Chitinases as Biomarkers of Disease Aggressiveness in Amyotrophic Lateral Sclerosis: Perspectives through the D50 Progression Model

Dissertation

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"Gradatim Ferociter"

"All major changes are like death. You can't see to the other side until you are there." – Michael Crichton, Jurassic Park

"And I knew exactly what to do. But in a much more real sense, I had no idea what to do" – Michael Scott, The Office

Dedicated to my family, both two-legged and four-legged, and above all else, to my most patient, supportive, and ardent cheerleader, my husband Mihkel. Your love is truly a gift.

Contents

1. List of Abbreviations

AD, Alzheimer's Disease

ALS, Amyotrophic Lateral Sclerosis

ALSFRS-R, Amyotrophic Lateral Sclerosis Functional Rating Scale (Revised)

AMCase, Acidic mammalian chitinase

AUC, Area under the curve

ΔFRS, delta-FRS or Disease Progression Rate

C9orf72, chromosome 9 open reading frame 72

CHIT1, Chitotriosidase 1

CHI3L1, Chitinase 3 like 1

CHI3L2, Chitinase 3 like 2

CNS, Central Nervous System

CSF, Cerebrospinal Fluid

DAPI, 4′,6-diamidino-2-phenylindole

DPR, Di-peptide repeat

DTI, Diffusion Tensor Imaging

FTD, Frontotemporal Dementia

GA-CFP, *Thy1* (GA_{149})-Cyan Fluorescent Protein

GFAP, Glial fibrillary acidic protein

GH, glycosyl hydrolase

HRE, hexanucleotide repeat expansions

Iba-1, Ionized calcium binding adaptor molecule 1

IFN-γ, Interferon gamma

IL-1α, Interleukin one alpha

IL-6, Interleukin 6

IL-8, Interleukin 8

LMN, Lower Motor Neuron

MCP-1, Monocyte Chemoattractant Protein 1

MHC, Major Histocompatibility Complex

MND, Motor Neuron Disease

MoMa, Monocyte-derived Macrophages

MRI, Magnetic Resonance Imaging

MS, Multiple Sclerosis

mt, Mutant

MUNIX, Motor Unit Number Index

NDCs, Non-Neurodegenerative Disease Controls

NDeg, Neurodegenerative Disease Controls

NeuN, Neuronal Nuclei

NfL, Neurofilament light chain

PET, Positron emission tomography

pNfH, Phosphorylated neurofilament heavy chain

rD50, Relative D50

ROC, Receiver operating characteristic

ROI, Region of Interest

SOD1, Superoxide dismutase 1

TDP-43, TAR DNA-binding protein 43

TNF, Tumor necrosis factor

UMN, Upper Motor Neuron

wt, Wild-Type

2. Summary

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative condition characterized by the vulnerability of upper and lower motor neurons. Limited disease-modifying therapies exist, as therapeutic development has been constrained by the disease's multi-factorial etiology and phenotypic heterogeneity. Precision biomarkers that reflect specific pathological processes can assist with patient stratification and provide readouts of treatment efficacy. Biomarkers of neuroinflammation are particularly relevant because non-cell autonomous mechanisms significantly exacerbate ALS pathology. Recent studies have focused on the chitinases; these are glycoside hydrolases that cleave chitin, a naturally occurring polysaccharide. Despite reports of key chitinase levels (CHIT1, CHI3L1, CHI3L2) being substantially upregulated in ALS patients, there are conflicting results on their clinical relevance, partially because of the use of outcome metrics with high variability, like the ALS functional rating scale (ALSFRS-R). The cellular sources that contribute to dysregulation in ALS also remain to be fully identified. Our literature review indicated that chitinase upregulation is not exclusive to ALS; rather it is a feature of several chronic inflammatory disorders, including neurodegenerative conditions. Further, owing to their roles as immunomodulators and autocrine and paracrine signaling networks, the chitinases can propagate neuroinflammation via a feed-forward loop, thus influencing disease severity.

Therefore, we used a translational approach combining a well characterized clinical cohort and preclinical models to investigate the biomarker potential of the chitinases in ALS and identify the contributing cellular sources. The novel D50 disease progression model was used as a validation framework because it provides independent descriptors for 1) individual disease aggressiveness (D50 – time taken in months for ALSFRS-R score to be halved) and 2) accumulated disease (relative D50 (rD50) – normalized time scale describing individual disease course).

Cross-sectional analyses using matched cerebrospinal fluid (CSF) and plasma samples from ALS patients, controls with other neurodegenerative diseases (NDegs), and controls with non-neurodegenerative diseases (NDCs) revealed that CSF levels of all three chitinases were significantly elevated in ALS patients relative to NDCs. However, only CHIT1 and CHI3L2, but not CHI3L1, were elevated relative to NDegs, confirming that chitinase upregulation is a feature of the neurodegenerative spectrum. No significant differences were noted in plasma, suggesting that chitinase dysregulation in ALS is primarily a feature of the central nervous system (CNS). All three chitinases correlated robustly with the neurofilament proteins (neurofilament light (NfL) and heavy chain (pNfH), which are established biomarkers of neuroaxonal damage in ALS, but did not diagnostically outperform them. We also showed that CHIT1 and CHI3L1, but not CHI3L2, were elevated in individuals with high disease aggressiveness and that this effect was independent of the

accumulated disease course. Moreover, regression analyses showed that CHIT1 and CHI3L1 could predict disease aggressiveness and significantly added to the prognostic power of neurofilaments alone. A hierarchical regression analysis combining pNfH, NfL, CHIT1 and CHI3L1 showed that the combination of NfL and CHI3L1 accounted for the highest amount of variation and had the most predictive power for disease aggressiveness. Finally, no significant differences in chitinase levels were observed across functional disease phases, in concordance with previous studies that have reported longitudinal stability.

To address which cellular sources contribute to chitinase dysregulation in ALS, we first examined the temporal expression of CHIT1, CHI3L1, and CHI3L2 in monocyte-derived macrophages (MoMas) from ALS patients and healthy controls (HCs) as these are a major physiological chitinase source. Although the temporal dynamics for CHIT1 and CHI3L1 were similar in both groups, profound differences were noted at later time-points i.e., when cells were fully differentiated. CHIT1 and CHI3L1 expression were significantly higher in MoMas from ALS patients at both the transcriptomic and protein level, with CHI3L1 levels also being influenced by age. This is the first report of circulating immune cells in ALS having an intrinsically augmented potential for chitinase production. To characterize chitinase sources in the CNS, we used the GA-CFP mouse model of ALS to perform quantitative immunostaining for CHIT1 and CHI3L1. This model was developed to understand of *C9orf72*-mediated pathology as mutations in C9orf72 are the most common genetic cause of ALS. Chitinase levels were upregulated in microglia and astrocytes in symptomatic GA-CFP+ mice, confirming results from prior post-mortem studies. Surprisingly, neurons robustly expressed both CHIT1 and CHI3L1, which has not been reported in the context of ALS. Further, neuronal CHIT1 levels were elevated in GA-CFP+ mice, indicating that this population is vulnerable to dysregulation in ALS. Qualitative assessment of the SOD1-G93A and rNSL8-hTDP-43 models of ALS showed that dysregulation was much more pronounced in these models, possibly because they present with substantial neuronal loss and a much more aggressive phenotype than the GA-CFP model. Therefore, ALS mouse model data aligns with our observation of CHIT1 and CHI3L1 being predictive of disease aggressiveness in human ALS patients.

To conclude, we show that key chitinase family members are dysregulated in ALS and multiple sources, including neurons, glia and circulating macrophages contribute to this dysregulation. The use of the D50 model showed that pNfH, CHIT1 and CHI3L2 are more reflective of ALS pathology and better suited for refining diagnoses while NfL and CHI3L1 are highly sensitive to disease aggressiveness and therefore apt for prognostic assessment.

3. Zusammenfassung

Amyotrophe Lateralsklerose (ALS) ist eine schwerwiegende neurodegenerative Erkrankung, die durch die Schädigung der oberen und unteren Motoneurone gekennzeichnet ist. Es existieren derzeit nur wenige krankheitsmodifizierende Therapieoptionen, da die Therapieentwicklung durch die phänotypische Heterogenität der Krankheit erschwert wird. Präzise Biomarker, die spezifische pathologische Prozesse widerspiegeln, können bei der Stratifizierung von Patienten helfen und Rückschlüsse auf die Wirksamkeit der Behandlung ermöglichen. Neuroinflammatorische Biomarker sind von besonderer Bedeutung, da nicht-zelluläre autonome Mechanismen die ALS-Pathologie deutlich verstärken. Jüngste Studien konzentrierten sich auf die Chitinasen; dabei handelt es sich um Glykosid-Hydrolasen, die Chitin, ein natürlich vorkommendes Polysaccharid, spalten. Neben Berichten, die zeigen, dass die Konzentrationen der zentralen Chitinasen (CHIT1, CHI3L1, CHI3L2) bei ALS-Patienten erheblich hochreguliert sind, gibt es widersprüchliche Ergebnisse zu deren klinischer Relevanz, die teilweise durch die Verwendung hoch variabler Datenerhebungen wie der ALS Functional Rating Scale (ALSFRS-R) begründet sein könnten. Die zellulären Quellen, welche zur Dysregulation bei ALS beitragen, müssen ebenfalls noch vollständig identifiziert werden. Unsere Literaturrecherche deutete darauf hin, dass die Hochregulierung von Chitinasen nicht nur bei ALS auftritt, sondern ein Merkmal verschiedener chronischer Entzündungskrankheiten, einschließlich neurodegenerativer Erkrankungen, ist. Darüber hinaus können die Chitinasen aufgrund ihrer Rolle autokrine und parakrine Signalnetzwerke die Neuroinflammation über eine Vorwärtsschleife verstärken und so den Schweregrad der Erkrankung beeinflussen.

Im Rahmen dieser Arbeit haben wir einen translationalen Ansatz gewählt, der eine gut charakterisierte klinische Kohorte und präklinische Modelle kombiniert, um das Biomarker-Potenzial der Chitinasen bei ALS zu untersuchen und die zugrunde liegenden zellulären Quellen zu identifizieren. Das D50-Krankheitsverlaufsmodell wurde als Validationsgrundlage verwendet, da es unabhängige Anhaltspunkte für 1) die individuelle Krankheitsaggressivität (D50 - Zeit in Monaten, die vergeht, bis der ALSFRS-R-Wert auf die Hälfte abfällt) und 2) die akkumulierte Erkrankung (relative D50 (rD50) - normalisierte Zeitskala, die den individuellen Krankheitsverlauf beschreibt) liefert.

Querschnittsstudien, in denen übereinstimmende Liquor- und Plasmaproben von ALS-Patienten, von Kontrollpersonen mit anderen neurodegenerativen Erkrankungen (NDegs) und von Kontrollpersonen mit nicht-neurodegenerativen Erkrankungen (NDCs) verwendet wurden, zeigten, dass die Liquorspiegel aller drei Chitinasen bei ALS-Patienten im Vergleich zu NDCs deutlich erhöht waren. Allerdings waren nur CHIT1 und CHI3L2, nicht aber CHI3L1, im Vergleich zu

NDegs erhöht, was bestätigt, dass die Hochregulierung von Chitinasen ein Merkmal des neurodegenerativen Spektrums ist. Im Plasma wurden keine signifikanten Unterschiede festgestellt, was darauf hindeutet, dass die Chitinase-Dysregulation bei ALS in erster Linie ein Merkmal des zentralen Nervensystems (ZNS) ist. Alle drei Chitinasen korrelierten stark mit den Neurofilamentproteinen (neurofilament light (NfL) und heavy chain (pNfH)), die als Biomarker für neuroaxonale Schäden bei ALS etabliert sind, waren ihnen aber diagnostisch nicht überlegen. Wir zeigten auch, dass CHIT1 und CHI3L1, nicht aber CHI3L2, bei Personen mit hoher Krankheitsaggressivität erhöht waren und dass dieser Effekt unabhängig vom akkumulierten Krankheitsverlauf war. Darüber hinaus deuteten Regressionsanalysen an, dass CHIT1 und CHI3L1 die Aggressivität der Erkrankung vorhersagen könnten und die prognostische Kraft der Neurofilamente signifikant ergänzten. Letztendlich wurden keine signifikanten Unterschiede in den Chitinase-Spiegeln zwischen den funktionellen Krankheitsphasen beobachtet, was mit früheren Studien übereinstimmt, die über eine longitudinale Stabilität berichtet haben.

Um herauszufinden, welche zellulären Ressourcen zur Dysregulation der Chitinasen bei ALS beitragen, untersuchten wir zunächst die zeitliche Expression von CHIT1, CHI3L1 und CHI3L2 in aus Monozyten hervorgegangenen Makrophagen (MoMas) von ALS-Patienten und gesunden Kontrollpersonen, da diese eine wichtige physiologische Chitinase-Quelle darstellen. Obwohl die zeitliche Dynamik von CHIT1 und CHI3L1 in beiden Gruppen ähnlich war, wurden zu späteren Zeitpunkten, d. h. wenn die Zellen vollständig differenziert waren, grundlegende Unterschiede festgestellt. Die Expression von CHIT1 und CHI3L1 war in MoMas von ALS-Patienten signifikant erhöht, wobei die CHI3L1-Werte auch vom Alter beeinflusst wurden. Dies ist der erste Bericht über zirkulierende Immunzellen bei ALS, die ein erhöhtes Eigenpotenzial für die Chitinaseproduktion aufweisen. Um die Chitinase-Quellen im ZNS zu charakterisieren, haben wir das GA-CFP-Mausmodell der ALS verwendet, um eine quantitative Immunfärbung für CHIT1 und CHI3L1 durchzuführen. Dieses Modell wurde entwickelt, um die *C9orf72*-vermittelte Pathologie zu verstehen, da Mutationen in C9orf72 die häufigste genetische Ursache von ALS darstellen. In symptomatischen GA-CFP+-Mäusen war der Chitinase-Spiegel in Mikroglia und Astrozyten erhöht, was die Ergebnisse früherer Post-mortem-Studien bestätigt. Überraschenderweise exprimierten die Neuronen sowohl CHIT1 als auch CHI3L1 stark, was im Zusammenhang mit ALS bisher nicht berichtet wurde. Darüber hinaus waren die neuronalen CHIT1-Spiegel in GA-CFP+-Mäusen erhöht, was darauf hindeutet, dass diese Population für Dysregulationen bei ALS anfällig ist. Die qualitative Bewertung der SOD1-G93A- und rNSL8-hTDP-43-Modelle der ALS zeigte, dass die Dysregulation in diesen Modellen viel ausgeprägter ist, was möglicherweise darauf zurückzuführen ist, dass diese Modelle einen einen viel aggressiveren Phänotyp aufweisen als das

GA-CFP-Modell. Die Daten aus dem ALS-Mausmodell stimmen daher mit unserer Beobachtung überein, dass CHIT1 und CHI3L1 einen prädiktiven Faktor bezüglich der Aggressivität der Krankheit bei ALS-Patienten darstellen können.

