth Factor 1 (IGF-1), Vascular Endothelial Growth Factor (VEGF), and Granulocyte-Colony Stimulating Factor (G-CSF). *In vivo* experiments performed in ALS models using single recombinant growth factors are described in this section.

CNTF, one of the first NTF studied in ALS models, injected intraperitonally in *pmn/pmn* mice, mouse model for human spinal motor neuron disease (11) or subcutaneously in wobbler mice (12) improved motor function and survival, and decreased neuronal degeneration and muscle atrophy (13). In addition, Mitsumoto et al. demonstrated a synergic effect of CNTF and BDNF, respectively, to arrest disease progression for 1 month (14).

The fusion protein BDNF with the c fragment of the tetanus toxin (BDNF-TTC) exhibited enhanced neuroprotective effect in $SOD1^{G93A}$ ALS mice model, but no synergic effect was observed compared to TTC alone (55). Recently, motor function improvement and less neuronal loss were observed in $SOD1^{G93A}$ mice treated with the flavonoid 7,8-dihydroxyflavone, a small-molecule mimicking the effect of BDNF (56). Two receptors binding the BDNF, $p75^{NTR}$ and TrkB.T1, were highlighted in $SOD1^{G93A}$: a decreased of $p75^{NTR}$ expression correlated with a delay of mortality and motor impairment (57); a deletion of the TrkB.T1 increased survival and delayed motor deficit (58).

Treatment with encapsuled GDNF-secreting cells in *pmn/pmn* mice did not impact motor neuron degeneration and lifespan (15). The authors suggest a combined treatment for GDNF with others NTFs. Recently, astrocytic GDNF triggered by the tumor necrosis factor α (TNFα) was highlighted in the $SOD1^{G93A}$ mice, and found to limit motor neuron degeneration and disease progression (59).

Intraperitoneal (16) or intracerebroventricular (17) injection of VEGF at doses of 1 g/kg/d and 0.2 μg/kg/d in $SOD1^{G93A}$ mice and rats, respectively, increased lifespan and improved motor performance. Similar data were observed in a sporadic model of ALS rats induced by excitotoxic administration of AMPA (60, 61).

Finally, protective properties of G-CSF were observed in $SOD1^{G93A}$ mice when delivered continuously at dose of 30 μg/kg/d (18). Indeed, disease progression was reduced and

TABLE 1 | Different routes of NTFs delivery and therapies in pre-clinical models.

NTF	Delivery route	Model	Outcomes	References
RECOMBINANT NEUROTROPHIC GROWTH FACTORS				
CNTF	I.P	*pmn/pmn* mice (20–21 d)	MP+, S+	(11)
	S.C	Wobbler mice	MP+, MC+	(12–14)
BDNF	S.C	Wobbler mice	MP+	(14)
GDNF	S.C	*pmn/pmn* mice (15–18 d)	No effect	(15)
VEGF	I.P	$SOD1^{G93A}$ mice (74 d)	MP+, DDO+, S+11 d	(16)
	I.C.V	$SOD1^{G93A/LSd}$ rats (60 d)	MP+, DDO+, S+10 d	(17)
	I.S.P	Excitotoxic model in rats	MP+, DDO+, S+10.5 d, +5 d	(18, 19)
Viral vector based gene therapy				
AAV-NTF				
IGF-1	I.M	$SOD1^{G93A}$ mice (90 d)	MP+, S+22 d	(20)
	I.S.P	$SOD1^{G93A}$ mice (60 d)	MP+, DDO+, S+12.3 d ♂	(21)
	In D.C.N	$SOD1^{G93A}$ mice (88–90 d)	MP+, S+14 d	(22)
	I.M	$SOD1^{G93A}$ mice (60 and 90 d)	MP+, DDO+, S+29 d and +15 d ♂, +24 d and +14 d ♀	(23)
	I.V	$SOD1^{G93A}$ mice (90 d)	MP+, S+10 d	(24)
	I.C.V	$SOD1^{G93A}$ mice (80–90 d)	DDO+, S+12 d	(25)
VEGF	I.C.V	$SOD1^{G93A}$ mice (80–90 d)	DDO+, S+9 d ♂, +20 d ♀	(25)
	I.T	$SOD1^{G93A}$ mice (90 d)	DDO+, S+12 d	(26)
GDNF	I.M	$SOD1^{G93A}$ mice (90 d)	MP+, DDO+, S+16.6 d	(27)
	I.V	$SOD1^{G93A}$ rats (25 d)	MP +/–, S–	(28)
G-CSF	I.S.P	$SOD1^{G93A}$ mice (70 d)	MP+, DDO+, S+	(29)
Stem cell based therapy				
AAV-NTF				
hSC-NSC	I.S.P	$SOD1^{G93A}$ rats (56–62 d)	MP+, DDO+, S+11 d	(30, 31)
gm hNSC line (VEGF)	I.T	$SOD1^{G93A}$ mice (70 d)	DDO+, S+12 d	(32)
hSC-NPC	I.S.P	$SOD1^{G93A}$ mice (40 d)	MP+, S+5 d	(33)
gm hNPC (GDNF)	I.S.P	$SOD1^{G93A}$ rats (∼80 d) rats (∼80 d)	MP–, S–	(34, 35)
	Cortex	$SOD1^{G93A}$ rats (∼80 d) macaques	DDO+, S+14 d	(36)
hBM-MSC	I.S.P	$SOD1^{(G93A)dl}$ mice (28 w)	MP+	(37)
		$SOD1^{G93A}$ mice	MP+	(38)
mBM-MSC	I.V	$SOD1^{G93A}$ mice (90 d)	MP+, S+17.3 d	(39)
gm hBM-MSC (GDNF, VEGF, GNDF/IGF-1, BDNF)	I.M	$SOD1^{G93A}$ rats (80 d)	MP+, S+28 d and +18 d for GDNF, + 13 d for VEGF, +28 d for GDNF/VEGF	(40, 41)
mBM	I.S.P and I.M	mdf/ocd mice (6 weeks)	MP+	(42, 43)
mASC	I.V	$SOD1^{G93A}$ mice (76–77 d)	MP+, S–	(44)
hASC	I.V and I.C.V	$SOD1^{G93A}$ mice (70 d)	MP+, DDO+, S+	(45)
hUCBC	I.V	$SOD1^{G93A}$ mice (56 d, 66 d)	DDO+, S+21 d, +38.5 d, +23.8 d	(46–48)
		$SOD1^{G93A}$ mice (60 and 90 d)	MP+, S+10 d	(49)
	I.T	$SOD1^{G93A}$ mice	No effect	(50)
	I.S.P	$SOD1^{G93A}$ mice (40 and 90 d)	MP+, S+6 d for 40 d mice	(51)
	I.C.V	$SOD1^{G93A}$ (70 d) Wobbler mice (28 d)	MP+, S+18 d MP+	(52)
gm hUCBC (VEGF, GDNF, and/or NCAM)	I.V	$SOD1^{(G93A)dl}$ mice	MP+, S+	(53, 54)

I.P, intraperitoneal; I.M, intramuscular; I.V, intravenous; I.C.V, intracerebrovascular; I.S.P, intraspinal; I.T, intrathecal; S.C, subcutaneous; DCN, deep cerebellar nuclei; gm, genetically modified for expression of NTFs in brackets; hSC-NSC: human spinal cord-neural stem cell; m/hBM-MSC, murine/human bone marrow-mesenchymal stem cell; m/hASC, murine/human adipose derived MSC; hUCBC, human umbilical cord blood cells. Main results are summarized as follow: MP, motor performance; DDO, delay of disease onset; S, survival. The age of the model at the treatment is noted in brackets (d, days old; w, weeks). +, improvement; –, deterioration. ♂, male; ♀, female.

survival increased by rescuing motoneurons. Similar results were obtained with subcutaneous injection of pegfilgrastim, a more stable analog of G-CSF (19).

As protein infusion has known drawbacks (invasive method of delivery, protein stability over time, short half-life) others strategies, such as viral vector-based gene therapy and stem cell-based therapy have been developed to express NTFs of interest and avoid chronic injection.

NTFs Delivery by Viral Vector-Based Gene Therapy

Many studies focused on IGF-1. The intramuscular injection of adeno-associated viral (AAV)-IGF-1 in SOD1^{G93A} mice before or at the time of disease symptoms delayed disease onset and increased lifespan (20). Intraparenchymal spinal cord delivery was also tested, showing higher expression of IGF-1 but partial rescue (21), whereas a stereotaxic injection into the deep cerebellar nuclei significantly extended mice lifespan (22). Recently the injection of self-complementary adeno-associated viral vector 9 (scAAV9), a more efficient transducing agent for IGF-1, extended survival, and motor performance of SOD1^{G93A} mice when injected either intramuscularly (23) or intravenously (24). Also, the intracerebroventricular injection of AAV4-VEGF was studied and gave similar results than AAV4-IGF-1 by slowing disease progression. No combined effect of these 2 constructions was observed in SOD1^{G93A} mice (25). Similarly the intrathecal injection of scAAV9-VEGF showed positive impact on lifespan and motor performance in mice (26). The AAV-GDNF, injected intramuscularly in SOD1^{G93A} allowed expression of the protein at the sites of injection, a retrograde transport in anterior horn neurons, and was associated with a delay in the onset and the progression of the disease (27). However, the systemic injection of AAV9-GDNF in SOD1^{G93A} rats showed limited functional improvement and no survival extension (28). Finally the efficacy of intraspinal delivery was showed for AAV-G-CSF in SOD1^{G93A} mice with minimal systemic effects (29).

NTFs Delivery by Stem Cell-Based Therapy

Different types of stem cells exist—based on their source, clonogenic capacity, differentiation potential and availability—and exert a paracrine effect, suitable for therapy in neurodegenerative disease such the ALS (62–65). We mainly focus here on stem cells with potential clinical application, engineered or used as such, e.g., a mix of NTFs.

Neuroprotection With Neural Stem Cells (NSC) and Neural Progenitor Cells (NPC)

Human NSC graft into lumbar protuberance of SOD1^{G93A} rats was shown to delay the onset and the progression of the disease, with their integration into the spinal cord (30, 31). Similarly, the intraspinal administration of human NPC delayed the progression of the disease in SOD1^{G93A} mice (33).

NSC were also engineered to secrete specific one. Intrathecal transplantation of human NSC overexpressing VEGF in SOD1^{G93A} mice delayed the onset of the disease and increased survival with an integration and differentiation of NSC-VEGF into the spinal cord (32). Human neural progenitor cells NPC (hNPC) were also genetically modified to secrete GDNF. The transplantation of such engineered cells in SOD1 rats were integrated into the spinal cord, limited motoneuron degeneration but failed to improve motor function (34, 35). However, the transplantation of hPNC-GDNF into the cortex extended the survival of SOD1^{G93A} rats and was safe for primates (36).

Mesenchymal Stromal Cells (MSC)

Bone marrow (BM) MSC (BM-MSC), when injected intraspinally (37, 38) or intravenously (39) in SOD1^{G93A} mice, allowed decreased motoneurons degeneration, improved survival and motor function, prevented pro-inflammatory factors. Indeed, MSC display immunomodulatory properties by secreting anti-inflammatory cytokines such as TGF-β or IL-10 (66) Since neuroinflammatory markers were detected in neural tissues of ALS patients (67) promising results can be expected with MSC based therapy. Moreover, intramuscular transplantation of human BM-MSC genetically modified to secrete GDNF in SOD1^{G93A} rats, showed a decrease in motoneuron loss and an overall increased lifespan (40). In addition they demonstrated a synergic effect of the combined intramuscular delivery of hMSC-GDNF and hMSC-VEGF with an increased survival, protection of neuromuscular junction and motoneuron degeneration, greater than either growth factor delivered individually (41). Even though human BM-MSC injections have positive effects on the disease progression, it should be noted that the whole BM intraspinally transplanted showed a greater improvement of motor functions than BM-MSC in *mdf/ocd* mice (42) and increased motoneurons survival when intramuscularly transplanted (43).

Others reported positive results with adipose derived MSC when administrated by systemic (44), or intracerebroventricular administration (45).

Human Umbilical Cord Blood (hUCB)

The first study performed on SOD1^{G93A} mice irradiated and transplanted intravenously with hUBC mononuclear cells (MNC), showed a delay in the onset of symptoms and increased the survival (46, 47). Transplanted cells integrated regions of motoneuron degeneration and expressed neural markers (48). Recently, the efficiency of chronic intravenous injections of UCB MNC in symptomatic SOD1^{G93A} mice was demonstrated, with increased lifespan and reduced inflammatory effectors (49). Similarly, the intraspinal as the intracerebroventricular injection of hUCB in pre-symptomatic SOD1^{G93A} or wobbler mice increased survival and motor performance (51, 52). However, intrathecal administration of hUCB did not affect the lifespan of motor function of ALS mice (50).

Some authors engineered hUCB MNC to secrete some NTFs or to enhance homing at the site of degeneration (68, 69). Recently, transplanted hUCB transduced with AAV encoding VEGF, GDNF and/or neural cell adhesion molecule (NCAM), led to a high rate of SOD1^{G93A} mice survival and improved motor function. Moreover, transplanted cells were detected 1 month after grafting into the lumbar spinal cord (53, 54).

CLINICAL TRIALS WITH GROWTH FACTORS: EVIDENCE AND HYPOTHESIS FOR THE FAILURE

Regarding the promising effects obtained in ALS animal models, clinical trials were conducted to examine the neuroprotective effects of these growth factors therapies in ALS patients (**Table 2**).

Trials Involving NTFs Protein Systemic Injections

CNTF

In 90's the ALS CNTF Treatment study group published results obtained in phase I (70) and phase II/III (72) clinical trials where enrolled patients received subcutaneous administration of recombinant human CNTF (rHCNTF) at different doses, 15 or 30 μg/kg, three times a week for 9 months. The phase II/III randomized, placebo-controlled evaluated the safety, tolerability, and efficacy. No statistically difference between rHCNTF-treated patients and placebo-treated patients were observed and side effects were sufficiently severe to limit dosing in many patients. A second trial, same year, did not show any positive effect either (71).

One year later, Penn et al. published results of a phase I clinical trial with intrathecal pump delivery (73). The disease progression was not modified either but no systemic side effects were observed. Thus, intrathecal administration may be the preferred route of administration. To our knowledge, no further clinical study are under investigation.

BDNF

Due to a promising phase I/II clinical trial showing the safety and efficacy of subcutaneous administration of BDNF in 1995, a phase III was designed (74). Results failed to demonstrate an effect on survival but *post-hoc* analyses showed that those ALS patients with early respiratory impairment showed benefit (75). One year later a phase I trial showed the feasibility of intrathecal method of delivery (76) but two other trials conducted in 2003 and 2005 felt to detect any efficacy (77, 78).

IGF-1

In the late 90's, two clinical trials used IGF-1 at a dose of 0.1 mg/kg/d by subcutaneous delivery and found contradictory and opposite results (79, 80). In 2008, a phase III showed no benefit of this route of delivery in 2 years of trials (82). In a pilot study conducted in 2005, intrathecal administration had beneficial effect using high doses of IGF-1 (3 μg/kg every 2 weeks) but it was not placebo-controlled (81).

G-CSF

Ten years ago, two pilot clinical trials with subcutaneous G-CSF administration at a dose of 5 μg/kg/d reported a trend for slowing down the disease progression (84) and a delay in motor decline (83). A Phase II clinical trial is under investigation but results are not yet available.

VEGF

Three clinical trials assessed the safety, tolerability, and the possible motor function improvement as well as survival time of the intracerebroventricular administration of 4 μg/d VEGF. To our knowledge, no results are published.

6- Failure Hypothesis

Most of the clinical trials based on direct protein administration gave disappointing outcomes in view of the promising preclinical results. Different hypotheses can be raised to explain those failures (70–84):

- The route of administration: subcutaneous injection seems less efficient than the intrathecal one
- The minimal ability of these growth factors to cross the blood brain barrier
- The dose: highest safe dose in humans can be lower than those determined in animals, as the clinical trial with CNTF demonstrated
- The treatment start time: in animals, treatment start before the onset of the disease whereas in humans the diagnosis is performed at later stage
- The need of synergic association of numerous neurotrophic factors

Trials Involving Adeno-Associated Viral Gene Therapy

To our knowledge, there is no reported clinical trial using adeno-associated viral gene therapy despite promising results obtained with $SOD1^{G93A}$ mice. AAV2 and AAV9 are vectors having the greatest potential, one specific for neuron tissue, one passing the blood brain barrier and exhibiting neuronal tropisms, respectively. One of the drawbacks of genes therapies for ALS can be the safety. Indeed to stop delivery will not be possible if serious adverse events occur during the treatment.

Trials Involving Stem Cell Therapy

Twenty-two trials involving stem cells-based therapy are registered on ClinicalTrials.gov. Most of them use MSC from different origins and few have results available. This section is an overview of all the known clinical trials.

Neural Stem Cells

In 2012, two trials sponsored by Neuralstem used NSC by intraspinal injection. The phase I did not show any adverse events (85, 86), but the phase II has an unknown status on the ClinicalTrials.gov website.

Recently, published results of a phase I trial, proposing transplantation of human NSCs into the lumbar spinal cord, demonstrated the safety and reproducibility of this cell therapy. Moreover, because the brain tissue used was from natural miscarriages, ethical concerns may be eliminated (87). An ongoing clinical trial concern neuronal progenitor cells engineered to produce GDNF. This is a phase I/IIA trial, active but not recruiting. No results are available for now.

TABLE 2 | Clinical trials with growth factors.

