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REVIEW SERIES: IMAGING IMMUNOLOGICAL PROCESSES IN NEUROINFLAMMATORY DISEASES

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Imaging immunological processes from blood to brain in amyotrophic lateral sclerosis

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Abstract

Neuropathology studies of amyotrophic lateral sclerosis (ALS) and animal models of ALS reveal a strong association between aberrant protein accumulation and motor neurone damage, as well as activated microglia and astrocytes. While the role of neuroinflammation in the pathology of ALS is unclear, imaging studies of the central nervous system (CNS) support the idea that innate immune activation occurs early in disease in both humans and rodent models of ALS. In addition, emerging studies also reveal changes in monocytes, macrophages and lymphocytes in peripheral blood as well as at the neuromuscular junction. To more clearly understand the association of neuroinflammation (innate and adaptive) with disease progression, the use of biomarkers and imaging modalities allow monitoring of immune parameters in the disease process. Such approaches are important for patient stratification, selection and inclusion in clinical trials, as well as to provide readouts of response to therapy. Here, we discuss the different imaging modalities, e.g. magnetic resonance imaging, magnetic resonance spectroscopy and positron emission tomography as well as other approaches, including biomarkers of inflammation in ALS, that aid the understanding of the underlying immune mechanisms associated with motor neurone degeneration in ALS.

KEYWORDS
amyotrophic lateral sclerosis, central nervous system, imaging, innate immune system, microglia, TSPO PET

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INTRODUCTION

Clinical features of amyotrophic lateral sclerosis (ALS)

While ALS is less prevalent compared to other neurodegenerative diseases, it is undoubtedly one of the most rapidly progressive diseases, with an average survival of 3 years from disease onset. Clinical features, including bulbar onset disease, disease progression rate and the presence of early cognitive changes, have so far been the most effective predictors of survival in ALS [1]. The marked clinical heterogeneity of ALS reflects the loss of motor neuronal system integrity and frontotemporal cortical function [2]. Early neuromuscular junction (NMJ) involvement is also a recognized clinical feature which, in combination with central pathology, leads to a wide spectrum of symptoms and signs including limb and/or bulbar muscle weakness, as well as cognitive and behavioural manifestations [3]. More than 30 genetic mutations have been linked to a small percentage (~20%) of ALS cases [4] of which some of the genetic variants are associated with a more severe disease course [5]. Importantly, novel features of the ALS pathobiology have more recently emerged as determinants of disease progression, notably age and changes of the innate as well as adaptive immunological responses [6].

Similar to other neurodegenerative and neuroinflammatory diseases, an older age of onset is associated with a more severe progression of ALS [7]. Ageing may therefore play a central role in the pathological process underpinning the rate of axonal and neuronal cell loss in ALS. The impact of ageing in ALS is probably due to the gradual accumulation of aberrant protein aggregations with time, but is also likely to be linked to a profound alteration of immunological responses collectively termed 'inflammaging' [8]. Immunosenescence and the potential loss of immune tolerance during ageing have emerged as potential effects of the reduction of T regulatory cell (T_{reg}) function, which has been reported in ALS individuals with a faster progression of the disease [9]. For example, a recent study has shown that the proteomes of blood mononuclear cells and plasma share a characteristic fingerprint of activation of molecular pathways linked to immune responses and senescence in ALS patients and animal models with a faster progression of the disease [10]. While therapeutic trial design in ALS remains challenging, the availability of novel prognostic and predictive biomarkers that act as surrogates of clinical assessment is likely to aid development of effective therapies for ALS. An important source of molecular targets for biomarkers discovery in ALS is the inflammatory response linked to neurodegeneration, a biological process that can be monitored through the chemical detection of immunological signals in biological fluids and by novel modalities of brain imaging. In addition, structural imaging and the study of diffusivity have yielded information on the anatomical spread of the disease and on structural changes arising from the pathological process. Imaging modalities including biomarkers could thus be used in the clinical setting to not only identify disease progression, but also rule out pathologies mimicking ALS.