Zusammenfassend konnte im Rahmen dieser Dissertation gezeigt werden, dass bedeutende Mitglieder der Chitinase-Familie bei ALS dysreguliert sind und mehrere Zelltypen, darunter Neuronen, Glia und zirkulierende Makrophagen, zu dieser Dysregulation beitragen. Die Verwendung des D50-Modells zeigt, dass pNfH, CHIT1 und CHI3L2 die ALS-Pathologie stärker widerspiegeln und sich somit für die Präzisierung von Diagnosen eignen, während NfL und CHI3L1 empfindlicher auf die Aggressivität der Krankheit reagieren und daher für die prognostische Beurteilung geeignet sind.

4. Introduction

4.1 Amyotrophic Lateral Sclerosis at a Glance

Amyotrophic Lateral Sclerosis (ALS) was first described as a distinct condition in 1869 by Jean-Martin Charcot; his seminal studies using the "*anatomo-clinical*" method delineated the link between progressive muscular atrophy and sclerosis of the lateral columns. ALS is a relentlessly progressive and fatal disorder with a pooled prevalence rate of 6.22 per 100,000 persons in European populations (Brown, Lally et al. 2021). While survival can range from a few months to several decades, population-based studies have consistently reported a median of 2-3 years from symptom onset, with most patients eventually succumbing to respiratory failure. Concomitant upper and lower motor neuron damage (UMN, LMN) are a hallmark of ALS, with deficits typically being focal at onset and progressively spreading outwards. Initial symptoms may include difficulties with speech and swallowing and twitching of the tongue muscles (bulbar-onset) or muscle weakness, cramps and fasciculations in the limbs (limb-onset) (Fig.1). Clinical manifestation is however notoriously variable and preceded by a protracted pre-symptomatic phase (Eisen, Kiernan et al. 2014). Additionally, no definitive diagnostic tests exist and neurologists must utilize a multi-modal repertoire of neuroimaging, electrophysiological and biochemical assessments to categorize individuals to **a)** a level of diagnostic certainty (revised El Escorial Criteria) (Ludolph, Drory et al. 2015) or **b)** as either "ALS" or "Non-ALS" (Gold Coast criteria) (Shefner, Al-Chalabi et al. 2020).

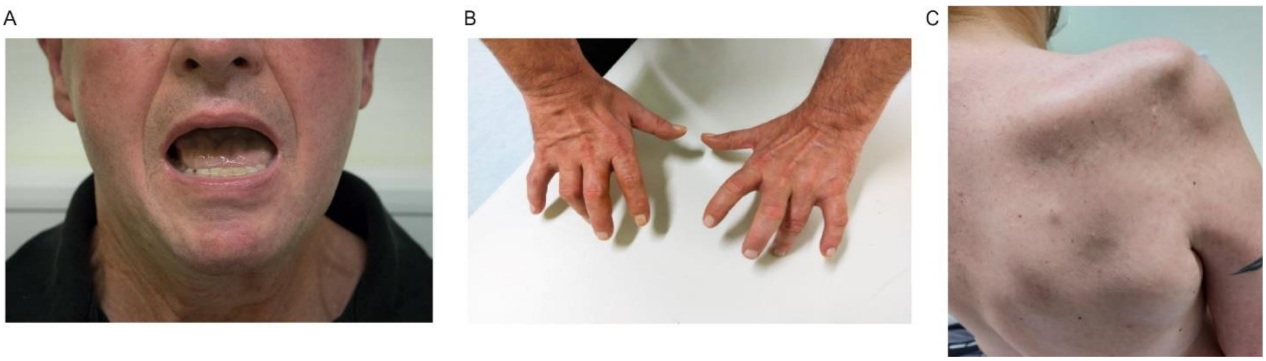

Figure 1: Images from patients displaying classical ALS symptoms including twitching of the tongue muscles **(A),** cramping of the hand muscles **(B)**, and muscle atrophy **(C)** *(Reproduced with consent from the Hans Berger Dept. of Neurology, Friedrich Schiller University Hospital, Jena)*

While initially conceptualized as a pure motor neuron disease (MND), ALS is now considered a heterogeneous syndrome positioned within the broader neurodegenerative spectrum. This is particularly evident from its overlap with frontotemporal dementia (FTD); close to 15% of ALS patients have comorbid FTD, with almost 50% of patients depicting cognitive changes within the FTD spectrum (Kiernan 2012, Phukan, Elamin et al. 2012, Elamin, Bede et al. 2013). Similarly, up

to 30% of FTD patients eventually develop motor symptoms (Burrell, Kiernan et al. 2011). Both diseases also share the key histopathological hallmark of cytoplasmic proteinaceous aggregates. Heterogeneity in ALS can stem from age-at and site-of onset, pattern of spread, the ratio of UMN/LMN deficits and degree of cognitive dysfunction. Indeed, the condition's genetic complexity is testament to its multi-systemic nature; only 5-10% of all ALS is familial, with most cases being sporadic. Over 30 genes that either cause or increase the risk for developing ALS have been identified, with mutations in *TARDP, C9orf72, SOD1 and FUS* and accounting for close to 70% of all familial cases (van Es, Hardiman et al. 2017). However, the substantial pleiotropy and incomplete penetrance noted in these Mendelian-pattern genes indicate that a sporadic/familial binarization may be too reductive. Next-generation sequencing studies have also established oligogenic/polygenic inheritance for sporadic ALS (van Blitterswijk, van Es et al. 2012, McCann, Henden et al. 2020). The adult-onset of the condition, despite the presence of even high penetrance mutations like *SOD1* from birth, reaffirms the need to look beyond simplistic genotype-phenotype extrapolations. Indeed, a multi-step hypothesis wherein interactions between environmental and genetic risk factors build over the lifespan and only become clinically evident once intrinsic compensatory mechanisms are breached, has already been proposed (Al-Chalabi, Calvo et al. 2014). Additionally, implicated genes span across a multitude of molecular processes, including mitochondrial dysregulation, neuroinflammation, cytoskeletal defects, RNA processing, oxidative stress regulation, and protein trafficking to name a few, further highlighting the multiple pathological mechanisms at play. While these mechanisms may ultimately converge on the common outcome of irreversible neuronal loss, they have important implications for therapeutic development. The observed clinical heterogeneity certainly suggests that different mechanisms may be at play to different degrees across individuals, making the success of a "one size fits all" treatment approach unlikely.

4.2 Tracking Heterogeneous Progression

In addition to its variable presentation, ALS is characterized by tremendous heterogeneity in progression. Certain risk factors that signal poorer outcomes are already established: for instance, weight loss, bulbar onset, increased age at diagnosis and early respiratory difficulties are all associated with shortened survival (Simon, Turner et al. 2014). Nevertheless, reliably quantifying and tracking progression has proven challenging. Typical clinical measures include tracking LMN loss via nerve conduction studies, muscle ultrasounds and neurophysiological tools like Motor Unit Number Index estimation (MUNIX) (Neuwirth, Barkhaus et al. 2017). Conversely, neuroimaging techniques like magnetic resonance imaging (MRI) are powerful measures of disease-associated

structural changes, cortical pathology and UMN damage (Steinbach, Batyrbekova et al. 2020, Steinbach, Gaur et al. 2021). Clinical trials have predominantly used survival and functional status as primary endpoints, as improvements in these domains are ultimately the most relevant outcomes for patients. However, using survival as an outcome is challenging, not in the least because it must be monitored for several years for any robust inferences to be made; this increases both costs and the risk of losing non-ambulatory patients to follow-up. The gold standard for measuring functional status remains the revised ALS functional rating scale (ALSFRS-R) (Cedarbaum, Stambler et al. 1999). Briefly, it is a multidimensional scale scored from zero to 48, with the latter indicating full functionality. It comprises 12 items spanning the domains of upper and lower limb, bulbar, and respiratory function. Several studies have also availed of the disease progression rate or ΔFRS which is calculated as (48-ALSFRS-R score at time of sampling)/(time elapsed since disease onset in months) and therefore incorporates a temporal axis (Kimura, Fujimura et al. 2006). These indices indeed predict survival (Gordon and Cheung 2006, Kimura, Fujimura et al. 2006), lend themselves to clinical staging systems (Tramacere, Dalla Bella et al. 2015) and have the obvious advantage of being simple and cost-effective. However, they have inherent limitations that directly affect their utility as trial outcomes. Firstly, although the ALSFRS-R is well suited to reflecting status in individual patients, inter-patient comparability is limited: individuals with identical scores may not be prognostically comparable owing to the scale's multidimensionality (Franchignoni, Mora et al. 2013). It also has a floor-effect, with patients surviving and discerning changes in physical function for several months after having reached a score of 0 (Wicks, Massagli et al. 2009). Certain questionnaire items, particularly those in the respiratory and bulbar domain, are associated with sudden large jumps in score and even reversals, typically around the time of intervention e.g. ventilatory assistance (Bakers, de Jongh et al. 2021). The ΔFRS is particularly problematic as trials designed around it are based on assumptions of linearity, when in fact progression in ALS is curvilinear (Gordon, Cheng et al. 2010) and has high inter-individual variability (Fig. 2A-B). Moreover, it assumes that the rate of decline within an individual is constant across the disease course, when in fact it is highly dynamic, particularly in individuals who have a higher ΔFRS to begin with (Fig. 2C). Categorization as a "rapid" or "slow" progressor is therefore entirely dependent on the ΔFRS calculated at that specific time-point and could result in inaccurate stratification for trials. There are also no universally agreed on thresholds for what constitutes a certain progression type; these are often derived from individual study cohorts and consequently entirely arbitrary.

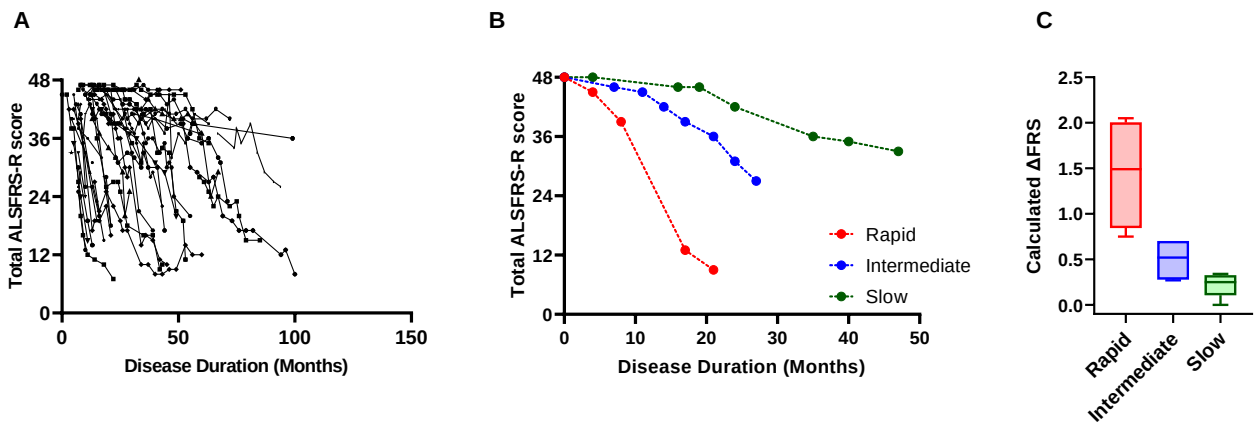

Figure 2: Total ALSFRS-R scores for a cohort of ALS patients (n = 39) **(A)** and for three representative progressor types **(B)** indicate the inter-individual variability and curvilinear functional decline. Data points reflect each instance of ALSFRS-R administration and the corresponding score. Calculated progression rates (ΔFRS) **(C)** show that progression is highly variable across the disease course even within the same individual.

We developed the D50 disease progression model to address these limitations. Its framework was initially conceived using our patient cohort at the University Hospital in Jena, Germany (400 individuals) and the PRO-ACT database (4838 individuals) (Atassi, Berry et al. 2014), as seen in poster format in Appendix 2. Further validation is described in the Dreger et al. manuscript attached to this thesis (Dreger, Steinbach et al. 2021). Briefly and as seen in Figure 3, the model uses a sigmoidal decay curve to describe the ALS disease course and yields the following key parameters:

- **D50** = time taken in months for ALSFRS-R score to drop to 24 i.e., 50% functional loss. This is a summative time-independent descriptor of overall disease aggressiveness.
- **rD50** = relative D50, derived by normalizing absolute disease duration to D50. This provides a unified and open-ended reference scale to describe an individual's functional disease course, wherein 0 signifies disease onset and 0.5 is the time-point of halved functionality. Individuals can also be grouped into mathematically contiguous disease phases: early semi-stable Phase I ($0 \leq rD50 < 0.25$), early progressive Phase II ($0.25 \leq rD50 < 0.5$), and late progressive and late stable Phases III/IV ($rD50 \geq 0.5$). rD50 can also be used to glean pseudo-longitudinal insights from cross-sectional datasets, which is particularly useful for a rapidly progressive condition like ALS (Prell, Gaur et al. 2020).

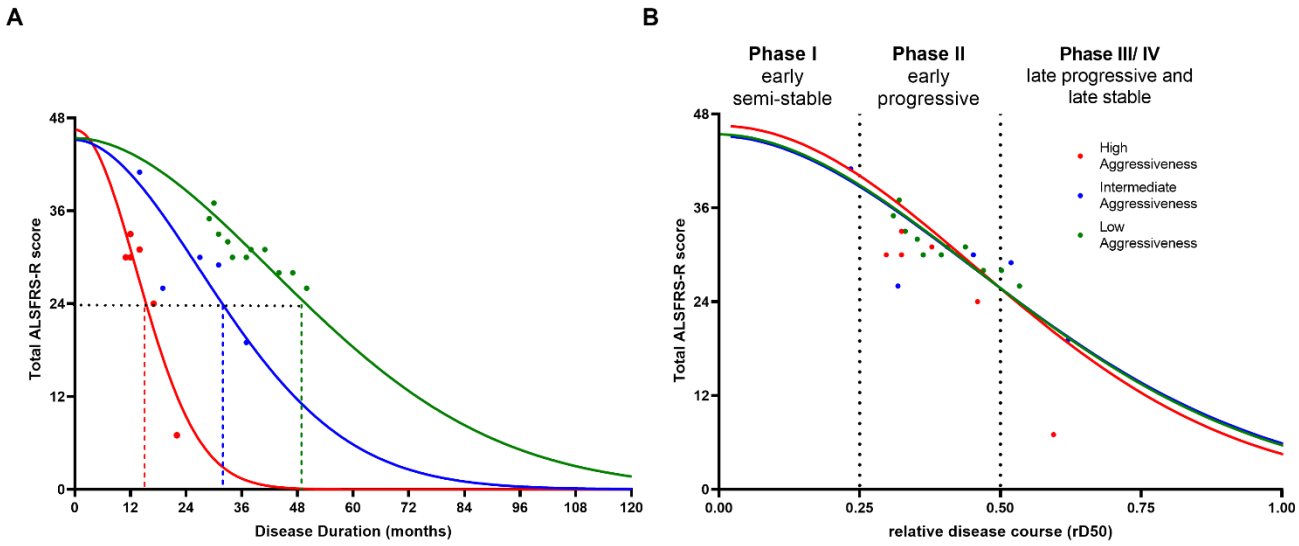

Figure 3: (A) D50 is the time taken in months for the ALSFRS-R score to drop from 48 to 24 (black dotted line) and is calculated by projecting a sigmoidal decay curve using actual ALSFRS-R scores (dots) for 3 representative progressor types (colored dashed lines) (high aggressiveness = D50 ≤ 20 months, intermediate aggressiveness = 20 < D50 ≤ 40 months, low aggressiveness = D50 > 40 months). **(B)** Normalization with rD50 allows for comparability between these different progressor types with different disease time scales. This metric shows that patients proceed through similar phases of functional decline irrespective of **1)** individual disease aggressiveness and **2)** how aggressiveness cut-offs are defined.