NCT number	NTF	Delivery method	Phase and status of the trial	Cohort size	Outcomes	References	Year
PROTEIN INFUSION							
Not provided	CNTF	SC	Phase I, terminated	57	No adverse neurologic effects, safe, and tolerated	(70)	1995
Not provided		SC	Phase I, terminated	570	No beneficial effect, adverse events dose related, increased number of death at the highest dose, no beneficial effect on ALS progression	(71)	1996
Not provided		SC	Phase II/III	730	Disease progression not modified, minor adverse side effects	(72)	1996
Not provided		IT	Phase I	4	Pain syndromes dose-related, no systemic side effect, no improvement, or worsen of motor function	(73)	1997
Not provided	BDNF	SC	Phase I/II, terminated	283	Tolerated, Trend of improved survival, less deterioration of predicted FVC	(74)	1995
Not provided	BDNF	SC	Phase III	1 135	Disease progression not modified, Patients with early respiratory impairment and with altered bowel function showed benefit	(75)	1999
Not provided	BDNF	IT	Phase I/II, terminated	25	Well tolerated, feasible	(76)	2000
Not provided	BDNF	IT	Phase III, terminated	17	No adverse events, no effect	(77)	2003
Not provided	BDNF	IT	Phase II/III, terminated	13	No effect	(78)	2005
Not provided	IGF-1	SC	Not specify	266	Slowed the progression of functional impairment, slow the decline in health-related quality of life	(79)	1997
Not provided		SC	Not specify	183	Safe and well-tolerated, no effect	(80)	1998
Not provided		IT	Not specify	9	No serious adverse effect, modest beneficial effect	(81)	2005
NCT00035815		SC	Phase III, completed	330	No benefit	(82)	2008
Not provided	G-CSF	SC	Phase I, terminated	13	Safe, less decline of ALSFRS score	(83)	2009
Not provided		SC	Phase I, terminated	39	Safe, no significative effect on ALSFRS score	(84)	2010
NCT00397423		Not specify	Phase II, completed	40	Not available		
NCT01999803	VEGF	ICV	Phase I, terminated	15	Not available		
NCT02269436		ICV	Phase I, terminated	11	Not available		
NCT01384162		ICV	Phase I/II, terminated	15	Not available		
STEM CELLS							
NCT number	**Type of stem cells**	**Delivery method**	**Phase and status of the trial**	**Cohort size**	**Results**	**References**	**Year**
NCT01348451	NSC	ISP	Phase I	12	No major adverse events	(85, 86)	2012
NCT01730716	NSC	ISP	Phase II, unknown status	18	Not available		
NCT02943850	NPC	ISP	Phase I/IIa, active, not recruiting	18	Not available		
NCT01640067	NSC	ISP	Phase I, completed	6	Safe approach, no increase of disease progression	(87)	2015
NCT00781872	MSC	IT, IV	Phase I/II, terminated	19	Safe and feasible, ALS-FRS score stable the first 6 months	(88)	2010
NCT03085706	PBMC	ISP	Phase NA, completed	14	Not available		
NCT01933321	HSC	IT	Phase II/III, completed	14	Not available		
NCT01609283	MSC	IT	Phase I, active, not recruiting	27	Not available		
NCT01142856	MSC	IT	Phase I, completed	1	Not available		

(Continued)

TABLE 2 | Continued

NCT number	NTF	Delivery method	Phase and status of the trial	Cohort size	Outcomes	References	Year
NCT00855400	MSC	ISP	Phase I/II completed	11	No severe adverse event, no acceleration in the rate of decline, possible neurotrophic activity	(89)	2012
NCT02286011	MC	IM	Phase I, active, not recruiting	20	Not available		
NCT00855400	MC	ISP	Phase I, completed	11	Safe approach, no worsening of the disease	(90)	2016
NCT03268603	MSC	IT	Phase II, recruiting	60	Not available		
NCT01254539	MSC	ISP, IT	Phase I/II, completed	63	Infusion of MSC produces spinal changes unrelated with clinical events and disease worsening	(91)	2013
NCT01363401	MSC	IT	Phase I/II, completed	64	Possible benefit lasting at least 6 months with safety	(92)	2018
NCT02917681	MSC	IT	Phase I/II, recruiting	28	Not available		
NCT02987413	MSC	IT	Phase I, completed	3	Not available		
NCT02290886	MSC	IV	Phase I/II, active, not recruiting	52	Not available		
NCT01051882	MSC	IM or IT	Phase I/II, completed	12	Safe and tolerated, no serious adverse event, possible benefits on ALS-FRS score, and percentage of FVC	(93)	2016
NCT01777646	MSC	IM + IT	Phase IIa, completed	14			
NCT03280056	MSC	IT	Phase III, Recruiting	200	Not available		
NCT02017912	MSC	IM, IT	Phase II, completed	48	Not available		
NCT01759797	MSC	IV	Phase I/II, completed	6	No adverse events, ALS-FRS score reduced, FVC percentage reduced	(94)	2019
NCT01771640	MSC	IT	Phase I, completed	8			

FVC, force vital capacity; HSC, hematopoietic stem cells; I, intramuscular; ISP, intraspinal; IT, intrathecal; IV, intravenous; MC, mononuclear cell; MSC, mesenchymal stem cells; NPC, neuronal progenitor cells; NSC, neural stem cells; NTF, neurotrophic factor, PBMC, peripheral blood mononuclear cell; SC, subcutaneous.

Blood Cells

Two clinical trials, one using autologous peripheral blood mononuclear cell for intraspinal transplantation and one in phase II/III using hematopoietic stem cells for intrathecal injection were conducted and completed but no results were reported to our knowledge. One trial using autologous bone marrow mononuclear cells (90) for intraspinal injection showed the safety of the procedure.

Mesenchymal Stromal Cells

Among 14 clinical trials using MSCs from diverse origin such as bone marrow, adipose tissue or engineered to secrete particular NTFs, through diverse types of delivery (intrathecal, intraspinal, intramuscular, intravenous, or intraventricular), 5 have no published results, 4 are ongoing, and 5 are completed with published results. All of them are listed in the **Table 2** and the last 5 are detailed below and involved the use of the bone marrow derived MSCs.

In 2012, a phase I/II, using autologous bone marrow MSCs administered by intraspinal delivery, was conducted. No severe adverse event were observed, no acceleration of the disease progression noticed and an increase of the motoneurons in the treated segments compared with the untreated segments for patients who died for unrelated reasons to the procedure. Thus, this trial demonstrates the safety of intraspinal infusion of MSCs and suggests their neurotrophic activity (89). In 2013, a phase I/II confirmed the safety of BM-MSC infusion (91).

In 2016, two clinical trials in small groups of patients, phase I/II, used bone marrow MSCs engineered to secrete NTFs. Intramuscular transplantation for early ALS patients and intrathecal transplantation for progressive ALS patients were evaluated. They concluded that both route of administration are safe and provide indications of possible clinical benefits that need to be confirmed on a bigger cohort (93).

In 2018, a phase I/II trial was initiated to evaluate the safety and efficacy of these cells through intrathecal delivery. A possible benefit seems to last at least 6 months with apparent safety (92). A phase II is required to evaluate long-term efficacy and safety.

Finally, recent phase I/II trials showed safety and feasibility of intravenous and intrathecal transplantation of autologous bone marrow MSCs (94). Indeed, no adverse events were reported and the ALS-FRS score and the force vital capacity percentage were significantly reduced. Additional trials with bigger cohort are needed.

To conclude, stem cells-based therapy as a future therapy to treat ALS patients is premature due to the lack of results. As for the protein infusion, some questions need to be considered:

- The delivery method
- The timing of intervention
- The number of cells to transplant to obtain a therapeutic efficacy
- The capacity of transplanted cells to migrate to the area of interest and to mature in the hostile environment
- The evaluation of the long-term efficacy

Nevertheless, trophic factors remain essential for neuronal maintenance and survival and remain a promising candidate to treat ALS patients. Another source of those factors can be the natural healing system, namely the platelet lysate, and a continuous infusion into the brain by intracerebroventricular (ICV) injection can be a route of administration, avoiding the potential problem with the blood brain barrier crossing.

HOW TO IMPROVE GROWTH FACTORS THERAPEUTICS IN ALS: A NEW THERAPEUTIC APPROACH BASED ON THE HUMAN PLATELET LYSATE

The lack of clinical efficacy of single NTF infusion, despite a good diffusion, required increasing the dose to a point where they finally induced poor tolerance (i.e., μg). A single NTF was therefore unable to induce the complex set of signaling pathways required to promote efficient neuroprotection. Platelets constitute abundant, natural sources of physiological balanced mixtures of many growth factors [e.g., Platelet Derived Growth Factor (PDGF), VEGF, IGF-1, EGF, or TGFβ) (95) and are used to enhance wound healing and tissue repair (96). In addition, they express adhesion molecules, secret chemokines (97) giving thus neuroinflammatory property to the platelate lysate that could be of an additional interest in ALS therapy. Interestingly, it was demonstrated that ICV injection of human platelet lysates significantly reduced infarct volumes in rats with permanent middle cerebral artery occlusion, improved motor function and promoted endogenous neural stem cells proliferation (98). Similar results were obtained with platelet rich plasma in ischemic rats (99). Moreover, intranasal (IN) administration of platelet lysates was demonstrated to be neuroprotective in Alzheimer and Parkinson's disease animal models (100, 101). To pursue with the neuroprotective potential of platelets lysate in neurodegenerative diseases, we developed a heated low protein human purified platelet lysate (HPPL) preparation, compatible with ICV and IN intermittent administration, to deplete fibrinogen, avoid thrombogenic, and proteolytic activities. We demonstrated its neuroprotective effect in *in vitro* and *in vivo* model of Parkinson's disease and its anti-inflammatory properties (102). To extend the concept to ALS, HPPL was tested on a motoneuron-like model and strongly protected from apoptosis and oxidative stress (103). Higher neuroprotection was obtained with HPPL compare to single growth factor or combination of 4 (PDGF, BDNF, BFGF, VEGF) and involved specific signaling pathway such as Akt and MEK (103). These results give a real hope for neuroprotective therapy and need to be confirmed in *in vivo* ALS model with ICV or IN administration of HPPL.

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CASE REPORT
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Amyotrophic Lateral Sclerosis and Primary Biliary Cirrhosis Overlap Syndrome: Two Cases Report

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Amyotrophic lateral sclerosis (ALS) is a disease of which the underlying etiology and pathogenesis are unknown. Numerous data indicate an important role of the immune system and mitochondrial function in the disease. Primary biliary cirrhosis (PBC) is an autoimmune liver disease resulting from a combination of genetic and environmental risk factors. Patients with PBC develop innate and adaptive immune reactions against mitochondrial antigens. Therefore, common mechanisms could exist in both diseases. We present two cases of ALS with PBC to explore the relationship between the two diseases from the immunological and mitochondrial aspects. Further attention should be given to immune-modulating therapy in ALS patients.

Keywords: amyotrophic lateral sclerosis, primary biliary cirrhosis, immunological mechanism, mitochondrial mechanism, ursodeoxycholic acid

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Zhang H, Liu Y, Li Z, Liang N, Zhou X, Nie X, Zhang T and Qi W (2019) Amyotrophic Lateral Sclerosis and Primary Biliary Cirrhosis Overlap Syndrome: Two Cases Report. Front. Neurol. 10:890. doi: 10.3389/fneur.2019.00890

INTRODUCTION

Primary biliary cirrhosis (PBC) is an autoimmune cholestatic disease characterized by the non-suppurative destruction of intrahepatic small bile ducts which can eventually progress to liver cirrhosis (1). It mainly affects middle-aged females, and the female to male ratio is about 10:1 (1). A diagnostic serum marker for PBC is an anti-mitochondrial antibody (AMA) which is positive in more than 90% of the patients (2). Autoimmunity plays an important role in the pathogenesis of PBC (3, 4).

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder involved with the upper (brain, brainstem, and spinal cord) and lower (cranial nerve nuclei, anterior horn cells of spinal cord) motor neurons (5). It is characterized clinically by progressive muscle atrophy, muscle weakness, and respiratory insufficiency with a fatal course (6, 7). The median survival duration is 3–5 years after the onset of the disease, while 10% of the patients can survive for over 10 years (8). The proposed hypotheses for the pathogenesis include glutamate excitotoxicity (9), mitochondrial dysfunction (10), gene defects (11), free radical-mediated oxidative stress (12) and immunological mechanism (13). To date, many patients with ALS and immune diseases (multiple sclerosis, myasthenia gravis, etc) have been reported (14), but ALS concomitant with PBC hasn't been reported.

Here we firstly report two cases of ALS with PBC and analyze the possible relationship between them, mainly from immunological, and mitochondrial aspects.

CASE REPORTS

Case 1

A 47-year-old female was admitted to the office with limbs weakness and dysarthria in February 2017. She firstly presented with left upper limb weakness in October 2016. Her symptoms deteriorated rapidly, and 2 months later, she suffered from mild dysarthria and sometimes choking while drinking water, difficulty in lifting and fastening buttons, and walking <100 meters. She reported no weight loss during the last 4 months. She had no remarkable past medical history.

During hospitalization, her vitals were stable. On neurological physical examination, the patient had no obvious muscular atrophy but had fasciculations noted in bilateral bicep and tricep muscles. Power was Medical Research Council (MRC) grade 3/5 in the bilateral upper extremities and 4/5 in the lower extremities. Tendon reflexes were 4+ in all extremities. She had hyperpharyngeal reflex and palmomental reflex. Bilateral Hoffman signs were positive. Neither sensory nor cerebellar dysfunction were identified. A complete blood count, serum biochemical studies, thyroid function, tumor marker showed normal results. Hepatitis panel was negative. Antinuclear antibody (ANA) was positive at a titer of 1:3,200 and AMA was over 1:40. The laboratory test showed that levels of immunoglobulins were within normal limits and alexins were almost within normal range. To rule out Sjogren's syndrome, we ordered Saliva Flow Rate (SFR), corneal fluorescein staining (CFS), breaking up time (BUT), Schirmer I test (SIT), anti-Sjogren syndrome A (SSA) antibody, and anti-Sjogren syndrome B (SSB) antibody. The results were all negative. The magnetic resonance imaging (MRI) of the brain and cervical spinal cord showed no abnormalities. Her chest computed tomography (CT) showed multiple subpleural inflammatory nodules. Considering the absence of cough and fever, we advised her to have a regular examination. The upper abdominal CT was suggestive of splenomegaly and liver cirrhosis. It showed that the morphology of the liver was abnormal, the velamen was not smooth, and the surface was rough. Multiple enlarged lymph nodes were observed near porta hepatis. No expansion or stenosis was observed in the intrahepatic and extrahepatic bile ducts. Electromyography (EMG) showed active and chronic denervation in all limbs, and in the sternocleidomastoid and paraspinal muscles. Nerve conduction studies (NCS) revealed decreased compound muscle action potential (CMAP) amplitudes of right median and ulnar nerve. Ursodeoxycholic acid (UDCA), a hydrophilic tertiary bile acid as the first line treatment of PBC, and riluzole were prescribed. In the late follow-up by telephone, she showed bed-bound at home, dysphagia, and weight loss of 40 kg a year after the symptom's onset.

Case 2

The second case was a 64-year-old woman diagnosed with PBC in June 2010, when she started UDCA 750 mg/d. Some months later, she was started on endoscopic sclerotherapy and injection of cyanoacrylate glue for gastric fundal varices. In December 2017, gastroesophageal varicose vein ligation and stripping were demonstrated for uncontrolled gastric bleeding. She had a 3-year history of type 2 diabetes mellitus, treated by keeping an appropriate diet for blood sugar. She developed dysphonia and weakness of the hands 3 months before admission. The symptoms gradually progressed. Two months later, she presented weakness in her lower limbs, therefore, she was admitted to our hospital in May 2018. She had lost 10 kg of weight over 3 months. She had no family history of neurodegenerative diseases.

Upon physical examination, muscle atrophy was observed bilaterally in the first dorsal interosseous muscles and the thenar, hypothenar muscles. Fasciculations and atrophy were evident in the tongue. Power was MRC grade 3/5 in the upper limbs and 4/5 in the lower limbs bilaterally. Deep tendon reflexes were brisk in all extremities. Positive bilateral Hoffman signs and hyperreflexia in the pharyngeal muscles were observed. No abnormality was observed in sensations and cerebellar function. AMA (>1:40) and ANA (1:320) were both positive. Hepatitis markers were negative, so were tumor markers. The levels of white blood cell count (1.80*109/L), red blood cell count (3.11*1012/L), hemoglobin (105 g/L), and platelet (30*109/L) all decreased. The biochemical results showed aspartate aminotransferase (AST) 41.3U/L and total bilirubin (TBIL) 26.0 umol/L. Baseline data of the patient was as follows (**Table 1**). There were some lacunar infarctions in bilateral frontal, temporal lobes, and left basilar ganglia on brain MRI. A thyroid ultrasound scan showed small nodules on the left and right lobes (**Figure 1**). Clear lungs were observed on her chest CT. Abdominal ultrasound visualized out of proportion hepatic lobes, mild heterogeneous decrease in echogenicity of the portal vein consistent with mural thrombus, splenomegaly (**Figure 2**) and dilated splenic vein, neither biliary obstruction nor space-occupied lesions. The CMAP amplitudes of right median and ulnar nerve decreased on NCS. EMG revealed florid active denervation changes in bulbar muscles and all limbs. Lumbar puncture was not executed because of her low platelet count. She was given UDCA, riluzole, and edaravone (Radicava), a new medication for ALS in 2017 approved by Food and Drug Administration (FDA) (15). At the follow-up, she had indwelled gastric tube and difficulty in ambulation in 8 months.

DISCUSSION

Our patients both presented dysarthria and limbs weakness. One was a limb onset, while the other was bulbar. The disease courses were short. On physical examination, pharyngeal reflex, and tendon reflexes were active or hyperactive. EMG showed denervation changes in all 4 body segments including bulbar, cervical, lumbar, and paraspinal. The clinically-definite diagnosis of ALS requires the presence of combined upper (UMN) and lower motor neurons (LMN) signa, and/or symptoms in at least 3 body segments (16). Therefore, our patients were consistent with clinically-definite ALS.

The diagnosis of PBC requires the exclusion of other liver diseases, no evidence of extrahepatic biliary tract obstruction on imaging, and two of the three criteria are met:(i) AMA titer higher than 1:40, (ii) alkaline phosphatase (ALP) over 1.5 times normal upper limit for 24 weeks at least, (iii) characteristic

TABLE 1 | Baseline data of the patient in case 2.

Laboratory values	
Blood routine	**Hepatitis screening**
White blood cell count, 1.80*10^9/L (3.50–9.50)	HBsAg ELISA Negative
Red blood cell count, 3.11*10^{12}/L(3.80–5.10)	Anti-HCV ELISA Negative
Hemoglobin, 105 g/L (115–150)	Anti-HBc Total Negative
Platelet, 30*10^9/L(125–350)	HIV ELISA Negative
Coagulation routine	**Immune indices**
International normalized ratio, 1.02 (0.80–1.20)	Immunoglobulin G, 15.5 g/L (7–16)
Activated partial thromboplastin time, 28.9s (25.4–38.4)	Immunoglobulin A, 2.21 g/L (0.7–4.0)
Thrombin time, 13.1 s (10.3–16.6)	Immunoglobulin M, 1.22 g/L (0.4–2.3)
Biochemistry	Antinuclear antibody, Positive (1:320)
Aspartate aminotransferase, 41.3 U/L (13–35)	Anti-mitochondrial antibody, Positive (>1:40)
Alanine aminotransferase, 31.4 U/L (7–40)	Alexin C3, 0.74 g/L (0.80–1.60 g/L)
r-Glutamyltransferase, 101 U/L (7–45)	Alexin C4, 0.18 g/L (0.16–0.38 g/L)
Alkaline phosphatase, 112 U/L (50–135)	**Thyroid function**
Albumin, 39.7 g/L (40–55)	Free triiodothyronine, 4.15 pmol/L (2.76–6.45)
Total bilirubin, 26.0 umol/L (3.4–17.1)	Free thyroxine, 12.80 pmol/L (8.75–22.00)
Creatinine, 48.1 umol/L (41–81)	Thyroid-stimulating hormone, 2.92 mIU/L (0.35–4.31)
Tumor marker	**Other indicators**
Alpha fetoprotein, <0.61 ng/ml(0–10.9)	Blood ammonia, 35 umol/L(9–30)
Carcino-embryonic antigen, 2.05 ng/ml(0–10)	Glycated hemoglobin, 8.00% (4.00–6.00)
Glycogen antigen CA125, 34.78 U/ml(0–35)	Folic acid, 3.43 ng/ml (>3.38)
Glycogen antigen CA199, 35.3 U/ml (0–37)	VitaminB12, 640 pg/ml (211–911)

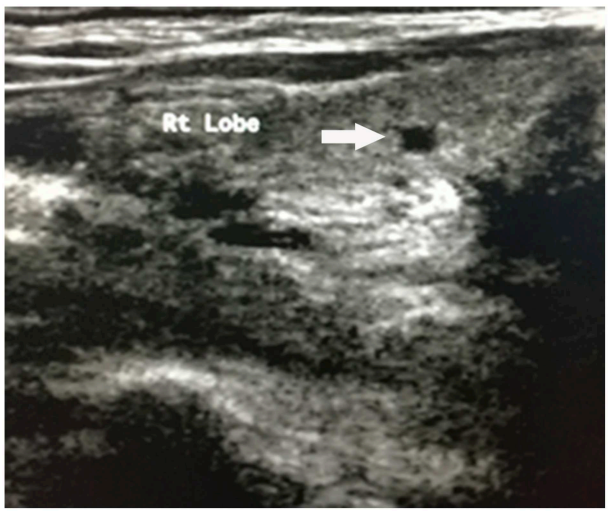

FIGURE 1 | Hypoechoic nodule of right lobe of thyroid (white arrow).