Pathology of ALS

The complex genetic landscape of ALS is a strong indication that the ultimate outcome of motor neurone degeneration represents the final common pathway of multiple converging biological alterations. This observation raises the importance of an individualized medicine approach to unravel the pathobiology of the disease, the only potentially successful strategy in the development of biological markers for the a priori clinical stratification in clinical trials [11,12]. The concept of ALS as a non-cell autonomous disorder and the report of a significant disruption of glutamate uptake, as well as of oxidative stress, has evolved towards the game-changing elucidation of the proteinopathy underpinning the pathological process. The identification of genetic defects and of the proteinopathy associated to TAR DNA-binding protein 43 (TDP-43), appearing as ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and ALS, is probably the most substantial step forward in ALS research [12]. As a highly conserved RNA binding protein (RBP), TAR DNA-binding protein 43 (TDP-43) interacts with other RBPs such as hnRNP A/B to regulate all aspects of RNA metabolism. Abnormal TDP-43 migration to the cytosol leads to loss of TDP-43 nuclear function and neuronal death [13]. Loss of TDP-43 in human iPSC-derived cortical-like neurones is associated with stathmin2 (STMN2) mis-splicing, resulting in cryptic exon inclusion and expression of a novel mRNA transcripts [14]. Of note is that RNA binding proteins associated with TDP-43 pathology are potentially responsible for the immunological dysregulation described in the progression of ALS. One line of evidence indicates that immune activation is induced by altered TDP-43 function. Over-expression of TDP-43 in glia and neurones of ALS patients has been shown to drive the pathogenic nuclear factor kappa B (NF-κβ) response, resulting in augmented production of proinflammatory factors [15,16]. A recent study showed that TDP-43 induces inflammation in ALS by triggering mitochondrial DNA release into the cytoplasm, inducing activation the of interferon genes [stimulator of interferon genes (STING)] pathway [17]. Loss of immune tolerance and the development of a chronic proinflammatory environment in the progression of ALS are probably the most obvious outcomes of abnormal protein accumulation and defective clearing mechanisms such as autophagy and proteostasis [18]. The result is the creation
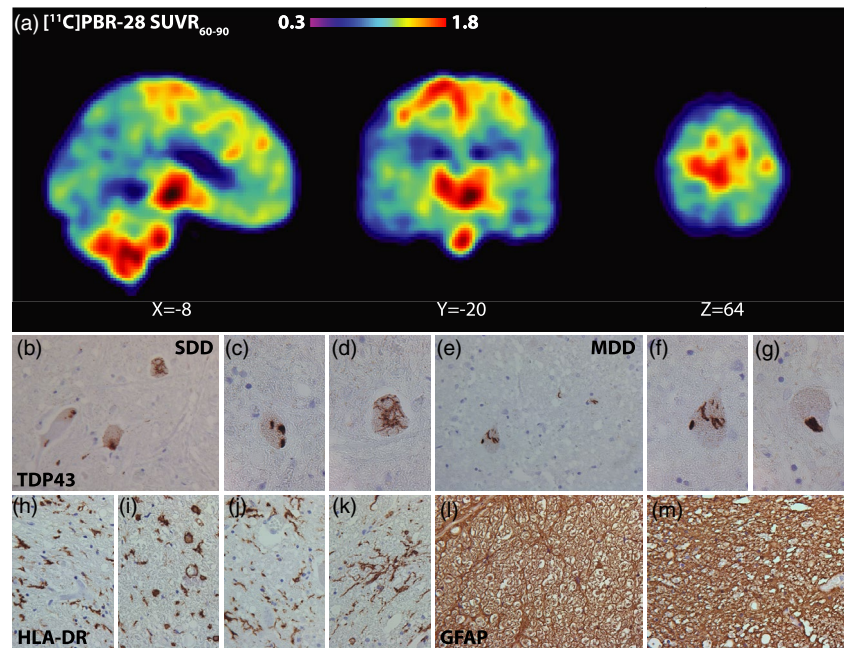
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FIGURE 1 Imaging and pathology of innate immunity in amyotrophic lateral sclerosis (ALS). Positron emission tomography (PET) imaging for translocator protein (TSPO) with peripheral benzodiazepine receptor ligand (PBR)-28 (a) shows increased uptake in the primary motor cortex in a patient with ALS. TAR DNA-binding protein 43 (TDP-43) pathology (b–g) in a patient with short disease duration (SDD) (b–e) and in a patient with moderate disease duration (MDD) (e–g). Microglia and macrophages are activated and show human leucocyte antigen D-related (HLA-DR) expression (h,i) in the ventral horns (h,i) and lateral columns (j,k) in both SDD (h,i) and MDD (j,k). Astrogliosis is present in SDD (l) and MDD (m) in patients with ALS

of a florid inflammatory environment in affected tissues where antigen-presenting cells, microglia, macrophages and astrocytes are active in clearing aggregated and aberrant proteins as well as producing immune mediators that contribute to the inflammatory milieu [19] (Figure 1).

