Progress towards a biotypic biomarker profile for amyotrophic lateral sclerosis–frontotemporal spectrum disorders

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Abstract

Determining the optimal timing of disease-modifying therapies for neurodegenerative disorders will necessitate identification of when the underlying pathobiological process becomes active, well in advance of the point at which clinical manifestions appear. Phenoconversion, the emergence of clinically manifest syndomes, may be preceded by years to decades of silent pathobiological activity that can only be mapped by an array of biomarkers.

ALS and FTD, traditionally identified as distinct clinical syndromes, are increasingly recognized to exist along a spectrum of clinical syndromes with shared genetic risk and shared underlying pathology. This clinicopathological spectrum is underpinned by cytoplasmic aggregation of TAR DNA-binding protein 43 (TDP-43) as the common neuropathological hallmark. In contrast, the majority of neuropathologically-defined frontotemporal lobar degeneration (FTLD) is associated with alterations in either TDP-43 metabolism (FTLD-TDP) or of the microtubule associated protein tau (FTLD-tau), with a smaller percentage associated with either autosomal dominant genetic mutations or impairments in the ubiquitin proteasome system. As the field of neurodegenerative disorders increasingly shifts towards the frameworks of a pathobiological definition of disease, there is a growing imperative to develop biomarkers that reflect the varied pathobiologies that underly these disorders, and to determine the sensitivity of such biomarkers to detect the presence of these pathobiologies before phenoconversion.

To that end, an international workshop was convened in London, Canada in 2025 to review the evidence for existing or evolving biomarkers suitable for (1) the detection of either ALS or FTD pathobiology prior to phenoconversion and/or (2) predict phenoconversion in at risk individuals. Such biomarkers might be conceptualized as “biotypic biomarkers”, capturing their ability to describe an underlying pathophysiology whilst being agnostic to the emergent clinical manifestations. Whereas no single biotypic marker is yet able to predict the emergence of ALS, FTD or their intersection, a multimodal approach to developing a biotypic biomarker profile holds promise for the detection of relevant pathobiological processes. The strength of such an approach would be augmented by also addressing issues of resiliency/susceptibility both in terms of genetic risk susceptibility profiles and developing sensitive biomarkers of genomic and cellular aging. By including such nontraditional markers of disease, a more robust picture of not only the degenerative process but also of those factors that might potentially mitigate or drive a heightened probability of disease can be derived.

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Introduction

As our understanding of the pathophysiological basis of amyotrophic lateral sclerosis (ALS), the frontotemporal dementias (FTD) and their intersection as the ALS frontotemporal spectrum disorders (ALS-FTSD) evolves, so too does the impetus to define each in terms of their respective biologies as opposed to discrete clinical entities[1]. To address this, thought leaders in each field convened in London, Canada in the spring of 2025 to examine whether the biological understanding of these disorders was sufficiently advanced to allow for the detection of a disease state prior to it becoming clinically manifest (phenoconversion). The impetus for this was, in part, the recognition that ALS and FTD are likely characterized by long subclinical latency that manifests only after some threshold of biological disease

burden has been crossed. While such latencies are likely variable, the ability to detect the presence of pathophysiologically relevant disease processes during the clinically silent and prodromal periods prior to phenoconversion will be critical as disease-modifying therapies for these disorders advance.

Doing so however presents a unique challenge in that the clinical and neuropathological intersection of ALS and FTD suggests that identifying a single biomarker that will reflect the core biology or biologies of these diseases will be unsuccessful. Because of this challenge and recognizing that current terminologies regarding biomarker classification are evolving, we have borrowed from recent advances in defining specific subgroups of psychosis. These use the terminology of biotypic biomarkers to specifically address biological markers that describe an underlying pathophysiology while being agnostic to the clinical manifestations or the different contexts of use of biomarkers as recognized by the BEST guideline (Biomarkers, EndpointS, and other Tools)[2-4]. The advantage of such an approach is the incorporation of a broad array of information in creating a biomarker profile that first and foremost characterizes the presence of a biological disease state well before it is clinically evident. There is precedent for such an approach for carriers of autosomal dominant mutations in *APP*, *PSEN1* and *PSEN2* in the detection of active pathobiological processes prior to phenoconversion to Alzheimer's disease (AD)[5]. In this, meaningful temporal mapping of the pathobiological activity underlying clinically silent AD can be accomplished using a suite of pathophysiologically relevant biomarkers that included measures of amyloid (Aβ) and tau deposition (AD relevant phospho-tau species), neuronal degeneration [microtubule associated tau protein (total tau; t-tau)] and axonal degeneration [neurofilament light chain (NFL)], alongside measures of synaptic degeneration (beta-synuclein), neuroinflammation, brain atrophy (magnetic resonance imaging; MRI) and cognition.

Such a multimodal biotypic biomarker profile by its nature is designed to inform the presence of a pathobiological process, but also allows for the incorporation of key factors such as markers of disease resiliency and susceptibility. The latter is relevant in that neurodegenerative diseases do not exist in genetic isolation but rather occur on a background of genetic risk factors [both monogenic and polygenic, including single nucleotide variants (SNPs) and epigenetic modifiers of gene expression] that may "heighten" the probability of disease induction. There are also genetic constructs that might "protect" an individual from the ravages of age-dependent neurological disorders and allow for longevity reaching well into the decades beyond traditional median ages of death – the superaging cohorts. Environmental exposures can additionally contribute to disease susceptibility; for example, up 45% of AD risk has been associated with modifiable factors such as education and air pollution[6]. While these are often categorized as

susceptibility/risk biomarkers, it can be strongly argued that they can also contribute to a biotypic biomarker profile.

One of the primary areas of focus for the symposium was an examination of whether the current suite of biomarkers for ALS, ALS-FTSD or FTD is sufficiently robust to permit detection of an active disease process during a stage of clinical silence and also predict phenoconversion. While biomarkers can also be utilized for prognostication, disease staging and the detection of a therapeutic response among those with clinically manifest disease, these latter entities have been critically reviewed elsewhere and were not the focus of the workshop[7]. Another limitation was the intentional minimal focus on biomarkers of imaging, electrophysiology and clinical outcomes (e.g., cognition, behaviour and signs of neuromuscular dysfunction) in the belief that this grouping of biomarkers would be deserving of a separate workshop for logistical reasons and robustness of discussion. As will be discussed, however, ultimately any biomarker suite will need to be inclusive of these critical modalities.

TDP-43 proteinopathies

Transactive response DNA-binding protein 43 (TDP-43) proteinopathies are a heterogeneous group of neurodegenerative disorders characterized pathologically by the presence of aberrant cytosolic aggregation with nuclear TDP-43 depletion, predominantly in the central nervous system although TDP-43 deposits or pathological phosphoTDP-43 (pTDP-43) isoforms have been reported in skin, peripheral blood monocytes, platelets and muscle (extensively reviewed elsewhere[8-12]).

TDP-43 is a DNA/RNA binding protein of the heterogenous nuclear ribonucleoprotein family encoded by the *TARDBP* gene. It consists of a highly structured N-terminal domain (NTD) with nuclear localization and nuclear export sequences (NLS and NES, respectively) and two sequential RNA-recognition motifs (RRM1 & 2) that assemble side by side into a positively charged groove that accommodates RNA, flanked by a glycine-rich intrinsically disordered C-terminus domain (CTD) (Figure 1)[13,14]. The NTD regulates physiological head-to-tail nuclear TDP-43 oligomerization in solenoid-like structures that mediate DNA/RNA interactions while the CTD plays a role in alternative splicing, mRNA stability, protein-protein interactions, the formation of phase-separated RNA granules (biomolecular condensates) and in pathological cytosolic TDP-43 oligomerization. Under physiological stress, TDP-43 undergoes a nucleocytosolic shift to become predominantly cytosolic and associated with stress granules[15-17]. Pathological TDP-43 expression, while still characterized by this nucleocytosolic shift, includes TDP-43 aggregate formation that is the neuropathological hallmark in 97% of all ALS cases and all FTLD-TDP cases[18,19]. These

condensates contain multiple RNA binding proteins (RBPs) and include highly toxic caspase-mediated TDP-43 C-terminal truncations of 25 and 35 kDa[20-22]. In undergoing liquid-liquid phase separation (LLPS) as a step towards solid hydrogel condensate formation, the intrinsically disordered region (IDR) of the CTD interacts with other IDR-containing RBPs. The IDR also exhibits the greatest density of ALS-associated mutations, the presence of which increase the likelihood of TDP-43 aggregation[23-30].

Pathological TDP-43 exhibits characteristic post-translational modifications (PTMs) that includes hyperphosphorylation, in this case particularly at serine residues 403/404 and 409/410, significantly affecting LLPS[12]. The insoluble condensates are also highly ubiquitinated, a modification associated with protein degradation pathways. Although other PTMs, including acetylation, SUMOylation, poly-ADP-ribosylation (PARylation) and oxidation of cysteine residues also influence TDP-43's localization, RNA-binding ability and aggregation propensity, they have not yet been the focus of biomarker development. Complicating matters further, while pathologically phosphorylated TDP-43 with molecular masses of 45, 50 and 60 kDa can be found in ALS tissue, multiple N-terminus and C-terminus fragments are also evident, with the suggestion that these fragments may be differentially expressed within the spinal cord and brain, along with an aggregation prone acetylated isoform[31].

A consequence of the loss of nuclear TDP-43 function includes the failure to repress the inclusion of intronic sequences, known as cryptic exons (CEs), into mature messenger RNAs (mRNAs)[32]. Normally, nuclear TDP-43 binds to "UG"-rich motifs in the introns flanking CEs, acting as a steric block to prevent the splicing machinery from incorporating these sequences into the final mRNA transcript. When TDP-43 is depleted from the nucleus, these CEs are de-repressed and included. The inclusion of CEs often introduces premature termination codons (stop codons) in the mRNA sequence, leading to the rapid degradation of the aberrant transcript through a quality control mechanism called nonsense-mediated decay (NMD) or to unstable RNA transcripts. This results in a functional loss of crucial neuronal proteins, such as stathmin-2 (STMN2) and UNC13A, that are vital for axon growth/regeneration and neurotransmitter release, respectively[33-37]. While less common, some CEs are spliced in-frame and escape NMD, leading to the translation of novel, aberrant proteins termed "cryptic peptides". These novel proteins, such as that derived from the gene encoding hepatoma-derived growth factor-like protein 2 (HDGFL2), are detectable in CSF[38-40].