4.3 The Case for Biomarkers in ALS

Over the last 20 years, more than 50 randomized clinical trials assessing 60 different molecules have yielded no treatments to either cure ALS or reverse neuronal damage (Kiernan, Vucic et al. 2021). Riluzole, the sole disease-modifying therapy available in both US and European markets, was approved over two decades ago, and only prolongs survival by a few months. Poor therapeutic translation is likely the cumulative result of several factors. To begin with, while animal models have greatly enhanced our understanding of pathological changes at the molecular level, particularly in pre-symptomatic disease, no single one has been able to faithfully recapitulate the phenotypic spectrum observed in humans (Ittner, Halliday et al. 2015). To illustrate, most preclinical studies have used the transgenic *SOD1-G93A* mouse model (Tu, Raju et al. 1996) even though it does not display a key pathological hallmark: nuclear to cytoplasmic TDP-43 mislocalization. Although multiple models now exist for different genes (e.g., *TDP43* and *C9orf72*), they are all ultimately only reflective of the very small proportion of individuals who carry these mutations. Next, limitations associated with the ALSFRS-R and ΔFRS undoubtedly contribute to poor trial outcomes, particularly if these indices are used for cohort stratification and clinical course prediction at recruitment. As outlined above, they only reflect the rate of decline at a circumscribed time-point. Arguably, one of the biggest contributing factors is that patients continue to be stratified based on external phenotypes, which, because of their profound variability,

inevitably create "noisy" cohorts. We posit that the inherent heterogeneity in complex disorders like ALS necessitates a paradigm shift in trial design: stratification should be based on the underlying disease mechanisms. Homogenous subgroups could then ideally be targeted with "mechanism-specific" therapies thereby reducing the risk of efficacy signals being occluded by noise. However, the creation of such subgroups requires quantitative biomarkers that reflect discrete disease mechanisms. The National Institute of Health defines a biomarker (biological marker) as "*a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention*" (Biomarkers Definitions Working 2001). Within the context of ALS these could assist with:

- Diagnostic Refinement i.e., earlier detection and confirmation of the underlying pathology and increased confidence when eliminating other diagnoses.
- Prognostication i.e., understanding how the **1)** presence of the mechanism and **2)** the magnitude of its influence (as captured by the biomarker) relate to certain clinical outcomes (e.g., survival, total ALSFRS-R score, ΔFRS, time until ventilation etc.)
- Identification/prediction of individuals most likely to respond to treatments aimed at modifying the specific underlying mechanism. Recent advancements in Alzheimer's disease (AD) provide the best evidence of the value of this approach. For instance, Positron emission tomography (PET) imaging of amyloid beta and/or tau are routinely used to screen patients for enrolment in drug trials targeting these proteins (Hansson 2021).
- Pharmacodynamic monitoring to show whether an intervention has elicited a biological response that reflects engagement of the mechanism being targeted.

In ALS, the neurofilament proteins are poised for routine clinical implementation and were most recently used as a secondary endpoint in a trial evaluating Tofersen, an antisense oligonucleotide targeting SOD1 (Miller, Cudkowicz et al. 2020). The neurofilaments are a class of intermediate filaments that are a major cytoskeletal component of neurons and critical determinants of axonal caliber and transport. They are heteropolymers comprising 4 subunits i.e., neurofilament light (NfL), medium (NfM), and heavy (NfH) polypeptides with α-internexin and peripherin in the central and peripheral nervous systems, respectively, and can undergo various post-translational modifications. Cerebrospinal fluid (CSF) neurofilament levels are increased in several conditions like AD, FTD and multiple sclerosis (MS), and are thus "generic markers" of neuroaxonal injury (van den Berg, Sorenson et al. 2019). However, both serum and CSF NfL and pNfH levels are substantially elevated in ALS relative to other neurological conditions and show diagnostic and predictive utility (Feneberg, Oeckl et al. 2018, Poesen and Van Damme 2018). The robust correlation between serum and CSF levels is particularly promising as non-invasive blood sampling

is easier to integrate into diagnostic workups. Despite ongoing debate about whether elevated neurofilament levels in ALS simply reflect large white matter tract degeneration or indicate continuous cellular turnover, possibly as a compensatory mechanism, (Turner and Gray 2016), there is promising prognostic potential. Levels are stable over time and higher levels are associated with shortened survival and worsened progression as measured by the ΔFRS (Lu, Macdonald-Wallis et al. 2015, Benatar, Zhang et al. 2020, Behzadi, Pujol-Calderon et al. 2021). Nevertheless, given the multiple and overlapping disease mechanisms at play in ALS, it is unlikely that a single biomarker can capture the full pathological spectrum. Indeed, differential diagnoses between ALS and other MNDs can still prove challenging and there is variability in progression that cannot be fully accounted for using the neurofilaments alone. Biomarker panels that simultaneously capture multiple mechanisms can help parse out this variability, improve specificity and sensitivity and ultimately guide clinical decision making. The D50 model is particularly well suited for the discovery of novel prognostic markers because unlike the ΔFRS, the model distinguishes between disease aggressiveness and accumulated degeneration. Hence, it provides a framework to examine the association of potential biomarkers with either of these outcomes without the confounding influence of the other. The use of a composite metric like D50 also circumvents problems associated with different sampling schedules. Indeed, several "wet" and "dry" biomarkers have been successfully evaluated within the framework of this model (Poesen, De Schaepdryver et al. 2017, Prell, Steinbach et al. 2019, Prell, Stubendorff et al. 2019); it was also recently used to identify patients with highly aggressive disease and plan care for them during the COVID-19 pandemic (Steinbach, Prell et al. 2020).

4.4 Neuroinflammation as a Disease Mechanism in ALS

Neuroinflammation is a pathogenic feature common to several neurodegenerative disorders (Gonzalez, Elgueta et al. 2014, Deleidi, Jaggle et al. 2015, Ferrer 2017, Molteni and Rossetti 2017): in its simplest form, it is defined as "*an inflammatory response within the brain and/or spinal cord*" (DiSabato, Quan et al. 2016). This response is mediated by a complex interplay between glial cells, including microglia and astrocytes, non-resident immune cells, chemokine and cytokine production, and other damage-related moieties like reactive oxygen species (Evans, Couch et al. 2013). While inflammation may not be the initial causative trigger for ALS, studies using mutant SOD1 mouse models have shown that it directly affects disease manifestation. Mutant protein expression in glia and concomitant glial activation are necessary—albeit not sufficient—for motor neuronal injury (Pramatarova, Laganiere et al. 2001, Zhao, Beers et al. 2010); furthermore, glia-specific protein deletion both delays onset and slows progression (Wang, Gutmann et al. 2011).

Data from patients confirm that pathology in ALS is indeed non-cell autonomous. Immunohistochemical analyses of post-mortem tissue have revealed extensive glial TDP-43 pathology throughout the CNS (Geser, Brandmeir et al. 2008), while PET imaging has shown significantly increased cerebral microglial activation *in vivo* (Turner, Cagnin et al. 2004, Corcia, Tauber et al. 2012). Additionally, higher CSF levels of pro-inflammatory mediators, including IL-6, TNF-α, and MCP-1, have been consistently reported in patients (Mitchell, Freeman et al. 2009, Vu and Bowser 2017). Inflammation in ALS is however not restricted to the CNS; there is compelling evidence for the involvement of the peripheral immune system. Several changes, including the functional alteration and pro-inflammatory phenotype of myeloid cells (Zondler, Muller et al. 2016, Zhao, Beers et al. 2017, Baufeld, O'Loughlin et al. 2018, Du, Zhao et al. 2020), reduction of regulatory T-cells in rapidly progressing patients (Beers, Henkel et al. 2011), dysregulated leukocytic chemokine receptor expression (Perner, Perner et al. 2018) and altered ratios of immune cell subsets (Murdock, Bender et al. 2016), have been reported. Immune cells can also "invade" the CNS, with a breach of the blood-brain barrier representing a potential route (Garbuzova-Davis and Sanberg 2014). For instance, activated macrophages and dendritic cells were detected in the spinal cord of patients, with the highest levels noted in patients with rapidly progressive disease (Henkel, Engelhardt et al. 2004). The adaptive immune system, particularly T lymphocytes, also participates in this infiltration (Kawamata, Akiyama et al. 1992, Beers, Henkel et al. 2008).

Disappointingly, therapeutic efforts aimed at inflammation have been largely unsuccessful, despite the abundant evidence for its contribution to disease activity. One reason for this may be the duality of glial responses to insult: these range on a continuum from neuroprotective to neurotoxic, with the latter dominating in advanced disease (Beers, Zhao et al. 2011, Liao, Zhao et al. 2012). Another factor may be timing: pre-clinical studies using mice typically initiate treatment regimens in the pre-symptomatic phase. However, owing to the diagnostic delay and rapidly progressive nature of ALS, patients are recruited much later in disease i.e., once the switch from acute to chronic inflammation has already occurred. Therefore, biomarkers that capture **1)** neuroinflammation at a stage when it is still amenable to therapeutic intervention and **2)** inter-individual variation in the immune response are urgently needed. To summarize, inflammation in ALS is not a passive consequence of motor neuronal injury; rather, it is a dynamic process that has different consequences for neuronal viability over time. Moreover, this non-cell autonomous cascade ensues regardless of the original etiology; genetic or sporadic; which has led some researchers to redefine ALS as a "systemic pro-inflammatory disorder" (Appel, Beers et al. 2021).

4.5 The Chitinases: Novel Inflammatory Markers in ALS?

Recent translational studies in ALS have focused on the biomarker potential of the chitinases, a group of enzymes that have been evolutionarily conserved across prokaryotic and eukaryotic organisms. The chitinases are glycoside hydrolases (GH) that cleave the β-(1-4) glycosidic bonds in polysaccharides like chitin (Chen, Jiang et al. 2020), a naturally abundant polymer that is the main structural component of the fungal cell wall and arthropod exoskeletons. Mammalian chitinases belong to the GH18 family, on the basis of their shared sequence homology, and employ substrate-assisted catalytic mechanisms (Funkhouser and Aronson 2007). Chitinases can be further split into "true" chitinases with enzymatic activity and the homologous chi-lectins or "chitinase-like" proteins (CLPs), which bind chitin with high affinity but are enzymatically inactive owing to a substitution in the active-site domain. Humans possess three active chitinases; chitotriosidase (CHIT1), acidic mammalian chitinase (AMCase), and di-N-acetylchitobiase; and several CLPs, including chitinase 3-like-1 (CHI3L1/YKL-40), chitinase 3-like-2 (CHI3L2/YKL-39), and oviductal glycoprotein 1. It is likely that the biological roles of the chitinases extend beyond innate immunity against chitin, given that humans neither synthesize nor metabolize it. In fact, phylogenetic studies have reported that the multiple gene duplication and diversification events in the evolution of the GH18 family drove substantial functional expansion (Funkhouser and Aronson 2007). Intriguingly, in humans, the majority of chitinase genes are located on chromosome 1, adjacent to the major histocompatibility complex (MHC) paralogon; the MHC gene family has a broad range of functions, including antigen presentation and processing. This synteny likely indicates a long "*organizational relationship*" (Funkhouser and Aronson 2007) between the families, and the earliest evidence for the interface between the chitinases and the immune system. Indeed, there is ample evidence that the chitinases are active immunomodulators. Immune cells like monocytes and macrophages are key (but not exclusive) sources of chitinases like AMCase, CHIT1, and CHI3L1 (Di Rosa, De Gregorio et al. 2013, Di Rosa, Malaguarnera et al. 2013, Di Rosa, Tibullo et al. 2016). Chitinase expression is also differentially regulated over time and by various cytokines and chemokines, including TNF-α, IFN-γ and members of the interleukin family, and immunogenic stimulants like LPS (Di Rosa, Musumeci et al. 2005, Di Rosa, Malaguarnera et al. 2013, Kunz, van't Wout et al. 2015). Crucially, the chitinases via autocrine and/or paracrine signalling can themselves regulate chemokine/cytokine production. For example, stimulation with CHI3L1 or CHIT1 significantly enhanced monocyte secretion of IL-8 and MCP-1 (Correale and Fiol 2011). Similarly, epithelial cells were both a source and target of AMCase, as stimulation with recombinant AMCase induced epithelial cell secretion of IL-8 and CCL17 (Hartl, He et al. 2008). Collectively, these data suggest that the chitinases may create "feed-back loops" that contribute to

inflammation. Several chitinase family members (Table 1) have been implicated in various conditions characterized by chronic inflammation, including cancer, diabetes, and atherosclerosis, and in some cases, been successfully recruited as biomarkers. For instance, AMCase is grossly elevated in asthma and is being explored as a therapeutic target (Zhu, Zheng et al. 2004), while elevated plasma CHIT1 activity is used for both disease severity and treatment response monitoring in Gaucher's disease, an inherited lysosomal storage disorder (Hollak, van Weely et al. 1994). The involvement of the chitinases in neurological conditions like MS, AD, and FTD to name a few, is therefore unsurprising given that neuroinflammation is a defining pathological feature of so many of them. In the context of ALS, several studies have consistently reported that CSF levels of CHIT1, CHI3L1 and CHI3L2 are substantially elevated relative to both healthy individuals and those with other neurodegenerative conditions (Thompson, Gray et al. 2018, Thompson, Gray et al. 2019, Vu, An et al. 2020). Interestingly, a 24 base pair duplication in exon 10 of the *CHIT1* gene leads to an enzymatically inactive form of the protein and directly affects CHIT1 activity and levels, with homozygous carriers displaying almost no activity (Boot, Renkema et al. 1998). While the genotype frequency does not differ between ALS patients and controls and does not associate with disease severity (Oeckl, Weydt et al. 2019), it may represent a potential confounding effect during interpretation.