Immunological aspects in ALS

A number of neuroinflammatory changes occur in the pathophysiology of ALS, including glial activation, myeloid and T cell infiltration in the central nervous system (CNS) as well as peripheral immune system activation (Figure 2). Such dysfunctional immune homeostasis has been associated with primary events in ALS pathogenesis, as well as disease progression and disease stage [20]. In support of this are the early presymptomatic changes in animal models of ALS in which various pathological and clinical signs are observed, characterized by the activation of astrocytes, microglia and inflammatory molecules [21].

In autopsy studies in humans, indicators of immune activation in the CNS in ALS include the up-regulation of Toll-like receptor (TLR)-4 signalling genes [22] and the persistent activation and nuclear translocation of transcription-3 [signal transducer and activator of transcription 3 (STAT-3)] in microglia [23]. Other glial proteins associated with the progression of the disease include

increased glial fibrillary acidic protein (GFAP) and connexin expression in astrocytes and CD68 in microglia [24–26]. Furthermore, increased expression of small heat shock proteins (HSPBs), predominantly in astrocytes in ALS, have been associated with disease duration [27] indicating reactive changes in response to tissue damage such as tau expression in motor neurones (Table 1).

Pathology studies of the CNS in human ALS reveal infiltration of monocytes/macrophages and T cells [28], as well as immunoglobulin and complement deposits associated with motor neurone destruction. Similarly, the presence of activated glia and T cell subpopulations in the brain parenchyma have been linked to motor neurone damage [29]. Also, CD34+ cells co-expressing the myeloid marker CD11b, which represents a subset of microglia, are observed adjacent to degenerating motor neurones in autopsied spinal cord from sporadic ALS subjects, but such cells are absent in control subjects [30] (Table 1).

Peripherally, the expansion of proinflammatory T helper type 1 (Th1)/Th17 cells and the decrease of anti-inflammatory Th2 and T_{regs} correlate with the presence of activated microglia [31] and ALS severity and progression [32]. In contrast, low levels of T_{regs} expressing forkhead box protein 3 (FoxP3), as well as reduced mRNA levels of transforming growth factor (TGF)-β, interleukin (IL)-4 and GATA binding protein 3 (GATA3) in T cells from ALS patients, predict reduced survival and rapid progression of disease [33].

BRAIN **DYSREGULATION** **MUSCLE NEUROMUSCULAR JUNCTION**

↑ iNOS/NOX, ROS, PGE, LTB4, SOD, TARDBP, C9orf72, FUS

Motor cells axonal loss

E — Change in expression ↑ Cytokines IL-8, IL-15, IL-6, TNF-α, NOGO-A ↑, Igf-1 ↓, N-CAM, MHC-I ↑, Bax-Bcl2 ↑, C4 ↓

A — ↑ Expression of molecules in glial cells in motor cortex and spinal cord

D — Schwann cells/-MHC I/II associated with infiltration of macrophages

B1 — Activation of microglia and astrocytes: TSPO (PET), myo-inositol (MRI)

B2 — Infiltration CD14⁺, T cells, mast cells, neutrophils, B cells, plasma cells

NMJ destruction Axonal degeneration and denervation

F — ↑ Antibodies in blood/CSF HMGB1, Voltage Ca²⁺ channels, GM1, Fas-R, fetal muscular proteins, vascular antigens, neurofilaments

IgM
IgG

Spinal cord

Motor cells axonal loss

P2X7 receptor ↑   ATP → Co-transmitter

Activation of AChR

C — ↑ Expression of pro-inflammatory factors MCP-1, IL-6, IL-13, IL-17, IL-18, IL-23, IL-β/α, IL-1RA, TNF-α, TNF-αR