While primary disorders of TDP-43 include the majority (97%) of ALS cases (regardless of the presence or absence of a genetic mutation) and approximately half of FTDs, they also include a broad range of other clinical or neuropathological disorders[8,9]. Amongst these, Limbic-predominant Age-related TDP-43 Encephalopathy Neuropathological Change

(LATE-NC) is of particular relevance to this discussion. LATE-NC is a neuropathologically-diagnosed TDP-43 proteinopathy that generally presents as an amnestic cognitive disorder with hippocampal atrophy, often in association with impairments in semantic memory while sparing other cognitive domains[41,42]. It frequently occurs concomitantly with AD in which case the clinical phenomenology is more varied and the course more rapid. However, the clinical correlate of LATE-NC (LATE) is commonly observed in isolation in individuals over age 65 years (> 10%) rising to over 25-40% in those aged 85 years or greater. It is significantly more frequent in APOE e4 carriers, but the degree to which this is independent of the association with Alzheimer's Disease remains to be fully clarified[41-44]. While the neuropathological differentiation of the most advanced variant of LATE-NC (LATE-NC Stage 3) from FTLD-TDP type A or B can prove challenging, recent advances in doing so have been made[44-46].

While the vast majority of FTLDs are either tauopathies or TDP-43 proteinopathies (FTLD-tau, FTLD-TDP, respectively), a small proportion are defined by intraneuronal inclusions of TAF15 (*TAF15* encoding TATA-box binding protein associated factor 15) together with the other two FET family of RNA binding proteins [FTLD-FET; *FUS* encoding the fused in sarcoma/translocated in liposarcoma RNA binding protein (FUS), *EWSR1* encoding Ewing Sarcoma RNA binding protein (EWS)] or dysfunction of the ubiquitin proteasome system (FTLD-UPS)[47]. Up to a half of all cases are familial with 10-15% inherited in an autosomal dominant manner with the majority due to monogenic mutations in the microtubule associated tau (*MAPT*) gene, the progranulin gene (*GRN*) or a pathological hexanucleotide repeat expansion of the gene encoding C9 open reading frame 72 (*C9orf72*)[47,48]. Rare instances of autosomal dominant FTD have also been associated with genes encoding for valosin-containing protein (*VCP*), chromatin-modifying protein 2B (*CHMP2B*), TDP-43 (*TARDBP*), FUS, p62 (*SQSTM1*), ubiquilin 2 (*UBQLN2*), Tank-binding kinase 1 (*TBK1*) and, although potentially as a risk modifier for FTD, triggering receptor expressed on myeloid cells 2 (*TREM2*)[49]. In the context of defining a biomarker profile for FTD, these genetic variants serve to define individuals at significantly elevated genetic risk.

It is perhaps not surprising, therefore, that the FTDs are a heterogeneous group of neurodegenerative diseases that share a common underlying distribution of pathology that manifests predominantly as frontal and temporal atrophy with neuronal loss and gliosis, but which can also be more widespread[50]. Although the clinical heterogeneity is significant, the core clinical elements are alterations in social behaviour, personality, speech and language with additional features of corticobasal or motor dysfunction in some patients (as discussed in the companion paper Benatar et al, this issue)[51,52].

Tau proteinopathies

Alterations in tau metabolism in FTLD

As noted earlier, pathological alterations in the metabolism of the microtubule-associated protein tau (MAPT; tau) occur in approximately half of all FTLD.[53] Human tau is an intrinsically disordered protein consisting of 6 isoforms ranging from 352 to 441 amino acids in length, with isoforms defined on the basis of the presence of 0, 1 or 2 N-terminal domain insertions generated through alternative splicing of exons 2 and 3 (0N, 1N or 2N tau) in combination with the presence or absence of exon 10 that encodes one of the four microtubule-binding repeats (generating a 3R or 4R isoform) (Figure 2)[54,55]. All 6 isoforms are expressed in the adult human brain with 3R and 4R expression being approximately equal[56]. While the interaction of tau with microtubules is crucial for microtubule stability and axonal transport, it also has an extensive array of functions in the nucleus (protecting against DNA damage, nuclear calcium homeostasis, chromatin dynamics), mitochondria and axon (maintenance of axonal integrity, regulation of synaptic plasticity and neuronal activity)[55-60].

Tau is a highly soluble protein predicted to adopt a paperclip-like structure in which it's N-terminus domain wraps around the C-terminus domain[61,62]. Alterations in its phosphorylation state can disrupt this structure, impeding its ability to interact with microtubules and driving it towards pathological fibril formation[63]. These pathological fibrils are derived from soluble, low molecular weight individual tau oligomers that form protofilaments that are assembled into insoluble higher molecular weight fibrils or filaments that are unique to individual tauopathies[64-66]. While the oligomeric species has been proposed to be the most toxic, it is this structural arrangement of the protofilaments that provides a signature to the individual tauopathies[67]. Although this level of structural specificity holds promise for the development of pathological tau biomarkers and although structurally-specific tau-PET tracers are being developed, the inability to define these structures in living patients currently limits their utility as a biomarker. Rather, this task falls to using disease-specific PTMs as highlighted by the recent deep proteomics study of Kumar et al [68] in which dementia-specific tau PTMs (both soluble and insoluble) were identified.

Physiological tau phosphorylation is necessary for regulating tau's function, while extensive phosphorylation at disease-specific residues can be associated with the induction of pathological tau isoforms with a heightened propensity to form ® -sheet-rich oligomers, the smallest toxic species[54,56]. For instance, the phosphorylation of tau at threonine 217 (pThr^{217} tau) is relatively specific for AD and correlates with tangle burden

with elevated plasma levels broadly discriminating against FTD/FTLD[69-73]. In contrast, the aberrant phosphorylation of tau by ERK2 at Thr^{175} (pThr^{175} tau), a phospho-epitope not observed in controls or with aging, has been observed in neuropathological studies of ALS with cognitive impairment (ALSci), FTD-MND and many, but not all, FTD/FTLDs[74-78]. The phosphorylation of Thr^{175} triggers a cascade of events resulting in the generation of pathogenic oligomeric pThr^{231} tau[74-78]. Critically, regardless of the process giving rise to pThr^{231} tau, it can exist as either *cis* or *trans* isomers with peptidyl-prolyl *cis-trans* isomerase (PIN1) driving the conversion of the aggregation-prone pathological *cis* isomer into its physiological *trans* conformation[79-81]. Loss of PIN1 expression and activity has been associated with several neurodegenerative disease states, including AD, vascular dementia and CTE[82,83]. However, while increases in CSF pThr^{231} have been shown to be amongst the earliest tau biomarkers in preclinical AD alongside increases in CSF pThr^{181} and pThr^{217}, the more pathologically important determination of *cis* vs *trans* isomerization of pThr^{231} tau is not readily available as a biomarker[84-87]. In the quest for tau biomarkers, the promise of defining unique patterns of tau transcripts, isomers, PTMs and ultimately structural species, is just beginning to fully unfold[68,88,89].

Alterations in tau metabolism in ALS

While over 97% of all variants of ALS demonstrate cytosolic aggregates of TDP-43 in affected neurons, pathogenic tau isoforms have been observed in both cortical and spinal motor neurons[90]. A proportion of patients with ALSci, FTD (ALS-FTD) or ALS in association with chronic traumatic encephalopathy (CTE-ALS) have pathological neuronal inclusions of tau, including pThr^{175} and pThr^{231} tau[75,78,91-96]. Nuclear and cytosolic immunoreactivity to pSer^{396} and cytosolic pSer^{214} and pSer^{404} tau, modifications that can be present in both healthy and neurodegenerative disease states, has been observed in ALS cervical spinal motor neurons and cortex while only rarely observed in controls[97]. Recently observed elevations in serum or plasma pThr^{181} and pThr^{217} tau suggest a possible peripheral source of tau in ALS, including denervated muscle[11,98,99]. These latter findings raise the issue that the biosample from which tau phospho-isoforms are assayed in ALS may reflect differing aspects of the disease process.

There is an increasing awareness of the co-occurrence of TDP-43 and tau proteinopathies with mounting evidence for a synergistic interaction between the two[42,96,100-108]. For instance, the co-occurrence of AD with a TDP-43 proteinopathy is associated with greater medial temporal atrophy, faster rates of hippocampal volume loss and more severe cognitive and memory impairment with some suggestion that concomitant TDP-43 deposition may impact the disease course as early as 10 years before death[109-112]. At the structural level, the interaction between tau and TDP-43 appears to be direct, in part driven

by hydrogen bonds, with the interaction between full length TDP-43 and tau enhancing the formation of cytosolic TDP-43 biomolecular condensates, although tau and TDP-43 aggregates typically do not colocalize in pathologic human tissue even if they are sometimes found within the same neuron[104,105,113,114]. With respect to the expression of 3R vs 4R tau isoforms, TDP-43 can mediate the alternative splicing of tau exon 10[115]. While these findings remain to be further explored, they raise the intriguing possibility that the intersection of ALS and FTD manifesting as ALS-FTSD is more than simply disease overlap but rather intrinsically shared pathobiology.

Blood and CSF biomarkers

This pathophysiological overlap between ALS and FTD presents as a challenge in the efforts to identify biomarkers that might discriminate between the two during clinically manifest disease, let alone in a presymptomatic state (Figure 3). Although there have been significant advances in the last several years in biomarker assay sensitivity, now reaching levels of femtomolar detection, there has been little consideration of their utility as biotypic biomarkers that are agnostic to the clinical state but rather focus on pathobiological processes (Supplementary Table 1). In the following section, using a narrative review approach, the current state of biomarkers *writ large* for the pathobiology that underpins ALS and FTD are examined individually and then collectively in the context of detecting pathophysiological dysfunction prior to phenoconversion.