Table 1: Chitinase family members frequently implicated in inflammatory conditions

Protein	Aliases	Gene	Expression
Chitotriosidase-1			
Homo Sapiens	-	*CHIT1*	✓
Mus Musculus	-	*Chit1*	✓
Acidic mammalian chitinase			
Homo Sapiens	-	*CHIA*	✓
Mus Musculus	Chia1	*Chia*	✓
Chitinase-3-like protein 1			
Homo Sapiens	hCGP-39, YKL-40	*CHI3L1*	✓
Mus Musculus	Brp39	*Chi3l1*	✓
Chitinase-3-like protein 2			
Homo Sapiens	YKL-39	*CHI3L2*	✓
Mus Musculus	-	-	x

The chitinases are considered proxies for reactive gliosis in ALS and here too, there is preclinical evidence of glial cells being both a chitinase source and target. Microglia, although a physiological source of CHIT1, are vulnerable to its dysregulation in ALS: microglial cultures exposed to CSF from ALS patients showed elevated CHIT1 expression (Varghese, Sharma et al. 2013). Intrathecal administration of recombinant CHIT1 to rats also resulted in micro- and astro-glial activation and

increased pro-inflammatory cytokine expression *in vivo* (Varghese, Ghosh et al. 2020). However, there remain several open questions; the most critical arguably being what the prognostic relevance of chitinase upregulation in ALS is. Studies have used outcome metrics ranging from the ΔFRS to survival and lung capacity and reported conflicting associations. Additionally, despite the increasing focus on the chitinases' biomarker potential, detailed studies on their cellular sources are limited.

5. Study Objectives

The body of work described in this doctoral thesis is based on the following multi-part hypothesis:

- The chitinases via their reported autocrine and paracrine effects exacerbate neuroinflammation in ALS and sustain it via a "feed-forward" loop (Fig. 4).
- This culminates in more aggressive disease in patients who, owing to either genetic predisposition and/or environmental risk factors, have a greater neuroinflammatory component
- Chitinase upregulation is not exclusive to ALS but can be used for prognostication
- The D50 model can help evaluate the prognostic utility of the chitinases in ALS.

This hypothesis was split into the research questions below and investigated using a multi-modal approach combining a clinical cohort and preclinical models:

- Can the chitinases be recruited as diagnostic biomarkers within ALS?
- What are the physiological sources for chitinases that are vulnerable to dysregulation in ALS?
- Can the D50 model provide a valid framework for biomarker assessment?
- Can the chitinases assist with prognostication in ALS i.e., do they associate with disease aggressiveness or accumulated disease as measured by the novel D50 disease progression model?

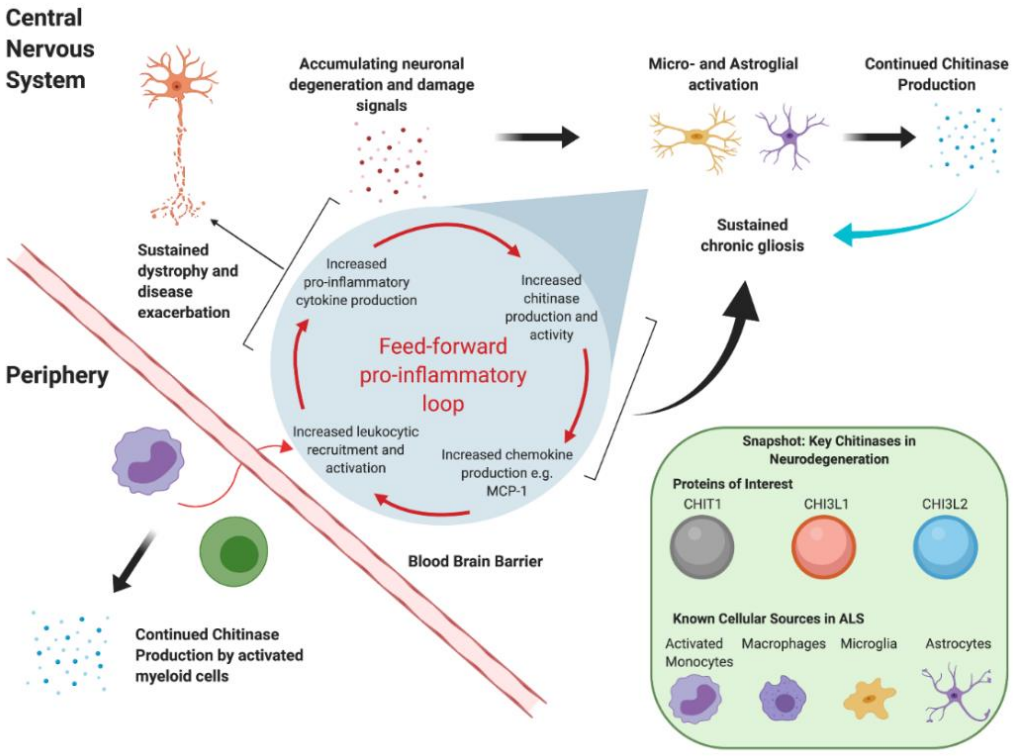

Figure 4: Hypothetical mutual regulation cascade of chitinases and the mammalian immune system that sustains neuroinflammation in ALS *(Adapted from Gaur et. al 2020)*

6. Supporting Manuscripts with Summaries

6.1 The Chitinases as Biomarkers for Amyotrophic Lateral Sclerosis: Signals from the CNS and Beyond **(Frontiers in Neurology, May 2020)**

This Review article was the first in the field to provide a summative overview of the existing literature on chitinases in ALS. Herein, we discussed the nature of the chitinases as immunomodulators, evidence for their diagnostic and prognostic utility, and broader implications for both ALS and the wider neurodegenerative field. Broadly, we concluded:

- Rather than being unique to ALS, chitinase dysregulation reflects neuroinflammation that is a part of the wider neurodegenerative process.
- Different neurodegenerative disorders may present with specific chitinase dysregulation patterns that reflect the differences in the underlying pathology.
- The evidence on the prognostic relevance of the chitinases in ALS is inconclusive, in part because of inadequate and differing outcome metrics.

Finally, this review articulated the core hypothesis of chitinases acting via a self-propagating loop to sustain and exacerbate neuroinflammation in ALS.

frontiers in Neurology

MINI REVIEW
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The Chitinases as Biomarkers for Amyotrophic Lateral Sclerosis: Signals From the CNS and Beyond

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Amyotrophic lateral sclerosis (ALS) is a late-onset neurodegenerative condition, most widely characterized by the selective vulnerability of motor neurons and the poor life expectancy of afflicted patients. Limited disease-modifying therapies currently exist, which only further attests to the substantial heterogeneity associated with this disease. In addition to established prognostic factors like genetic background, site of onset, and age at onset, wide consensus on the role of neuroinflammation as a disease exacerbator and driver has been established. In lieu of this, the emerging literature on chitinases in ALS is particularly intriguing. Individual groups have reported substantially elevated chitotriosidase (CHIT1), chitinase-3-like-1 (CHI3L1), and chitinase-3-like-2 (CHI3L2) levels in the cerebrospinal, motor cortex, and spinal cord of ALS patients with multiple—and often conflicting—lines of evidence hinting at possible links to disease severity and progression. This mini-review, while not exhaustive, will aim to discuss current evidence on the involvement of key chitinases in ALS within the wider framework of other neurodegenerative conditions. Implications for understanding disease etiology, developing immunomodulatory therapies and biomarkers, and other translational opportunities will be considered.

Keywords: neurodegeneration, biomarker (BM), neuroinflammation, chitinases, amyotrophic lateral sclerosis (ALS)

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INTRODUCTION

ALS and Neuroinflammation

Amyotrophic lateral sclerosis (ALS) is the most prevalent form of adult-onset motor neuron disease and clinically presents with the relentless destruction of primarily (but not exclusively) upper and lower motor neurons (UMN, LMN). Riluzole, the sole treatment available, confers only modest effects via a median increase of 2–3 months in survival; most patients eventually succumb to respiratory failure. Although there is a pressing need for treatment modalities that tackle disease aggressiveness, therapeutic development has been severely constrained by the disease's characteristic heterogeneity; this stems from age-at-onset and site-of-onset, presence of disease-associated mutations, and comorbidities, including frontotemporal dementia (FTD) (1). Progression and survival rates are also highly variable; while the median survival is 2–3 years from symptom onset, some patients present with a disease duration of over 10 years (2). Cellular and animal studies have provided elegant evidence that neuroinflammation contributes to ALS pathology and that concomitant glial dysregulation is necessary for motor neuronal

degeneration (3–5). Numerous immunological changes, including the functional alteration and pro-inflammatory phenotype of circulating myeloid cells (6), dysregulated leukocytic chemokine receptor expression (7), the reduction of regulatory T cells (8), and cytotoxic T cell infiltration, have also been reported in patients (9).

Despite this, there remains a paucity of biological tools that adequately capture the neuroinflammatory response across the disease; this may partially explain the failure of immunomodulatory therapies to date. Biomarkers that reflect target engagement and assess the efficacy of novel treatments are therefore crucial. Although molecular imaging studies of microglial activation are underway, fluid-based biomarkers are more accessible and can provide important insights into disease pathomechanisms. For instance, cerebrospinal fluid (CSF) and humoral levels of the neurofilament proteins have been validated as robust diagnostic and prognostic markers for ALS. Several inflammatory cytokines have also been reported as dysregulated in ALS, including TNF-α, MCP-1, and IL-6 (10–12). In lieu of this, recent reports of elevated chitinase levels in ALS are particularly interesting, as these have already been reported as surrogate markers of a chronic inflammatory response in non-neuronal conditions.

Mammalian Chitinases: Novel Players in Neurodegeneration?

The chitinases belong to the family 18 glycosyl hydrolases (GH18) and are characterized by their ability to cleave chitin, a natural polysaccharide found in the coating of various pathogens. The GH18 family is ubiquitously expressed across a wide range of organisms, from bacteria to humans; evolutionary conservation in the latter is particularly interesting, given the lack of endogenous chitin synthesis. This has led to the view that chitin is a defense target for the mammalian immune system or an "immune stimulator." Indeed, it is recognized by several pattern recognition receptors and can trigger associated immune responses in a fragment-size and tissue-dependent manner (13). Mammalian chitinases include the enzymatically active chitinases chitotriosidase (CHIT1) and acidic mammalian chitinase (AMCase) that can degrade chitin, and the chi-lectins (CLs) chitinase 3-like 1 and -like 2 (CHI3L1, CHI3L2). Despite being able to bind chitin with high affinity, the CLs possess no chitinolytic activity, owing to the absence of the catalytic motif. CHIT1 is primarily expressed by cells of myeloid lineage, particularly mature macrophages (14, 15). Like CHIT1, CHI3L1 is absent in monocytes and strongly upregulated during later stages of macrophage differentiation (16). CHI3L1 is also produced by reactive astrocytes and associated with chronic neuroinflammation, as will be further discussed in the Section Chitinases Across the ALS-FTD Spectrum (17–19). While CHI3L2 hasn't been as extensively studied, expression has been noted in chondrocytes, synoviocytes, and alternatively activated "M2" macrophages (20).

Although the exact roles of these moieties remain to be fully elucidated, it is clear that they extend beyond innate immunity against chitin-containing pathogens. Chitinases have been reported in the context of adaptive Th2 response mediation (21, 22), tissue remodeling and repair, and, most recently, oligodendrogenesis (23). Dysregulated chitinase levels have been reported in several chronic neurodegenerative conditions, including Alzheimer's disease (AD) and FTD. *In vitro* evidence suggests that, at least in ALS, they may act in a "feed-forward" loop that sustains neuroinflammation and exacerbates disease, as illustrated in **Figure 1**. For instance, in a transgenic rat model, TDP-43 induced astrocytic *CHI3L1* up-regulation; in turn, synthetic CHI3L1 caused neuronal death in a dose-dependent manner (19). Similarly, Raju et al. reported that CSF from ALS patients impacted cell viability and upregulated CHIT1 expression in murine microglial cultures (24). Subsequent exposure to CHIT1 itself caused microglial activation, indicating again a "self-propagating" inflammatory mechanism (25).

This review, while not exhaustive, will summarize current evidence for chitinase dysregulation in ALS and its implications for understanding disease etiology and progression, and therapeutic and biomarker development. CHIT1, CHI3L1, and CHI3L2 will be focused on, since these have been most extensively studied in a neurodegenerative context.

THE CHITINASES IN ALS

Evidence Concerning Diagnostic Potential

Varghese et al. (26) were the first to report chitinases in the context of ALS; using quantitative mass spectrometry (MS) and ELISA-based validation in an independent cohort, they showed that CSF levels of CHIT1, CHI3L1, and CHI3L2 were significantly elevated in ALS patients relative to healthy controls (HCs). This elevation has since been confirmed by several studies using a range of proteomic and transcriptomic methods (27–33). Recent studies have predominantly focused on assessing discriminatory power with regard to mimic conditions, and other neurodegenerative diseases. However, studies have differed with respect to (a) the chitinases and secondary targets investigated; (b) cohort demographics; (c) bio-fluids assessed, and (d) experimental and analytical methods used (**Table 1**). Thompson et al. subsequently investigated all three chitinases and reported that they were significantly higher in the CSF of ALS patients relative to HCs, mimics, and asymptomatic mutation carriers (MCs) and that increased CHIT1 levels corresponded to active forms of the enzyme. While all three could reliably distinguish ALS from HCs and mimics, they were outperformed by pNfH. Furthermore, all three chitinases performed poorly in distinguishing ALS from primary lateral sclerosis (PLS) (28). A

Abbreviations: AD, Alzheimer's Disease; ALS, Amyotrophic Lateral Sclerosis; ALSFRS-R, Amyotrophic Lateral Sclerosis Functional Rating Scale Revised; CHIT1, Chitotriosidase; CHI3L1, Chitinase 3-Like 1; CHI3L2, Chitinase 3-Like 2; CLs, Chi-lectins; fALS, Familial ALS; FTD, Frontotemporal Dementia; gALS, genetic ALS; GH18, family 18 Glycosyl Hydrolases; HCs, Healthy Controls; IFN-γ, Interferon gamma; IL-6, Interleukin 6; LMN, Lower Motor Neuron; LPS, Lipopolysaccharide; MCs, Mutation Carriers; MCP-1, Monocyte Chemoattractant Protein 1; MS, Mass Spectrometry; NDCs, Neurological Disease Controls; NfL, Neurofilament Light Chain; PLS, Primary Lateral Sclerosis; pNfH, Phosphorylated Neurofilament Heavy Chain; PR, Progression Rate; sALS, sporadic ALS; sAPPβ, soluble Amyloid Precursor Protein Beta; Th2, T Helper Type 2; TNF-α, Tumor Necrosis Factor alpha; TREM2, Triggering Receptor expressed on Myeloid Cells 2; UMN, Upper Motor Neuron.