G — Antibodies against AChR, Agrin, LRP4, voltage Ca²⁺ channels, GM1 ganglioside

Nerve

Ca²⁺ imbalance Mitochondria permability transition Impaired axonal transport

Disease development

In line with these studies, the suppressive dysfunction of T_{regs} is most marked in fast-progressing ALS patients compared to slow-progressing patients and healthy donors [9]. These observations have been extended in clinical trials aimed to increase the suppressive effect of regulatory T lymphocytes. In support of this, autologous infusions of T_{regs} into patients at early and later stages of ALS have been shown to be safe, and importantly reported to slow the progression rate of disease [34]. Emerging data have also linked myeloid cell signatures to clinical features of ALS [35]. For example, within the peripheral motor system, the frequency of myeloid cell populations and expression of CD14 and human leucocyte antigen D-related (HLA-DR) are associated with

bulbar symptoms and clinical severity [36]. Furthermore, genetic variants of triggering receptors expressed on myeloid cells (Trem2) expressed on myeloid cells have been associated with increased risk of ALS [37]. However, this finding remains controversial, as specific variants can influence the effect of Trem2 on inflammation, as reported in other studies [38]. The presence of autoantibodies directed to neuromuscular junction (NMJ) antigens have also been detected in ALS as well as other disorders of neuromuscular transmission, showing upper and lower motor neurone involvement [39–41]. For example, antibodies to low-density lipoprotein-related receptor protein 4 (LRP4) are involved in neurological symptoms affecting LRP4-containing tissues

FIGURE 2 Proposed immune involvement in the central nervous system and neuromuscular junction in amyotrophic lateral sclerosis (ALS). Inflammatory factors, e.g. reactive oxygen species (ROS), prostaglandin E2 (PGE), leukotriene B4 (LTB4), inducible nitric oxide synthase (iNOS), NADPH oxidase (NOX) and genetic factors participate in motor neurone death in ALS [134,135]. In the central nervous system in ALS (A) reactive microglia expressing high levels of leucocyte common antigen (LCA), Fc-gamma receptor 1 (FcγRI) and lymphocyte function-associated antigen-1 (LFA-1), complement receptors CR3 and CR4 and major histocompatibility complex (MHC)-II molecules present in the primary motor cortex and motor nuclei of the brain stem and spinal cord [136,137] (B1). Activated microglia and astrocytes; translocator protein (TSPO) and myo-inositol are increased in affected brain regions as determined by positron emission tomography (PET) and magnetic resonance imaging (MRI). (B2) Infiltrating T cells (CD4$^+$ and CD8$^+$) and natural killer (NK) cells occur near degenerating corticospinal tracts in ALS and superoxide dismutase (SOD)-1 mutant mice [138]. Infiltrating interleukin (IL)-17A$^+$ CD8$^+$ cells and IL-17A$^+$ mast cells and neutrophils in the spinal cord [139]. (C) Macrophage chemoattractant protein-1 (MCP-1), IL-1β and tumour necrosis factor (TNF)-α expression precedes oxidation and apoptosis gene expression [122]. (D) Infiltration of mast cells, neutrophils in the neuromuscular junction (NMJ) [46]. MHC-I/II on Schwann cells are associated with recruitment of macrophages (CD11b$^+$CD169$^+$, CD68$^+$, Iba1$^+$) in peripheral nerves in SOD-1 mice and ALS [140]. Up-regulation of MCP-1 and down-regulation of C-C chemokine receptor type 2 (CCR2). (E) Changes in structure and function in muscle and NMJs in ALS. Over-expression of Bax and B cell lymphoma 2 (Bcl-2) at the post-synaptic domain of NMJ are associated with muscle degeneration [141]. Up-regulation of inflammatory cytokines at the NMJs. (F) Serum and cerebrospinal fluid (CSF) antibodies to high-motility group box 1 (HMGB1), ganglioside 1 (GM1), fas receptor (Fas-R) (CD95), fetal muscular proteins, vascular antigen and neurofilament proteins (Nf). (G). Antibodies to LRP4, GM1 ganglioside, voltage-gated calcium channels (VGCC), acetylcholine receptor (AChR) in presynaptic motor nerve terminals and NMJs linked to muscle denervation [39,43,104,107,112,142]

and are found more frequently in ALS than in myasthenia gravis MG [39,41,42]. Whether LRP4 antibodies may have a pathogenic activity in ALS in the denervation process is unclear [41]. At the NMJ, LRP4, agrin proteins, muscle-specific kinase (MuSK) and acetylcholine receptors (AChR clustering), which make up the synapses between spinal motor neurones and the skeletal muscle, are the main targets of the immune system [43,44] (Figure 2, Table 1).

In ALS, peripheral local immune responses along the degenerating motor axons in sciatic nerves and skeletal muscle are observed [45]. Additionally, activated monocytes, mast cells, neutrophils and inflammatory mediators are involved in the immune cell recruitment in the neuromuscular compartment [35,46].