Biomarkers of ALS

The use of TDP-43 as a biomarker of any type of TDP-43 proteinopathy but specifically ALS has proven challenging for several reasons, including its propensity to self aggregate, its extensive PTMs, the existence of multiple splice variants and cleavage fragments, poor CSF-plasma correlation and the marked variability between various assays (reviewed extensively elsewhere[7,31,116,117]). While in general TDP-43 or pTDP-43 levels are found to be elevated in CSF or blood samples, the aforementioned issues drive significant variability rendering interpretation difficult[9,118]. Suggesting a potential role in a biomarker profile, when CSF TDP-43 is evaluated in combination with one or more of CSF t-tau, pThr^{181} tau or NFL, AUC (Area Under the Receiver Operating Characteristic Curve) values were significantly improved compared to TDP-43 alone, a result not observed in combining plasma and CSF composites[117]. This latter finding held true for FTD as well[119]. At the moment therefore, direct measures of TDP-43 have not proven to be of value.

The use of t-tau or phospho-tau (ptau) as a biomarker in ALS has only recently begun to be evaluated. In a comparison study of ALS (including C9*orf*72 and *mt*SOD1) against AD and

controls using the Quanterix single molecule array (Simoa) platform, pThr^{181} tau levels were observed to be elevated in ALS plasma but not CSF with a tendency for spinal onset LMN disease to have the highest pThr^{181} tau levels[99]. In a large multicentre study using the nucleic acid linked immune-sandwich assay (NULISA) CNS disease panel platform, significant increases in plasma MAPT (reflecting total tau), pThr^{181} tau, pThr^{217} tau, pThr^{231} tau and pTDP-43 that correlated with elevated NFL levels were observed in ALS patients[120]. Consistent with earlier studies, pThr^{181} tau levels were highest in those patients with spinal onset[99]. Also using the NULISA platform, serum levels of pThr^{181} tau and pThr^{217} tau have been found to be statistically higher in ALS and AD patients than disease controls, with elevations positively associated with age and a sex effect[11]. No differences were observed between ALS and AD serum pThr^{181} tau levels. AD patients showed higher serum levels of pThr^{217} tau than ALS patients as well as higher levels of t-tau compared to both ALS and controls, the latter not being significantly different from each other. Using AUC analysis, the authors concluded that serum pThr^{181} tau could discriminate ALS from controls, but not ALS from AD, whereas pThr^{217} discriminated AD from both controls and ALS.

However, the use of t-tau or ptau as biomarkers suffers from methodological issues of variability. For instance, in a number of studies, only total CSF tau and not pThr^{181} tau discriminated ALS from controls while both total t-tau and pThr^{181}tau/t-tau have been shown to be either significantly increased or decreased (respectively) compared to controls with AUC analysis for both suggesting varying degrees of sensitivity and specificity for detecting ALS[121-124]. This variability in t-tau, pThr^{181} or pThr^{181}/t-tau as reported biomarkers of ALS is complex and in part may relate to poor concordance rates between CSF and plasma values[85,125]. For example, whereas plasma pThr^{181} tau levels have been found to be elevated in ALS patients (with proposed greater sensitivity to lower motor neuron loss) compared to controls, CSF pThr^{181} has been observed to be reduced relative to controls and AD[99,125]. In a single study, ALS with concomitant amyloid pathology (as indicated by lower CSF A® $_{42/40}$ ratio than ALS alone, and comparable to AD alone) showed plasma pThr^{181} levels significantly greater than ALS alone[125]. This latter study also failed to show a correlation between CSF and plasma pThr^{181} values.

Perhaps more challenging in interpreting t-tau levels in ALS is a more fundamental and unexplored issue in that it is unknown the degree to which tau fragments are present in the CSF, such as that in AD[126]. Linked to this, the tau amino acid (aa) sequence recognized by any given tau antibody used in the detection of CSF tau can have a significant influence on the level of tau detected[126]. For example, ptau antibodies recognizing aa sequences containing pThr^{181} [aa 159 – 181 (using tau^{441} as the reference sequence)] and pThr^{231} (aa 159 – 231) can discriminate AD from controls although the use of a pThr^{181} antibody recognizing most of the N-terminus domain of tau (aa 9 – 181) is less discriminatory. The

method of CSF tau isolation or sample enrichment will also influence tau levels (ie., the use of direct assays vs tau enrichment through immunoprecipitation or HPLC purification). Moreover, few studies have accounted for the presence or absence of cognitive impairment, the severity of disease at the time of testing or the extent of corticospinal tract degeneration as assessed by neuroimaging measures[123,127]. However, if the intent is to use t-tau as an indicator of an active pathobiological process driven by a loss of neuronal integrity or tau dysmetabolism (i.e., as a biotypic biomarker), then these issues are surmountable.

In contrast to assays of TDP-43 and tau, there is consistency across virtually all biomarker studies of ALS, including several systematic reviews and meta-analyses, that elevations of CSF, blood, plasma or serum NFL levels, including NFL peptides, possess a high level of sensitivity in discriminating ALS from healthy controls and ALS mimics[11,99,117,121,124,125,127-140]. Correcting for age-adjusted increases in plasma NFL levels, both in terms of absolute levels and rate of change over time, is crucial[141]. While CSF and serum levels of NFL tend to show a high degree of correlation, in a large meta-analysis, average serum concentrations tended to be moderately (approximately 17%) higher than in plasma[131,132,134]. It is less clear whether plasma NFL levels alone can differentiate ALS from FTD, although a recent peptidomic analysis demonstrated that elevated CSF NFL peptide levels differentiated ALS from AD, bvFTD, PD and healthy controls, albeit with small sample sizes[135,138].

The role of phosphorylated NFH (pNFH) as a biomarker is less clear. In general, there is a trend towards elevated CSF pNFH levels in ALS with both NFL and pNFH being able to discriminate between ALS and ALS mimics, including early in the disease course with blood or serum NFH/pNFH levels being similarly elevated albeit with somewhat greater variability[11,130,133,136,142-144].

Although the cellular origin of the chitinase family of proteins within the CNS remains an open question (microglial, astrocytic or both), CHI3L1 (YKL-40), Chitotriosidase-1 (CHIT1) and Chitinase-3-like protein 2 (CHI3L2 or YKL-39) are elevated in ALS CSF when compared to control cases and ALS-mimics[121,137,145-148]. CHIT1 levels in CSF, but not serum, were significantly elevated compared to AD, PD and FTLD, suggesting a potential role as biomarkers[146]. However, no significant difference has been observed in any CSF chitinase level between asymptomatic 'at risk' ALS and healthy controls, suggesting that it will likely not have utility in a biotypic biomarker profile[147]. Although a single paper has suggested that CSF levels of TREM2 are significantly elevated in early stage levels of ALS over late stage and healthy controls, absent the values of one outlier in the early ALS group, this differentiation is lost[149]. The use of proinflammatory molecules as biotypic biomarkers thus, at this moment, has not been demonstrated.

Biomarkers in predicting phenoconversion in ALS

The utility of biomarkers in predicting phenoconversion to clinically manifest ALS has only recently come under study[150]. In a prospective longitudinal study of carriers of ALS-associated gene mutations ("at risk" individuals) both serum and CSF pNFL levels have been shown to be elevated within a 12 month window of time prior to the appearance of ALS[128]. Serum and CSF pNFL levels were found to be highly correlated and stable over time apart from those individuals who phenoconverted. In contrast, CSF and serum pNFH levels were poorly correlated. While, serum or CSF NFL levels have been observed to increase prior to phenoconversion, a higher rate of change of serum pNFH over time was observed only among those who phenoconverted[151]. This latter finding is supported by the observation of elevated serum pNFH levels in a small cohort of pre-diagnostic ALS patients as early as 26 months prior to diagnosis[143]. It is unknown whether this is reflective of the generation of autoantibodies against pNFH or the contribution of pNFH aggregates to either assay variability[152]. The measurement of plasma pNFH, at least experimentally, can be confounded by the 'hook effect' whereby pNFH aggregates may bind soluble pNFH and give rise to falsely low levels on immunoassays, a challenge that might be obviated by more harsh solubilization[153].

Although the numbers were small, both Benatar studies suggested an impact of underlying disease genotype[128,151]. In eight (8) presymptomatic *C9orf72* hexanucleotide expansion carriers, no differences from baseline CSF NFH or NFL levels were observed in a 4 month window prior to disease onset, although statistically significant increases in both were observed coincident with the appearance of clinically overt ALS[133]. In this context, it is relevant to note that in a longitudinal study of individuals "at risk" for AD (members of families carrying autosomal dominant mutations in *APP*, *PSEN1* or *PSEN2*), while CSF and serum levels of NFL became significantly elevated on average 6.8 years before symptom onset, the rate of change of serum NFL levels became statistically different from controls on average 16.2 years before symptom onset[154]. Similar observations of age-adjusted increases in the annual rate of change of serum NFL levels in asymptomatic "at risk" FTD genetic mutation carriers at the time of phenoconversion have been reported, but do not differentiate between pre-symptomatic and post-symptomatic rises[155]. These findings strongly suggest that single point measures of either CSF or serum NFL levels will not be of value in detecting phenoconversion beyond establishing baseline levels for an individual and that monitoring the rate of change over time will be equally if not more important.

Biomarkers of FTD/FTLD

The literature surrounding the use of biomarkers to detect FTLD is a complex web of individual studies of FTD alone, comparator studies against AD, disease mimics and

controls, or less frequently, studies to differentiate amongst subsets of FTD. Virtually all focus on the issue of clinical detection or discrimination and are thus generally (mistakenly) viewed as diagnostic biomarkers. With the exception of reduced serum, plasma or CSF progranulin levels in FTD patients harbouring a *GRN* null mutation (FTD-*GRN*), including pre-symptomatic carriers, there is no single specific biomarker of FTD. Rather, as will be discussed, there is a need for a combination of biomarkers with careful attention to the nature of the biosample [e.g., CSF, blood (whole blood, serum, plasma)], to the bioassay methodology employed and, crucially, the clinical population to which they are being applied[156-158].

Plasma biomarkers have proven to be of value in discriminating FTD from AD/MCI (Alzheimer's disease and A® -PET-positive mild cognitive impairment), Lewy body dementia, progressive supranuclear palsy (PSP) and nondemented controls[159]. Using the Simoa platform, only increased plasma NFL levels discriminated FTD from controls, while increased levels of NFL, GFAP, pThr[181] tau and reduced A® $_{42/40}$ differentiated AD/MCI from FTD and PSP. These results are consistent with earlier studies showing the utility of plasma pThr[181] tau to differentiate AD (elevated) from FTD (not elevated) and controls[160-162], with the specificity enhanced when combined with measures of A® $_{42}$ protein and NFL[158,163,164]. The use of CSF NFH levels does not add further specificity[165]. Further differentiation between FTLD-tau and FTLD-TDP can be achieved using CSF pThr[181]/t-tau ratios, with a significantly lower ratio in FTLD-TDP than observed in controls and FTLD-tau[166-168] although this level of discrimination has not been observed in all studies[158].