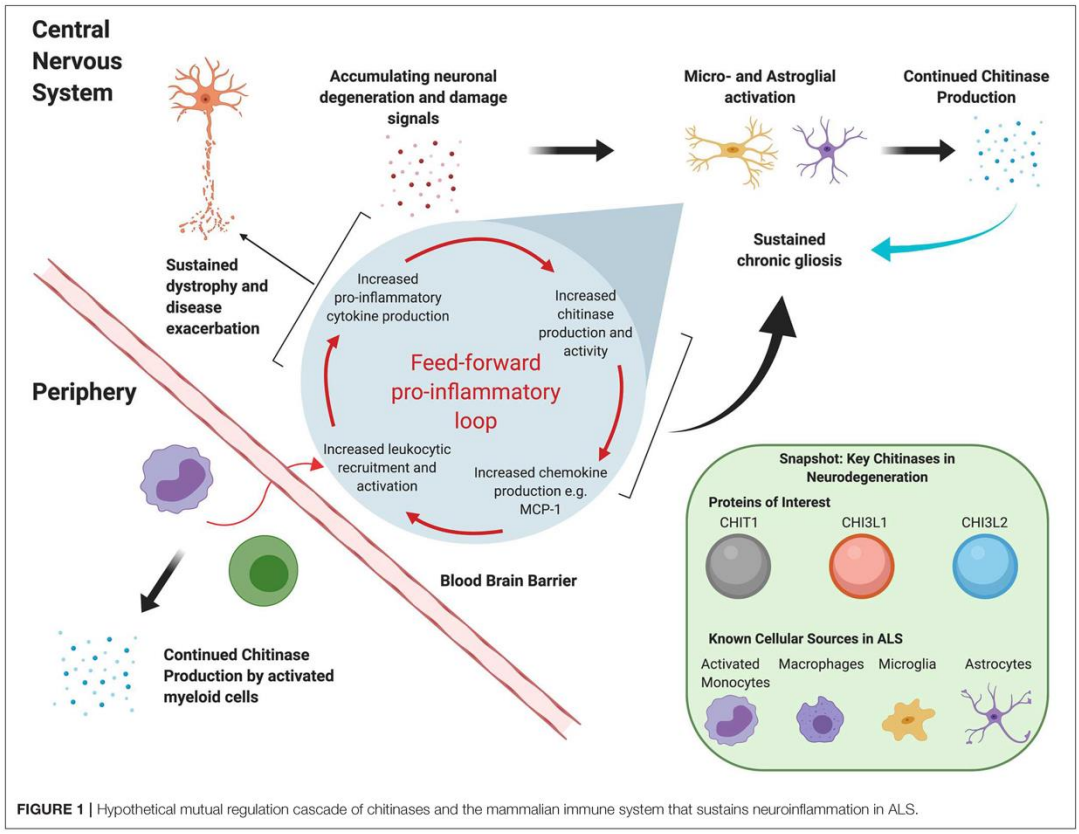

FIGURE 1 | Hypothetical mutual regulation cascade of chitinases and the mammalian immune system that sustains neuroinflammation in ALS.

prior MS-based study by the same group also noted a modest fold change for only CSF CHIT1 and CHI3L2 between ALS and PLS (31). Similarly, while Steinacker et al. (33) recommended CHIT1 as a potential differential diagnostic marker for ALS, they also noted that levels were increased in other neurodegenerative conditions and that pNfH and NfL had superior discriminatory power. In the same vein, Gille et al. (10) reported that elevated CSF CHIT1 and CHI3L1 levels were only weakly specific to ALS patients relative to neurological disease controls (NDCs). Observations of significant ALS-associated chitinase elevations in blood have been limited, barring one study that reported significantly elevated CHIT1 activity in dried blood spots (30) and another that noted higher CHIT1 levels in a genetic ALS (gALS) cohort (27) (both relative to HCs). This, coupled with reports of poor correlations between peripheral and CSF chitinase levels, makes a blood-based marker unlikely.

Applicability as stand-alone diagnostic markers is also likely to be constrained by the effect of functional variants. For instance, polymorphisms in the *CHI3L1* locus contribute to almost 15% of the variance in CSF CHI3L1 levels (34). Likewise, duplication in exon 10 of the *CHIT1* gene reduces both expression and activity; although this polymorphism is highly prevalent in European populations, no significant differences in genotype frequency have been observed between ALS patients and healthy individuals (27, 30). Additionally, presence of the *CHIT1* polymorphism has no influence on neurofilament levels or age of onset in patients, making a causative role in ALS pathogenesis unlikely. Importantly however, both CHIT1 expression and activity are significantly elevated in ALS patients (relative to HCs) independent of genotype and other factors like gender and age, indicating that disease status—rather than the presence of the polymorphism—determines the extent of dysregulation (12, 27, 30).

Evidence Concerning Prognostic Potential

The prognostic potential of the chitinases has been examined in relation to several clinical outcomes, including disease severity (overall ALSFRS-R score), the ALSFRS-R-derived progression rate (PR), survival, and disease duration, with several conflicting results as discussed below. The majority of the results discussed here focus on CSF, as almost no robust and consistent links between blood chitinase levels and prognostic factors have been

TABLE 1 | An overview of recent studies investigating chitinases in the context of ALS.

Study PMID	Study targets	Participants				Sample type	Methods used	Relative expression in ALS*	Correlation with neurofilament levels*	Other targets	Proposed bio mechanism	Proposed utility
		ALS	HCs	Mimics NDegs, NDCs	Other groups							
31178189	CHIT1 and CHI3L1	105		16 mimics, 102 NDCs		CSF and serum	ELISA and chemiluminescent assays	CSF CHIT and CHI3L1 ↑NDCs,Mimics	All with pNH and NfL (in CSF)	MCP-1	Surrogate markers of Gliosis	Monitoring therapy response and stratification
31123140	CHIT1, CHI3L1, CHI3L2	82	25	12 mimics, 10 PLS	5 asym gALS VCs	CSF and serum	ELISA, enzymatic activity assay	CSF All ↑HCs,Mimics,VCs, CSF CHI3L2-↑PLS	All with pNH (in CSF)	GFAP	Glial activity	Adjunctive predictor of progression, monitoring glial response to therapy
30224549	CHIT1 CHI3L1	70 sALS, 65 gALS	36 HCs	25 sFTD, 23 gFTD	25 asym. gALS VCs	CSF and blood	ELISA	CSF CHIT1↑HCs,MCs,gFTD, Blood CHIT1↑HCs, CSF CHI3L1 for gALS, and gFTD↑HCs,MCs	All with pNH and NfL (in CSF)	GFAP	Microglial activity and astrogliosis. Neuroinflammation is common to gALS and sALS	
30215803	CHI3L1	IHC 11, ELISA 85, RT-qPCR 12 all sALS	IHC 23, ELISA 21, RT-qPCR 10			CSF, blood, spinal cord and frontal cortex PMT	RT-qPCR, IHC, ELISA, IB	PMT CHI3L1, CHI3L1 ↑HCs, CSF CHI3L1↑HCs	NfL	AIF1, CD68, IBA1, ChAT	Increased astroglial activity	Potential prognostic marker when used with Nfl
29142136	CHIT1	ELISA 60, IHC 3	ELISA 25, IHC 2	ELISA 46 NDCs and 135 NDegs, IHC NDegs 4		CSF, blood, spinal cord, PM	ELISA, IHC	CSF CHIT1↑HCs, NDCs, NDegs, Spinal cord, PM, CHIT1↑NDegs,HCs	pNH and NfL (↑ CSF)	IBA1, CD68	Microglia / macrophage activation	Biomarker for immune activation, can be used to monitor efficacy of immunomodulatory therapies
29331073	CHIT1, CHI3L1, CHI3L2	43	25	6 PLS, 12 mimics, 20 NDeg		CSF	High throughput VS. ELISA (for pNfH)	CHIT1, CHI3L2 ↑HCs,Mimics,NDegs,PLS, CHI3L1↑HCs,Mimics,NDeg	All with pNH		Microglial activity	Distinguishing between ALS and ALS mimic conditions
30134252	CHIT1	29	36			CSF and blood	ELISA, chemiluminescence, enzymatic activity assay	CSF CHIT1 activity↑HCs		CCL18, TNF-α, IL6, CHIT1	Microglial activation	Neuroinflammation biomarker
30291183	CHI3L1	38	49	86 FTD		CSF	ELISA	CHI3L1↑HCs	NfL	sAPPβ		Staging and prognosis along ALS–FTD spectrum
29863789	CHIT1	75	108			Blood	Enzymatic activity assay	CHIT1 activity↑ HCs		Lysosomal enzyme activity	Microglial activity and possible chronic triggering of the innate immune system	
27634542	CHIT1	40 sALS		40 NDCs		CSF	ELISA	CHIT1↑NDCs	pNfH	S100-β, Cystatin C		Improves diagnostic potential when used with pNfH

(Continued)

TABLE 1 | Continued

Study PMID	Study targets	Participants				Sample type	Methods used	Relative expression in ALS*	Correlation with neurofilament levels*	Other targets	Proposed bio mechanism	Proposed utility
		ALS	HCs	Mimics NDegs, NDCs	Other groups							
24265388	CHIT1, CHI3L1, CHI3L2	26 sALS	23			CSF	LC-MS/MS, ELISA, enzymatic activity assay	CHIT1, CHI3L2↑^{HCs}, CHIT activity ↑^{HCs}		Osteopontin	Microglial activity and potential response to deposition of chitin-like substances in CNS aggregates	

*Reported results were statistically significant.

AIF-1, Allograft inflammatory factor 1; asym, asymptomatic; CCL-18, Chemokine ligand 18; C-RP, C Reactive Protein; CSF, cerebrospinal fluid; ELISA, Enzyme-linked Immunosorbent assay; FTD, frontotemporal dementia; gALS, genetic Amyotrophic Lateral Sclerosis; GFAP, glial fibrillary acidic protein; gFTD, genetic Frontotemporal Dementia; HCs, Healthy Controls; IB, Immunoblotting; IHC, Immunohistochemistry; IL-6, interleukin 6; LC-MS, Liquid Chromatography Mass Spectrometry; MCs, Mutation Carriers; MCP-1, Monocyte chemoattractant protein 1; MS, Mass Spectrometry; NDCs, Neurological Disease Controls; NDegs, Neurodegenerative Disease Controls; NfL, neurofilament light chain; PLS, Primary Lateral Sclerosis; PMT, Post-mortem Tissue; pNfH, phosphorylated neurofilament heavy chain; RT-qPCR, Real-time Quantitative Polymerase Chain Reaction; sALS, sporadic ALS; sAPPβ, soluble amyloid precursor protein beta; sFTD, sporadic Frontotemporal Dementia; TNF-α, tumor necrosis factor alpha.

reported. It is worth noting, however, that studies have only now begun to examine CHIT1 enzymatic activity in addition to protein levels and that links between the periphery and prognostic factors, as reported by Pagliardini et al. (30), may yet emerge.

Links With Disease Severity and Progression

Evidence for a link with disease severity and progression has been tenuous at best. Martinez-Merino et al. (12) controlled for *CHIT1* genotype and reported that while ALS patients had significantly elevated CHIT1 activity, it correlated with neither disease severity nor progression. Thompson et al. (28) reported a significant albeit modest correlation between CHIT and CHI3L2 levels—but not CHI3L1—and PR after controlling for gender, age at onset, and site of onset; however, a stronger correlation was noted for pNfH. Conversely, Illán-Gala et al. (32) and Andres-Benito et al. (35) reported that CSF CHI3L1 levels correlated with PR to almost the same degree as CSF NfL levels.

Gille et al. (10) noted that both CSF CHIT1 and CHI3L1 only weakly correlated with PR at time of sampling; however, "fast" progressors had significantly higher levels of CHIT1 and CHI3L1 than "slow" progressors. One study reported that CSF CHIT1 also significantly correlated with both disease severity and PR (inversely) and to almost the same magnitude as NfL and pNfH. However, these correlations did not persist when patients were stratified based on PR, despite "fast" progressors having significantly higher levels of CHIT1 (33). Chen et al. (29) too reported no significant differences in CHIT1 levels between PR-stratified patients.

It is worth noting that establishing any association between the chitinases and PR is likely confounded by the lack of any external consensus on the thresholds for "high" or "low" PR. These are often arbitrarily set based on individual cohorts, thus constraining inter-study comparability and potentially occluding genuine biological signals.

Links With Disease Duration

Evidence for an association with disease duration has also been inconsistent, even by the few studies that have included longitudinal sampling. CSF CHIT1 activity did not significantly differ between patients stratified based on time since onset to sampling (12). A MS-based study reported a small increase in CSF CHI3L1 levels over time in patients who had low levels at onset (31). However, a subsequent ELISA-based verification noted that CSF chitinase levels in ALS and PLS patients did not significantly increase over a follow-up period of ∼2 years, even when patients were stratified by PR (28). Similarly, no significant associations between CSF CHIT1 and CHI3L1 and disease duration were observed in a cohort of 105 ALS patients (10). Indeed, evidence from asymptomatic ALS and FTD MCs suggests that chitinase elevation is a feature of the early symptomatic phase of the disease and is unlikely by itself to trigger disease onset, given that no significant differences were observed between patients with either genetic or sporadic disease (27).

Links With Survival and Mortality

Studies examining survival have also reported discrepant results. Di Rosa et al. analyzed microarray datasets and reported that patients with a shorter survival had significantly higher *CHI3L1* and *CHI3L2* in their motor cortex than those that survived longer; levels also inversely correlated with survival in the entire patient cohort (36). Cox proportional hazards analyses have also revealed a significant association between CSF CHIT1 levels and mortality, while one study reported that the association was independent of pNfH levels, another by the same group reported the opposite (28, 31). However, neither study had data on other prognostic factors, e.g., respiratory and *C9orf72* status, thus precluding a definitive conclusion on the influence of CHIT1. In contradiction, Gille et al. (10) reported that CSF CHI3L1, but not CHIT1, significantly affected mortality; this is compelling because they included data for eight established prognostic markers. The authors did not however compare how the chitinases performed relative to neurofilaments. Building on this, Illán-Gala et al. (32) also reported that increased CSF CHI3L1 levels were associated with shortened survival, even after adjustment for sex, age at onset and site of onset, NfL levels, and ALSFRS-R score at time of sampling. Taken together however, the currently available evidence doesn't unequivocally establish the degree to which the chitinases influence survival and whether they outperform established prognostic factors.