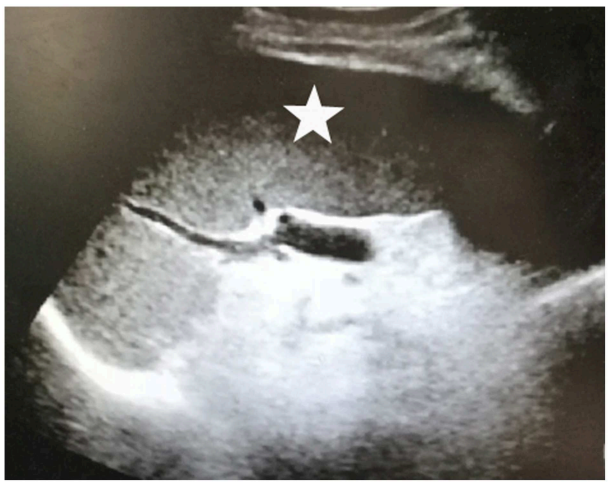

FIGURE 2 | Splenic enlargement (white asterisk).

liver histology, especially non-suppurative cholangitis and interlobular bile duct injury (17, 18). Our first patient was a middle-aged female diagnosed with PBC during hospitalization. Considering her negative results of hepatotropic virus, we ruled out cirrhosis caused by long-term hepatotropic virus infection. And no obstruction was found in extrahepatic bile duct on abdominal CT. AMA was positive in 95% of patients with PBC and ANA was 70%. In our case, AMA was positive (> 1:40) and ANA tire reached up to 1:3,200. The results of SFR, CFS, BUT, SIT, rheumatism factor, and lupus anticoagulant were all negative which excluded Sjogren's syndrome, systemic lupus erythematosus, and rheumatoid arthritis. The patient refused a liver biopsy. It had been found that up to 0.5% of the population in screening studies were AMA positive, typically, 50% of those having normal liver biochemistry (19). Our patient was initially diagnosed as PBC though her liver enzymes were normal. The second case had a history of PBC, and started a long-term use of UDCA. She presented end-stage liver performance, like liver cirrhosis, portal hypertension, hypersplenism, bleeding disorder, esophageal-gastric varices, and abnormal blood ammonia risk of hepatic encephalopathy (20).

It had been reported that 70% of PBC patients had extrahepatic diseases, but none with ALS. One study showed that PBC patients with overlapping characteristics of autoimmune hepatitis (AIH) would progress rapidly to cirrhosis and liver failure (21). Whereas, another study suggested that patients with superimposed features were more prone to develop esophageal varices, ascites and liver failure compared with typical PBC patients (22). Therefore, we analyzed the disease characteristics of ALS overlapped with PBC: (i) both patients were female, possibly due to the significant female susceptibility to PBC (1), and some scholars suggested that the gender difference might

be related to the presence of genes to control immune tolerance on X chromosome (23) and sex hormone levels (24), (ii) both had a short course of disease, 4 and 3 months, respectively, (iii) clinical signs were involved with upper and LMN, and EMG showed wide denervation, (iv) in view of the follow-up, they both progressed quickly. The second patient might progress more rapidly than the first one as a consequence of end-stage liver disease.

The incidence of ALS ranges between 1.5 and 2.5 for 100,000 per year in the general population of the world (25) and the incidence of PBC is 4–40 per 100,000 people (26). Thus, the probability of co-occurrence of the two diseases in a single patient is statistically speaking very low, which may indicate common unknown mechanisms between the two diseases. The robust evidence points to a crucial role of urinary tract infection (UTI) caused by *Escherichia coli* (*E. coli*) in increasing the risk of PBC. *E. coli* infection is a key factor in the breaking of mitochondrial autoantigen immune tolerance, leading to the generation of specific AMA (27). Human PDC-E2 shares a significant homology with *E. coli* PDC-E2 which may reason for it. Besides, hepatocytes and bile epithelial cells in the liver of PBC patients express large amounts of human leucocyte antigen classes I and II molecules. Therefore, both CD4+ and CD8+ autoreactive T cells also play a crucial role in PBC (28). Thus, the pathogenesis of PBC is associated with the interaction between mitochondrial autoantigens and anti-mitochondrial antibodies and T cell-mediated toxicity (29). Changes in the immune system have also been observed in the spinal cord and cortical motor areas of ALS patients (30). The activity of CD8+ T cells could be found in both PBC and ALS. In the early stage, T cell subsets and M2 microglias are activated to prevent the neurodegenerative process. In the late stage, the activity of M1 microglia and CD8+ increases leading to decreased numbers of regulatory T cells. To some extent, the neurotoxic effect exceeds the neuroprotective effect, which results in the loss of neurons (31, 32). Association between the two diseases may be driven by dysregulation of the immune system particularly in CD8+ T cells.

The cumulative data shows that structural and functional abnormalities of mitochondria play an important role in ALS (33–35). PBC ensues from loss of mitochondrial antigen immune tolerance, and the mitochondrial autoantigens are found in all nucleated cells. Although it's said that the attack is predominantly for PDC-E2 expressed by bile epithelial cells, it is still under debate (36). Therefore, we speculate that immune attack of PBC may also impair other parts of the body, like motor neurons. Thus, (i) the normal process of electron transport chains is disturbed, causing less production of ATP (37), (ii) the destruction of Ca2+ homeostasis, resulting in synaptic dysfunction and neuronal damage (38), (iii) the apoptotic signaling is perturbed (39), leading to ALS. The hypothesis remains to be demonstrated.

With regards to treatment, UDCA is the approved medical treatment to reduce progression of disease in PBC and riluzole in ALS. However, there is no literature on specific medical doses in patients with ALS-PBC overlap syndrome. We recommended our patients to take the medications in regular doses and reexamine one time every 3 months, because most liver enzyme levels are elevated within the first 3 months of riluzole treatment (40). The patients responded satisfactorily except for intermittent nausea in the first patient. Her nausea was relieved by taking the drug in combination with food probably due to less abrupt rises in plasma concentrations. Our case report fills a gap in the researches on ALS-PBC overlap syndrome but the treatment about it has yet to be further studied. PBC is associated with immune-mediated destruction of intrahepatic bile ducts. In ALS, the immune system also plays a pivotal role. UDCA, as an immunomodulatory agent, protects cholangiocytes from bile acid toxicity in PBC patients and takes therapeutic effect on ALS (41). Recently, it has been showed that tauroursodeoxycholic acid (TUDCA) can slow progression in ALS patients (42). Compared with the first patient, our second one developed ALS after a long time with PBC, which may be related to her use of UDCA. Thus, we speculate that immune-modulating therapy for prior ALS, like UDCA, may have some protective or suppressive effect to delay the onset of motor neuron damage. However, it should be noted that this is still speculation based on a clinical phenomenon, and further studies are needed to verify the hypothesis.

CONCLUSION

The coexistence of ALS and PBC indicates a relationship between the two diseases from immunological and mitochondrial aspects. The pathomechanisms of them and the effects of immune-modulating therapy at an early stage before onset of ALS symptoms remain to be elucidated combined with more clinical data.

ETHICS STATEMENT

This study was approved by the Ethics Committee of Clinical Research of the Second Hospital of Hebei Medical University (Shijiazhuang, China). Written informed consent was obtained from the participants for the publication of this case report.

AUTHOR CONTRIBUTIONS

HZ: plan of the case reports, data acquisition and analysis, follow-up with patients, and writing of the initial manuscript. YL: work-up of the patients, investigation and organization of the work, manuscript review, and revision. ZL, NL, XZ, XN, TZ, and WQ: clinical investigations of patients, supplement to the argument, review, and revision.

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frontiers in Neuroscience

MINI REVIEW
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Kynurenine Pathway Metabolites as Biomarkers for Amyotrophic Lateral Sclerosis

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Amyotrophic Lateral Sclerosis (ALS) currently lacks a robust and well-defined biomarker that can 1) assess the progression of the disease, 2) predict and/or delineate the various clinical subtypes, and 3) evaluate or predict a patient's response to treatments. The kynurenine Pathway (KP) of tryptophan degradation represent a promising candidate as it is involved with several neuropathological features present in ALS including neuroinflammation, excitotoxicity, oxidative stress, immune system activation and dysregulation of energy metabolism. Some of the KP metabolites (KPMs) can cross the blood brain barrier, and many studies have shown their levels are dysregulated in major neurodegenerative diseases including ALS. The KPMs can be easily analyzed in body fluids and tissue and as they are small molecules, and are stable. KPMs have a Janus face action, they can be either or both neurotoxic and/or neuroprotective depending of their levels. This mini review examines and presents evidence supporting the use of KPMs as a relevant set of biomarkers for ALS, and highlights the criteria required to achieve a valid biomarker set for ALS.

Keywords: kynurenine pathway, amyotrophic lateral sclerosis, biomarker development, neurodegeneration, motor neuron disease, neuroinflammation and neurodegeneration, tryptophan

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AMYOTROPHIC LATERAL SCLEROSIS

The diagnosis of amyotrophic lateral sclerosis (ALS) can only be fully confirmed by the post mortem detection of ALS-associated protein inclusions such as TDP-43 and SOD1 (Turner et al., 2013). Coupled with the spectrum of symptoms seen in the clinical presentation of ALS, the diagnosis of ALS relies on presentation to a neurologist and the elimination of other neurological and/or muscular diseases such as Kennedy's disease or myasthenia gravis, based on the El Escorial criteria that requires the assessment of disease progression (Brooks et al., 2000; Lambrechts et al., 2007; Al-Chalabi et al., 2016; Hardiman et al., 2017). This results in the average time from onset of symptoms after diagnosis of ALS being 10 months, in a disease with survival of 24–48 months (Chiò et al., 2009; Hardiman et al., 2017).

Abbreviations: 3HAA, 3-hydroxyanthranilic acid; 3HK, 3-hydroxykynurenine; 5-HT, serotonin; AA, anthranilic acid; ALS, amyotrophic lateral sclerosis; BBB, blood brain barrier; BH4, tetrahydrobiopterin; ChAT, choline acetyltransferase; CNS, central nervous system; CSF, cerebrospinal fluid; GCMS, gas chromatography mass spectrometry; HPLC, high performance liquid chromatography; IHC, immunohistochemistry; KP, kynurenine pathway; KPM, KP metabolites; KTR, kynurenine: tryptophan Ratio; KYN, kynurenine; KYNA, kynurenic acid; LC-MS/MS, liquid chromatography tandem mass spectrometry; MS, multiple sclerosis; NAD+, nicotinamide adenine dinucleotide; NMDA, *N*-methyl-D-aspartate; PIC, picolinic acid; QUIN, quinolinic acid; QUINA, quinaldic acid; SOD1, Cu/Zn superoxide dismutase; TRP, tryptophan; XA, xanthurenic acid.

Defined as characteristic that is objectively measured and evaluated as an indicator of normal biological process, pathogenic process, or a pharmacogenomic process to therapeutic intervention, biomarkers include genomics, proteomics, metabolomics, neurophysiology, and neuroimaging (Ganesalingam and Bowser, 2010; Turner et al., 2011). The lack of a reliable biomarker for ALS hampers a rapid, definitive diagnosis of disease, determination of ALS subtypes, monitoring of disease progression in patients, and limits the ability of clinicians and scientists to achieve an unbiased assessment of the efficiency of new treatments (Turner et al., 2009; Ganesalingam and Bowser, 2010). For patients and their families, a sensitive and specific biomarkers could allow detection of ALS at early stages, and allow the prognosis of the clinical subtype of ALS to predict disease aggressivity and subtype (Ganesalingam and Bowser, 2010; Al-Chalabi et al., 2016). This research gap in biomarker discovery and development for ALS comes not only as an impediment for patients and their families, but also at a cost to the pharmaceutical industries, through the monitoring of drug effects and disease progression in clinical trials. In particular, the repeated failure of drugs demonstrating clinical efficacy, and the inability to detect improvements, or non-improvements rapidly (Aggarwal and Cudkowicz, 2008; Ganesalingam and Bowser, 2010; Petrov et al., 2017).

THE KYNURENINE PATHWAY

One of the hallmarks of ALS is the presence of neuroinflammation and the kynurenine pathway (KP) is known to be strongly induced by inflammatory cytokines such as IFN-γ (McGeer and McGeer, 2002; Moffett and Namboodiri, 2003; Chen et al., 2010; Oxenkrug, 2011). The KP is the major route of tryptophan (TRP) catabolism, and feeds into the serotonin pathway, immune related tetrahydrobiopterin (BH4) pathway, glycolysis, and *de novo* nicotinamide adenine dinucleotide (NAD+) pathway (**Figure 1**) (Stone, 1993; Grant et al., 2010; Oxenkrug, 2013; Sasaki, 2019); linking it to fatigue, depression, inflammation, and decrease in energy metabolism (Sandyk, 2006; Grant et al., 2010; Oxenkrug, 2013).

The essential amino acid tryptophan originates from the diet, if which up to 85% is bound to albumin in blood circulation, and 99% metabolized in the liver (Quagliariello et al., 1964; Yuwiler et al., 1977; Badawy, 2017). Activation of the KP is achieved by the triggering of the first enzyme of the pathway, indoleamine 2,3 dioxygenase (IDO1) (Guillemin et al., 2005c; Badawy, 2017). This results in the production of several neuroactive metabolites such as the excitotoxins quinolinic acid (QUIN), and 3-hydroxykynurenine (3HK) by activated monocytic cells (Guillemin et al., 2003b); and the neuroprotective kynurenic acid (KA) and picolinic acid (PIC) by astrocytes and neurons, respectively (Heyes et al., 1988; Beninger et al., 1994; Guillemin et al., 2001, 2007; Badawy, 2017). The KP is active in most cell types, particularly in the liver (Takikawa et al., 1986; Heyes et al., 1997), and is highly activated in monocytic cells during inflammation (Jones et al., 2015). Only a limited number of KP can cross the blood brain barrier (BBB). TRP, Kynurenine (KYN), 3HK, anthranilic acid (AA) are actively transported by the large neutral amino acid carrier system; and others via passive diffusion (Fukui et al., 1991; Ruddick et al., 2006). This indicates that peripheral activation of the KP by inflammation can be translocated to the central nervous system (CNS), altering immune regulation and increasing neurotoxicity (Owe-Young et al., 2008). In the CNS, most cells contain the complete set of KP enzymes, and are capable of degrading TRP (Guillemin et al., 2005c; Lee et al., 2017). However, neurons, astrocytes and oligodendrocytes are incapable of synthesizing QUIN, only activated microglia and infiltrating macrophages produce QUIN (Guillemin et al., 2000; Lim et al., 2007).

The concept of using kynurenine pathway metabolites (KPMs) as markers for diseases dates back to the 1950s (Musajo et al., 1955; Tompsett, 1959), where excretion of KPMs were observed in the urine of patients diagnosed with cancer, rheumatoid arthritis, cardiovascular events and fevers (Musajo et al., 1955; Takahashi et al., 1956; Tompsett, 1959; McMillan, 1960; McManus and Jackson, 1968; Mawatari et al., 1995). More recently, the KP is investigated mostly in other liquid biopsies such as serum and plasma (Lewitt et al., 2013). The levels of the KPMs has been shown to be well correlated between the cerebrospinal fluid (CSF) and blood (Curzon, 1979; Chen et al., 2010; Myint, 2012; Jacobs et al., 2019), however, they are not always identical; and only few studies (Curzon, 1979; Widner et al., 2002; Chen et al., 2010; Zuo et al., 2016; Havelund et al., 2017; Lim et al., 2017; Jacobs et al., 2019) correlate the KP levels in different biofluids from the same patients at the same time. KPMs have been historically measured using thin layer chromatography, and detected under UV light, or via radioactive metabolites (Musajo et al., 1955, 1956; McMillan, 1960; McManus and Jackson, 1968; Shibata, 1988). Today, KPMs are more often measured using more sensitive methods and equipment such as high performance liquid chromatography (HPLC), Gas chromatography mass spectrometry (GCMS), and liquid chromatography tandem mass spectrometry (LC-MS/MS) (Heyes and Markey, 1988; Bizzarri et al., 1990; Smythe et al., 2003; de Jong et al., 2009; Pedersen et al., 2013; Miller et al., 2018). The most commonly measured KPMs are TRP, KYN, and KYNA, and are often presented as ratios. As they are small molecules, the KPMs such as KYN, KYNA, Xanthurenic acid (XA) and AA have been shown to be stable. With the exception of 3-hydroxy anthranilic acid (3HAA), which is known to be particularly unstable over time and sensitive to light (Darlington et al., 2010; Midttun et al., 2014).

Ex vivo, the KPMs have been measured using immunohistochemistry (IHC) in tissue sections (Guillemin et al., 2005a; Steiner et al., 2011; Lim et al., 2013). More recently, techniques such as tissue-based Matrix-assisted laser desorption/ionization (MALDI) Mass spectrometry Imaging (MSI) and tissue microarray has been used to not only detect, but localize the presence of TRP and KYN in tumors *ex vivo* (Puccetti et al., 2015; Ait-Belkacem et al., 2017). This specific localization will allow for focal observation of KPMs changes within tissue, and targeted applications of monitoring and altering of the KP if this can be translated *in vivo*.