A major advance has been the recent use of the NULISA CNS panel to differentiate amongst AD (including A® positive MCI), FTD (including bvFTD, nonfluent variant PPA, semantic variant PPA), PSP and Lewy body disease [including diffuse Lewy body disease (DLB), Parkinson's disease (PD) with dementia], revealing that elevated serum pThr[231]tau and GFAP levels differentiated MCI, AD, LBD and PSP from healthy controls while elevated pThr[217] levels differentiated MCI, AD and LBD from healthy controls[169]. Serum NFL levels were nonspecifically elevated across all degenerative states[135]. In differentiating PSP and FTD from healthy controls, elevated plasma NFL and reduced corticotrophin releasing hormone (CRH) serum levels were significant. Markers of inflammation assayed using the NULISA inflammation panel were elevated across all disease states compared to controls. These latter observations support the concept that any panel of biotypic biomarkers will need to broadly span neuronal injury, pathologically modified proteins and inflammatory markers.

Few studies have attempted to correlate antemortem CSF tau levels, alone or as part of a suite of bioassays, with postmortem neuropathological findings. Irwin et al[170] examined the

correlation between CSF t-tau, pThr^{181} tau and A® $_{1-42}$ with t-tau (albeit using AT8, a marker for pSer^{202}/pThr^{205} tau) cerebral load postmortem in FTLD-tau (n=31), FTLD-TDP (n=49) and AD (n=26). Across all groups, average cerebral tau correlated with CSF pThr^{181} tau but not t-tau, with a nonsignificant trend towards lower CSF pThr^{181} tau in FTLD-TDP. In addition, AD co-pathology with FTLD was independently associated with higher pThr^{181} tau. Excluding this latter group, pure FTLD-TDP patients had significantly lower pThr^{181} tau than pure FTLD-tau patients. In all instances, lower CSF A® $_{1-42}$ levels were associated with AD pathology postmortem as would be expected[171]. This is consistent with earlier studies demonstrating that a combination of elevated pThr^{181} tau with low (below control) CSF A® $_{1-42}$ levels is specific to AD, while reduced or normal levels of pThr^{181} in association with reduced A® $_{38}$, A® $_{40}$, A® $_{42}$ or A® $_{38/42}$ is specific to FTD[172]. Others, however, have observed increased A® $_{42}$ CSF levels to be characteristic of FTD, although with wide variability[158]. Despite limited numbers, a trend towards higher CSF NFL levels in neuropathologically confirmed tau negative FTLD (n=7) than in FTLD-tau (n=3) cases has been observed, a trend that is augmented when FTLD with motor neuron degeneration (ALS-FTD) is included amongst the tau-negative (TDP-43) cases[164,173]. Moreover, CSF pThr^{181}/t-tau ratios have been observed to be lower in neuropathologically confirmed FTLD-TDP than in FTLD-tau[173]. The addition of CSF NFL levels to the National Institute on Aging-Alzheimer's Association ATN framework (CSF A® $_{1-42}$, pThr^{181}tau, t-tau) in a neuropathologically confirmed series of AD and FTLD cases significantly increased the ability of the framework to correctly identify those individuals with FTLD (in whom NFL levels were increased)[174]. Plasma pThr^{181} levels differentiate neuropathologically confirmed AD from both FTLD-tau and FTLD-TDP with no difference amongst carriers of *MAPT*, *GRN* or *C9orf72* mutations[175]. All of which suggests that differentiating FTLD-tau, FTLD-TDP and AD from healthy controls and each other will be heavily reliant on the combination of NFL, pThr^{181} (either singly or as a ratio to t-tau) and A® fragments.

Used in isolation, NFL levels in serum, plasma or CSF can be helpful in discriminating FTD from cognitively intact controls and psychiatric patients with greater consistency for CSF biosamples[129,135,176]. The ability to discriminate mild cognitive impairment and disease mimics was less robust. FTD patients as a whole demonstrate a negative correlation between CSF and serum NFL levels on the one hand and brain atrophy as measured by grey matter density or atlas based volumetry on the other hand[177-179].

Biomarkers in association with genetic variants of FTD/FTLD

With respect to the individual genetic variants of FTD, CSF NFH levels have been found to be elevated in FTLD-*C9orf72* and FTLD-*MAPT* variants over FTLD-*GRN*, whereas NFL levels were consistently observed to be significantly elevated in FTLD-*GRN* over the former two

variants[141,158,180]. In an untargeted proteomics CSF analysis of symptomatic carriers of *C9orf72* pathological hexanucleotide expansion, *GRN* or *MAPT* mutations in whom NFL, NFM, CHI3L1 and NFH expressions were significantly upregulated[181].

In modelling disease progression in asymptomatic carriers of these genetic FTD variants, a multimodal approach that included MR imaging allowed for a more nuanced detection of disease[182]. Amongst all three FTD genetic variants, while regional brain atrophy and elevated NFL plasma levels were the initial indicators of disease presence, the period of latency from the initial appearance of a biomarker to disease manifestation varied markedly, ranging from 30 years for NFL elevations in C9*orf*72 patients to 15 years in *GRN* patients and just prior to clinical manifestations in *MAPT* patients. In contrast to these results, no association between autopsy confirmed *C9orf72* pathological expansion and any biomarker was observed in a recent study, although potentially confounded by a small sample size (n=8)[183]. The role of plasma NFL levels in predicting phenoconversion amongst *C9orf72* expansion carriers is less clear[141].

Predicting phenoconversion in FTD/FTLD

There is limited data with respect to the use of biomarkers to predict phenoconversion in FTD/FTLD. Age-adjusted increases in the rate of change of serum or plasma NFL levels have been shown to occur coincident with phenoconversion in genetically "at risk" FTD[135,141,155,180]. Rojas et al[180] examined the role of plasma NFL levels (Simoa platform) in distinguishing 'at risk" individuals (*C9orf72*, *GRN*, *MAPT* mutation carriers) from family members without genetic risk across asymptomatic, prodromal and full phenotype, including both an original cohort and a replication cohort with a multimodal approach that included disease severity and neuropsychological scales, CSF biomarkers (NFL, neurogranin) and brain MRI. Consistent with earlier reports, symptomatic mutation carriers exhibited significantly higher plasma NFL levels with excellent correlation to CSF NFL levels, while for those patients asymptomatic at baseline who subsequently phenoconverted, median baseline NFL concentrations were higher in both the original and validation cohorts. The latter group also exhibited lower frontal and temporal brain volumes after 2 years with neuropsychological decline. Finally, while CSF levels of soluble TREM2 are significantly elevated in both symptomatic and asymptomatic *GRN* mutation carriers, its value in a potential suite of biotypic biomarkers directed at predicting phenoconversion is not yet clear[184].

Biomarkers of ALS concomitantly with FTD/FTLD

All the challenges described above become amplified when both clinical syndromes are present. In a comparison of FTLD-TDP with or without ALS to FTLD-tau, AD and a control

group with subjective memory complaints, while CSF NFL levels were increased across all neurodegenerative states, the greatest were amongst those with FTLD-TDP and ALS followed by FTLD-TDP without ALS versus all others, suggesting that the presence of concomitant ALS was a significant driver of elevated NFL levels[168]. This finding is consistent with subsequent observations, including in neuropathologically confirmed FTLD with or without ALS[174,180]. It is also consistent with elevated CSF and plasma NFL levels observed when compared to a cohort of patients with primary psychiatric disorders, suggesting utility as a diagnostic biomarker in differentiating the latter from the former[185]. Elevated plasma GFAP/NFL ratios have been shown to correlate with pathologically-confirmed tau burden and to discriminate between sporadic FTLD-tau and FTLD-TDP, such that it is elevated in the former although levels in both were observed to be less than healthy controls[186]. None of these measures, however, speak to the existence of biotypic biomarkers sensitive to the underlying pathophysiology of ALS-FTSD.

In summary, there is very little in the literature regarding biotypic biomarkers in FTD, in part related to the heterogeneous nature of the disorder and in part due to the understandable focus and the development of diagnostic biomarkers. However, such a broad view does yield a number of specific consistencies that will be of assistance in informing the development of a biotypic biomarker panel:

(i) Reduced CSF and plasma progranulin levels are specific to FTLD-*GRN*.

(ii) In general, while there is significant variability across studies, FTD/FTLD can be differentiated from AD and controls using a panel of biomarkers. These include NFL (which, although lacking specificity when used in isolation, is most consistently elevated in CSF and blood, serum or plasma when compared to cognitively intact controls), pThr^{181}tau (most consistently reduced in comparison to controls and AD) and measures of Aβ protein that can include $A\beta_{38}$, $A\beta_{42}$, $A\beta_{38/42}$ or $A\beta_{42/40}$ (consistently elevated in CSF from FTD/FTLD and decreased in AD).

(iii) Lower CSF pThr^{181}/t-tau ratios with increased NFL levels may differentiate FTLD-TDP from FTLD-tau (increased t-tau and pThr^{181} with increased pThr^{181}/Ab_{42} ratio and no change in NFL)[183] although this finding warrants further study.

(iv) The technology underlying the Simoa and NULISA platforms represents a significant advance in the ability to detect trace levels of biomarkers at femtomolar concentrations with both showing good correlations for key CNS proteins measured in plasma and serum[169]. However, both are still reliant on an antibody:antigen interaction, albeit at single molecule levels, so issues of antigenic specificity, the nature of protein fragments being detected, and the collection conditions remain present. Addressing these issues will remain paramount as untargeted omics approaches within validation workflows in the identification of novel biomarkers.