Links With Additional Indices

Although data are limited, some studies have also begun to examine a wider range of clinical outcomes; for instance, peripheral CHIT1 activity was significantly inversely correlated with forced vital capacity (30). Additionally, CSF CHIT1 and CHI3L1 levels correlated with the number of regions clinically affected by both UMN and LMN and only UMN degeneration, respectively (10, 28). Frontotemporal cortical thickness, as assessed by structural MRI, directly correlated with the CSF sAPPβ:CHI3L1 ratio in both ALS and FTD patients (32). Finally, whether chitinase levels also reflect the poorer outcomes associated with factors like bulbar onset or genetic status (e.g., C9orf72) needs further investigation.

CHITINASES ACROSS THE ALS-FTD SPECTRUM

Studies focusing on the broader ALS-FTD spectrum have noted that the two conditions present with specific chitinase dysregulation patterns. When examined alongside glial activation markers, these suggest different underlying inflammatory processes: increased microglial (as evidenced by CHIT1) and astroglial (as evidenced by CHI3L1) activation in ALS and FTD, respectively.

For instance, although CSF CHIT1 is elevated in FTD patients relative to both HCs and asymptomatic MCs, it is significantly higher in ALS patients (27, 33). Furthermore, CHIT1 immunostaining in post-mortem spinal cord tissue was observed only in ALS cases, where it co-localized with IBA1-positive microglia and CD68-positive macrophages, and not in other neurodegenerative disorders, including FTD and AD (27, 33). Conversely, despite considerable overlap, CSF CHI3L1 levels were higher in patients with sporadic FTD relative to those with sporadic ALS, albeit only slightly. CHI3L1 elevation also correlated with cognitive dysfunction, as assessed by the Edinburgh Cognitive and Behavioral ALS Screen (ECAS), suggesting that it skews more closely to the FTD phenotype (28). Illán-Gala et al. (32) reported that although neither absolute CSF CHI3L1 levels nor the sAPPβ:CHI3L1 ratio significantly differed between FTD and ALS patients, CHI3L1 and global cognitive performance only correlated in the FTD subgroup. Furthermore, a robust inverse correlation was noted between the sAPPβ:CHI3L1 ratio and the FTD-Clinical Dementia Rating score in FTD patients. CHI3L1 immunoreactivity has been observed in astrocytes, but not microglia and neurons; its expression correlates with GFAP, particularly in acute inflammatory conditions like multiple sclerosis, suggesting that CHI3L1 is indicative of reactive astrocytosis (18, 19, 37). Crucially, negligible CHI3L1-positive astrocytes were observed in post-mortem ALS cortical tissue and no significant differences in *GFAP* mRNA in the spinal cord were noted between ALS patients and HCs (18, 35). CSF GFAP levels were also significantly increased in FTD patients while they were unaffected in ALS patients (27).

In summary, while the chitinases may not be specific markers for either condition, they allude to distinct neuroinflammatory profiles. If corroborated by other modalities, e.g., PET imaging (38), these profiles could help delineate the underlying pathology and provide specific targets for immunomodulatory therapy.

CHITINASES IN THE BROADER NEUROINFLAMMATORY AND NEURODEGENERATIVE MILIEU

While much remains unknown about their cellular origin, it is evident that chitinase expression is not exclusive to ALS. It has been noted in multiple neurodegenerative conditions, where it predicts both clinical severity and long-term risk (39–41). The chitinases also robustly correlate with established neurodegenerative markers, including, e.g., the neurofilaments (10, 27, 31) and both total and phosphorylated tau (40, 42). Studies investigating multivariate panels have additionally reported close links to other inflammatory mediators. For instance, CSF chitinase levels correlated with MCP-1, and C-reactive protein in ALS patients and soluble TREM2 in cognitively unimpaired individuals (10, 28, 43). Transcriptomic studies have shown that *CHIT1* correlates with *IL-16, IL-18*, and *CHI3L1* and *CHI3L2* with complement *C1s* subcomponent (36, 41). Therefore, it is probable that the chitinases reflect the inflammation that is characteristic of the wider neurodegenerative process. Given the evidence from post-mortem co-localization studies and that significant dysregulations have been primarily observed in CSF rather than blood, we further speculate that the chitinases are proxies for reactive gliosis. It is worth noting, however, that systemic conditions may also influence chitinase levels, potentially "masking" alterations in blood.

While there is considerable overlap between neurodegenerative conditions, expression patterns differ, underscoring the different pathomechanisms at play; for instance, while CSF CHI3L1 increases as cognitive deficits worsen along the AD continuum, no similar associations have been noted with the ALSFRS-R, the primary indicator of disease severity in ALS (40). However, limitations with using the ALSFRS-R and derived parameters have been previously described (44). Instead, disease progression models could be particularly informative, as they allow interpretation of biomarker profiles within the disease course.

It is also imperative to expand beyond studying the chitinases as just fold changes within a case–control paradigm, given the evidence that they act as active immune modulators rather than just passive indicators of pathology. For instance, TNF-α, LPS, and IFN-γ stimulation increased both *CHIT1* expression and activity in human macrophages (45). Conversely, CHIT1, CHI3L1, and AMCase stimulation increased the transmigratory capacity of leukocytes from patients with multiple sclerosis (46).

In conclusion, studies should address how immune activation—vis-à-vis chitinase elevation—presents across the ALS disease course, whether it differs between glial cell types and what the functional consequences are. Studies also need to account for physiological aging, given multiple reports that it influences chitinase levels (27, 31, 47).

CONCLUSIONS AND FUTURE DIRECTIONS

What can be concluded of the chitinases holds true for all biomarkers; no single molecule can capture all the pathogenic processes at play in a disease as heterogeneous as ALS. This is particularly relevant in the case of inflammatory markers: these cannot be viewed in isolation because of their functional abundance and intricate signaling networks. It is the interaction with the disease microenvironment and the interplay between different cell types that drives pathology, rather than the singular action of a specific target. Multivariate biomarker panels are more likely to capture the dynamic immune signatures associated with different functional disease phases and identify optimal treatment windows and patients who would most benefit from immunomodulatory therapies. Therefore, the chitinases represent valuable additions to the current immuno-biomarker repertoire; while their diagnostic and prognostic efficacy is unlikely to supersede that of the neurofilaments, they can assist with subtle distinctions between different neurodegenerative conditions and delineate the mechanisms underlying glial dysregulation. Additional mechanistic studies could focus on how the chitinases reflect the dynamicity of glial cell responses across the disease. For instance, current evidence already indicates that the chitinases reflect a neuroinflammatory component that is common to both genetic and sporadic forms of ALS (27). Future prospective studies could focus on recruiting MCs and following them as they transition to clinical disease to better understand how chitinase elevation manifests, what triggers it, and how it relates to other modalities.

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6.2 Cerebrospinal Fluid Neurofilament Light Chain (NfL) Predicts Disease Aggressiveness in Amyotrophic Lateral Sclerosis: An Application of the D50 Disease Progression Model ((**Frontiers in Neuroscience, April 2021)**

This study provides an **1)** in-depth explanation of the D50 model, **2)** its parameters and how they relate to traditional metrics and **3)** how it can be used to evaluate candidate biomarkers. We focused on NfL as it is an established marker of neuronal damage; it was assayed in the CSF of 156 patients with ALS and the model was used to assess its prognostic utility. We showed that NfL was significantly associated with disease aggressiveness independent of several co-variates, most significantly functional disease phase. Here, we also used the D50 model to introduce the critical concept of the "sampling shift" in ALS.

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ORIGINAL RESEARCH
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Cerebrospinal Fluid Neurofilament Light Chain (NfL) Predicts Disease Aggressiveness in Amyotrophic Lateral Sclerosis: An Application of the D50 Disease Progression Model

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Amyotrophic lateral sclerosis (ALS) is a relentlessly progressive neurodegenerative disorder. As previous therapeutic trials in ALS have been severely hampered by patients' heterogeneity, the identification of biomarkers that reliably reflect disease progression represents a priority in ALS research. Here, we used the D50 disease progression model to investigate correlations between cerebrospinal fluid (CSF) neurofilament light chain (NfL) levels and disease aggressiveness. The D50 model quantifies individual disease trajectories for each ALS patient. The value D50 provides a unified measure of a patient's overall disease aggressiveness (defined as time taken in months to lose 50% of functionality). The relative D50 (rD50) reflects the individual disease covered and can be calculated for any time point in the disease course. We analyzed clinical data from a well-defined cohort of 156 patients with ALS. The concentration of NfL in CSF samples was measured at two different laboratories using the same procedure. Based on patients' individual D50 values, we defined subgroups with high (<20), intermediate (20–40), or low (>40) disease aggressiveness. NfL levels were compared between these subgroups via analysis of covariance, using an array of confounding factors: age, gender, clinical phenotype, frontotemporal dementia, rD50-derived disease phase, and analyzing laboratory. We found highly significant differences in NfL concentrations between all three D50 subgroups ($p < 0.001$), representing an increase of NfL levels with increasing disease aggressiveness. The conducted analysis of covariance showed that this correlation was independent of gender, disease phenotype, and phase; however, age, analyzing laboratory, and dementia significantly influenced NfL concentration. We could show that CSF NfL is independent of patients' disease covered at the time of sampling. The present study provides strong evidence for the potential of

NfL to reflect disease aggressiveness in ALS and in addition proofed to remain at stable levels throughout the disease course. Implementation of CSF NfL as a potential read-out for future therapeutic trials in ALS is currently constrained by its demonstrated susceptibility to (pre-)analytical variations. Here we show that the D50 model enables the discovery of correlations between clinical characteristics and CSF analytes and can be recommended for future studies evaluating potential biomarkers.

Keywords: amyotrophic lateral sclerosis, neurofilaments, NfL, cerebrospinal fluid, prognostic biomarker

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder that is predominately characterized by the progressive loss of motor neuron function. The clinical presentation of the disease varies significantly among patients, with atrophy and weakness as well as spasticity and fasciculations in limb, bulbar, and thoracic muscles. Despite constant efforts to develop new disease-modifying therapies, survival for most patients with ALS is still restricted to 2–5 years after symptom onset (Paganoni et al., 2014).

As phenotypic variability and disease course variability represent major constraints to clinical management and therapeutic trials in ALS, the search for biomarkers that can accurately predict progression is a current research priority. Previous therapeutic trials predominantly employed clinical measures such as long-term survival rates and linearly approximated declines of the ALS Functional Rating Scale-Revised (ALSFRS-R) as outcome measures (Petrov et al., 2017). The detection of significant treatment effects in these trials requires large sample sizes and consumes time and resources, which could be improved by specific pharmacodynamic or prognostic/predictive biomarkers. The importance of such biomarkers has been underlined in the recently revised Airlie House consensus criteria for clinical trial development in ALS (Van Den Berg et al., 2019).

Cerebrospinal fluid (CSF) neurofilaments are promising candidate biomarkers with prognostic implications in ALS. Neurofilaments constitute the main structural components of motor axons. Following neuroaxonal damage, increased concentrations of neurofilament light chain (NfL) and phosphorylated neurofilament heavy chain (pNfH) have been reported in both CSF and blood in various neurologic disorders (Khalil et al., 2018). While CSF pNfH has demonstrated greater diagnostic accuracy (Poesen et al., 2017), the concentration of NfL in the CSF of ALS patients reportedly correlates with both survival (Zetterberg et al., 2007; Pijnenburg et al., 2015; Gaiani et al., 2017; Gong et al., 2018; Illán-Gala et al., 2018; Rossi et al., 2018; Scarafino et al., 2018; Schreiber et al., 2018; Kasai et al., 2019; Abu-Rumeileh et al., 2020) and the disease progression rate (Tortelli et al., 2012; Lu et al., 2015; Menke et al., 2015; Steinacker et al., 2016, 2018b; Gaiani et al., 2017; Poesen et al., 2017; Andres-Benito et al., 2018; Gong et al., 2018; Rossi et al., 2018; Scarafino et al., 2018; Schreiber et al., 2018; Abu-Rumeileh et al., 2020). These findings suggest that CSF NfL concentrations at baseline may allow early stratification of patients in clinical trials according to anticipated progressiveness, thereby reducing clinical heterogeneity and enabling the detection of significant treatment effects even in smaller ALS patient cohorts.

However, the exact role of NfL in ALS is not yet entirely understood, and several challenges hamper its routine use as a biomarker in clinical trials. CSF NfL has been reported to correlate not only with the rate of disease progression but also with the clinical status at the time of lumbar puncture, as assessed by clinical scores or imaging measures of disease severity (Tortelli et al., 2012; Steinacker et al., 2016, 2018b; Gong et al., 2018; Scarafino et al., 2018). This raises the question of whether CSF NfL reflects cumulative neuroaxonal damage rather than the rate of neuroaxonal breakdown. As patients with faster disease courses have typically reached a more advanced disease stage at the time of sampling (sampling shift), these factors are inextricably intertwined in ALS patient cohorts. The temporal profile of CSF NfL throughout the disease course remains to be more precisely elucidated. The few available longitudinal studies on CSF NfL in patients with ALS comprised rather small sample sizes and reported inconsistent results (Lu et al., 2015; Steinacker et al., 2016; Poesen et al., 2017; Skillbäck et al., 2017; Benatar et al., 2018; Huang et al., 2020). Furthermore, the concentration of NfL in the CSF may be influenced by several other factors, including the presence of frontotemporal dementia (FTD) (Illán-Gala et al., 2018; Steinacker et al., 2018a), different ALS genotypes (Zetterberg et al., 2007; Huang et al., 2020), or the predominant affection of upper motor neurons (UMNs) rather than lower motor neurons (LMNs) (Rosengren et al., 1996; Gaiani et al., 2017; Schreiber et al., 2018).

An additional concern is the interlaboratory variation of CSF NfL measurements (Oeckl et al., 2016; Gray et al., 2020), as validation of biomarkers and translation into clinical trials require multicenter confirmation.

In an attempt to address the mentioned uncertainties regarding the prognostic role of CSF NfL in ALS, we applied the D50 disease progression model (Poesen et al., 2017; Prell et al., 2019; Steinbach et al., 2020) in a large-scale cross-sectional cohort. As the model addresses the phenotypic heterogeneity inherent to the disease and reduces the noise associated with the ALSFRS-R, this approach may help uncover the effect of

Abbreviations: ALS, amyotrophic lateral sclerosis; ALSFRS-R, Amyotrophic Lateral Sclerosis Functional Rating Scale-Revised; ANCOVA, analysis of covariance; CSF, cerebrospinal fluid; ELISA, enzyme-linked immunosorbent assay; FTD, frontotemporal dementia; LMN, lower motor neuron; MiToS, Milano–Torino Staging System; NfL, neurofilament light chain; pNfH, phosphorylated neurofilament heavy chain; rD50, relative D50; SD, standard deviation; UMN, upper motor neuron.

disease aggressiveness on CSF neurofilament levels in a clinically diverse ALS patient cohort, while simultaneously controlling for the potential influence of disease accumulation at the time of sampling.