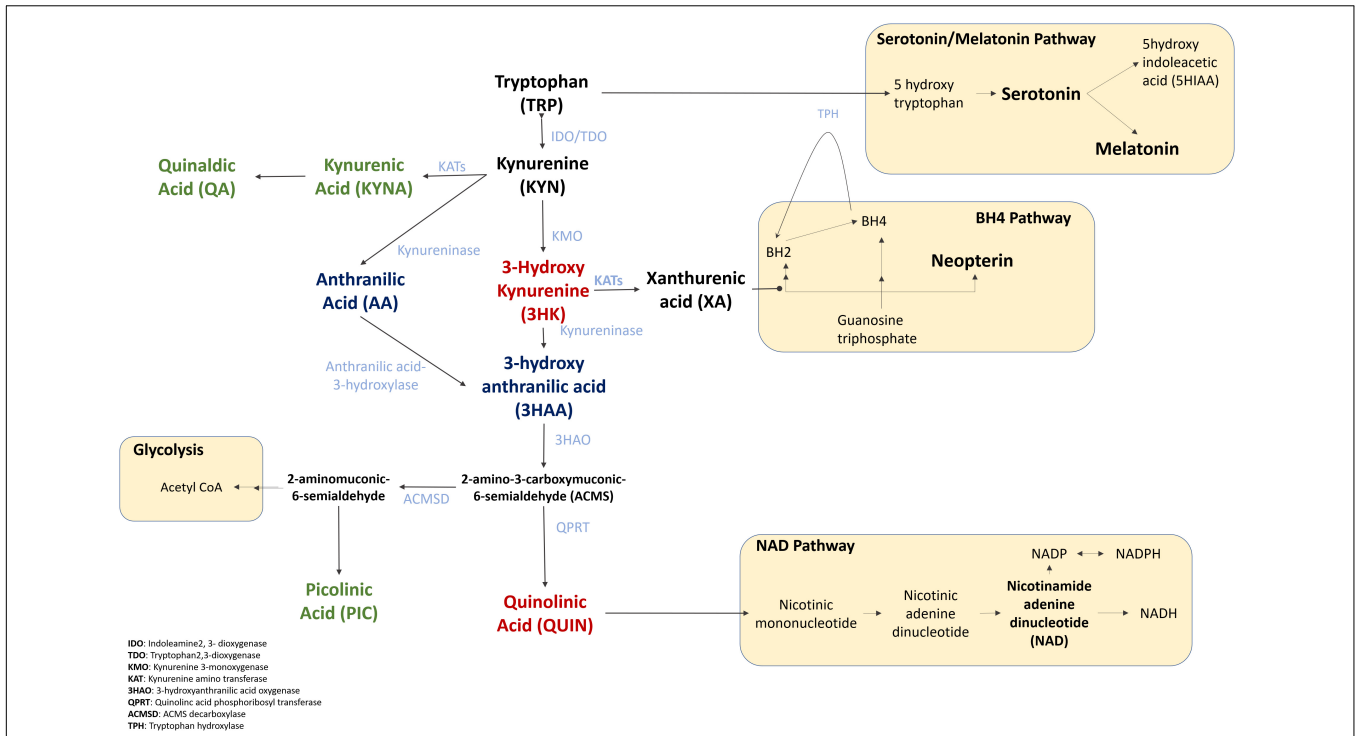

FIGURE 1 | The Kynurenine Pathway and its downstream pathways of Serotonin/Melatonin, BH4, Glycolysis, and NAD+. Tryptophan is converted into serotonin and melatonin, that regulate mood and sleep. The tetrahydrobiopterin (BH4) pathway interacts with the KP in three ways, (1) the sharing of the enzyme TPH that degrades tryptophan, (2) the inhibition of a key BH4 pathway enzyme, sepiapterin reductase, (3) both BH4 and KP are induced by inflammatory cytokines. Tryptophan also feeds into the glycolysis cycle via ACMS, affecting ATP production. Finally, the KP is the *de novo* synthesis pathway of NAD+ which is associated with cellular energy, repair and fatigue. The key KPMs are bolded, neurotoxic metabolites represented in red, neuroprotective metabolites in green, and dual functioning in blue.

Using the levels of KPMs, the activity of their associated enzymes can be derived as a proxy of the concentrations of direct derivatives of the metabolites as a ratio (Darlington et al., 2007; Sathyasaikumar et al., 2011; Lim et al., 2013) – most commonly measuring IDO1 via the Kynurenine: Tryptophan ratio (K/T ratio; KTR); or via direct enzymatic assays (Sathyasaikumar et al., 2011). Although few studies have looked at the direct correlation between metabolite ratio and enzymatic concentrations (Baran et al., 1999).

In vitro, neurotoxic KPMs such as QUIN and 3HK, have been shown to induce neurodegeneration and neuronal cell death through excitotoxicity, *N*-methyl-D-aspartate (NMDA) receptor antagonism, increased glutamate release, and the production of reactive oxygen species (Kim and Choi, 1987; Koh and Choi, 1988; Khaspekov et al., 1989; Nakagami et al., 1996; Shoki et al., 1998; Guidetti and Schwarcz, 1999; Leipnitz et al., 2007; Guillemin, 2012b; Kalonia et al., 2012; Pierozan et al., 2015). The neurotoxic mechanisms of QUIN is well established, and overlaps with mechanisms of neurodegeneration in ALS such as excitotoxicity, hyperphosphorylation, and protein dysfunction (Pierozan et al., 2010; Guillemin, 2012a; Lee et al., 2017). Some of the KPMs such as KYNA, PIC, and 3HAA have neuroprotective and immunomodulatory properties (Foster et al., 1984; Behan and Stone, 2000; Grant et al., 2009; Krause et al., 2011; Lugo-Huitrón et al., 2011). Other KPMs such as 3HAA, have both neurotoxic and neuroprotective functions depending on their relative concentrations (Colín-González et al., 2013; Pérez-González et al., 2017). The KPMs can influence each other levels (Perkins and Stone, 1982; Jhamandas et al., 1990), and the balance of KPMs is crucial for managing the equilibrium between neurotoxicity and neuroprotection. The dysregulation of KPMs, especially excessive QUIN production, has been correlated with variations of other neuroinflammatory markers (Heyes et al., 1992; Guillemin et al., 2003a; Kalonia et al., 2011), making the modulation of KPMs a plausible target for the regulation of the immune response within the CNS (Stone et al., 2012; Bohár et al., 2015; Jacobs and Lovejoy, 2018).

Using these modern techniques, the KP has been investigated as a marker for progression, severity, and prognostic for diseases such as systemic lupus erythematosus (Perl, 2015; Åkesson et al., 2018), cancers (Jin et al., 2015; Zuo et al., 2016; Xie et al., 2017; Huang et al., 2018; Liu et al., 2018; Khan et al., 2019), cardiovascular disease (Sun et al., 2013; Zuo et al., 2016), lung cancer and chronic obstructive pulmonary disease (Chuang et al., 2014; Zinellu et al., 2018), chronic kidney disease and diabetes (Hirayama et al., 2012; Zhao, 2013), acquired immunodeficiency syndrome (AIDS) and HIV-dementia (Fuchs et al., 1990; Heyes et al., 1991; Sardar et al., 2002; Guillemin et al., 2005b; Favre et al., 2010; Lee et al., 2016; Wang et al., 2019), pancreatic cysts (Park et al., 2013), acute myeloid leukemia and lymphomas

(Giusti et al., 1996; Finger et al., 2017), vitamin levels (Midttun et al., 2014), tuberculosis (Weiner et al., 2012; Feng et al., 2015), malaria (Medana et al., 2003), irritable bowel syndrome (IBS) (Clarke et al., 2012; Gupta et al., 2012), rheumatoid arthritis (Spiera and Vallarino, 1969; Schroecksnadel et al., 2003), growth deficits (Kosek et al., 2016), obesity (Mangge et al., 2014), and preeclampsia (Nilsen et al., 2012). In the nervous system, the KP has been shown to associate with pathologies such as stroke (Darlington et al., 2007), schizophrenia (Müller and Schwarz, 2006; Kegel et al., 2014; Oxenkrug et al., 2016), Parkinson's (Ogawa et al., 1992; Widner et al., 2002; Lewitt et al., 2013; Havelund et al., 2017), neuropsychiatric disorders such as depression and stress (Mackay et al., 2009; Gabbay et al., 2010; Olsson et al., 2010; Steiner et al., 2011; Kocki et al., 2012; Erhardt et al., 2013; Comai et al., 2016; Küster et al., 2017; Huang et al., 2018; Kuwano et al., 2018), suicide (Erhardt et al., 2013; Bay-Richter et al., 2015; Brundin et al., 2016), multiple sclerosis (Rejdak et al., 2002; Lim et al., 2017), Alzheimer's disease (Guillemin et al., 2005a; Hartai et al., 2007), Huntington's disease (Schwarcz et al., 1988; Beal et al., 1990; Stoy et al., 2005; Byrne and Wild, 2016), brain tumors (Adams et al., 2012, 2014), Autism Spectrum Disorders, and Attention Deficit Hyperactivity Disorder (ADHD) (Aarsland et al., 2015; Bryn et al., 2017). More recently, studies have demonstrated that the KPMs could be used for the prognosis of MS, and also to differentiate between disease subtypes (Aeinehband et al., 2015; Lim et al., 2017).

AMYOTROPHIC LATERAL SCLEROSIS AND KYNURENINE-ASSOCIATED PATHWAYS

The levels of KPMs are known to be dysregulated in the serum, CSF, and tissue of ALS patients (Ilzecka et al., 2003; Chen et al., 2010) (**Table 1**). The first study by Ilzecka et al. (2003) investigated the presence of KYNA in ALS patients and matching healthy controls. Broadly, the results did not show any significant differences in the levels of KYNA between patients and controls in either serum or CSF. However, CSF KYNA was higher in (1) patients with severe clinical status; and (2) in patients with bulbar onset, compared to patients with limb onset. The authors concluded that this increase likely associated with the neuroprotective role of KYNA. The authors also showed that the concentrations of KYNA in CSF and in serum were not correlated, indicating that KYNA in the CNS is mostly produced in the brain by astrocytes (Guillemin et al., 2001), and this confirms that KYNA is able to cross the BBB and may be imported from the PNS. This is supported by the presence of astrogliosis as part of the neuroinflammatory features found in ALS brain. In 2010, our team reported increased levels of TRP, KYN, and QUIN in both CSF and serum. This study did not investigate KYNA or astrogliosis as Ilzecka et al. did, however, does confirm the neuroinflammatory status in ALS patients, with presence of activated microglia and activation of the KP in the motor cortex.

Other studies have indirectly reported associations between ALS and the KPMs. Jhamandas et al. (1990) showed that injections of the excitotoxin QUIN and 3HAA, directly into the rat brain, triggers a decrease in choline acetyltransferase (ChAT) activity, and that KYNA, PIC, quinaldic acid (QUINA), and AA co-injections could antagonize the QUIN-induced neurotoxicity. In addition, QUIN injections were associated with neuronal loss, but also glial proliferation, highlighting the important roles played by KPMs in neuroinflammation and glial activation in ALS.

Aside from the KP, tryptophan is also metabolized by pinealocytes into serotonin (5-HT), and then melatonin, a serotonin downstream metabolite. Pinealocytes are external to the BBB, and thus directly affected by the KP in the periphery, but not directly by the KP in the CNS (Ruddick et al., 2006). Within the brain, serotonin is modulated by tryptophan levels. A decrease in serotonin levels has been linked to depression through tryptophan depletion (Owens and Nemeroff, 1994; Ruddick et al., 2006; Maes et al., 2011), and with the decrease of melatonin, and sleep disturbances, which are both symptoms in ALS patients (Sandyk, 2006). Furthermore, motoneurons affected in ALS are heavily innervated by serotoninergic neurons; whereas those resistant to ALS-associated degeneration are less innervated by serotonin neurons, possibly linking serotonin with induction of neuronal excitability and neurodegeneration. The roles of serotonin in ALS has been reviewed (Sandyk, 2006). Melatonin has been shown to confer neuroprotection in ALS patients and Cu/Zn superoxide dismutase (SOD1) mice models, likely by decreasing systemic oxidative stress, caspase activation, and by increasing ATP availability to increase cell repair mechanisms to limit neuronal death (Weishaupt et al., 2006; Zhang et al., 2013).

The metabolic pathway of tryptophan degradation also feeds into the cell's energy metabolism through the production of NAD+ and glycolysis. Its dysregulation increases the risk for the development of neurodegenerative diseases as many repair and neuroprotective systems perform at a suboptimal level. NAD+ depletion can lead to fatigue (Procaccini et al., 2016; Camandola and Mattson, 2017; Sasaki, 2019). Altered energy metabolism has also been investigated in ALS (Dupuis et al., 2004; Ngo and Steyn, 2015), and has been shown to be altered by QUIN via the respiratory chain and Krebs cycle (Ribeiro et al., 2006; Colín-González et al., 2015). The NAD+ pathway represents an important therapeutic avenue, and is being targeted using precursors such as nicotinamide phosphoribosyl transferase, or nicotinamide ribosyl directed at ageing, neurodegeneration, and in particular, axonal degeneration (Sasaki et al., 2006; Imai and Yoshino, 2013; Verdin, 2015; Pehar et al., 2018).

A pathway that has been understudied in ALS is the tetrahydrobiopterin (BH4) pathway (**Figure 1**). Interconnected to the KP via the modulatory effect of XA, and as a co-substrate for tryptophan hydroxylase (Zhang et al., 2006; Cronin et al., 2018), studies on BH4 have largely focused on inflammation, pain and neuroprotection (Oxenkrug, 2007; Ghisoni et al., 2015; Cronin et al., 2018). BH4 is strongly associated with neuroinflammation, and is also an essential co-factor in nitric oxide synthases in oxidative stress (Sakai et al., 1995; Guix et al., 2005; Cronin et al., 2018) - both pathological features present in ALS. Several reports have associated BH4 with neurodegeneration, such as the differential methylation of BH4

TABLE 1 | Summary of Kynurenine Pathway metabolite levels in controls and ALS patients collated from Ilzecka and Chen.

References	KPM	Population	Serum	CSF	Trend observed	
					Serum	CSF
Ilzecka	Kyna pmol/ml	Control (*n* = 14)	59.6 ± 20.5	2.41 ± 1.7		
		ALS (*n* = 16)	57.8 ± 35.0	1.59 ± 0.9	Mild > Severe	Control < Bulbar
		Bulbar (*n* = 6)	59.5 ± 39.3	3.61 ± 2.0	Control > Severe	Bulbar > Limb
		Limb (*n* = 10)	59.6 ± 31.2	1.70 ± 1.0	clinical status	Control < Severe
		Mild clinical status (*n* = 6)	81.6 ± 41.2	1.75 ± 09		clinical status
		Severe clinical status (*n* = 8)	39.9 ± 14.7	3.26 ± 2.1		
Chen	TRP (μM)	Control (*n* = 17)	75.0 ± 10.5	2.58 ± 0.16	Control < ALS**	Control < ALS**
		ALS(140)	143.3 + 5.6	5.0 ± 0.2		
		sALS (*n* = 133)	133.3 ± 6.0	4.67 ± 0.19		
		fALS (*n* = 7)	166.4 ± 20.7	5.20 ± 0.87		
		Bulbar (*n* = 31)	128.2 ± 10.6	4.58 ± 0.33		
		Limb (*n* = 109)	137.3 ± 6.9	4.73 ± 0.22		
	KYN (μM)	Control (*n* = 17)	2.52 ± 0.19	0.027 ± 0.00	Control < ALS**	Control < ALS**
		ALS(140)	4.0 + 0.2	0.23 + 0.02		
		sALS (*n* = 133)	4.05 ± 0.21	0.22 ± 0.01		
		fALS (*n* = 7)	3.24 ± 0.36	0.26 ± 0.05		
		Bulbar (*n* = 31)	3.99 ± 0.29	0.22 ± 0.02		
		Limb (*n* = 109)	4.00 ± 0.24	0.21 ± 0.03		
	PIC (μM)	Control (*n* = 35)	2.4 ± 0.4	0.51 ± 0.11	Control > ALS*	Control > ALS ($p = 0.09$)
		ALS(140)	1.4 + 0.1	0.36 + 0.03		
		sALS (*n* = 133)	1.46 ± 0.13	0.35 ± 0.07		
		fALS (*n* = 7)	1.80 ± 0.51	0.60 ± 0.21		
		Bulbar (*n* = 31)	1.45 ± 0.16	0.30 ± 0.06		
		Limb (*n* = 109)	1.49 ± 0.10	0.35 ± 0.07		
	QUIN (μM)	Control (*n* = 35)	0.30 ± 0.03	0.038 ± 0.004	Control < ALS*	Control < ALS*
		ALS(140)	0.37 + 0.02	0.053 + 0.005		
		sALS (*n* = 133)	0.38 ± 0.02	0.05 ± 0.01		
		fALS (*n* = 7)	0.36 ± 0.04	0.04 ± 0.01		
		Bulbar (*n* = 31)	0.43 ± 0.04	0.04 ± 0.01		
		Limb (*n* = 109)	0.36 ± 0.02	0.05 ± 0.01		
	IDO Activity (K/T ratio)	Control (*n* = 17)	0.039 ± 0.004	0.011 ± 0.001		Control < ALS
		ALS (*n* = 40)	0.037 ± 0.0025	0.044 + 0.002		
		sALS (*n* = 133)	0.04 ± 0.00	0.04 ± 0.00		
		fALS (*n* = 7)	0.02 ± 0.00	0.04 ± 0.01		
		Bulbar (*n* = 31)	0.04 ± 0.00	0.04 ± 0.00		
		Limb (*n* = 109)	0.04 ± 0.00	0.04 ± 0.00		

*Mild clinical status defined as mild to moderate according to Munsat, Severe clinical status defined as severe to terminal according to Munsat. KMP, KP metabolites; Kyna, kynurenic acid; TRP, tryptophan; KYN, kynurenine; PIC, picolinic acid; QUIN, quinolinic acid; IDO, indoleamine dioxygenase; K/T ratio, kynurenine/tryptophan ratio; sALS, sporadic ALS; fALS, familial ALS. *$p < 0.05$; **$p < 0.0001$.*

in monozygotic twins discordant for ALS (Young et al., 2017); and particularly in Parkinson's Disease (Choi et al., 2004; Foxton et al., 2007; Yoon et al., 2010).

With all these evidences associating the KP in ALS, especially the unbalance between neuroprotective and neurotoxic metabolites, the KPMs represent a relevant set of biomarkers to characterize disease subtypes and to assess disease progression. As mentioned previously, such biomarkers are lacking especially for the response to treatments and for testing new drugs in clinical trials. One of the main reasons supporting the role of KPMs as a biomarker for ALS is its association with neuroinflammation. The KTR (indication of IDO activity, and thus KP activation) is a very sensitive and specific marker for inflammation. This KTR ratio is well suited as a surrogate progressive, or end-point marker for neuroinflammation. Apart from CSF, body fluids such as blood and urine are easiest to collect. Measurement of KPMs levels in blood present a rapid and reliable set of markers as there are validated quantification methods, and they are stable. However, there are still some limitations using the KPMs as a biomarker for diseases.