Future research directions

Cryptic peptides as biotypic biomarkers of nuclear TDP-43 depletion

The discovery that CE-containing transcripts or the resulting cryptic peptides (CP) can serve as specific CSF or blood biomarkers of nuclear TDP-43 depletion represents a significant advance in our understanding of ALS, ALS-FTD, FTLD-TDP and LATE-NC[33-39,42,187,188]. The presence of specific cryptic exons/peptides (e.g., STMN2, UNC13A, and HDGFL2) is highly specific to TDP-43 proteinopathies and generally absent in healthy controls or other neurodegenerative diseases like FTLD-tau. In postmortem tissue, there is a high degree of concordance between HDGFL2-CE protein (in contrast to the wild-type protein: HDGFL2-*wt*) and pTDP-43 burden in ALS-FTD, FTLD-TDP and AD-TDP[40].

In a comprehensive study of brains from individuals aged 21 – 109 years, Chang and colleagues[42] observed that TDP-43 nuclear clearance accompanied by HDGFL2-CE protein expression precedes the appearance of TDP-43 neuronal cytoplasmic inclusions by a decade, suggesting that HDGFL2-CE protein expression may be a viable biotypic marker of a presymptomatic TDP-43 proteinopathy. Using a proteomics approach, Seddighi and colleagues[39] observed the expression of six CPs, including HDGFL2, in CSF of ALS-FTD patients. Supporting the potential role for CPs in presymptomatic testing, elevated levels of cryptic HDGFL2 in both CSF and plasma have been reported in pre-symptomatic ALS and ALS-FTD carriers of pathological *C9orf72* hexanucleotide expansions, though conclusions should be tempered by poor assay sensitivity[38]. Although the numbers studied were small, longitudinal data were very limited, and a validation study is clearly needed, these authors hypothesize that elevations in CSF cryptic HDGFL2 would precede the elevations in NFL or pNFH in predicting phenoconversion.

Extracellular vesicles (with relevance to pTDP-43, tau and miRNAs)

Extracellular vesicles (EVs), while challenging to isolate, are increasingly being studied as tools in the quest for biotypic biomarkers given their core role in both physiological and pathological intercellular communication by transporting proteins, nucleic acids and lipids between cells, including those associated with pathological aggregate formation[189-200]. Relevant to this discussion, both tau and TDP-43 can undergo cell-to-cell transfer via EVs and are capable of recruiting endogenous proteins into existing aggregates[104,201-204]. Recent studies have provided evidence for relevant differences in biophysical properties of EVs between ALS and healthy controls, including their cargos [ie., pTDP-43, tau isoforms (3R, 4R), HSP90 or cyclophilin A (PPIA)][203,205,206]. While these measures remain to be further explored and validated, their specificity and direct biological relevance make EVs an attractive biosample.

An example of a potential role for EVs is in the quantification of neural-specific microRNA (miRNA) expression. These endogenous small non-coding RNAs have unique expression profiles depending on the cell or tissue of origin and are crucial regulators of gene expression by interacting directly with mRNA 3'UTRs to drive, in the majority, but not always, degradation of the mRNA transcript[207-209]. The challenge using miRNAs as a biomarker has been the marked variability in defining specific miRNAs, either individually or in clusters, based on the tissue specificity, stage of disease or the failure to adequately define the efficiency of the EV isolation methodology. The use of standardized methodologies to isolate and evaluate EVs from CSF or blood may help resolve this issue[210-215]. Suggesting that such studies have potential in the development of a biotypic biomarker profile, significant alterations in a panel of 24 miRNAs have been identified as downregulated in fALS patients (mt*SOD1*, mt*FUS*, *C9orf72* hexanucleotide expansions) versus healthy controls as early as two decades prior to symptom onset[216]. Whether the up-regulation of miR-206 and miR-151a-5p observed in "mild" stages of ALS are reflective of phenoconversion remains to be seen[217].

Telomeric length as a marker of biological aging

Telomeres are repeat sequences of TTAGGG in association with the protein complex shelterin that cap the ends of chromosomes and serve to maintain DNA integrity by preventing the loss of gene sequences[218]. Because telomeric DNA cannot be completely replicated by DNA polymerases, telomeres are gradually shortened with each replication cycle until a critical point is reached at which genomic instability occurs that in turn leads to senescence and cell death. Although telomerases add nucleotides to telomeres, serving to maintain telomere length, somatic cells generally have low levels of telomeric activity resulting in progressive shortening over time[219]. Telomere shortening, or attrition, is thus thought to be a marker of aging[220,221]. In the context of women tending to have longer telomeres than men, it is interesting that the lifetime risk for ALS is higher for men and that male:female ratios for ALS change significantly during aging[222-226].

Although there is no association between telomere length and the risk of developing ALS, perhaps counter-intuitively telomere length in ALS has been observed to be significantly longer than in age and sex-matched controls with those individuals amongst the ALS cohort with shorter telomere length tending to have a longer median survival[226-228]. This lengthening has been proposed to be due to the rate of attrition being slower as opposed to active lengthening by telomerases[226]. Of interest, longer telomeres have also been associated with FTD, including ALS-FTD, although the numbers studied were small and not replicated across all studies[229-231]. It remains to be determined whether, as a component of

a multimodality tool, age- and sex-adjusted telomeric measurement as an index of resiliency might be a valuable addition to a biotypic biomarker profile.

Epigenetic markers of biological risk

Epigenetics refers specifically to those alterations in gene expression that occur independently of alterations in the DNA sequence and are largely held to reflect changes in DNA methylation, histone posttranslational modifications, chromatin remodelling and modifications of the interactions of RNA with DNA, ultimately affecting gene transcription[232-234]. The majority of DNA methylation, regulated by DNA methyltransferases, occurs at carbon-5 of the pyrimidine ring of cytosine (5-mC) when followed by a guanine repeat (CpG dinucleotide), resulting in inhibition of gene transcription when adjacent to promoter regions[235,236]. CpG islands, regions of greater than 500 basepairs with high CpG content often clustered in promoter regions and typically hypomethylated, are hypermethylated with age leading to transcriptional silencing[235].

DNA methylation is increased in genomic DNA extracted from the blood of ALS patients with *SOD1* mutations and in brain DNA derived from the frontal cortex of sALS patients[237,238]. In the former, DNA methylation levels in presymptomatic carriers were similar to control, raising the question as to the exact timing of alterations in DNA methylation status in the context of phenoconversion. It is noteworthy that increased CpG island methylation near the promoter region of *C9orf72* has been observed in C9*orf*72 ALS, ALS-FTD and FTD, predominantly in large repeat expansions $(G_4C_2)_n$ ($n > 90$) with reductions in promoter activity and moreover, that the $(G_4C_2)_n$ methylation was evident in both blood and brain samples, including asymptomatic carriers[239-241]. Emerging work suggests that epigenetic profiling of cell-free DNA (cfDNA) may offer a minimally invasive route to brain-derived biomarkers. Tissue-of-origin–specific DNA methylation patterns in cfDNA have been used to infer the presence of neurodegenerative diseases including ALS and FTD, by identifying neuron subtype specific DNA methylation signatures[242-244].

Epigenetic drift, referring to age-related changes in the patterns of DNA methylation (either hypo- or hypermethylation) has been observed in symptomatic ALS cases when compared to neurologically intact controls[245]. DNA methylation can also be used to provide a biological age estimate (DNAm age), sometimes termed the “epigenetic clock” in which cumulative methylation levels across 353 CpGs measured using a genome-wide array [of which, using the “clock” developed by Horvath[246], 193 CpGs increase methylation levels with age while 160 decrease][246]. While acceleration of DNAm age has been linked to earlier disease onset and shorter survival in both sporadic and C9*orf*72 ALS[241,247,248], it’s potential role as a modifier with a biotypic biomarker profile has yet to be addressed.

Beyond DNA methylation signatures of disease and biological ageing, other epigenetic mechanisms, particularly histone modifications, represent a promising but underexplored avenue for ALS and FTD biomarker discovery. Studies in AD and PD have shown that disease-associated alterations in histone acetylation and methylation can be detected in both brain tissue and peripheral cells and may track with pathological burden and clinical progression[249-252]. Translating similar approaches to ALS and FTD could help identify histone-based signatures that reflect disease processes such as early neuronal dysfunction, synaptic disturbance, or neuroinflammation[253]. More broadly, epigenetic marks across multiple layers may encode the cumulative imprint of environmental and lifestyle exposures (e.g. smoking, metabolic factors, toxins, physical activity) on disease-relevant pathways[254-256].

Polygenic Risk Score (PRS)

The awareness that neurodegeneration occurs on the background of a genetic risk profile in which the absence of specific genetic risk variants or the presence of genetic variants associated with healthy cognitive aging or enhanced longevity, respectively, is fundamental to any discussion of a biotypic biomarker profile[257]. This concept has been central to the construct of a polygenic risk score (PRS) from large genome-wide association studies (GWAS) evaluating single nucleotide polymorphisms (SNPs) as a methodology to aggregate genetic markers that do not individually reach significance[258,259]. For example, when applied to cognitively healthy centenarians, a number of novel protective alleles were identified (*ANKH*, *GRN*, *TMEM106B*, *SORT1*, *PLCG2*, *RIN3*) in addition to APOE genotype that could be attributed to reducing AD risk by greater than 5 fold.[260] When used in combination with age, sex and the expression of the APOE-ε4 allele, a PRS [also termed a genetic risk score (GRS)] constructed from the European Alzheimer & Dementia Biobank with 83 genome-wide significant variants predicted conversion to AD in population-based studies and from MCI to AD in MCI cohorts, with the risk for both cohorts rising with an increased number of risk alleles independent of non-APOE risk variants[261].

In FTD, the *rs1990622*^{G} allele of TMEM106B shows both protective effects on age at phenoconversion, as well as effects on several of the biomarkers discussed above[262-264]. In *GRN* carriers and *C9orf72* carriers, the *rs1990622*^{G} allele is associated with reduced atrophy, lower NFL levels, and/or better cognitive performance[265]. Many of these differences are observed at baseline assessments even in presymptomatic carriers, highlighting the potential impact at the neurodevelopmental level a complex interplay between risk and protective factors across the lifespan which will influence models of phenoconversion prediction.