MATERIALS AND METHODS

Participants

All participants were recruited from the neuromuscular center at the University Hospital of Jena, Germany, between 2013 and 2020. The participants provided written and informed consent prior to study commencement, and the study was approved by the local ethics committee (Nr 3633-11/12). Two hundred seventy-three participants with available CSF NfL measurements were identified from our local specialized neuromuscular disease database. Based on clinical disease histories, a total of 238 participants could be allocated to one of the four following condition groups: (a) non-neurological controls (n = 15); (b) disease controls (n = 56), suffering from neurologic disorders other than ALS; (c) ALS mimics (n = 11), with other conditions that shared symptomatology with an ALS disease course; and (d) patients with ALS (n = 156) (**Supplementary Table 1**). Of the initial 185 ALS patients, 29 patients were excluded, either because fewer than two ALSFRS-R assessments were available (n = 16), or because the Gold Coast criteria for the diagnosis of ALS (Shefner et al., 2020) were not fulfilled (n = 13). From a total of 62 disease controls, six were excluded because of an uncertain diagnosis (n = 5) or acute intracranial bleedings (n = 1).

Diagnosis and Phenotypic Characterization of Patients With ALS

One hundred fifty-six patients fulfilled the recently defined Gold Coast criteria for the diagnosis of ALS at the time of CSF sampling (Shefner et al., 2020) and had a minimum of two ALSFRS-R scores obtained throughout the disease course. According to the revised El Escorial criteria at the time of lumbar puncture, ALS patients had either suspected, possible, laboratory-supported probable, probable, or definite ALS (Brooks et al., 2000). According to the evaluation of the entire disease history of these patients, they presented with one of the following clinical phenotypes: classic, bulbar, pyramidal, flail arm, flail leg, or respiratory or pure LMN, according to the classification by Chió et al. in 2011 (Chiò et al., 2011). The diagnosis of clinically overt frontotemporal dementia (FTD) was made by experienced neurologists at the University Hospital Jena based on clinical observations. All 6 patients diagnosed with FTD fulfilled the original Strong diagnostic criteria for the diagnosis of FTD (Strong et al., 2009, 2017).

We also estimated the number of regions (bulbar, cervical, thoracic, or lumbar) with UMN and/or LMN involvement at the time of CSF sampling. The four regions were evaluated clinically and electromyographically according to the revised El Escorial and Awaji criteria (Brooks et al., 2000; de Carvalho et al., 2008). Hence, ALS patients were divided into categories of one (none or one region), two (two regions), or three (three or four regions) regions affected by UMN and/or LMN degeneration. ALS patients were also classified according to (a) the King's staging system (Roche et al., 2012) and (b) the Milano–Torino Staging System (MiToS) (Chio et al., 2015), both calculated using the ALSFRS-R closest to the time of CSF sampling. The King's staging system allocates patients to stages I (involvement of one clinical region) to IV (respiratory or nutritional failure), whereas the MiToS System describes stages 0 (functional involvement) to IV (loss of independence in four domains) (Roche et al., 2012; Chio et al., 2015).

The D50 Disease Progression Model

To assess the impact of clinical characteristics of patient's ALS disease course on CSF NfL, the D50 disease progression model was applied (Poesen et al., 2017; Prell et al., 2020; Steinbach et al., 2020). The D50 model was chosen because it provides quantitative measures of disease aggressiveness, distinct from parameters of disease accumulation, and thus provides a framework to interpret associations with any biomarker (**Figure 1**). The model uses regularly assessed ALSFRS-R scores of each individual patient to calculate a sigmoidal state transition from full health to functional loss. Here, we applied an adaptation of the model that allows a variable presymptomatic phase of supratotal functionality up to 6 months prior to symptom onset. This approach accounts for the known uncertainties in the exact time point of first symptoms as reported by the patients, as well as a presymptomatic breakdown of motoric functional reserves. The resulting sigmoidal curve can be characterized by (a) the value D50, which describes the time taken in months from symptom onset to reach halved functionality, and (b) the dx, the time constant of functional decline. Because dx and D50 correlate linearly (**Figure 1C**), the D50 value alone provides a meaningful descriptor of patients' overall disease aggressiveness.

The ALS patient cohort could thus be divided into three groups of (a) high ($0 \leq D50 < 20$), (b) intermediate ($20 \leq D50 < 40$), and (c) low ($40 \leq D50$) disease aggressiveness. A normalization of patient's real-time sigmoidal disease trajectory to D50 yields the parameter relative D50 (rD50), an open-ended reference scale where 0 signifies disease onset and 0.5 the time point of halved functionality (**Figure 1B**). The rD50 provides an individualized time scale of accumulated disease (independent of disease aggressiveness) and was calculated for the individual time point of lumbar puncture. Patients with ALS could thus also be grouped into one of the following three phases: the early semistable phase I ($0 \leq rD50 < 0.25$), the early progressive phase II ($0.25 \leq rD50 < 0.5$), and the late progressive and stable phase (III/IV) ($0.5 \leq rD50$).

For comparability with former studies, we also calculated the more traditionally used linear disease progression rate at the time of CSF sampling, defined as (48 - ALSFRS-R at sampling)/disease duration in months (**Figure 1E**).

CSF Collection and Analysis

All CSF samples were collected via lumbar puncture at the Department of Neurology, Jena University Hospital. The samples were centrifuged, aliquoted, and stored at −80°C within 2 h after lumbar puncture. NfL concentration was assessed using the commercially validated IBL International enzyme-linked

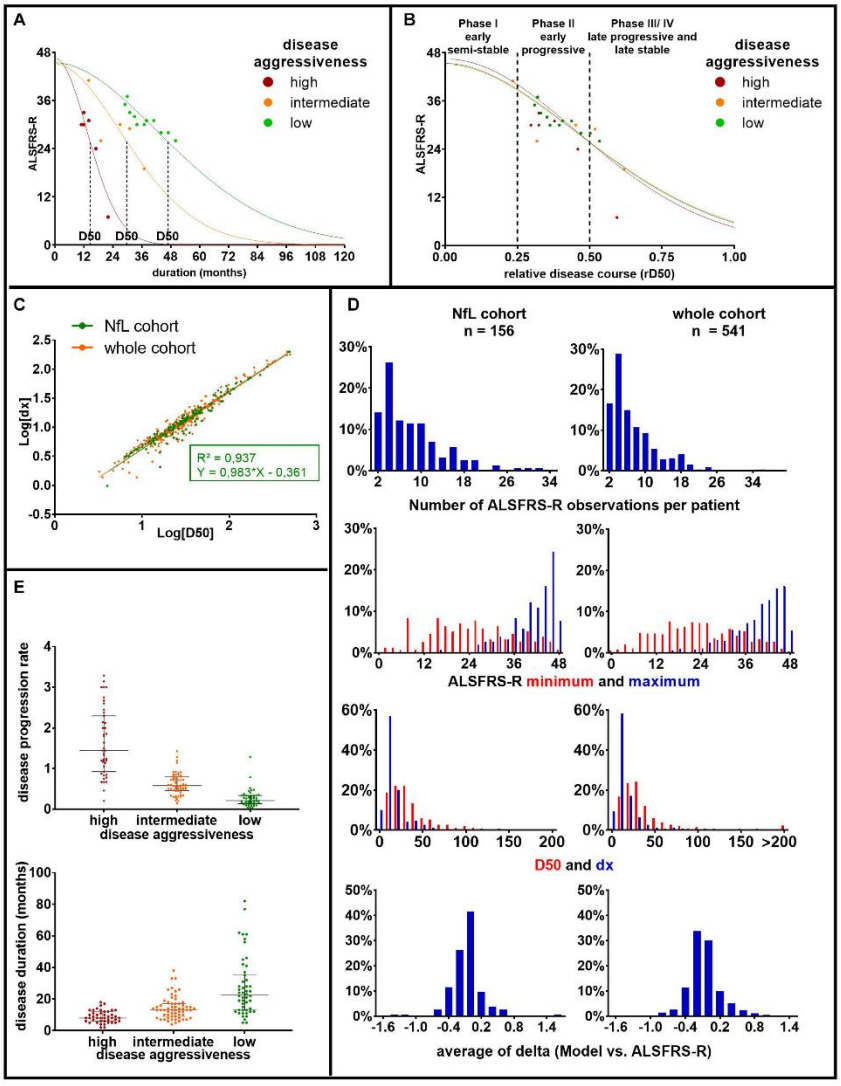


FIGURE 1 | Principles and parameters of the D50 model: **(A)** based on consecutively obtained ALSFRS-R scores (dots), a sigmoidal functional decline curve is calculated. The value D50 depicts the individual time in months since symptom onset until halved functionality, indicating the overall disease aggressiveness of each individual patient. The curves represent three example patients with either high (D50 = 14.56 months, in red), intermediate (D50 = 29.88 months, in orange), or low disease aggressiveness (D50 = 46.84 months, in green). **(B)** Normalization of patient's individual sigmoidal curves by D50 yields the parameter relative D50 (rD50). rD50 describes the individual disease covered and facilitates the comparison of vastly differing progression types. **(C)** The parameter D50 linearly correlates with the time constant of ALSFRS-R decline (dx) in this, as well as in other ALS cohorts. Thus, D50 alone can be used to describe patients' disease aggressiveness. **(D)** Histograms of pertinent disease variables for the patients of the current cohort (NfL cohort), as well as all ALS patient data available in our center (whole cohort). It illustrates that the current cohort well coincides with the entire ALS patient cohort treated at our center. **(E)** Scatterplots of patients' disease progression rate and disease duration at the time of sampling, subdivided by the three D50 subgroups in our cohort: (a) high ($0 \leq D50 < 20$, in red), (b) intermediate ($20 \leq D50 < 40$, in orange), and (c) low ($40 \leq D50$, in green) disease aggressiveness. It illustrates large variations of the disease progression rate, especially within the high aggressive subgroup. Bars indicate median and interquartile range. ALS, amyotrophic lateral sclerosis; ALSFRS-R, Amyotrophic Lateral Sclerosis Functional Rating Scale-Revised; rD50, relative D50; NfL, neurofilament light chain.

immunosorbent assay (ELISA) kit at two different European laboratories: (a) in Germany (n = 140) and (b) in Belgium (n = 99). All samples and standards were assayed in duplicate and in accordance with manufacturer instructions; intra-assay and interassay variations were ≤10%, and ≤20%, respectively.

Statistical Analysis

Statistical analyses were performed using the SPSS® Statistics software program (v27.0.0.0 IBM®, Chicago, IL, United States). For graphical representation of data, GraphPad Prism was used (v9.0.0 for Windows, GraphPad Software, San Diego, CA, United States). Normal distribution of variables was assessed with the Shapiro–Wilk test. Normal distribution of NfL concentration was achieved via log transformation, and log[NfL] was used for parametric testing. Two-sample t-tests were used for comparison of Log[NfL] concentrations between ALS patients and control groups. Receiver operating characteristic curves were used to calculate the sensitivity and specificity of CSF NfL for differentiating ALS from the control groups. The optimal cutoff was calculated with the Youden Index.

To evaluate differences in NfL concentrations between different ALS subgroups, a one-way analysis of covariance (ANCOVA) was applied, followed by pairwise *post hoc* tests with Bonferroni correction. For the comparison of low, intermediate, and high disease aggressiveness subgroups, the following covariates were applied: age, sex, FTD, laboratory of NfL measurement, clinical phenotype, and disease phase.

In our ALS cohort, a significant sampling shift occurred, which was previously observed in other cohorts analyzed using the D50 model (**Table 1**): patients with slow and intermediate progression were still in the earlier phases of the disease at the time of sampling, whereas patients with fast progression had already reached later disease phases by the time they were referred to our center, and lumbar puncture was performed. Therefore, the covariate disease phase did not meet the assumption for ANCOVA of homogenous distribution over the three subgroups. We therefore conducted an additional ANCOVA in a filtered ALS cohort, in which patients of all disease phases were equally distributed throughout the three aggressiveness subgroups (**Supplementary Table 2**).

A one-way ANCOVA was conducted to compare CSF Log[NfL] concentration of the three disease phases, while controlling for disease aggressiveness, FTD, clinical phenotype, age, gender, and laboratory of measurement.

Linear regression analysis and Spearman correlation was used to assess correlations between NfL, D50, and rD50 at the time of sampling. Pearson correlation was used to assess correlation between paired Log[NfL] measurements from the two centers in Germany and Belgium. Differences between CSF NfL concentrations of ALS patients with and without FTD were tested with a Mann–Whitney U test.

For survival analyses, ALS patients were divided into three groups with low (Log[NfL] < 3.651), intermediate (3.651 ≤ Log[NfL] < 4.149), and high (4.149 ≤ Log[NfL]) CSF NfL concentrations, with cutoffs derived from the estimated marginal means of our previously described ANCOVA (comparing disease aggressiveness subgroups). The Kaplan–Meier method was used for survival analyses, and subgroups were compared with a log–rank test. 97 patients (13 with low, 51 with intermediate, and 33 with high CSF NfL levels) reached the endpoint death or tracheostomy, whereas the remaining 59 patients were censored. Statistical significance was defined as $p < 0.05$.

RESULTS

Diagnostic Performance of CSF NfL in ALS

Cerebrospinal fluid Log[NfL] levels were significantly higher in the ALS group (mean = 3.87, SD = 0.37) as compared to the non-neurological control (mean = 2.72, SD = 0.27, $p < 0.001$), disease control (mean = 3.18, SD = 0.38, $p < 0.001$), and ALS mimic groups (mean = 3.20, SD = 0.19, $p < 0.001$). When distinguishing ALS from disease controls, the area under the curve (AUC) was 0.895 (0.849–0.9413), sensitivity was 87.8%, and specificity was 78.6% at a cutoff of 2,946.00 pg/mL. For the differentiation between ALS and ALS mimics, the AUC was 0.941 (0.897–0.985), sensitivity was 91.0%, and specificity was 90.9% at a cutoff of 2,259.55 pg/mL. A cutoff of 1,620.5 pg/mL distinguished ALS patients from non-neurological controls with a sensitivity of 96.15% and specificity of 100% [AUC = 0.993 (0.984–1.002)] (**Figure 2**).

Cerebrospinal fluid NfL levels did not significantly differ between different ALS phenotypes [$F(6,149) = 0.925$, $p = 0.479$]. Patients with FTD had significantly higher CSF NfL levels relative to those without FTD ($U = 208.0$, $Z = -2.23$, $p < 0.05$).