Firstly, a potential pitfall using the KPMs as a biomarker for neurological diseases and psychological disorders is that KP activation is not specific of one disease as it is present in all neuroinflammatory diseases. Thus, the KP cannot be used a

diagnostic marker, but is relevant as a prognostic/progression marker, and to identify disease subtypes. Diagnostically, the KPMs still have a great potential as a confirmatory biomarker in conjunction with a shortlisted clinical diagnosis, or subtype. For example in MS, we were able to differentiate MS subtypes from patients diagnosed with MS (Lim et al., 2017). Similarly, when a patient is suspected to have ALS, or has been diagnosed with ALS by a neurologist, the KPMs can be used to differentiate between disease subtypes (e.g., bulbar or lower motor neuron symptoms) and be able to differentiate between patients predicted to be fast or slow progressors. The addition of other inflammatory markers such as cytokines, chemokines, C Protein Reactive, etc, in combination with KPMs would increase the sensitivity and specificity of the biomarker set.

Secondly, the biological functions of all the KPMs are not fully understood – it is a very complex system that is intertwined other regulatory pathways such as BH4 (Cronin et al., 2018), and ultimately regulate the immune system. Further, there is only a limited direct correlation between enzymatic activities and the metabolite formation and their ratios. This is not a key issue in using the KP as surrogate biomarkers, as the crux is that the KPM ratios (ratios of the bioactive metabolites) are what confer biological activity and biomarker association; rather than the function of measuring the enzyme activity. The levels of KPMs in the general population has been directly investigated by Zuo et al. (2016) (n = 7015) and Gostner et al. (2015) (n = 100), which showed that some KPMs are influenced by both age (KTR, KYN, HAA), and gender (TRP) (de Bie et al., 2016a,b). Further, tryptophan has been shown to increase through to adolescence (Lepage et al., 1997) and in adulthood (Mangge et al., 2014). An earlier study by Medana et al. (2003) investigated the KPMs in Malawian children and Vietnamese adults who were affected by Malaria, showing that increases in QA and PIC in both populations could predict a fatal outcome. On the contrary, differences in KA levels in Malawian children as compared to Vietnamese adults (Medana et al., 2003), although it is unclear if this difference was attribute to age, disease, or ethnicity. Further, the correlation of the KPMs in different biofluids need to be better established for correlation and pathway studies. Urine represents the ideal biofluid as it is non-invasive. However, it is not homeostatic, and apart from early studies when the KP was discovered in urine (Musajo et al., 1955, 1956; Tompsett, 1959; McMillan, 1960; Mawatari et al., 1995), only few recent studies have analyzed the KPMs in urine (Fukuwatari et al., 2004; Pedersen et al., 2013; Dolina et al., 2014). Recent research mostly use serum or plasma to assess the KPMs (Darlington et al., 2007; Favre et al., 2010; Hirayama et al., 2012; Aarsland et al., 2015; Comai et al., 2016; Oxenkrug et al., 2016; Lim et al., 2017), and some when available, CSF (Erhardt et al., 2013; Havelund et al., 2017; Sühs et al., 2019). Further, the KP has been proposed as therapeutic intervention for neurodegenerative diseases such as ALS, and has been well reviewed (Füvesi et al., 2012).

As for other potential candidate biomarkers for ALS, the need for defined classifications of ALS subtypes or stages of disease progression (Gil et al., 2017) is critical. Standardized operation procedures for a defined analysis of progression rate, imaging, biopsy retrieval and storage, and biomarker analysis techniques need to be implemented to ensure consistency across centers to achieve an objective assessment. Biobanks storing clinical and biopsies of patient and control samples will be crucial to achieving the aim of a clinically applicable biomarker for ALS.

CONCLUSION

Overall, the KPMs have potential to be used as a sensitive and specific biomarker for patients diagnosed with ALS. Such markers would also have the ability to be used for surrogate clinical and prognostic biomarkers as we previously demonstrated for MS (Lim et al., 2017) and Alzheimer's disease (Chatterjee et al., 2018; Jacobs et al., 2019). The strong correlation of the KP with neuroinflammation, depression, and immune regulation makes it a valid candidate as a surrogate biomarker for ALS, for disease progression (fast/slow progressors) and possibly disease subtyping. Combining the KPM levels together with (1) other markers of inflammation or neurodegeneration, (2) clinical information, and (3) imaging would strongly increase both sensitivity and specificity of the biomarker set.

AUTHOR CONTRIBUTIONS

VT wrote the first draft of the manuscript. Both authors contributed to manuscript revision and read and approved the submitted version.

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Structural Connectivity Alterations in Amyotrophic Lateral Sclerosis: A Graph Theory Based Imaging Study

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Background: Amyotrophic lateral sclerosis (ALS) is a relentlessly progressive neurodegenerative disorder. Diffusion magnetic resonance imagining (MRI) studies have consistently showed widespread alterations in both motor and non-motor brain regions. However, connectomics and graph theory based approaches have shown inconsistent results. Hub-centered lesion patterns and their impact on local and large-scale brain networks remain to be established. The objective of this work is to characterize topological properties of structural brain connectivity in ALS using an array of local, global and hub-based network metrics.

Materials and Methods: Magnetic resonance imagining data were acquired from 25 patients with ALS and 26 age-matched healthy controls. Structural network graphs were constructed from diffusion tensor MRI. Network-based statistics (NBS) and graph theory metrics were used to compare structural networks without *a priori* regions of interest.

Results: Patients with ALS exhibited global network alterations with decreased global efficiency (Eglob) ($p = 0.03$) and a trend of reduced whole brain mean degree ($p = 0.05$) compared to controls. Six nodes showed significantly decreased mean degree in ALS: left postcentral gyrus, left interparietal and transverse parietal sulcus, left calcarine sulcus, left occipital temporal medial and lingual sulcus, right precentral gyrus and right frontal inferior sulcus ($p < 0.01$). Hub distribution was comparable between the two groups. There was no selective hub vulnerability or topological reorganization centered on these regions as the hub disruption index (κ) was not significant for the relevant metrics (degree, local efficiency and betweenness centrality). Using NBS, we identified an impaired motor subnetwork of 11 nodes and 10 edges centered on the precentral and the paracentral nodes ($p < 0.01$). Significant clinical correlations were identified between degree in the frontal area and the disease progression rate of ALS patients ($p < 0.01$).

Conclusion: Our study provides evidence that alterations of structural connectivity in ALS are primarily driven by node degree and white matter tract degeneration within an extended network around the precentral and the paracentral areas without hub-centered reorganization.

Keywords: ALS, MRI, connectivity, DTI, hub, graph theory

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative motor neuron disorder characterized by progressive upper and lower motor neuron degeneration, leading to severe motor disability and death due to respiratory failure within few years (Kiernan et al., 2011). While the etiology of ALS remains elusive, ALS is now widely regarded as a multisystem disorder with considerable extra-motor involvement (Al-Chalabi et al., 2016; Christidi et al., 2018). Neuroimaging studies in ALS have consistently captured clinico-radiological correlations in the central nervous system (Cirillo et al., 2012; Bede and Hardiman, 2014).

Structural changes are relatively difficult to ascertain in ALS with conventional, clinical magnetic resonance imagining (MRI) sequences, therefore research studies rely on quantitative techniques, such as diffusion tensor imaging (DTI; Grolez et al., 2016), cortical thickness mapping (Schuster et al., 2017; Consonni et al., 2019) or MRI spectroscopy (Kalra, 2019). DTI studies have highlighted fractional anisotropy (FA) reductions in both motor and extra-motor regions (Foerster et al., 2013) and FA proved to be a sensitive DTI metric for both diagnostic (Tang et al., 2015; Bede et al., 2017) and progression modeling (Menke et al., 2012; Müller et al., 2016). Tractography studies in ALS readily detect white matter tract degeneration principally in the corticospinal tracts (Agosta et al., 2010). These studies have described anatomical patterns of white matter degeneration, but the impact of focal white matter changes on brain network integrity has not been fully characterized to date (Bede, 2017).

Structural connectivity studies based on graph theory offer a valuable tool to analyze the topological organization of cerebral networks and elucidate how different brain regions relate to each other (Bullmore and Sporns, 2009). In connectomics, cortical and subcortical brain regions can be parcellated in nodes with white matter tracts between them representing the edges of a mathematical graph. The human connectome has been extensively studied with graph theory in physiological and pathological contexts and it exhibits non-random features such as the presence of highly connected regions, named hubs (Achard, 2006). Connectivity hubs of the human brain support integrative processing and adaptive behaviors with high metabolic demands and represent vulnerable foci of neurodegeneration (Buckner et al., 2009; van den Heuvel et al., 2013; Proudfoot et al., 2019).

In ALS, few structural connectivity studies have reported both motor (Verstraete et al., 2011) and extra-motor node impairment (Verstraete et al., 2014; Buchanan et al., 2015; Dimond et al., 2017) and corticobasal connectivity is seldom evaluated specifically (Bede et al., 2018). However, due to methodological differences, the findings of these studies are inconsistent; some authors have described decreased (Dimond et al., 2017), while others reported preserved global efficiency (Buchanan et al., 2015). While many brain disorders, such as Alzheimer disease (Buckner et al., 2009; Verstraete et al., 2011) or schizophrenia (Rubinov and Bullmore, 2013) exhibit a hub-centered pattern, this finding is not evident in ALS connectivity studies (Crossley et al., 2014). It is still not clear if the clinical manifestations of ALS are primarily driven by white matter degeneration or by hub topology alterations.

Accordingly, the main objective of this prospective MRI study, is the characterization of structural connectivity in ALS using graph theory methods at different scales: global, local (nodal) and network analysis using statistical methods such as the Network-based Statistics (NBS; Zalesky et al., 2010) allowing us to analyze both cortical and white matter integrity.

MATERIALS AND METHODS

Ethics

This prospective imaging study was approved by the regional Ethics Committee (Sud Mediterranee I). All subjects provided informed consent in accordance with the principles of the Declaration of Helsinki.

Participants

Twenty-five consecutive patients with ALS, diagnosed according to the revised El Escorial criteria (Brooks et al., 2000), were recruited from the ALS Center of Marseille University Hospital, France. Comorbid neurological conditions or coexisting frontotemporal dementia (FTD; Rascovsky et al., 2011) were considered as exclusion criteria. Twenty-six healthy volunteers were also recruited as radiological controls. Healthy controls had no history of prior head injuries, neurological or psychiatric diagnoses and had a normal clinical examination.

Clinical Evaluation

All ALS patients underwent a standardized clinical examination on the day of the MRI. The recorded demographic and clinical parameters included: disease duration, site of onset, revised ALS functional rating scale (ALSFRS-R) scores (Cedarbaum et al., 1999) and disease progression rate defined as (48-ALSFRS-R)/disease duration (months).

Abbreviations: ALS: amyotrophic lateral sclerosis; ALSFRS-R: revised ALS functional rating scale; BC: betweenness centrality; CSF: cerebrospinal fluid; DTI: diffusion tensor imaging; Eglob: global efficiency; Eloc: local efficiency; FA: fractional anisotropy; FTD: frontotemporal dementia; NBS: network-based statistics; ROI: region of interest.

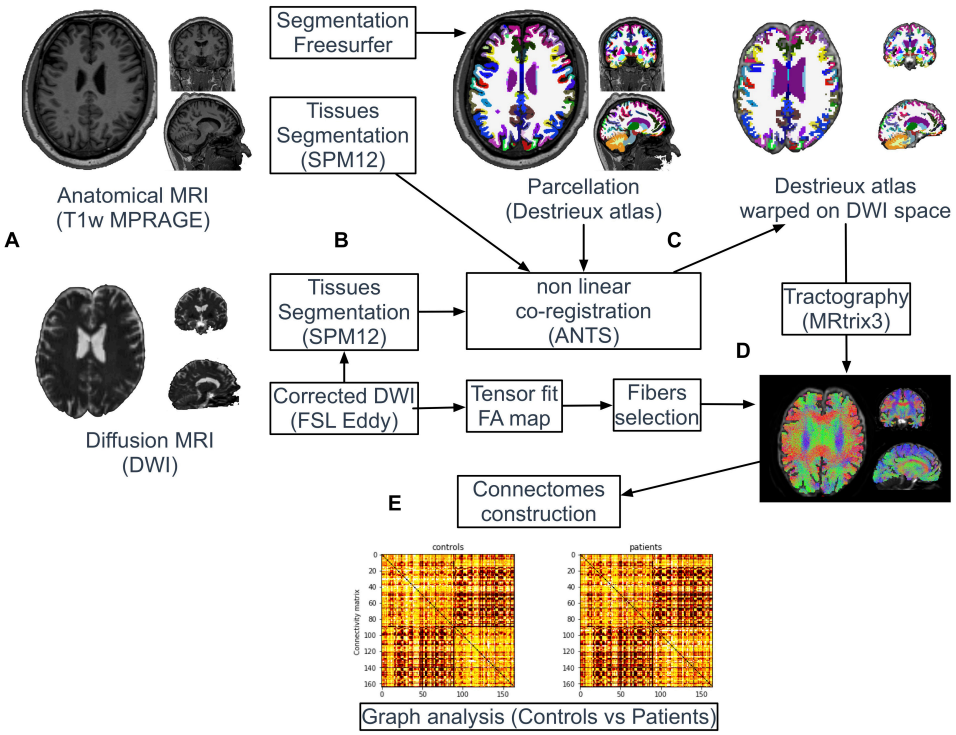

FIGURE 1 | Pipeline of image processing and graph analysis. T1w and diffusion MRI sequences were acquired from all patients and controls **(A)**. Following automatic parcellation of the structural images into cortical and subcortical regions **(B)**, the two set of images were non-linearly co-registered **(C)**. White matter tracts were reconstructed based on diffusion data using the probabilistic algorithm of MRtrix **(D)**. Individual brain network maps were generated using nodes and white matter connections resulting in a FA-weighted connectivity matrix for each subject. Graph analyses were performed on the FA-based connectivity matrix **(E)**.

MRI Acquisition

Magnetic resonance imagining data were acquired on a 3T MAGNETOM Verio system (Siemens, Erlangen, Germany) using a 32-channel phased-array ^{1}H head coil. The protocol included a high-resolution ^{1}H T1-weighted (T1w) 3D-Magnetization-Prepared Rapid Acquisition Gradient-Echo (MPRAGE) sequence (TE/TR/TI = 3/2300/0.9 ms, 160 slices, resolution of 1 × 1 × 1 mm^3, acquisition time = 6 min), and a single shot echo-planar imaging DTI sequence (64 encoding directions, b = 1000 s/mm^2 and b0, TE = 95 ms, TR = 10700 ms, slice thickness 2 mm, 60 contiguous slices, resolution of 2 × 2 mm^2, acquisition time = 12 min). A T2-weighted 3D-Fluid-Attenuated Inversion Recovery (FLAIR) sequence (TE/TR/TI = 395/5000/1800 ms, 160 slices, 1 × 1 × 1 mm^3 spatial resolution, acquisition time = 6 min) was also performed in both patients and controls and systematically reviewed to ensure the absence of vasculopathic white matter abnormalities which may affect focal diffusivity parameters.

Image Processing (Figure 1)

Anatomical Cortical and Subcortical Parcellation

Cortical and subcortical brain regions of each subject were parcellated using the Freesurfer software[1] (v5.0) with the Destrieux atlas, based on the T1w MPRAGE images to obtain 164 regions of interest (Destrieux et al., 2010). Subcortical regions included the thalamus, caudate nucleus, putamen, pallidum, hippocampus, amygdala, nucleus accumbens and ventral diencephalon.

Co-registration Between T1w Images and Diffusion Images

Tissues-type segmentation was performed based on T1w and b0 diffusion images using the Statistical Parameters Mapping software[2] (SPM12) to extract cerebrospinal fluid (CSF) maps (**Figure 1B**). T1w and b0 CSF maps were used as source and target images, respectively, to estimate geometric distortions with a non-linear registration procedure (Syn Model of ANTs library (Avants et al., 2008; **Figure 1C**). The resulting deformation map obtained from this co-registration was applied on the parcellation mask (Destrieux labels). Nearest neighbor interpolation was applied on the mask to keep the integer values of the original labels.

Diffusion Image Preprocessing

Each diffusion dataset was aligned to its b0 image using affine registration to correct for head movement and Eddy-currents using FSL[3] (version 5.0.8, FMRIB Software Library, Destrieux et al., 2010).

[1]http://surfer.nmr.mgh.harvard.edu

[2]https://www.fil.ion.ucl.ac.uk/spm/software/spm12/

[3]https://fsl.fmrib.ox.ac.uk

TABLE 1 | Demographic and clinical parameters of ALS patients and controls.

Demographic and clinical variables		ALS patients	Healthy controls	*P*-value
n (male/female)		25 (16/9)	26 (15/11)	0.64
Age mean ± SD (years)		55 ± 10	51 ± 10	0.17
Handedness (right/left)		23/2	21/5	0.24
Disease duration mean ± SD; [range] (months)		18 ± 15; [5–61]	N/A	N/A
Disease onset site	Spinal	*n* = 19 (left LL: *n* = 6, right LL: *n* = 7, left UL: *n* = 1, right UL: *n* = 5)	N/A	N/A
	Bulbar	*n* = 6	N/A	N/A
Revised El-Escorial criteria	Definite	*n* = 5	N/A	N/A
	Probable	*n* = 10	N/A	N/A
	Probable Laboratory Supported	*n* = 5	N/A	N/A
	Possible	*n* = 5	N/A	N/A
ALSFRS-R mean ± SD; [range]		39 ± 6; [23–47]	N/A	N/A
Disease progression rate mean ± SD; [range]		0.9 ± 0.9; [0.1–3.6]	N/A	N/A

ALSFRS-R, revised ALS functional rating scale; LL, lower limb; N/A, not applicable; UL, upper limb.

Tractography and Connectomes Construction

White matter tracts were reconstructed adopting a whole-brain probabilistic fibertracking approach using MRtrix[4] (Brain Research Institute, Melbourne, Australia). The method has been previously presented in detailed (Besson et al., 2014). A combined bi-hemispheric white matter mask was 1 mm dilated and defined as the region of interest (ROI) for the tracking algorithm (**Figure 1D**). One million fibers were then generated from all voxels included in this ROI with a probabilistic tracking algorithm [tckgen command, FOD model (Behrens et al., 2003)]. The algorithm generated one million fibers with a minimum length of 20 mm. Default tracking parameters included a minimum radius of curvature of 1 mm, a FOD cutoff of 0.1 and a step size of 0.2 mm. Finally, FA values were interpolated at each point of the fibers and the FA-based connectivity matrix (size 164 × 164) were generated from all streamlines and the parcellation mask (164 labels) previously warped in the diffusion dataset space (tck2connectome command, MRtrix) (**Figure 1E**).