A similar approach using single variant GWAS to identify novel risk genes and foci specific to FTLD-TDP pathological subtypes identified a genome-wide significant signal at the *UNC13A* locus for all FTLD-TDP variants[266]. The most significant locus for FTLD-TDP type was *GRN* in addition to several new significant loci (*TINAG*, *MZT1*, *FARP2*). The most significant association with FTLD-TDP type B was for the *UNC13A* locus, with three new risk loci (*TNIP1*, *RCL1* and *PDS5B*). For neuropathologically-confirmed FTLD-TDP type C, four genome-wide significant loci were identified, including *LRP1B*, *COL22A1*, *TMEM135* and *TRPC4* [having excluded semantic variant Primary Progressive Aphasia (svPPA)]. When the expression of these subtype specific gene expression profiles was compared to published loci for Alzheimer's disease and related disorders (ADRD) and to an ALS GWAS, FTLD-TDP subtypes presented a distinct genetic signature with the exception of *UNC13A* colocalization with ALS and *TNIP1* with both ALS and ADRD. For the first time, this provides a window of opportunity to consider a biotypic biomarker profile with specificity to individual FTLD-TDP subtypes antemortem.

Although few studies have examined the role of PRS in ALS, an increase in PRS over controls is associated with an increased risk of ALS and moreso for those individuals without rare ALS-causative variants[267,268]. While the risk contribution to those harbouring ALS-associated rare variants appeared nonsignificant, sample size was limited. These findings were consistent with prior observations that genome-wide significant loci could account for only 0.2% of heritability in ALS[269]. Of note, a higher PRS for cognitive impairment in ALS was associated with both neuroimaging evidence of cortical thinning and neuropathological features (neuronal loss, TDP-43 pathological inclusions) in domains known to be associated with cognitive dysfunction[270]. Several of the SNPs were shared risk loci for FTD (in or near *MOBP*, *NSF*, *ATXN3*, *ERGIC1* and *UNC13A*). Although no inference from these studies can be made with respect to predicting those individuals at risk for phenoconversion, future studies will need to consider a broader range of genetic risk profiles in both ALS and FTD, including both monogenic and oligogenic inheritance in addition to PRS profiles[271,272].

Conclusions

The challenge in utilizing existing biomarkers to predict phenotypic conversion is that the majority of biomarkers to date are linked to clinical phenotype, not as biotypic biomarkers linked to the underlying biology. However if existent biomarkers are viewed in the context of their ability to detect the process of neurodegeneration, even while being agnostic to the exact nature of the disease itself (i.e., biotypic biomarkers), then this may well be sufficient if the goal is to identify 'at risk' individuals who have entered into a phase of subclinical

biological activity (Figure 4). In this conceptualization, evolving biomarkers such as those linked to alterations in lipid metabolism[273-276] or the previously discussed genetic markers (telomeric length, epigenetic modifications and PRS) might be considered most appropriately as susceptibility or risk biomarkers. Amongst all of the biomarkers currently available, the use of CSF, blood or plasma levels of NFL appears to be the most sensitive to the presence of such an underlying neurodegenerative process although it lacks disease specificity[171,277]. If one is simply wishing to detect the presence of prodromal disease and in doing so sacrifice specificity, then NFL would seem to be the current leader[177,277].

If however the goal is to identify biotypic biomarkers that reflect the underlying proteinopathy and then determine whether these biomarkers predict divergent phenotypes of clinical disease, then no single biomarker has sufficient specificity and sensitivity to be considered a "gold standard" for the characterization or definition of an individual neurodegenerative disease process. The cumulative weight of the literature suggests that a multimodal biotypic biomarker profile will be required, not only in the prediction of phenoconversion, but in the identification of the underlying pathobiology irrespective of the conversion. Such a profile is likely to be influenced by issues of genetic tolerance and susceptibility. Although not discussed in detail in this review, such a biotypic biomarker profile will necessitate the integration of clinical data, neuroimaging and biophysical studies[278-282]. Given these considerations, as biotypic biomarker profiles are developed and replicated, a number of key issues need to be considered.

First and foremost, there is a limited availability of large cohorts of deeply endophenotyped presymptomatic individuals who have been followed longitudinally over time using an array of both biochemical biomarkers (as reviewed in this article) with detailed clinical markers as referenced in the preceding paragraph. As reviewed by Benatar et al[283], such priority studies are challenging on multiple fronts, including ensuring clear operational definitions of disease stage, ethical considerations, and ultimately, cost. Moreover, as the evidence in support of biomarkers reflective of involvement of the peripheral nervous system and muscle evolve, such biomarkers will need to be evaluated as potential sensitive indicators of early metabolic remodelling and inflammatory signalling in distinguishing ALS from FTD phenotypes.

Secondly, if the concept of a multimodal biotypic biomarker profile is to have utility, its individual constituents should have relevance to the underlying pathobiological processes. Ideally, such a profile should be developed with age- and sex-adjusted normative data used to establish appropriate cut-off values. For individual proteins that are known to undergo extensive PTMs, efforts should be made to ensure that disease-specific epitopes are addressed. A case in point is the need to more clearly define the tau fragment(s) on which

t-tau assays are reported and in accurately characterizing disease-specific CSF fragments of NFL. Doing so will provide unique protein or peptide signatures beyond simply reflecting nonspecific features of axonal degeneration[138,284]. The same can be said for TDP-43 although the advent of cryptic peptides as a biotypic biomarker may mitigate this need. Once extracted, the candidate biomarkers should be biologically stable during storage or, at minimum, the impact of storage over prolonged periods of time corrected for.

There should be a significant correlation between CSF and blood, serum or plasma values for any given biotypic biomarker included in the profile that is thought to reflect pathophysiological processes arising within the central nervous system. For biosamples arising from outside of the CNS (e.g., skin, gut or muscle), this might not apply although a greater understanding of the correlation with CNS pathobiological processes will be crucial.

While the bulk of attention of this review has been on identifying biotypic biomarkers capable of predicting phenoconversion in 'at risk' individuals, the challenge of predicting those individuals whose existent clinical disease state will evolve from a "pure" ALS or FTD phenotype into an ALS-FTSD also exists. Further research with a focus on cohorts investigating these questions are necessary to solve this.

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Competing interests

A.A. reports consultancies or advisory boards for Amylyx, Apellis, Biogen, Clene Therapeutics, Cytokinetics, GenieUs, GSK, Lilly, Mitsubishi Tanabe Pharma, Novartis, OrionPharma, Quralis, Sano, Sanofi, Vesalic, Voyager Therapeutics, and Wave Pharmaceuticals, and the following patents under consideration or approved: Low dose human interleukin-2 for the treatment of amyotrophic lateral sclerosis in a subgroup of patients WO2024121173A1; IL-2 for the treatment of frontotemporal dementia disorder EP25306172.5. B.B. has served as an investigator for clinical trials sponsored by Alector, Cognition Therapeutics, EIP Pharma/Cervomed, and Transposon. He serves on the Scientific Advisory Board of the Tau Consortium, which is funded by the Rainwater Charitable Foundation. He receives research support from the NIH, Lewy Body Dementia Association, American Brain Foundation, Mayo Clinic Dorothy and Harry T. Mangurian Jr. Lewy Body Dementia Program, Little Family Foundation, and Ted Turner and Family Foundation. E.F. reports advisory fees from Takeda/Denali, Vigil Therapeutics Inc., Ionis and Biogen, and research support from CIHR and the Weston Foundation. H.H. reports support by ABOARD, a public-private partnership receiving funding from ZonMw (73305095007) and Health Holland, Topsector Life Sciences & Health (LSHM20106), the Hans und Ilse Breuer Stiftung (2020), the HorstingStuit Foundation (2018), and the Dutch Research Council (Aspasia premie 015.016.059). E.L. reports consulting fees from Eli Lilly. M.S. reports research support from the Canadian Institutes of Health Research (CIHR grant SOP-160442) and the Temerty Family Foundation. MJS is a member of the Scientific Advisory Board of the Saudi Arabia National Institutes of Health M.O. received research support from the German Federal Ministry of Education and Research (projects: FTLDc 01GI1007A), the EU: Moodmarker, PredictFTD, Synapsing, the ALS Association, the foundation of the state Baden-Württemberg, Boehringer Ingelheim Ulm University BioCenter, and the Thierry Latran Foundation and EU-MIRIADE, EU-PredictFTD, Synapsing, Moodmarker and the Roux-programme of the Martin Luther University Halle (Saale); MO. received consulting fees from Biogen, Axon, Roche, and Grifols; and participates on the Biogen ATLAS trial board, all unrelated to the work presented in this paper; is a speaker of the German FTLD consortium, is involved in an unpaid role with the German Society for CSF Diagnostics and Neurochemistry, and is involved without pay with the Society for CSF Diagnostics and Neurochemistry. L.P. is a consultant for Expansion Therapeutics. L.P. is supported by the National Institutes of Health (R01NS120992, U54NS123743, R35NS097273), the Target ALS Foundation. M.P. reports no conflicts. M.P. is supported by the National Institutes of Health (R01NS120992, U54NS123743), the Target ALS Foundation (#9), the BrightFocus Foundation (A2024017S), and the Kissick Family Foundation (MILKEN#5). T.S. has served as a consultant for Blue Rock Therapeutics, Centessa, Critical Path for Parkinson's

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Supplementary material

Supplementary material is available at *Brain* online.

Appendix 1

Workshop attendees

Samir Abu Rumeileh, Ammar Al-Chalabi, Sharon Abrahams, Megan Barker, Michael Benatar, Alberto Benussi, Bradley Boeve, Robert Bowser, Idnu Navar Binghan, Danae Campos-Melo, Sharon Coleman, Johnathon Cooper-Knock, Kathryn Cousins Joke De Vocht, Cristian Droppelmann, Elizabeth Finger, Leah Forsberg, Nicole Harris, Lee Freiburger, Jenna Gregory, Henne Holstege, Edward Huey, Clifford R. Jack, Jr. Murray Junop, Sanjay Kalra, Brianna Kaplanis, Raffaella Klima, Edward Lee, David Lester, Irene Litvan, Alessandra Longo, Sarah Marzi, Caroline McHutchison, Crystal McLellan, Roison

McMackin, Corey McMillan, Christopher M Morrow, Veronica Noches, Panteleimon Oikonomou, Nicholas Olney, Bjorn Oskarsson, Chiadi U. Onyike, Markus Otto, Frederica Palacino, Francesca Paron, Dung Pham, Yolande Pijnenburg, Barbara Poletti, Kathleen Poston, Mercedes Prudencio, Janice Robertson, Angela Roberts, Alex Salazar, Niyatee Samudra, Christen Shoesmith, Vincenzo Silani, Isis So, Tomas Solomon, Michael Strong, Carmella Tartaglia, David Taylor, Martin Turner, Michael Van Es, Juan-Camilo Vargus-Gonzalez, David A. Wolk.