In summary, inflammation in the periphery and also the CNS are constant features of both early and late stages of ALS. Immune cells, activation markers and molecules related to the disease pathology and the application of positron emission tomography (PET) and magnetic resonance (MR) imaging to study physiological changes of target tissues in ALS are illustrated and summarized in Figure 2 and Table 1.

Animal models of ALS

The most common ALS-associated mutations occur in *SOD1*, *TARDBP*, *FUS* and *C9ORF72*, and thus genetically modified animals expressing mutations of these genes have been developed to understand pathological mechanisms leading to neuronal death and develop therapeutic strategies [47,48]. Local glial activation, T cell infiltration and immunoglobin deposition in the CNS, as well as systemic immune system activation in animal models and ALS patients, support the concept that immunological

pathways contribute to disease progression. To identify immune biomarkers of disease, imaging modalities have been applied to monitor these pathways during disease. While imaging of ALS patients presents fewer problems than imaging small animals, linking imaging studies to pathological features of ALS in humans is difficult, and is limited to post-mortem MR imaging (MRI) and pathology studies. By contrast, models including bi- and triple transgenic mice lend themselves to the study of specific immune factors. As an example, monocyte chemoattractant protein 1 (MCP1) and C-C chemokine receptor type 2 (CCR2) genetically labelled with monomeric red fluorescent protein-1 (mRFP) and enhanced green fluorescent protein (eGFP) [49] crossed into the human superoxide dismutase 1 (hSOD1G93A) mouse has allowed visualization of the timing and the extent of innate immune response in the CNS during disease initiation and progression [49]. Similarly, regional neurochemistry alterations have been examined in SOD mice using localized *in-vivo* magnetic resonance spectroscopy (MRS), revealing changes in creatine, N-acetylaspartate (NAA), glutamate, glutamine, γ-aminobutyric acid (GABA) and myoinositol [50] Mouse models also lend themselves to *in-vivo* PET imaging studies to study synaptic glutamatergic function [18F]FPEB ([18F]3-fluoro-5-[2-pyridylethynyl]- benzonitrile) [51] and inflammatory response using [11C]PBR28 (peripheral benzodiazepine receptor ligand). While neuroimmunological processes in animal models of ALS can be easily visualized, the development of transgenic mice in which immune markers are tagged using fluorescent probes, and the availability of *in-vivo* imaging, has expanded the imaging possibilities in the ALS field. For example, multi-photon *in-vivo* microscopy (MPM), in which tissue localized deeper within the organism is accessed through 'imaging windows' similar to one-photon microscopy,

TABLE 1 Immune aspects of ALS in the CNS, PNS and NMJ

Immune aspect	Neuroinflammation (CNS)	Peripheral inflammation (PNS)	Inflammation at the NMJ	References
Antibody	HMGB1	LRP4	Agrin	[39,42]
	GM1 ganglioside	Voltage-gated calcium channels: P/Q-type, N-type, L-type	LRP4	[102–104]
		Fas-R (CD95)	MuSK	[105]
		Fetal muscular proteins	AChRs	[106]
		GM1 ganglioside		[107,108]
		HMGB1		[109–111]
		Neurofilament proteins		[43]
		AChR		[112]
Cellular response	Microglia activation TSPO (PET), myoinositol (MRI)	Macrophages (CD68$^+$, CD11b$^+$ CD169$^+$), Iba1$^+$	Macrophages (CD68$^+$/ CD11b$^+$)	[113]
	FcgR1 expression	NK cells in sciatic nerves.	Schwann cells: CSF1 and IL-34	[114]
	Macrophages in ventral nerve root	CD4$^+$, CD8$^+$, NK cells	CSF-1R expressing monocyte/ macrophages	[103,115,116]
	Macrophages (CD68, CD34, TLR-4, COX-2$^+$), Mast cells and T cells in brain cortex and spinal cord	Neutrophils Th17, Th1, monocytes		[117,118]
	DCs, MCP-1, microglia, (CD68$^+$, TLR-4), astrocytes (GFAP, HSPBs)			[119,120]
	Corticospinal tract infiltration: CD4$^+$, Th17, Th1, CD8$^+$ (suppressor and cytotoxic), NK cells, monocytes			[45,121]
				[122,123]
				[124,125]
				[118,126–128]
Cytokines and other inflammatory factors	IL-1β, IL-6, TNF-α, H$_2$O$_2$, ROS, NO (microglia and astrocytes)	PBMC: IL-13$^+$, IL-6$^+$, IL-15, TNF-α, IL-33↓	TNF-α	[119,129,130]
	RANTES in CSF	Eosinophil-derived neurotoxin in serum	Complement activation	[131]
			C1q (MAC), CD55 and CD59	[132]
				[133]