Network Construction and Graph Theory Based Analysis

We modeled the structural undirected brain network of each participant using the reconstructed white matter tracts and the parcellated brain regions obtained in the previous steps (Bullmore and Sporns, 2009). Each region was used to define a node of a network graph. Edges were determined by tractography streamlines connecting any pair of nodes. An edge was considered present between two nodes if a streamline was generated with start and end points in each region. Network metrics were computed using the Brain Connectivity Toolbox (Rubinov and Bullmore, 2013). We investigated measures of global network architecture of each subject with global efficiency and mean degree. Local structural alterations were evaluated based on local efficiency, degree and betweenness centrality of each region.

TABLE 2 | Global network analysis.

Graph metrics	ALS patients	Healthy controls	*P*-value
Eglob	0.3395	0.3507	0.0348
Degree mean ± SD	106.15 ± 29.77	111.46 ± 29.10	0.0523
Eloc mean ± SD	0.3775 ± 0.0249	0.3854 ± 0.0244	0.12
BC mean ± SD	169.80 ± 245.61	170.06 ± 209.02	0.98

Statistical significance was set at $p < 0.05$. Eglob, global efficiency; Eloc, local efficiency; BC, betweenness centrality.

As defined by Rubinov and Sporns (2010), global efficiency (Eglob) is the average inverse shortest path length in a network and is inversely related to the characteristic path length. It is a metric of functional integration that reflects on the brain's ability for specialized processing across distributed brain areas. Node degree is defined as the number of edges connected to the node and is a fundamental basic network measure to assess the central role of a brain region among a network.

Mean degree of the whole network was also computed and defined for one subject as the mean of each node degree of this subject. Betweenness centrality (BC) is the fraction of all shortest paths in the network that pass-through a given node. The local efficiency (Eloc) is the global efficiency computed on the neighborhood of the nodes.

Hub Analysis

In connectomics, hubs are described as highly connected nodes with topological centrality and a critical role in integrative processes and adaptive behaviors. Hubs are typically defined as nodes with the highest degree, i.e., a degree one standard deviation higher than the average degree of all nodes in the studied population (van den Heuvel and Sporns, 2011; Llufriu et al., 2017).

[4]http://www.brain.org.au/software/

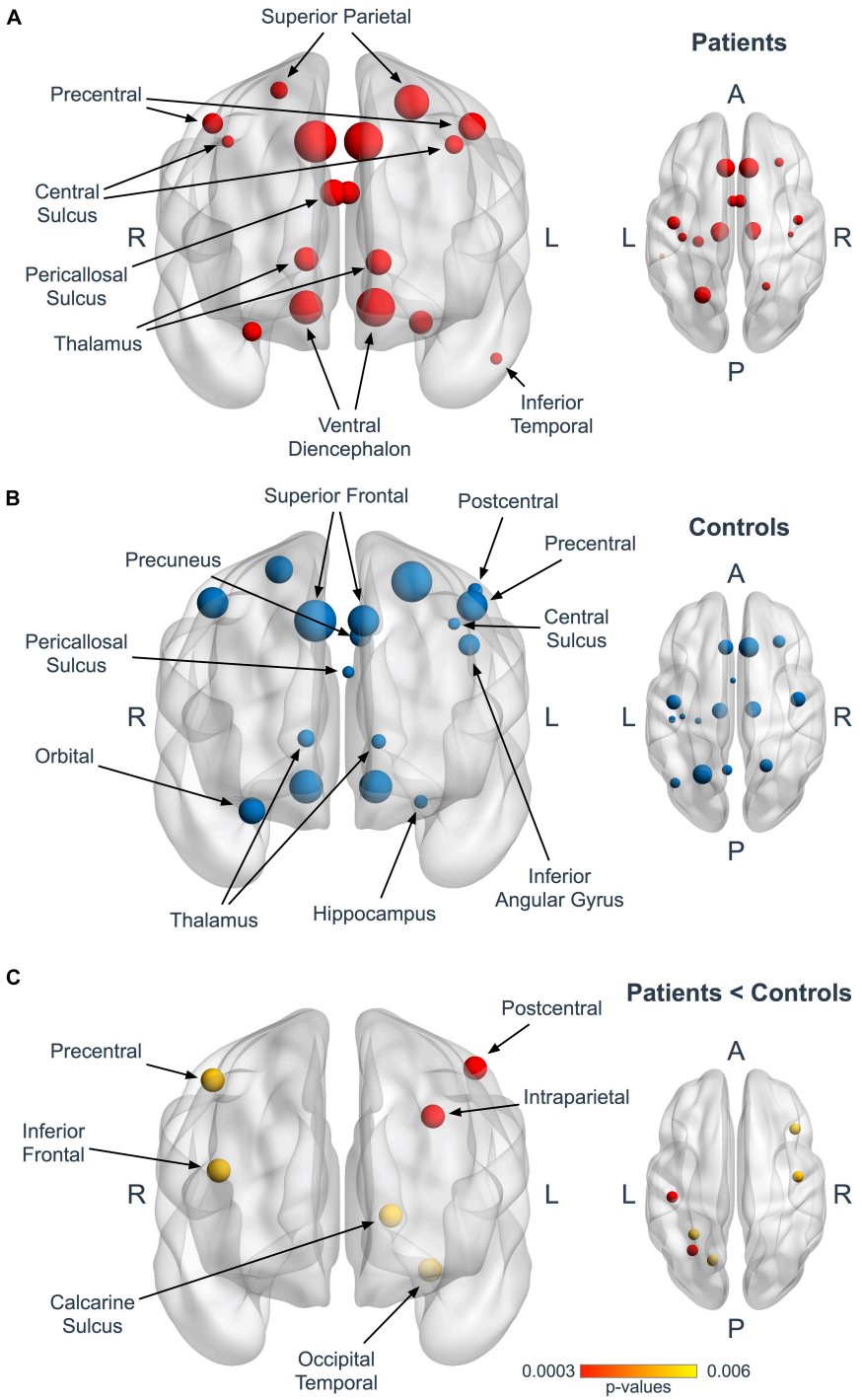

FIGURE 2 | Hubs in ALS patients and controls. Panels **(A,B)** represent coronal and axial views illustrating the hubs in patients (**A**, red) and controls (**B**, blue). The size of the nodes is proportional to their degree values. Panel **(C)** shows the 6 nodes with significantly reduced mean degree in patients at the Bonferroni corrected threshold (red, $p < 0.0003$) and at the 1/n exploratory threshold (yellow, $p < 0.006$).

To assess if the topologic reorganization was more prevalent in hubs, we also calculated the hub disruption index, κ, as defined by Achard (Achard, 2006). κ index is the gradient of a straight line, plotting the mean value at each node in the healthy controls group (x-axis) versus the difference between patient and control groups at each node, for any given metric (y-axis). For example, a negative κ index, crossing the zero line on the y-axis, reveals a trend for high-value regions in

controls to decrease in the studied subject, and low-value nodes to increase.

Network Based Statistics

Considering a 164-node network, 26732 network connections can theoretically be generated. As a result, standard statistical tests for nodal analyses may be underpowered after correcting for multiple comparisons. Accordingly, we used the NBS approach to identify impaired subnetworks in patients compared to controls. The NBS methodology improves the statistical power by controlling for type I error (Zalesky et al., 2010). The NBS network was computed using the parameters detailed in a previous ALS study (Verstraete et al., 2011) permitting the identification of an altered network derived from the FA-based connectome. The mean FA of each tract of this isolated subnetwork was calculated for each subject and the total FA sum (NBS FA sum) was compared between the two groups.

Statistical Analyses

Statistical analyses were performed using JMP 9.0.1, SAS Institute Inc (JMP®, Version 9. SAS Institute Inc., Cary, NC, 1989–2019). Group characteristics were compared using the non-parametric Kruskal-Wallis test for age and Chi-squared test for gender and handedness. Differences in global connectivity between patient and control groups were assessed using pairwise non-parametric Wilcoxon signed-rank tests for multiple comparisons with uncorrected $p < 0.05$ being considered statistically significant. For each node, group differences in the mean degree, Eloc, and Eglob were also explored with non-parametric Wilcoxon tests ($p < 0.05$).

At the nodal scale, we used two thresholds to study differences between patients and controls: a first $p = 0.05/164 = 0.0003$ threshold corresponding to Bonferroni corrections applied with 164 brain regions, and a p exploratory threshold 1/n ($p < 0.006$) (Ridley et al., 2015).

The connectivity metrics with significant differences between patients and controls were then correlated with clinical parameters including ALSFRS-R score and disease progression rate, using a Spearman Rank test with multiple corrections. A $p < 0.0125$ for global metrics (Eglob and mean degree) and a $p < 0.004$ for local metrics (6 nodes with significant connectivity differences between patients and controls) were considered statistically significant.

RESULTS

Demographic and Clinical Parameters

The demographic and clinical profile of the participants is presented in **Table 1**. Twenty-five patients with ALS (mean age 55; SD 10 years; 16 males and 9 females, 2 left-handed) and 26 healthy controls (mean age 51; SD 10 years; 15 males and 11 females, 5 left-handed) were included. There was no statistical difference in age ($p = 0.17$), gender ($p = 0.64$) and handedness ($p = 0.24$) between the study groups. The mean ALSFRS-R score in the ALS cohort was 39 ± 6.

Global Metrics

Global network analyses are presented in **Table 2**. Compared to controls, ALS patients showed a significant decrease in Eglob (0.3395 vs. 0.3507, $p = 0.0348$). A trend of reduced mean degree of the whole brain was found in ALS patients (106.15 vs. 111.46, $p = 0.0523$).

Nodal Analysis

Using the Bonferroni corrected threshold, we found a significant decrease in ALS patients for the left postcentral gyrus ($p < 0.0001$) and for the left interparietal and transverse parietal sulcus ($p < 0.0001$) mean degree. No significant differences were identified in Eloc or BC in any nodes at this threshold. Furthermore, using the exploratory threshold, we found a significant decrease in the mean degree of the left calcarine sulcus ($p = 0.0021$), the left occipital temporal medial and lingual sulcus ($p = 0.0009$), the right precentral gyrus ($p = 0.0021$) and the right frontal inferior sulcus ($p = 0.0009$) (**Figure 2C** and **Table 3**).

Hub Analysis

Seventeen regions among 164 were defined as hubs both in patients and controls. The results are illustrated in **Figure 2**. Three hubs in controls were not identified as hubs in ALS patients: the left precuneus, the left parietal inferior angular gyrus and the left postcentral gyrus (**Figure 3**), but regarding the hub disruption index, no significant differences were found in κ-Eloc ($p = 0.1270$), κ-BC ($p = 0.6511$) or κ-degree ($p = 0.6647$) (**Figure 4**).

Network-Based Statistics

Network-based statistics highlighted a subnetwork (11 nodes, 10 edges) of impaired connectivity in the ALS group ($p = 0.015$) (**Figure 5**). This network included the left precentral gyrus, the left paracentral gyrus, the left caudate nucleus, the left suborbital sulcus, the left inferior temporal sulcus, the left

TABLE 3 | Significant mean degree nodes differences between ALS patients and controls.

Nodes	ALS patients	Healthy controls	*P*-value [a,b]
Left postcentral gyrus	133.64 ± 9.30	142.35 ± 8.16	0.0001[a]
Left interparietal and transverse parietal sulcus	126.68 ± 12.93	138.81 ± 7.93	0.0001[a]
Right precentral gyrus	139.52 ± 9.88	147.31 ± 6.83	0.0021[b]
Right frontal inferior sulcus	98.04 ± 15.00	110.69 ± 11.94	0.0009[b]
Left calcarine sulcus	76.52 ± 19.76	96.69 ± 21.03	0.0021[b]
Left occipital temporal medial and lingual sulcus	97.36 ± 12.13	109.11 ± 13.15	0.0009[b]

Values are reported as mean ± SD. [a]Statistical significance was set using Bonferroni corrections based on 164 brain regions ($p < 0.0003$). [b]Statistical significance was set using an exploratory threshold 1/n ($p < 0.006$).

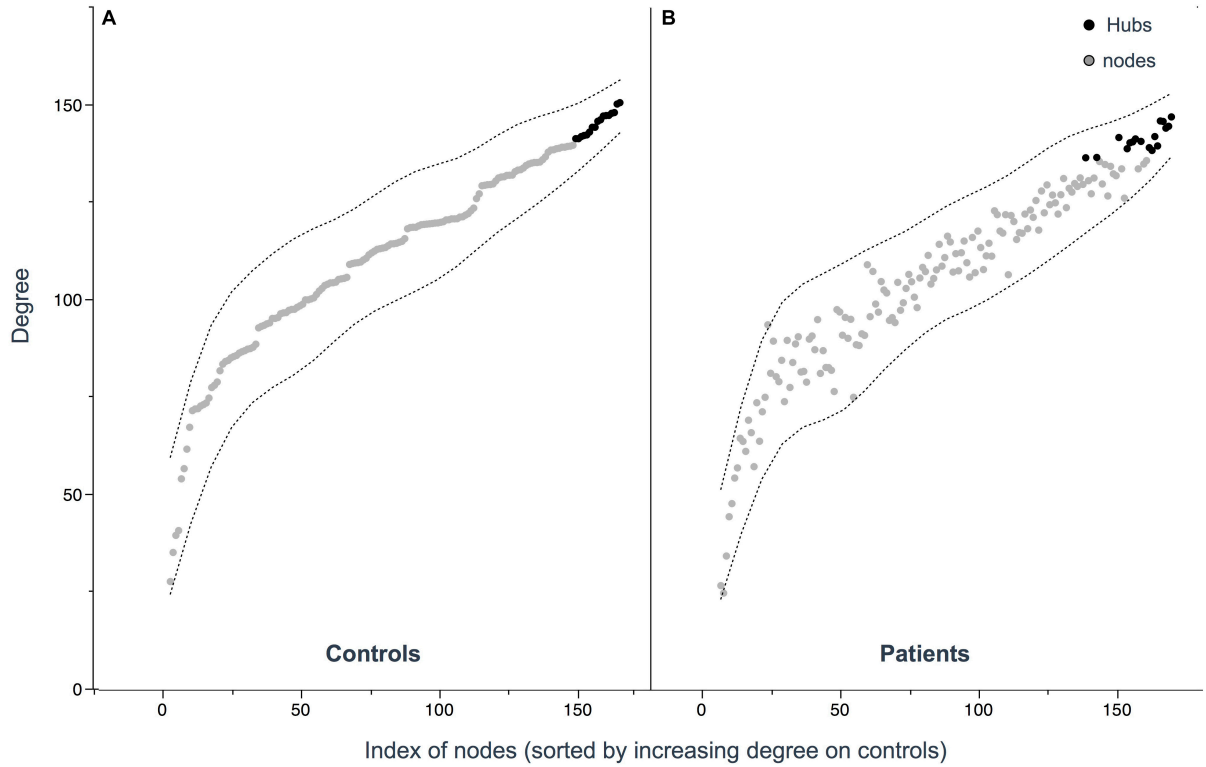

FIGURE 3 | Hub distribution patterns among controls **(A)** and patients **(B)**. Hubs were defined as nodes with a degree one standard deviation higher than the average node degree. 17 hubs were identified in patients and 17 hubs in controls. Nodes **(A,B)** are sorted out by increasing values of degree in controls showing similar distribution.

cingulate marginal gyrus, the right middle frontal gyrus, the right pallidum, the right accumbens area, the right anterior cingulate gyrus and sulcus and the right inferior superior parietal gyrus. The total FA sum of the edges in this subnetwork (NBS FA sum) was significantly reduced in ALS compared to controls ($p < 0.0001$).

Correlation Between Connectivity Metrics and Clinical Parameters

Among the six nodes identified in the Nodal Analysis results section, degree in the right frontal inferior sulcus was correlated with the disease progression rate ($p = 0.0089$, rho = −0.5396) and a trend of association was also identified with ALSFRS-R scores ($p = 0.0456$, rho = 0.4033).

No significant correlations were found between the other nodes and ALSFRS-R score or disease progression rate. There were no significant associations between Eglob and ALSFRS-R score or disease progression rate.

DISCUSSION

This study provides evidence of disease-specific structural connectivity changes in ALS. We report global efficiency reductions in structural networks in ALS characterized by a significant decrease in Eglob and a trend of mean degree reduction. The use of the graph theory enables a topological study of the entire cerebral network, without targeting *a priori* regions of interest. Our results are in line with the reports of decreased of Eglob in previous studies (Zhang et al., 2019). Our findings also support the notion of widespread, multisystem, multi-network degeneration in ALS which has been conceived based on other structural (Keil et al., 2012; Menke et al., 2012; Bede et al., 2016; Müller et al., 2016) and functional methods (Agosta et al., 2011; Douaud et al., 2011; Geevasinga et al., 2017; Dukic et al., 2019; Nasseroleslami et al., 2019). Reduced global connectivity underpins the impaired integration of multiple cerebral circuits in ALS, which may be driven by long-range connectivity changes (He et al., 2009). Previous structural connectivity studies did not capture significant reductions in global parameters (Verstraete et al., 2011; Buchanan et al., 2015) which may be explained by differences in post processing pipelines which were used to generate structural connectomes (deterministic fiber tracking algorithm, number of tracts or different parcellation atlas).