For further details, see the Supplementary material.

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Figure legends

Figure 1 Schematic illustration of TDP-43 structure and post-translational modifications. (A) Prediction of intrinsic disorder tendency for full length TDP-43 (PrDOS; protein disorder prediction system [(https://prdos.hgc.jp/cgi-bin/top.cgi) with prediction validated using UniProt (https://www.uniprot.org) based on Q13148 TADBP_Human]. Values of greater than 0.5 are indicative of being intrinsically disordered (unstructured). Typical of RNA binding proteins, both RRM1 and RRM2 are highly structured while virtually the entirety of the C-terminus domain (the glycine rich domain) is unstructured and capable of considerable flexibility. (B) Schematic illustration of full length TDP-43 including intrinsically disordered regions (IDR; yellow), RNA binding domains (RRM1 & 2; green); nuclear localization sequence (NLS; blue) and a putative nuclear export sequence (NES; blue). Posttranslational modifications are indicated according to the figure inset, while crucial phospho-epitopes observed in ALS tissues are noted. (C) Using AlphaFold3 and the Q13148 TDP-43 structure, the predicted structure for TDP-43 shows the highly structured N-terminus domain with RRM1 & RRM2 (green) in close approximation while the intrinsically disordered C-terminus [glycine-rich, Prion-like domain (PrLD)]) is unstructured with the exception of an amyloidogenic α-helical segment. The majority of ALS-associated mutations are located within the PrDL 282-287.

Figure 2 Tau protein structure and assembly. (A) Tau protein, encoded by the MAPT gene (chromosome 17q21), is composed of four domains, including the N-terminal domain (spanning a.a 1-150), Proline-rich domain (a.a 151-243), Microtubule-binding domain (a.a 244-369), and C-terminal domain (a.a 370-441). Within the N-terminal domain are two N-terminal repeats, N1 and N2, which are encoded by exons 2 and 3, spanning a.a 45-103. The microtubule-binding domain consists of four imperfect repeats, R1-R4, encoded by exons 9-12, respectively. With respect to the longest tau isoform (2N4R), various epitopes are used as recognition sequences for utilization as diagnostic biomarkers, including the N-terminal and Microtubule-binding domain for t-tau, and pThr175, pThr181, pThr217, and pThr231 tau for phosphorylation-specific alterations. (B) In the human central nervous system, six unique isoforms of tau are produced through alternative splicing of exons 2, 3

and 10, resulting in isoforms varying with the inclusion and exclusion of the two N-terminal repeats and the second Microtubule-binding domain. In general, tau isoforms are classified as either 3R or 4R, based on the absence or presence of the second microtubule binding repeat. (C) The phosphorylation and assembly of pathogenic tau fibrils occur through an ordered process, beginning with the phosphorylation of microtubule-bound tau, leading to its dissociation from the microtubule and loss of structural integrity. Phosphorylated-unbound tau has an increased propensity to misfold and form soluble oligomers, which in turn leads to the aggregation of disease-specific protofilaments and fibrils. Created in BioRender. Donison, N. (2026) https://BioRender.com/6kp89kl.

Figure 3 Intersection of ALS-FTSD Pathobiology. (A) ALS, ALS-FTSD and FTD are characterized by regional brain atrophy compared to healthy controls. In ALS and FTD, evidence of frontal lobe atrophy is present, with the addition of temporal atrophy in FTD cases. (B) There is considerable overlap in the cellular mechanisms driving the progression of ALS-FTSD and FTD in the brain and spinal cord with aggregation of TDP-43 and tau in neuronal cytosol, leading to disruption of cellular homeostasis, synaptic dysfunction, and ultimately neuronal death. This process is reflected by the presence of a broad range of biomarkers, most commonly neurofilaments NFL and NFH in the CSF and blood. Disruption of RNA homeostasis is reflected by the presence of cryptic exon encoded peptides and miRNA species. A widespread neuroinflammatory response is defined by TREM2-positive microglia and GFAP-positive reactive astrocytes. Biomarkers in CSF or blood, either in isolation or contained within extracellular vesicles, include TDP-43, Tau, Aβ, NFL, NFH, CHI3L1, TREM2, GFAP, EVs, and mRNA, in addition to the absence of progranulin, is indicative of certain ALS-FTSD disease phenotypes. (C) In ALS-FTSD, progressive loss lower motor neurons in the brainstem and spinal cord often results in muscular atrophy, leading to clinical features of muscle weakness, and swallowing and speech impairments while upper motor neuron loss is reflected in spasticity. In affected neurons, nuclear TDP-43 depletion leads to the expression of cryptic exons and the production of cryptic proteins, which are often truncated or functionally altered. Recently, the presence of cryptic exons and proteins has been used as a biomarker for TDP-43 proteinopathies, including ALS-FTSD. Created in BioRender. Donison, N. (2026) https://BioRender.com/u7m606a.

Figure 4 The conceptual positioning of biotypic biomarkers. The progression of neurodegenerative diseases can be broadly characterized by a clinically manifest phase

that is readily recognized at the bedside and a presymptomatic phase that consists of both a clinically silent and a prodromal phase as described by Benatar and colleagues[283,285]. The clinically silent phase can be further conceptualized by a "biologically at risk state" until its transition to a "biologically active disease state". Over time, the cumulative impact of the neurodegenerative processes in this latter phase leads to phenotransition and heralds the onset of the prodromal phase characterized by the insidious development of minimal clinical deficits, in this case in the domains of motor (MMI), cognitive (MCI) and behavioural (MBI). Ultimately, a threshold is reached beyond which further pathobiological progression is accompanied by the emergence of a clinically manifest syndrome (the point of phenoconversion). Disease progression in each phase and in each axis of neurological impairment can be highly asynchronous and not necessarily co-existent (ie., in the case of isolated motor degeneration in ALS without neuropsychological impairments). Biotypic biomarkers (yellow highlight) occupy a unique niche in this conceptual timeline in being agnostic to the specific underlying disease process. Such biomarkers reflect the cumulative effect of a wide range of risk or susceptibility factors that drive the initiation of the neurodegenerative process well before the period of phenotransition. Whether such biotypic biomarkers can also be utilized to more robustly characterize the underlying pathophysiology during the prodromal disease state through to clinically manifest disease remains to be seen, although if so, reclassification as a diagnostic biomarker may be considered.