Abbreviations: CNS = central nervous system; PNS = peripheral nervous system; NMJ = neuromuscular junction; CSF = cerebrospinal fluid; PBMC = peripheral blood mononuclear cells. Markers identified by MRI = magnetic resonance imaging; MRS = magnetic resonance spectrometry; PET = positron emission tomography; LRP4 = lipoprotein-related protein 4; MuSK = muscle-specific tyrosine kinase; AChRs = acetylcholine receptors; HMGB1 = high-motility group box 1; FcgRI = immunoglobulin receptor FcγR1; Fas-R = Fas receptor; GM1 = gangliosides; COX-2 = cyclo-oxygenase-2; TLR = Toll-like receptor; MCP-1 = macrophage chemoattractant protein-1; Th = T helper type cells; HSPBs = small heat shock protein; GFAP = glial fibrillary acidic protein; CSF1 = colony-stimulating factor-1; CSF-1R = colony-stimulating factor-1 receptor; RANTES = regulated on activation normal T cell expressed and secreted; MAC = membrane attack complex. ↑ = up-regulation and ↓ = down-regulation of response.

could be applied to ALS models. Sekiguchi *et al.* successfully implemented MPM to image calcium responses of astrocytes in murine spinal cords [52]. Such applications in suitable transgenic mice models of ALS, as discussed above, could allow for detailed analysis of the disease process in time in *in-vivo* models. An alternative animal

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model for live imaging in neurodegenerative diseases is the zebrafish. Because of its translucency there is no need for invasive surgical procedures such as implanting imaging windows. Furthermore, several genes associated with ALS, e.g. SOD1, are highly conserved in the zebrafish [53]. In addition, many transgenic zebrafish lines in which fluorescent reporters have been inserted are suitable for ALS research. These include Tg(spi1b:GAL4,UAS:TagRFP) and Tg(mnx1-GFP), fluorescent proteins specific to glial cells and motor neurones, respectively [54]. Linking these approaches in animal models to the conventional imaging modalities in humans, as discussed below, will be critical to more clearly understand the disease process in humans.

IMAGING MODALITIES

Several imaging modalities have been used to monitor neuroinflammation and pathological changes in ALS and experimental animal models of ALS [46]. One approach is PET imaging, which has the advantage of being able to interrogate various disease mechanisms using specific molecular ligands to directly study the CNS, peripheral nervous system (PNS) or neuromuscular junction [55] Furthermore, PET allows direct visualization of neuroinflammation in early stages of disease as well as recurrent analysis to monitor disease progression. Importantly, this approach lends itself to study the efficacy of therapies targeting molecular pathways that can be visualized by specific ligands [55]. PET imaging may thus serve as a marker of disease progression or a prognostic or predictive biomarker, while allowing a detailed analysis of molecular alterations key to the pathogenesis of ALS, such as cerebral blood flow, glucose metabolism, neuroinflammation, neuronal dysfunction and oxidative stress [56]. Most studies utilizing PET for ALS use 18F-fluorodexyglucose (FDG), a radiotracer that only detects glucose metabolism but does not directly detect inflammatory processes in the CNS. By contrast, translocator protein (TSPO) PET imaging is a promising molecular imaging technique that represents a multi-cellular neuroinflammatory reaction, with a potential use as pharmacodynamic biomarker for ALS therapies [57,58]. TSPO is expressed on microglia and astrocytes in the CNS under neuroinflammatory conditions, and TSPO-targeting PET detects high densities of microglial activity [59]. Early studies have used first-generation TSPO ligands [11C]-PK11195 (1-[2-chlorophenyl]-N-[1-methyl-propyl]-3-iso-quinoline carboxamide) and [18F]-DPA-714 (N,N-diethyl-2-[4-(2-fluoroethoxy)phenyl]-5,7-dimethylpyrazolo[1,5-a] pyrimidine-3-acetamide), while more recent studies use second-generation TSPO tracers [11C]-PBR28 (N-[(2-methoxyphenyl)methyl]-N-(2-phenoxyphenyl)acetamide), which have improved uptake and binding affinity compared to older tracers [60]. A higher TSPO PET signal was found in

motor cortex, supplementary motor and temporal cortex in ALS patients with bulbar onset compared to healthy controls using [18F]-DPA-714 [61]. A study using [11C]-PK11195 showed increases in binding in motor cortex, pons, frontal lobe regions and the thalamus in ALS patients compared to healthy controls, regardless of bulbar or limb onset [62].