At a local scale, we evaluated parcellated brain regions with multiple metrics (Eloc, degree and betweenness centrality) to determine if the global disturbances were due to the alteration of specific nodes. Nodal analysis demonstrated a significant reduction of degree in 6 out of 164 regions in ALS patients. These six nodes are located in pathognomonic brain regions closely associated with ALS. Precentral and frontal structural degeneration is a hallmark feature of ALS confirmed by a

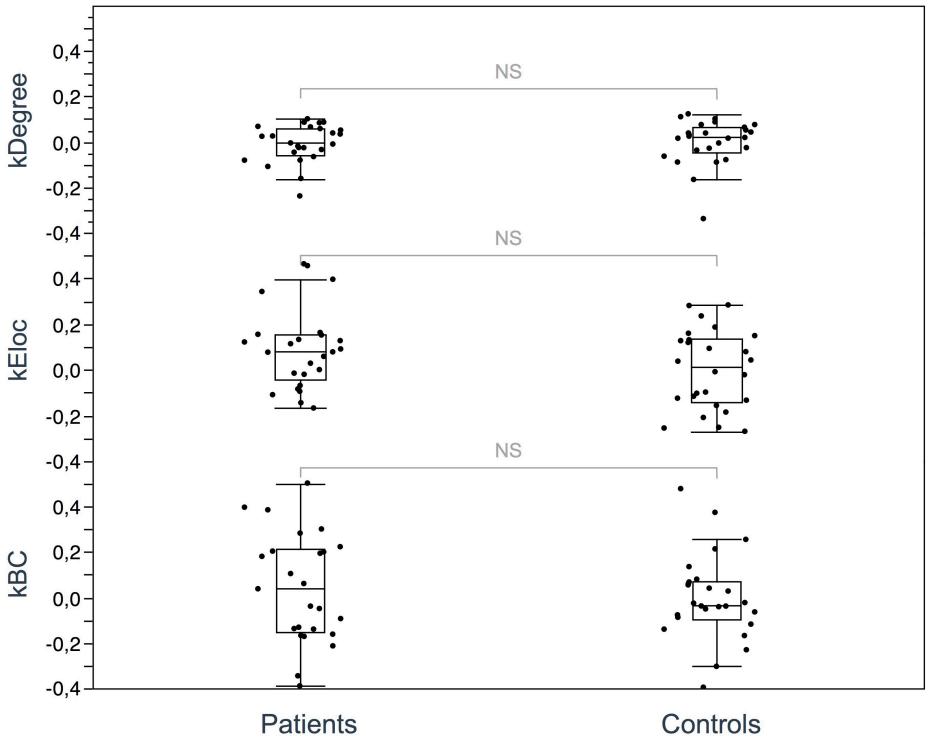

FIGURE 4 | Hub disruption index. The box plots show the κ index of each subject for 3 metrics: κ-degree, κ-Eloc, κ-BC. No significant differences were identified between ALS patients and controls. Differences were considered significant at $p < 0.05$ for each metric.

multitude of cortical gray and subcortical white matter studies (Kasper et al., 2014; Schuster et al., 2016; Mazón et al., 2018). The intraparietal sulcus is part of the supplementary motor areas which is also affected in ALS based on both functional and structural studies (Abidi et al., 2019). The involvement of the temporal lingual sulcus may be the substrate of the spectrum of cognitive changes observed in ALS (Abrahams et al., 2004; Phukan et al., 2007). Intrahemispheric connectivity alterations and the degeneration of the sensorimotor network have also been previously reported in ALS by studies demonstrating widespread pre- and postcentral FA reductions (Rose et al., 2012).

To explore internodal interactions, we used NBS (Zalesky et al., 2010). This tool permits the integrated analysis of a subgroup of nodes that belongs to a single altered network. In our study, we identified an impaired subnetwork centered on motor nodes: precentral, paracentral and frontal gyri. This subnetwork is comparable to the ones described in previous studies (Verstraete et al., 2011; Buchanan et al., 2015). Our finding of parietal and temporal lobe involvement is in line with recently proposed pathological staging systems (Brettschneider et al., 2013), and longitudinal connectivity studies (Verstraete et al., 2014).

Interestingly, no major topological reorganization of hubs was evidenced in ALS in the present study. The human connectome is known to follow characteristic topological patterns based on hubs, defined by a subnetwork of highly connected nodes with a high number of tracts playing a central role in the brain's structural architecture. Due to their anatomical position and their high metabolic demands, these regions are considered particularly vulnerable in many neurological and psychiatric disorders (Buckner et al., 2009; Sharma et al., 2011; Rubinov and Bullmore, 2013; Gollo et al., 2018). Previous studies have shown that the selective hub degeneration occurs in a disease-specific pattern in several neurological and psychiatric conditions such as Alzheimer's disease or schizophrenia (Crossley et al., 2014). Hub pathology in ALS, however, has not been specifically investigated to date and no graph theory based structural studies have been performed to evaluate the distribution of hubs and the extent to which they are reorganized in ALS patients relative to controls. We showed here that both the number and the distribution of hubs are similar between patients and controls. To our knowledge, this first use of the hub disruption index in ALS demonstrating the absence of hub reorganization, suggests that disease propagation in ALS does not follow hub-based patterns. Despite motor network changes evidenced by the NBS analysis, the precentral nodes kept their hub properties. Impairment of the global efficiency seems to be related to local structural abnormalities rather than a complex compensatory hub reorganization as reported in other disorders such as multiple sclerosis and epilepsy (Ridley et al., 2015; Faivre et al., 2016; Tur et al., 2018).

Noteworthy, we found associations between structural connectivity metrics and clinical parameters, such as disease

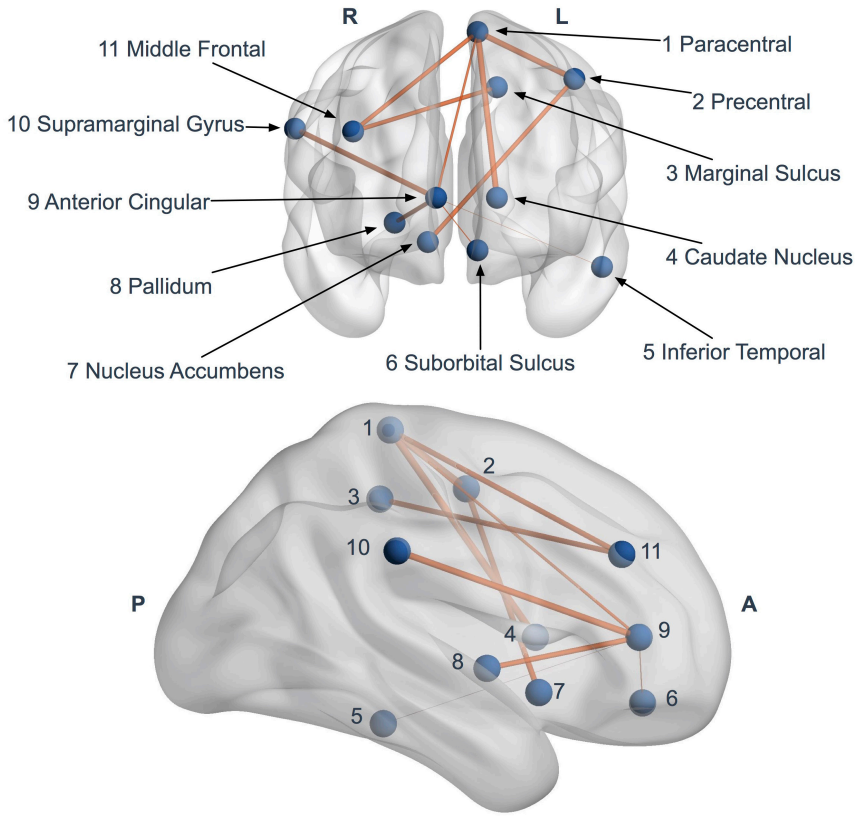

FIGURE 5 | Network based statistics (NBS) impaired subnetwork. Coronal and sagittal views of impaired subnetwork nodes and interconnections identified by NBS (11 nodes and 10 edges). Edge diameter is based on the FA reduction (the larger connections are the more affected, with significant FA reduction).

progression rate and ALSFRS-R score. Correlations between brain imaging metrics and clinical parameters are often difficult to establish in ALS (Chipika et al., 2019) because motor disability is not merely due to upper but also lower motor neuron degeneration and the considerable clinical heterogeneity of the disease precludes direct clinico-radiological associations (Verstraete et al., 2015). It is therefore not surprising that no direct correlations were identified between clinical parameters and global connectivity measures. Node degree and their white matter integrity indexes seem to better explain the clinical deficits observed in ALS.

This study is not without limitations. The sample size of our ALS cohort is relatively limited which did not allow the phenotypic stratification of ALS patients into subgroups. Nonetheless, our study presents compelling evidence that graph-analyses and connectomics provide meaningful non-invasive insights into the degenerative changes of ALS. Furthermore, genetic information was not available for all patients which is a limitation as certain ALS genotypes may be associated with specific white matter alterations and more extensive network impairment (Menke et al., 2016; Floeter and Gendron, 2018). The lack of standardized cognitive evaluation is another limitation given the evidence of phenotype-specific morphometric changes along the ALS-FTD continuum (Omer et al., 2017). Future studies should therefore include ALS-FTD patients and ALS patients with behavioral and executive dysfunction to characterize the connectivity signature of these cognitive phenotypes. An additional limitation is the significantly higher proportion of male ALS patients in the present study. Given the evidence of gender-associated developmental and radiological features in ALS and healthy populations (Menzler et al., 2011; Vivekananda et al., 2011; Bede et al., 2014), future connectivity studies should seek to recruit gender-balanced cohorts.

CONCLUSION

In conclusion, our structural connectivity study highlights a diffuse, non-focal network impairment in ALS without selective hubs vulnerability or topological network reorganization. These results suggest that clinical manifestations in ALS could be more driven by tract degeneration than by hub topology alterations. Based on these observations, a prospective longitudinal study is required to characterize dynamic progressive connectivity changes. Recent imaging studies have shown evidence of considerable presymptomatic structural changes in ALS-associated mutation carriers (Schuster et al., 2015; Querin et al., 2019). A connectomic analysis of such cohorts

could identify early network alterations in the most vulnerable circuits and provide invaluable insights on disease propagation.

DATA AVAILABILITY STATEMENT

Anonymized datasets generated in this study may be available on request from the corresponding author, but are subject to institutional and EU data handling procedures.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Sud Mediterranee I Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors contributed scientifically to this manuscript. Clinical evaluations were performed by A-MG, AV, and SA. The methodological design was optimized by A-MG, MG, J-PR, and WZ. Image processing was undertaken by EF, AL, and BR. Data analyses were conducted by EF, A-MG, BR, J-PR, and WZ. The manuscript was drafted by EF, A-MG, and WZ. All co-authors have reviewed the manuscript for intellectual content.

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MINI REVIEW
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An Overview of MicroRNAs as Biomarkers of ALS

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Amyotrophic lateral sclerosis (ALS; MND, motor neuron disease) is a debilitating neurodegenerative disease affecting 4.5 per 100,000 people per year around the world. There is currently no cure for this disease, and its causes are relatively unknown. Diagnosis is based on a battery of clinical tests up to a year after symptom onset, with no robust markers of diagnosis or disease progression currently identified. A major thrust of current research is to identify potential non-invasive markers ("biomarkers") in body fluids such as blood and/or cerebrospinal fluid (CSF) to use for diagnostic or prognostic purposes. Non-coding RNAs (ncRNAs), including microRNAs (miRNAs), are found at detectable and stable levels in blood and other bodily fluids. Specific ncRNAs can vary in levels between ALS patients and non-ALS controls without the disease. In this review, we will provide an overview of early findings, demonstrate the potential of this new class as biomarkers, and discuss future challenges and opportunities taking this forward to help patients with ALS.

Keywords: amyotrophic lateral sclerosis, motor neuron disease, biomarkers, non-coding RNA, microRNA

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is the most prevalent adult onset form of motor neuron disease. As a result of progressive death of motor neurons in the primary motor cortex, brainstem and spinal cord, there is atrophy of the muscles that are innervated by these neurons. This results in muscle weakness and paralysis with death usually occurring within 3–5 years. Over the last decade, significant progress has been made in identifying the genes responsible for familial cases of ALS (fALS). Of these, the most frequently mutated genes are chromosome 9 open reading frame 72 (*C9orf72*), superoxide dismutase 1 (*SOD1*), TAR DNA-binding protein 43 (*TARDBP*; TDP-43), and FUS RNA binding protein (*FUS*), accounting for over 70% of fALS cases (1). Nevertheless, fALS constitutes approximately 10% of all cases, with the genetic underpinnings of sporadic ALS (sALS) mostly unknown, though *C9orf72* is known to account for 5% of sALS cases.

The lack of a common cause has resulted in difficulties not only in timely disease diagnosis resulting in delay of treatment, but in developing drugs and treatments for the disease. Thus, identifying useful biomarkers as tools for early diagnosis, for determining subgroups in relation to pathogenesis and/or phenotype, and as indicators of treatment response, are urgently required. Development of biomarkers that are minimally invasive to obtain, simple to undertake, and time efficient are key and those derived from biofluids, such as blood, are well suited for this. Further, it is not necessary for the biomarkers to underlie the pathology of the disease if it correlates strongly and specifically to the disease. Indeed, this is more difficult to assess in diseases such as ALS where the underlying molecular causes of pathology is unknown or unclear.

One class of molecules increasingly investigated as potential biomarkers are short ncRNA species (those under 100 nucleotides long), which include tRNA, rRNA, piwi-RNA (piRNA), and microRNA (miRNA). MiRNA have been the main focus of most studies to date, driven by a good understanding of their biogenesis and function, an ease in profiling their expression with a range of techniques including microarray, RNA-seq, and RT-qPCR, a relatively simple structure, increased stability from RNase degradation and freeze-thaw cycles, and a presence in a range of biofluids including blood, cerebrospinal fluid (CSF), and urine (2, 3). To date, a number of studies have shown that miRNAs are differentially expressed in ALS patients when compared to controls in a variety of biofluids, including CSF, and in the blood-derived components plasma and serum (4–18) (summarized in **Table 1**). This review will aim to present recent work identifying miRNA-based biomarkers in biofluids, the possibility of using other ncRNA as biomarkers, and the next steps required to move this into a clinical setting.

EXISTING CIRCULATING RNA BIOMARKERS FOR ALS

Serum-Based Biomarkers

Freischmidt and colleagues have undertaken a number of studies to identify potential miRNA-based biomarkers in the ALS patient serum (4, 6, 8). Their first study selected ten miRNAs previously identified to regulate the ALS-related gene *TARDPB* and found five miRNAs were differentially expressed in serum of sALS patients (4). Their later study focused on miRNA expression in serum from fALS patients using Affymetrix miRNA array chips, and found downregulation of a set of 30 potential miRNA biomarkers for the disease [**Table 1**; 3]. Four miRNA were selected based on their false discovery rate (FDR)-adjusted *p*-value (MIR1915-3p, MIR3665, MIR4530, MIR4745-5p) and their downregulation validated with RT-qPCR in the fALS patients, and all but MIR1915-3p were further observed to be downregulated in sALS patients. While increased variability was observed in sALS patients, this suggested that there may be similarities in the miRNA profile between the two groups. Curiously, these three miRNAs (MIR3665, MIR4530, MIR4745-5p) were found not to be differentially expressed in their most recent study using sALS patients, which described only MIR1234-3p and MIR1825 as being downregulated (8). An interesting aspect of their 2014 study was investigating miRNA expression in non-symptomatic patients who had ALS-related genetic mutations, but predicted to present disease symptoms within the next 20 years. These predicted pre-symptomatic carriers shared 91.7% of the downregulated miRNA of symptomatic patients, although to a lesser dysregulation. This suggests that these biomarkers may be present before symptoms and could be used to identify potential ALS cases. Furthermore, considering there were differences between pre-symptomatic and symptomatic patients in the degree of dysregulation, this may suggest that these biomarkers could change with time. However, further work would be needed to determine this and if it would apply to sALS cases along with whether these biomarkers are specific to ALS itself.

Other studies have also identified potential biomarkers that may be differentially expressed in serum from ALS patients. The upregulation of MIR143-3p and MIR206, and the downregulation of MIR374B-5p were observed in 23 sALS patients and were further validated in an additional 27 sALS patients (13). Of these, 22 samples were in a longitudinal study and MIR143-3p and MIR374B-5p both became more dysregulated, suggesting a link to disease progression, though MIR206 remained stable for at least 3 months later. Another study using patient serum also found MIR206 upregulation in ALS patients along with MIR106b, differences that were reflected in a SOD1-G93A mouse model of ALS (7). MIR206, described as a myoMiR due to its high abundance in skeletal muscle tissue, is one of the few miRNA biomarkers identified across multiple studies, including those described below in serum and plasma (11, 12). The working hypothesis has been that as a result of muscle death, MIR206 is released from the muscle fibers and into the blood stream as a waste product (19). However, MIR206 has been identified as a blood-based biomarker for other muscle-related diseases and therefore not specific to ALS (20, 21). Nonetheless, it could play an important role in helping to identify ALS patients if used in conjunction with other biomarkers to help distinguish from ALS-like conditions. Lastly, one study has investigated the exosomes present in serum, and investigated a single miRNA (MIR27A-3p) based on the research group's previous work with myoblast exosomes (18). However, the normalization to MIR16-5p may limit the interpretation of this data as it has been shown to be dysregulated in ALS (15, 22) and no evidence was shown that MIR16-5p was stable. Nonetheless, with a fuller investigation, identifying dysregulated miRNA present in exosomes in ALS may provide clues as to the source, destination, and thus function of circulating miRNA in ALS.

Plasma-Based Biomarkers

Two studies have investigated biomarkers in sALS patients using plasma; the portion of blood which contains clotting factors. Using microarray analysis followed by RT-qPCR, Takahashi et al. (9) found significant upregulation of MIR4649-5p and downregulation of MIR4299 in ALS patients compared to healthy controls. Interestingly, this study incorporated a follow up analysis of the expression of miRNAs in seven of the patients, including one patient 24 months later. However, no significant change in the expression of any of the miRNAs were found, although there was a trend for an increase of MIR663b over time. Similarly, in another study, while MIR424 and MIR206 were found to be overexpressed in plasma of sALS patients, they did not show significant changes over 6 and 12 months in a cohort of sALS patients (11). This lack of change in MIR206 over time is consistent with the above results of Waller et al. (13). This suggests that for these miRNA, their expression levels are not correlated with disease progression and changes in the patient condition. This may mean that they may only be suitable as diagnostic markers and not useful in tracking treatment responses in disease.

TABLE 1 | Circulating miRNA-based biomarkers found to be differentially expressed in biofluids.