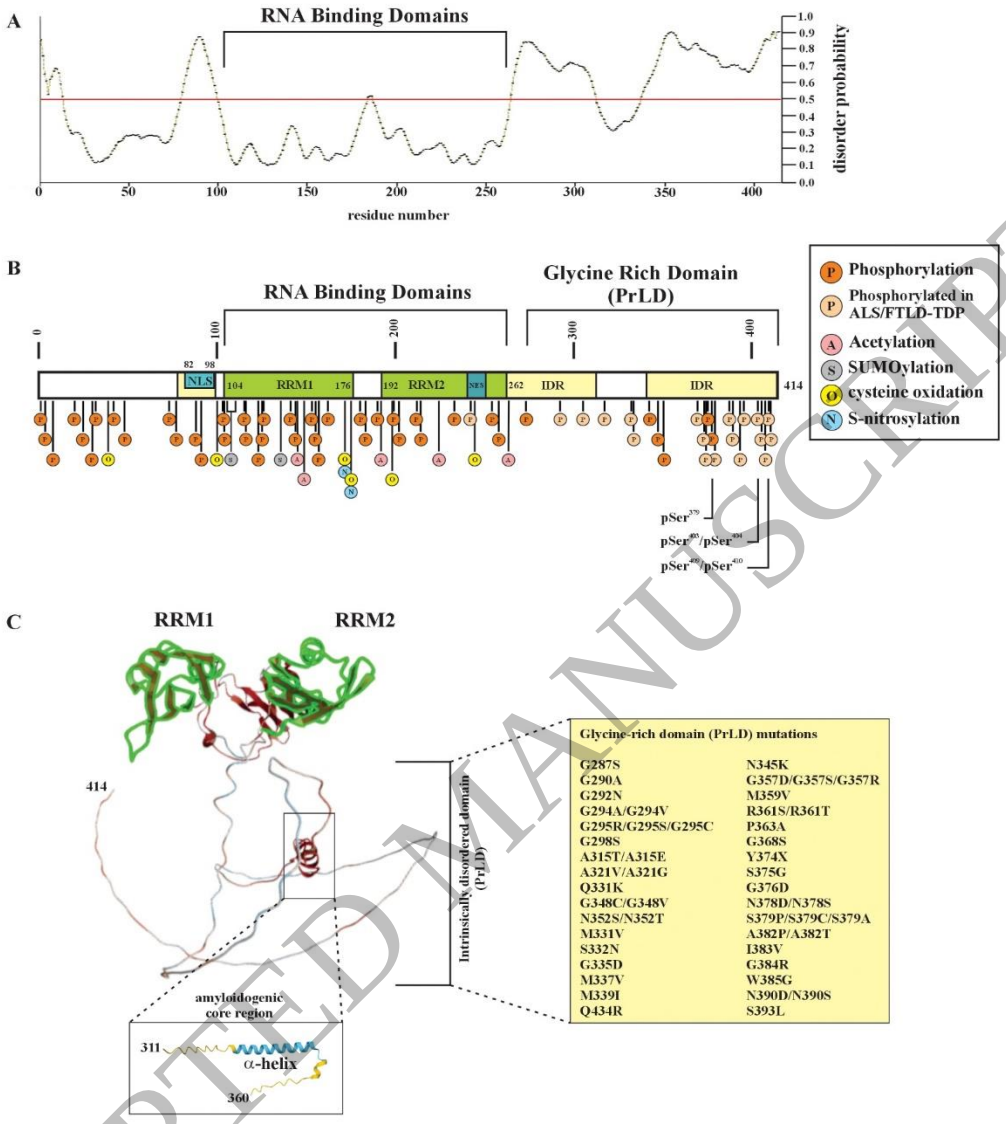

Figure 1
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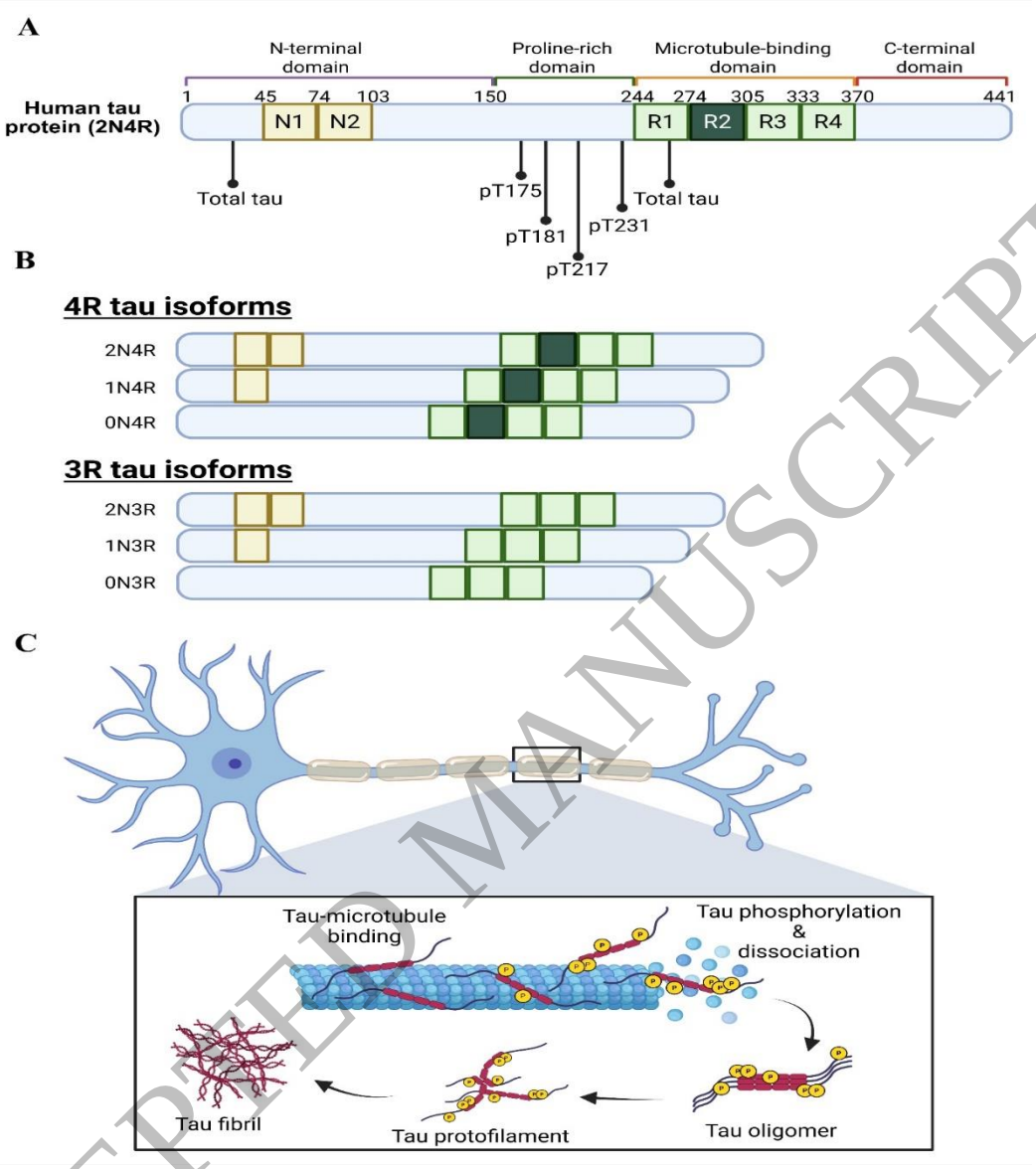

Figure 2
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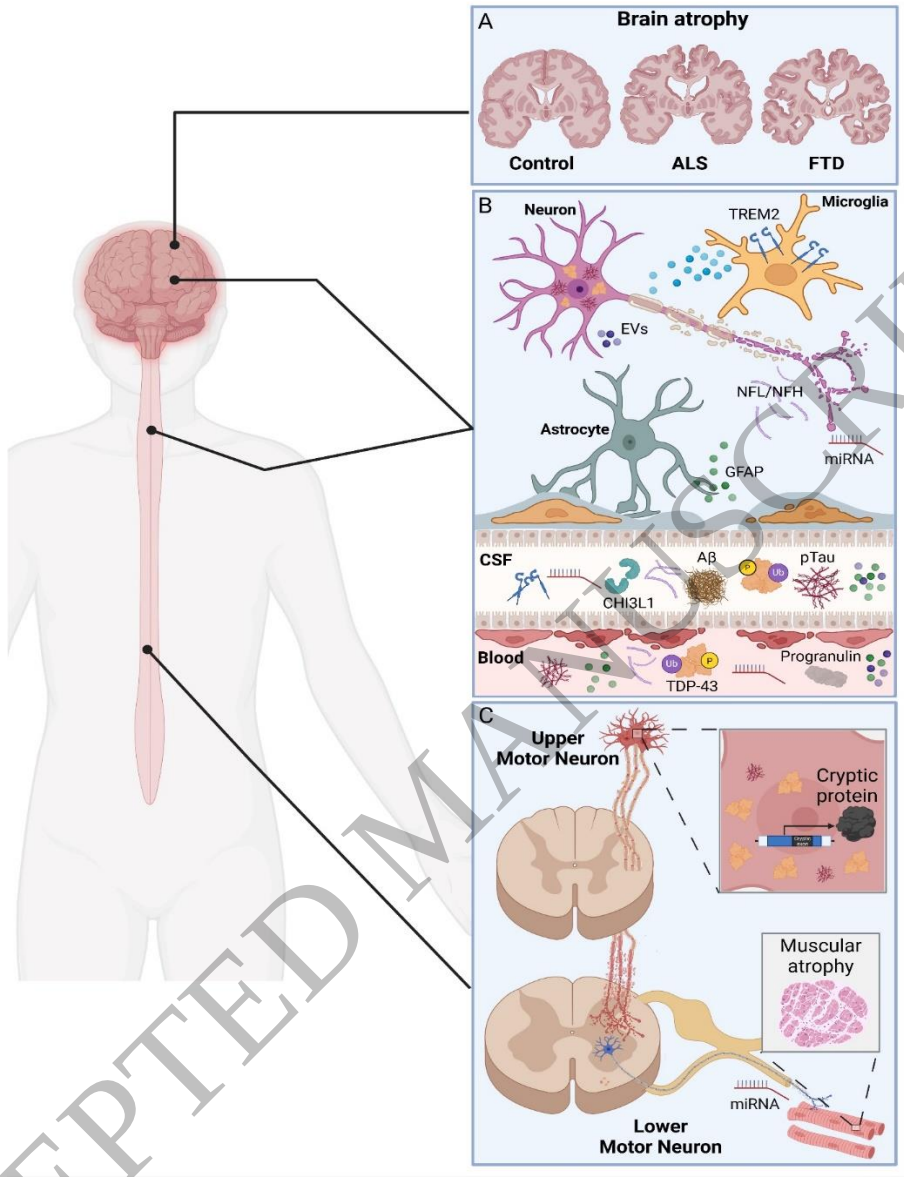

Figure 3
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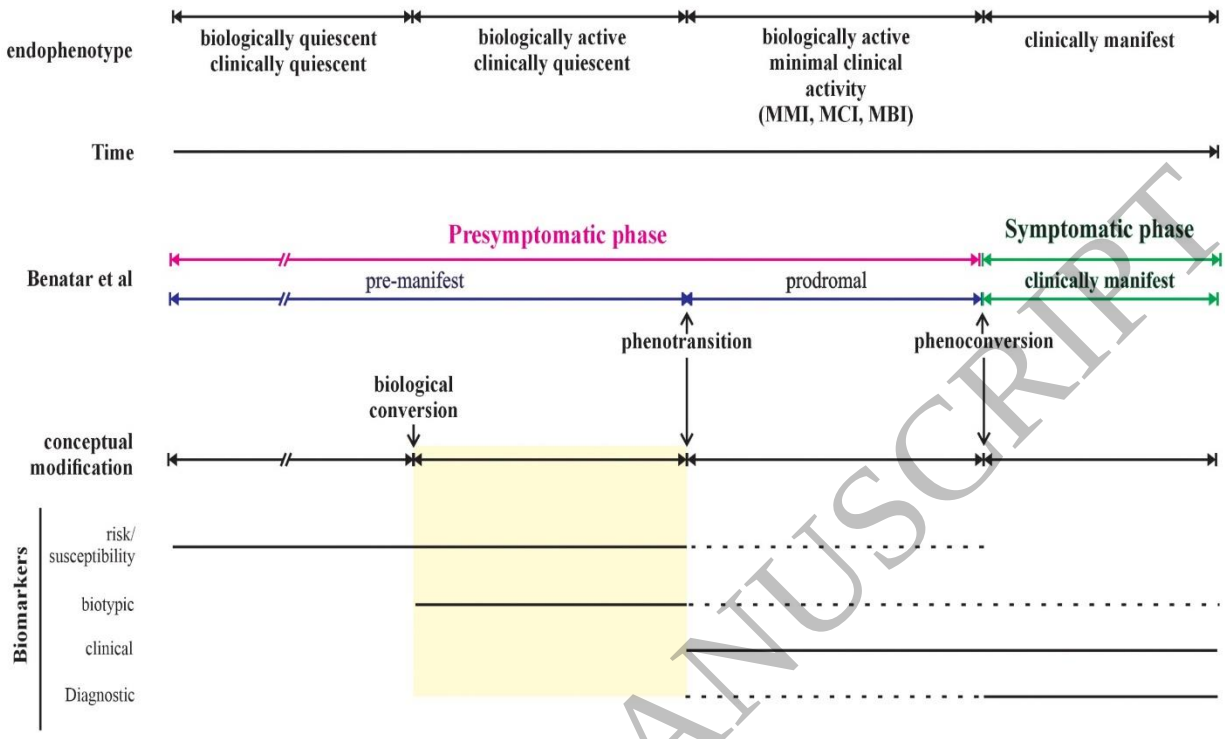

Figure 4
271x139 mm (x DPI)