More recent studies utilizing [11C]-PBR28 report increased binding of TSPO PET in the motor cortex, but also in upper regions of the corticospinal tracts [63–65]. More specifically, increased binding was found in precentral gyri for limb-onset ALS patients and in the brain stem for bulbar-onset ALS patients, indicating that glial activation is present in clinically relevant neuroanatomical areas [63,64] and providing an atlas of the disease neuropathology with a close relationship to the clinical phenotype. Correlating altered PET binding with MRI has shown that increased binding in the precentral gyri was associated with cortical thinning, reduced fractional anisotropy (FA) and higher diffusivity [66,67]. However, no changes were found in [11C]-PBR28 uptake during 6 months of disease progression. While TSPO PET originally was initially thought to reflect pathogenic microglia activation, a recent study identified that TSPO PET reflects microglial density rather than the microglial activation state in multiple sclerosis (MS) [68]. Overall, PET studies have found similar affected regions in ALS patients (motor cortex and the upper corticospinal tracts), and there is some evidence that astrocytes are also affected in the white matter and the pons of ALS patients showing increased binding of [11C]-DED (deuterium-L-deprenyl), which binds to the monoamine oxidase B (MAO-B) present on mitochondria of reactive astrocytes [57]. Furthermore, while there is no lack of PET studies in ALS [56] most do not focus upon glial cells and have limitations that restrict the ability to draw strong conclusions about the immunopathology in ALS. In addition, few studies involve large sample sizes or follow their subjects longitudinally.

Another approach is MRI, frequently used to identify novel so-called 'dry biomarkers' which, together with the use of functional scales, task performance and electrophysiology, has become an established field of investigation in ALS [69]. The use of MRI is one of the most relevant tools to address the lack of understanding of the origin and progression of the disease, with most models based on the assumption of a disease originating and propagating from the cortex or the spinal cord and later developing into a diffused neuromuscular disease [70]. Technical limitations, such as the lack of homogeneity due to the proximity of the spinal cord to bone, air and soft tissue, as well as the cerebrospinal fluid (CSF) flow, have limited the ability of MRI to detect changes in the spinal cord in ALS [71]. Furthermore, imaging of the spine is not only limited by motion of the CSF but also physiological motion due to breathing [71,72]. MRI has clear value in the evaluation of structural changes, and can be applied to

retrieve information on a range of pathological processes and their anatomical distribution.

MRI techniques include voxel-based morphometry (VBM), diffusion tensor imaging (DTI), magnetic resonance spectroscopy (MRS) and other iron-sensitive sequences, including T2, susceptibility weighted imaging (SWI) and functional MRI (fMRI). These methodologies make it possible to extend the investigation from structural into functional changes. For example, MR structural imaging has highlighted volumetric and cortical changes in the precentral gyrus and somatosensory areas, changes associated with the progression of ALS, although the degree of volume and substance loss has not always correlated with functional parameters of disease progression [73]. Together with structural changes and analysis of connectivity of different CNS regions, a deeper insight into the loss of function in key areas of ALS pathology has come from the use of DTI, allowing the identification of low fraction anisotropy along the corticospinal tracts in patients with ALS [74]. Such studies, e.g. diffusion tensor MRI, have also been applied in a mouse model of ALS to evaluate axonal injuries across different axonal tracts [75].

To more clearly correlate imaging studies with pathology, combined post-mortem T1 sequence and histological assessment in ALS patients have been applied. These studies show changes between cortical grey matter and subcortical white matter in the motor cortex, in somatosensory areas and in the primary visual cortex. In part, these changes are attributed to neurone loss but also to increased numbers of astrocytes correlating with findings from PET imaging [57,76,77]. MR studies in ALS has also revealed a link between tissue inflammation and iron deposition in the motor cortex in ALS patients, as well as animal models. Motor cortex hypointensity on MRI images in ALS is associated with increased iron accumulation by microglia [78], although it is unclear how iron contributes to the disease process [79]. Further imaging studies, such as proton magnetic resonance spectroscopy, have enabled the detection of aspartate metabolites, choline, the glial marker myoinositol, amino acids and compounds related to cellular bioenergetic and oxidative status. In ALS such metabolites, including glutamate, N-acetyl aspartate and gamma aminobutyric acid present in the supplementary motor area, have been suggested to act as a surrogate biomarker for ALS [78,80]. A novel imaging technique, chemical exchange saturation transfer (CEST), provides high-resolution and sensitivity to image-specific metabolites and was already shown to differentiate ALS by reduced CEST signal intensity in the motor cortex [81]

In summary, the use of PET and MR imaging has revealed that TSPO and myoinositol, a spectroscopic marker for glial activity, are increased in the motor cortex of ALS patients and represent promising biomarkers of neuroinflammation [56,81–84].