	Authors	ALS type	*n*	Validated changes		Controls	RNA extraction	Profiling technique	RT-qPCR validation	RT-qPCR Normalization
				Increase	Decrease					
Serum	Freischmidt et al. (4)	Sporadic	22	–	MIR132-5p MIR132-3p **MIR143-5p** **MIR143-3p** LET7B-5p	Age-matched healthy controls	miRNeasy Mini	–	Ncode VILO EXPRESS SYBR GreenER	Spiked in cel-MIR39-3p
	De Felice et al. (5)	Sporadic	72	MIR338-3p	–	Age-matched healthy controls	Trizol	–	miScript RT-qPCR	LET7A
	Freischmidt et al. (6)	Familial	22	–	MIR1825 MIR1915-3p MIR3665 MIR4530 MIR4745-5p	Age-matched healthy controls	QIAzol and miRNeasy Mini	Affymetrix GeneChip 3.0 Array	miScript RT-qPCR	Spiked in cel-MIR39-3p
		Sporadic	14	–	MIR3665 MIR4530 MIR4745-5p					
	Toivonen et al. (7)	–	12	**MIR106B** MIR206	–	Age-matched healthy controls	Norgen Total RNA	Affymetrix GeneChip 2.0 Array	TaqMan miRNA RT-qPCR	Spiked in cel-MIR39-3p
	Freischmidt et al. (8)	Sporadic	18	–	MIR1234-3p MIR1825	Age-matched healthy controls/ Alzheimer's/ Huntington's	QIAzol and miRNeasy Mini	Affymetrix GeneChip 3.0 Array	miScript RT-qPCR	Spiked in cel-MIR39-3p
	Waller et al. (13)	Sporadic	50	**MIR143-3p** MIR206	MIR374B-5p	Age-matched healthy controls/ disease mimics	Norgen Circulating Nucleic Acid Isolation	TaqMan Low Density RT-qPCR arrays	miScript RT-qPCR	MIR17-5p MIR24 MIR223-3p
	Matamala et al. (16)	Sporadic	20	MIR142-3p	MIR1249-3p	Age-matched healthy controls	Trizol LS and miRNeasy Serum/ Plasma	Illumina TruSeq Small RNA on Illumina MiSeq	TaqMan miRNA RT-qPCR	Spiked in cel-MIR39-3p

(Continued)

TABLE 1 | Continued

	Authors	ALS type	*n*	Validated changes		Controls	RNA extraction	Profiling technique	RT-qPCR validation	RT-qPCR Normalization
				Increase	Decrease					
	Raheja et al. (17)	Sporadic/ Familial	23	Screen only	Screen only	Healthy controls	miRcury	miRNA LNA RT-qPCR arrays	–	–
	Xu et al. (18)	–	10	–	MIR27A-3p	Healthy controls	Trizol or miRNeasy Micro	–	miDETECT A Track miRNA RT-qPCR or TaqMan miRNA RT-qPCR	MIR16-5p
Plasma	Takahashi et al. (9)	Sporadic	48	MIR4649-5p	MIR4299	Age-matched healthy controls	miRNeasy Serum/ Plasma	3D-Gene Human miRNA oligo chip	miScript RT-qPCR	MIR4516
	de Andrade et al. (11)	Sporadic	39	MIR424 MIR206	–	Aged match healthy control	miRVana PARIS	Affymetrix GeneChip array (on muscle)	TaqMan miRNA RT-qPCR	MIR16-5p
	Sheinerman et al. (12)	–	50	MIR206/MIR338-3p MIR9/MIR129-3p MIR335-5p/MIR338-3p		Age-matched healthy controls	Trizol and Ambion Glass fiber Columns	Literature search	TaqMan miRNA RT–qPCR	–
Cerebrospinal Fluid	Freischmidt et al. (4)	Sporadic	22	**MIR143-5p** MIR574-5p	MIR132-5p MIR132-3p **MIR143-3p**	Age-matched healthy controls	miRNeasy Mini	–	Ncode VILO EXPRESS SYBR GreenER	Spiked in cel-MIR39-3p
	De Felice et al. (5)	Sporadic	72	MIR338-3p	–	Age-matched healthy controls	Trizol	–	miScript RT-qPCR	MIR24
	Benigni et al. (10)	Sporadic	24	MIR181A-5p	LET7A-5p LET7B-5p LET7F-5p MIR15b-5p MIR21-5p MIR195-5p MIR148A-3p	Age-matched healthy controls	miRNeasy Mini	Human miFinder 384HC miRNA PCR array	SYBR Green RT-qPCR	Spiked in cel-MIR39-3p MIR608 MIR328-3p
	Waller et al. (14)	Sporadic	32	Screen only	Screen only	Age-matched healthy controls/disease mimics	miRVana PARIS	Illumina TruSeq Small RNA on Illumina HiScanSq	miScript II RT-qPCR	Spiked in cel-MIR39-3p MIR30A-5p

(Continued)

TABLE 1 | Continued

	Authors	ALS type	*n*	Validated changes		Controls	RNA extraction	Profiling technique	RT-qPCR validation	RT-qPCR Normalization
				Increase	Decrease					
Whole Blood	Liguori et al. (15)	Sporadic	56	-	LET7A-5p LET7D-5p LET7F-5p LET7G-5p LET7I-5p MIR15A-5p MIR15B-5p MIR151A-5p MIR151B MIR16-5p MIR22-3p MIR23A-3p MIR26A-5p MIR26B-5p MIR27B-3p MIR28-3p MIR30B-5p MIR30C-5p MIR93-5p MIR103A-3p **MIR106B-3p** MIR128-3p MIR130A-3p MIR130B-3p MIR144-5p MIR148A-3p MIR148B-3p MIR182-5p MIR183-5p MIR186-5p MIR221-3p MIR223-3p MIR342-3p MIR425-5p MIR451A MIR532-5p MIR550A-3p MIR584-5p	Age-matched healthy controls	PAXgene Blood RNA	Illumina TruSeq Small RNA on Illumina HiSeq2500	TaqMan Advanced miRNA RT-qPCR	MIR484

Those miRNA underlined show consistent directional changes between control and ALS cases while those in bold show contrasting directional changes between control and ALS cases.

Cerebrospinal Fluid-Based Biomarkers

In addition to serum and plasma, differential expression of ncRNA has also been investigated in CSF. Although CSF is not as easily obtainable as blood, changes in expression may potentially be more insightful due to its close proximity to the central nervous system. Using RT-qPCR, De Felice et al. (5) not only found MIR338-3p to be over-expressed in serum, but also in CSF, blood leukocytes, and spinal cord tissue in ALS patients compared to controls and other patient groups (including patients with Alzheimer's and Parkinson's disease). *In situ* hybridization staining of spinal cord tissue post mortem found that MIR338-3p was localized in the dorsal root gray matter and overexpressed in ALS patients, suggesting a potential source of the miRNA. In contrast, Freischmidt et al. (4) used the biomarkers identified in their serum work to find out if there were similar changes in the CSF. While four of those miRNAs were dysregulated, only MIR143-3p showed a significant correlative relationship between the serum and CSF, suggesting there is low correlation in miRNA expression between these two biofluids. Combined with generally higher concentrations of miRNA in the serum, the authors concluded that there might be separate regulatory mechanisms underlying the levels of miRNAs in these two body compartments. This is supported by other papers looking into CSF which have shown very little overlap with other serum studies, but studies that have looked at both within the same sample groups are limited.

EMERGING THEMES IN ALS BIOMARKER DISCOVERY

Recently, two main themes are starting to emerge in biomarker discovery, including in those for ALS. Firstly, it is becoming evident that seeking to identify singular biomarkers for disease is unlikely, underscored by the minimal overlap demonstrated by the above studies. In a study to identify miRNA biomarkers in CSF, using ratios between the expression of two miRNA as determined by RT-qPCR increased sensitivity and specificity in identifying sALS cases compared to using a single miRNA (10). The study pointed out that the use of more than one miRNA as a "biomarker signature" is preferable as it reduces the dependency on variation between individuals. The pairing of the upregulated MIR181A-5p, with either of their two downregulated miRNA, MIR21-5p and MIR15B-5p, increased both the sensitivity and the specificity, with MIR15B-5p increased by 15% on average. Another study has also used this concept for miRNA present in serum, using a number of pairs to identify not only patients with ALS, but other neurological disorders such as Alzheimer's disease, frontotemporal dementia, and Parkinson's disease (12). Having identified 37 brain- or inflammation-enriched miRNA, they found the combination of the three pairs of miRNAs (MIR206/MIR338-3p, MIR9/MIR129-3p, and MIR335-5p/MIR338-3p) were able to clearly distinguish between ALS and control patients in their cohort with a sensitivity of 84% and a specificity of 82%. Furthermore, other paired combinations were able to differentiate between other neurodegenerative diseases and ALS. Sheinerman et al. (12) found an 8-fold increase in MIR206 levels in the plasma of ALS patients when compared to the controls and this was enough to distinguish ALS patients from controls by itself. Therefore, on the whole, pairs of miRNA were able to distinguish between the various diseases and controls with higher accuracy than could be achieved by an individual miRNA.

Secondly, recent advances have improved the generation of high quality libraries from small amounts of starting RNA, allowing unbiased screening of potential ncRNA biomarkers by the RNA-seq technique. In one of the first studies, following on from their work with serum, Waller et al. (14) used RNA-seq to profile miRNA expression in the CSF of ALS patients. While they were able to successfully sequence the miRNA and identify potential candidates, they were unable to confirm those with RT-qPCR because of technical issues. Nonetheless, it supports the conclusion of the above studies that differences in miRNA can be detected in CSF and that CSF could be a source of biomarkers.

More recently, one study has used total blood to screen for miRNA biomarkers in ALS using RNA-seq (15). Following identification of 42 differentially expressed miRNA in the discovery cohort, 38 were validated using RT-qPCR, most of which have been previously reported in other papers. Interestingly, seven of the miRNAs (MIR30B-5p, MIR30C-5p, MIR106B-3p, MIR128-3p, MIR148B-3p, MIR186-5p, MIR342-3p) were able to distinguish between spinal and bulbar onset, with decreased expression present for those with spinal onset. Furthermore, this study also carried out RNA-seq on mRNAs in the same samples to help identify targets that could be regulated by the miRNA. The use of total blood, however, limits the interpretation of these results due to the presence of red blood cells in the samples and the possibility of variable numbers of different types of white blood cells between patient and control groups.

Matamala et al. (16) also utilized RNA-seq for the identification of ALS biomarkers, but started by profiling serum samples from transgenic mouse models of ALS, followed by RT-qPCR validation in human samples. While a number of miRNAs were found to change in levels between the ALS model and controls, there was limited cross-validation when this was taken forward to the human studies. Two miRNA that did show differences between ALS patients and controls in the human studies were MIR142-3p and MIR1249-3p. The authors found that MIR142-3p seemed to correlate negatively with a decline in clinical disability scale ALSFRS-R in patients, thus suggesting that this could be used to measure the effect of any disease-slowing treatment. Further, it was found to potentially target the expression of the ALS genes *TARDBP* and *C9orf72*. Interestingly, Matamala et al. (16) also briefly described the detection of non-miRNA ncRNA with their RNA-seq, but did not state if they were differentially expressed or whether they were investigated further. As such, there may be a range of potential biomarkers that have not yet been identified. Indeed, several other ncRNA species have been detected in serum including rRNA and tRNA (23–25). These have also been highlighted as potential biomarkers in diseases other than ALS in blood (26–29) and other tissues (24, 30–32). To this end, we are currently using RNA-seq to identify potential biomarkers in

ALS within the full cohort of ncRNA species, and early results suggest that we have potential candidates, which include miRNA, piRNA, and tRNA.

CHALLENGES AND OPPORTUNITIES

Across these studies, there is very little overlap in the miRNA species as potential biomarkers in the biofluids (see **Table 1**), and there are multiple potential reasons for this. Firstly, as these are mostly from elderly human patients, some of these patients could have other conditions which could alter the miRNA composition of the biofluids themselves, thus confound the detection of ALS-specific biomarkers; careful screening of patients therefore is required. Further, some of these studies do not include patients from ALS disease mimics to help identify ALS-specific markers. This is important as some biomarkers identified such as MIR206 are not specific to ALS as described above. Additionally, most of these studies have been carried out on samples from one population group. As differences may exist between different populations with the disease, the lack of cross-validation of changes in miRNA expression between studies may be reflective of differences in the patient population, whether that be mediated genetically and/or environmentally. The number of patients also differ, from 12 to 72 ALS patients, and so the statistical power for some of these biomarkers is limited.

Alternatively, the causes could be related to the methodology of the study, from the extraction of the biofluids and RNA, through to the screening and validation of the miRNA biomarkers. As seen in **Table 1** and **Figure 1**, a range of different workflows have been undertaken across all the studies, all of which may contribute to differences in the changes that are detected. In addition, some of these factors potentially could affect the strength of some of these studies. For example, how the samples were collected and processed may vary. Some of the studies did not describe their collection procedures, and it is well known that differences in the centrifugation time post-collection, speed of centrifugation, and temperature can all affect the quality and quantity of RNA in the samples (33, 34).

Further, techniques used to normalize the RT-qPCR could be considered questionable in a number of studies. Due to the minimal and varied amount of RNA in biofluids, and the resultant difficulty in quantifying the RNA concentration, most miRNA RT-qPCR kits used fixed sample volumes instead of fixed total RNA amounts. Therefore, miRNA RT-qPCR normalization must control for input RNA, not just for technical variation, by crucially using a reliable target as a normalizer. However, no universal normalizer for biomarker work exists, and identification of a suitable normalizer is a problem across

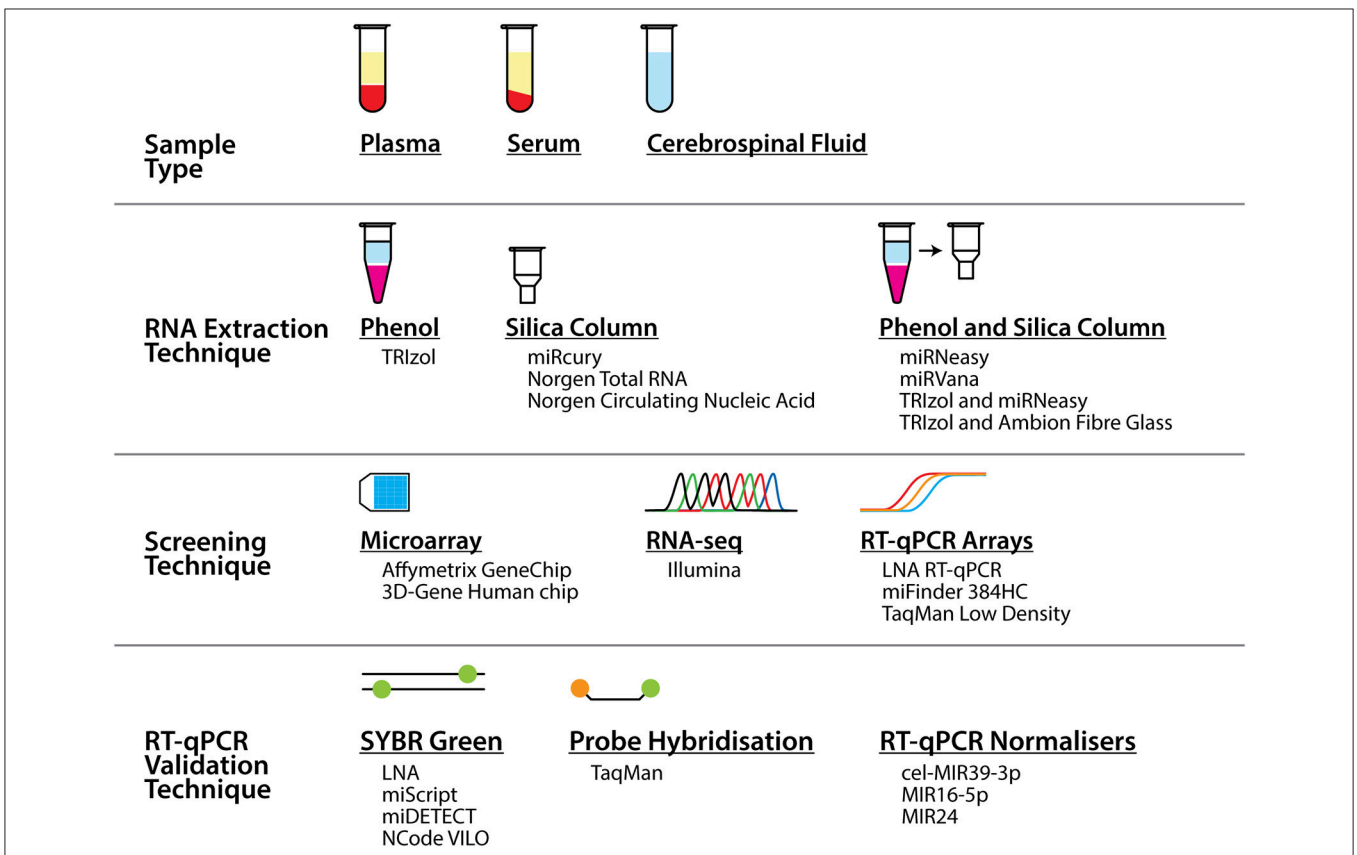

FIGURE 1 | Summary of the different sample types, techniques, and tools that were used to profile miRNA biomarkers in ALS in the studies presented in this review. As can be seen alongside **Table 1**, numerous different combinations of these sample types and tools across the techniques were employed across a number of studies.

all biofluid biomarker studies (35, 36). Normalizers should be selected per study from those ncRNA with the most stable expression in their screen and then validated. Instead, a number of the above studies have used the synthetic spike-in cel-MIR39B, but this would only control for technical variation introduced from RNA extraction onwards, not for the total RNA amount in the starting volume. Others have used miRNA recommended as normalizers such as MIR16-5p, but as described above, it has been shown to be regulated in ALS (15, 22), and also in stress and in red blood cells (37, 38). Indeed, as some studies did not check for hemolysis in their samples, the observed changes may be due to released miRNA from the lysed red blood cells. Taken together, this underlies why there may be limited cross-validation between studies and thus careful consideration of identifying objective normalizers are required.

One question is how do these miRNA-based biomarkers compare to other biomarkers for ALS? While the properties of miRNA as biomarkers as described above are ideal, there are other molecules such as DNA, RNA, protein, and metabolites that could also be used. One of the most commonly used biomarkers in ALS is the neurofilament proteins, which form part of the cytoskeleton of neurons and has been found to be present in both CSF and serum. Studies have shown that neurofilaments are able to help with identification of ALS cases but like MIR206 are not specific to ALS, and rather a measure of axonal death. As such, it is likely that singular miRNA or neurofilaments by themselves will not be able to help with ALS diagnosis or prognosis, but they could form part of any potential biomarker signature. Therefore, it is likely that an integrative approach is required, using data on the levels of a number of ncRNA biomarkers, as has been shown for other diseases (39). Such approaches include utilizing multiple biomarkers, including both miRNA and non-miRNA based biomarkers, and integrating them into a signature model such as a discriminant model, or by using ratios of miRNA expression and using them to help with classification of the disease state, and a number of the above studies have done this. Together, this may help allow ALS patients to be specifically identified, not only from healthy controls but from disease mimics. Therefore, taking this work forward into larger cohorts of patients is vital to test integrating these biomarkers together.

Indeed, opportunities from well-designed studies to validate their biomarkers in separate and larger cohorts could allow for these biomarkers to be used clinically. Further, these studies have been designed first and foremost to find biomarkers for ALS with little attempt to determine the biology underlying these changes, as presence alone does not infer function. Nonetheless, considering the wide and varied biological roles of miRNAs, determining their biological function will be important. Future studies need to include their source and destination, potentially by investigating exosomes and their contents and function. These studies would provide new insights into the mechanisms that may underlie ALS. Therefore, not only do larger cohorts need to be screened but proper experimental design needs to be undertaken to ensure that results are valid and can be used to progress the field further.

What is ultimately being sought is a set of biomarkers that are able to help with the diagnosis and prognosis of ALS patients. Diagnosis and prognosis of patients based on an ncRNA biomarker could assist with the development of tailored and targeted treatments to extend or improve patients' quality of life. As such, these studies have shown that there is potential here for ncRNA-based biomarkers to be identified, and with careful consideration, future work will help to further refine this to progress this to the clinical setting.

AUTHOR CONTRIBUTIONS

GJ wrote and edited the manuscript. MH, PL, and SN critically reviewed and edited the manuscript.

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