Biological fluid biomarkers

A number of potential biomarkers relevant to both CNS and/or neuromuscular pathology ALS include biological fluid-based neurochemical biomarkers, neuroimaging techniques, electrophysiological parameters, electrical impedance myography and clinical features that are captured by a number of functional rating scales [85]. The development of new highly specific imaging approaches for visualizing immune activation will be critical for the integration of imaging and biofluid-based inflammatory biomarkers.

Studies of CSF, blood, urine and saliva in ALS have facilitated the identification and quantification of disease-specific molecules that show higher clinical utility when analysed in combination. For example, levels of neurofilament proteins (Nf) present in CSF and blood samples have been extensively characterized as diagnostic and prognostic markers of disease [86–89] and thus have potential utility as pharmacodynamic biomarkers [86], supporting their potential implementation into clinical practice. For example, a recent longitudinal study showed a panel of blood markers of neuronal integrity, e.g. Nf light chain, phosphorylated NF heavy chain, DNA oxidation, lipid peroxidation, IL-6 and iron status, have a high predictive value of ALS progression [90], whereas gene expression of copper chaperone for superoxide dismutase (CCS) and genes linked to mitochondrial respiration are associated with survival [91]. Other CSF and blood-based biomarkers, whose increased levels are associated with the degree of neuronal damage, include circulating microRNAs, inflammatory markers (IL-8, IL-5, IL-10, IL-15 and IL-2), granulocyte–macrophage colony-stimulating factor (GM-CSF), TDP-43 and metabolites (glutamate and lysine), and SOD1 proteins in familial and sporadic disease [92,93]. Recent data also indicate that levels of chitinase expressed in activated astrocytes reflect neuroinflammation in both genetic and sporadic forms of ALS [94,95]. This is supported by the finding that a gradual rise in CSF levels of chitinase correlates with the presymptomatic and early symptomatic phases of ALS, suggesting its potential as a target of immune therapeutic interventions [96]. Despite the finding that several markers such as Nf-L in the CSF levels differentiate ALS from inflammatory peripheral neuropathies, urinary p75 neurotrophin receptor extracellular domain (p75NTRECD) is currently the only biological fluid-based biomarker of disease progression [97].

Given the substantial heterogeneity of the disease and variability in progression rates in ALS, identification of biological fluid biomarkers for the clinical stratification of the disease remains challenging and is currently not strongly linked with local pathology. In light of this, electrophysiological biomarkers such as nerve conduction study parameters and needle electromyography, that

measures the intrinsic electrical activity of muscle fibres, together with the clinical examination, are the main approach in the diagnosis of ALS [98]. In addition, the strong link between ALS disease progression and NMJ dysfunction suggests that biomarkers of NMJ damage such as SMAD proteins elevated in ALS muscle or muscle-specific microRNAs could potentially serve as markers of disease progression or regression [99,100]. In line with this, markers that reflect muscle atrophy and denervation have been associated with poor prognosis and a short survival time in ALS patients [101].

FUTURE PERSPECTIVES

The development of imaging approaches to visualize disease progression, as well as to examine and determine pathological mechanisms operating in disease, will continue to be key to discriminate ALS from non-ALS diseases as well as segregate subgroups of ALS patients. Important considerations when applying imaging modalities to distinguish different forms of ALS, disease progression or response to therapy include the size of cohorts used, as well as the appropriate control cohorts. For example, small or heterogenous ALS cohorts or inadequately controlled cohorts are probably reasons for the inconsistent findings in published studies. The emerging technology in ultra-high field and resolution functional MRI and time-of-flight PET/MRI offers new possibilities in human imaging. Advances in imaging performance with increased sensitivity and specificity will enable higher spatial and temporal resolution and detection of targets with low endogenous expression.

CONFLICTS OF INTEREST
All authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS
All authors contributed equally and all approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT
Data are freely available upon reasonable request.

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