Cohort of Patients With ALS

Detailed demographic and clinical data of ALS patients are shown in **Table 1**. Age, gender, and laboratory of analysis did not significantly differ between patients with high, intermediate, or low disease aggressiveness. The rD50 at the time of lumbar puncture, as well as the rD50-derived disease phase, showed significant differences between these three subgroups, as patients with lower disease aggressiveness were still in the earlier phases of the disease due to the sampling shift. Accordingly, the more traditionally used disease metrics, namely, the ALSFRS-R, the King's and MiToS stages, the stage of diagnostic certainty according to the revised El Escorial criteria (Brooks et al., 2000), the disease duration (time between symptom onset and lumbar puncture), and the disease progression rate, differed significantly between the three subgroups. Other disease characteristics, such as ALS phenotype, presence of FTD, or Riluzole intake, were homogenously distributed throughout the three subgroups.

CSF NfL Predicts Disease Aggressiveness

The ANCOVA showed a significant main effect for CSF Log[NfL] (pg/mL) concentrations of the three disease aggressiveness subgroups [$F(2,147) = 30.055$, $p < 0.001$]. *Post hoc* pairwise comparisons of the estimated marginal means showed that CSF Log[NfL] was highest in the highly aggressive disease subgroup

TABLE 1 | Demographic and clinical data for patients with ALS (n = 156).

		Disease aggressiveness			p
		High (D50 < 20)	**Intermediate (20 ≤ D50 < 40)**	**Low (D50 ≥ 40)**	
n		43	61	52	
Neurofilament light chain (NfL) measurements					
NfL (pg/mL)[$]		14,500.0 (7,883.0–24,680.0)	8,959.67 (4,410.5–12,157.5)	4,426.69 (2,879.5–7,333.5)	<0.001[˜]
Laboratory: Germany/Belgium		29/14 67.4%/32.6%	35/26 57.4%/42.6%	40/12 76.9%/23.1%	0.253
Demographics					
Age at lumbar puncture		64.3 ± 9.54	63.33 ± 10.47	61.42 ± 10.95	0.384
Gender:Male/female		23/20 53.5%/46.5%	37/24 60.7%/39.3%	34/18 65.4%/34.6%	0.514
D50 disease progression model parameters					
D50[$]		13.62 (9.40–16.14)	28.81 (23.07–31.73)	62.58 (46.12–96.61)	–
rD50[$]		0.37 (0.23–0.45)	0.23 (0.17–0.32)	0.18 (0.10–0.32)	<0.001[˜]
Phase	I (rD50 < 0.25)	11 (25.6%)	32 (52.2%)	33 (63.5%)	0.001[*]
	II (0.25 ≤ rD50 < 0.5)	27 (62.8%)	27 (44.3%)	19 (36.5%)	
	III/IV (rD50 ≥ 0.5)	5 (11.6%)	2 (3.3%)	0 (0%)	
Traditional disease metrics					
ALSFRS-R at lumbar puncture[$]		35 (29–40)	41 (38.50–44)	42 (39.25–45.75)	<0.001[˜]
Disease progression rate[$]		1.64 (1.05–2.30)	0.60 (0.46–0.74)	0.21 (0.13–0.33)	<0.001[˜]
Disease duration at lumbar puncture (mo)[$]		8 (2–18)	13 (4–38)	23.50 (5–212)	<0.001[˜]
King's stage	I	10 (23.3%)	20 (32.8%)	21 (40.4%)	0.008[*]
	II	11 (25.6%)	24 (39.9%)	24 (46.2%)	
	III	17 (39.5%)	12 (19.7%)	7 (13.5%)	
	IV a	3 (7%)	1 (1.6%)	0 (0%)	
	IV b	2 (4.7%)	4 (6.6%)	0 (0%)	
	V	0 (0%)	0 (0%)	0 (0%)	
MiToS stage	0	21 (48.8%)	52 (85.2%)	46 (88.5%)	<0.001[˜]
	I	18 (41.9%)	7 (7%)	6 (11.5%)	
	II	4 (9.3%)	2 (3.3%)	0 (0%)	
	III–V	0 (0%)	0 (0%)	0 (0%)	
ALS phenotype	Classic	21 (48.8%)	38 (62.3%)	33 (63.5%)	0.058
	Bulbar	18 (41.9%)	19 (31.1%)	9 (17.3%)	
	Pyramidal	3 (7%)	4 (6.6%)	5 (9.6%)	
	Respiratory	1 (2.3%)	0 (0%)	0 (0%)	
	Flail arm	0 (0%)	0 (0%)	3 (5.8%)	
	Flail leg	0 (0%)	0 (0%)	1 (1.9%)	
	Pure LMN	0 (0%)	0 (0%)	1 (1.9%)	
Revised El Escorial criteria	Definitive	10 (23.3%)	3 (4.9%)	1 (1.9%)	<0.001[˜]
	Probable	22 (51.2%)	33 (54.1%)	19 (36.5%)	
	Laboratory-supported probable	8 (18.6%)	20 (32.8%)	18 (34.6%)	
	Possible	3 (7%)	3 (4.9%)	9 (17.3%)	
	Suspected	0 (0%)	2 (3.3%)	5 (9.6%)	
Presence of FTD: yes/no		2/41 4.7%/95.3%	3/58 4.9%/95.1%	1/51 1.9%/98.1%	0.671

(Continued)

TABLE 1 | Continued

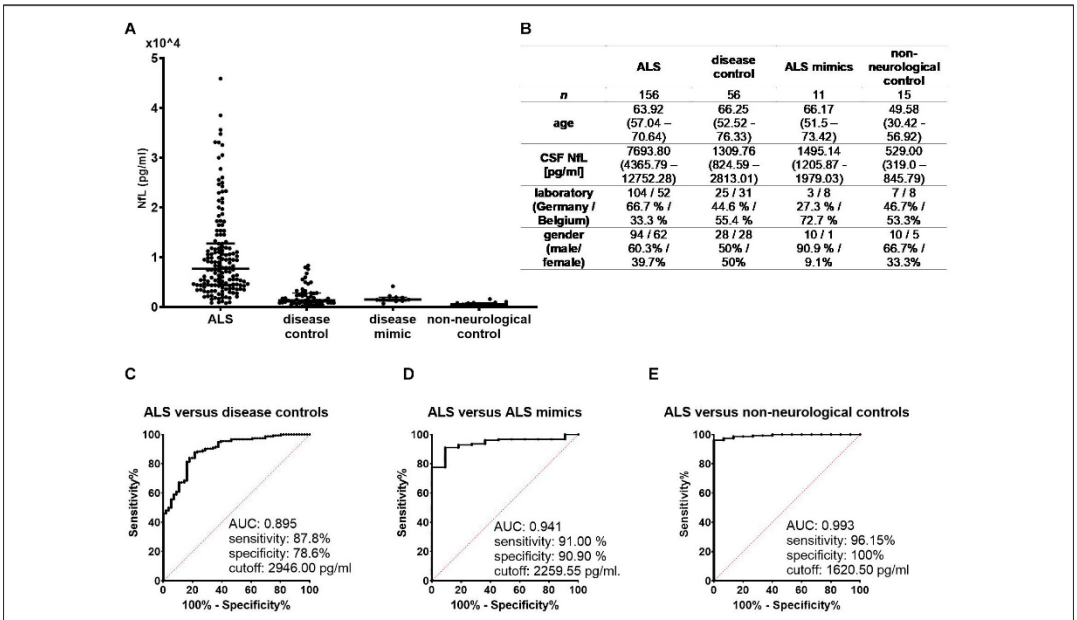
	Disease aggressiveness			*p*
	High (D50 < 20)	**Intermediate (20 ≤ D50 < 40)**	**Low (D50 ≥ 40)**	
Riluzole treatment: yes/no	42/1 97.7%/2.1%	60/1 98.4%/1.6%	49/3 94.2%/5.8%	0.671

Continuous variables with normal distribution are expressed as mean with standard deviation. Categorical variables are expressed as number and percentage. For the comparison of demographic and clinical variables among the three aggressiveness subgroups, analyses of variance, Kruskal–Wallis tests, χ^2 tests, or Fisher–Freeman–Halton exact tests were applied where appropriate.

$Non-parametric nominal variables, represented as median and interquartile range.

**Statistical significance at $p < 0.05$.*

ALS, amyotrophic lateral sclerosis; ALSFRS-R, Revised ALS Functional Rating Scale; FTD, frontotemporal dementia; LMN, lower motor neuron; MiToS, Milano–Torino Staging System; NfL, neurofilament light chain; rD50, relative D50.



	ALS	disease control	ALS mimics	non-neurological control
n	156	56	11	15
age	63.92 (57.04 – 70.64)	66.25 (52.52 - 76.33)	66.17 (51.5 – 73.42)	49.58 (30.42 - 56.92)
CSF NfL [pg/ml]	7693.80 (4365.79 – 12752.28)	1309.76 (824.59 – 2813.01)	1495.14 (1205.87 - 1979.03)	529.00 (319.0 – 845.79)
laboratory (Germany / Belgium)	104 / 52 66.7 % / 33.3 %	25 / 31 44.6 % / 55.4 %	3 / 8 27.3 % / 72.7 %	7 / 8 46.7% / 53.3%
gender (male/ female)	94 / 62 60.3% / 39.7%	28 / 28 50% / 50%	10 / 1 90.9 % / 9.1%	10 / 5 66.7% / 33.3%

FIGURE 2 | (A) NfL concentrations in cerebrospinal fluid were significantly higher in the ALS group compared to each control group ($p < 0.001$ for each pairwise comparison). **(B)** Demographic and clinical data of the four condition groups are expressed as either median with interquartile range or as number and percentages. Receiver operating characteristic curves illustrate the diagnostic performance of NfL in distinguishing ALS from disease controls **(C)**, ALS mimics **(D)**, and non-neurological controls **(E)**. ALS, amyotrophic lateral sclerosis; AUC, area under the curve; NfL, neurofilament light chain.

(mean = 4.149), lower in the intermediate aggressiveness subgroup (mean = 3.857) and lowest in patients with low disease aggressiveness (mean = 3.651; $p < 0.001$ for all pairwise comparisons) **(Figure 3)**. The covariates age, [$F(1,147) = 12.451$, $p < 0.001$], laboratory of analysis [$F(1,147) = 13.748$, $p < 0.001$], and FTD [$F(1,147) = 6.176$, $p = 0.014$] were also significantly related to CSF Log[NfL], whereas gender, disease phenotype, and phase showed no impact.

The main effect of disease aggressiveness on Log[NfL] remained in a similar ANCOVA for the filtered cohort (with homogenous distribution of disease phases over the three aggressiveness subgroups). Most importantly, the disease phase did not have a significant effect on Log[NfL] concentrations (**Supplementary Table 3**).

There was a negative correlation between the D50 parameter and CSF NfL ($p < 0.001$, $\rho = -0.553$) **(Figure 4A)**. The linear regression analysis showed that 31.3% of the variation in CSF NfL can be explained by the D50 parameter ($R^2 = 0.313$, Log[NfL] = 4.734 - 0.581 × Log[D50], $p < 0.001$). This correlation remained significant when analyzing patients in disease phases I and II separately (phase I: n = 76, $p < 0.001$, $\rho = -0.528$, phase II: $n = 73$, $p < 0.001$, $\rho = -0.521$). Patients in

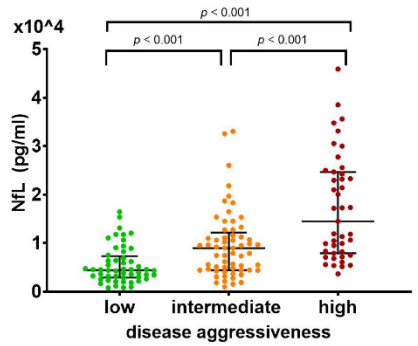


FIGURE 3 | CSF NfL differs significantly between patients with (a) high ($0 \leq$ D50 < 20, in red), (b) intermediate ($20 \leq$ D50 < 40, in orange), and (c) low ($40 \leq$ D50, in green) disease aggressiveness. This effect remained significant after controlling for clinical phenotype, presence of frontotemporal dementia, age, gender, disease phase, and laboratory of measurement in an ANCOVA ($p < 0.001$). *Post hoc* pairwise comparisons of the estimated marginal means confirmed an increase of NfL levels with increasing disease aggressiveness (low: 4,477.13, intermediate: 7,194.49, high: 14,092.89; $p < 0.001$ for all pairwise comparisons). Bars indicate median and interquartile range. ANCOVA, analysis of covariance; NfL, neurofilament light chain.

phase III/IV showed a similar tendency of negative correlation, but did not reach statistical significance, most likely due to the small sample size ($n = 7$, $p < 0.337$, $\rho = -0.429$).

CSF NfL Is Independent of Disease Phase and Number of Affected Regions

There was no significant main effect of disease phase on Log[NfL] concentrations [$F(2,147) = 1.692$, $p = 0.188$] in the respective ANCOVA, but the covariates disease aggressiveness $F(1,147) = 61.032$, $p < 0.001$), age [$F(1,147) = 13.603$, $p < 0.001$], laboratory of analysis [$F(1,147) = 13.927$, $p < 0.001$], and FTD [$F(1,147) = 6.284$, $p = 0.013$] showed a significant impact.

For the whole ALS patient cohort, a correlation between CSF NfL and rD50 was noted ($p = 0.005$, $\rho = 0.224$); however, this did not retain significance when stratifying patients into the three D50 subgroups (**Figure 4B**). This calculated correlation of CSF NfL with rD50 is thus likely attributable to the aforementioned cohort-specific intercorrelation between the parameters rD50 and D50, resulting from the sampling shift ($p < 0.001$, $\rho = -0.432$) (**Supplementary Figure 1**).

There were no significant differences in the CSF Log[NfL] concentration when stratifying patients according to the number of regions with UMN [$F(2,153) = 2.858$, $p = 0.060$] or LMN [$F(2,153) = 0.659$, $p = 0.519$] involvement at the time of sampling. Also, in combination, the number of regions with UMN and/or LMN affection did not have a significant effect on the CSF Log[NfL] concentrations [$F(2,153) = 1.403$, $p = 0.249$] (**Supplementary Table 4**).

CSF NfL Predicts Survival in Patients With ALS

Kaplan–Meier survival curves and log–rank tests showed significant differences in survival [$\chi^2(2) = 56.505$, $p < 0{,}001$], when trichotomizing the ALS patients into groups with high ($n = 36$), intermediate ($n = 77$), and low ($n = 43$) CSF Log[NfL] concentrations based on disease aggressiveness–adjusted marginal means (**Figure 5**).

Interlaboratory Variation and Paired Sample Comparison

Cerebrospinal fluid samples from 57 patients with ALS were pairwise analyzed in both laboratories. The mean coefficient of variation of CSF NfL measurements between laboratories was 21.19% (SD = 24.75) for these 57 samples. There was a strong positive correlation between paired CSF Log[NfL]

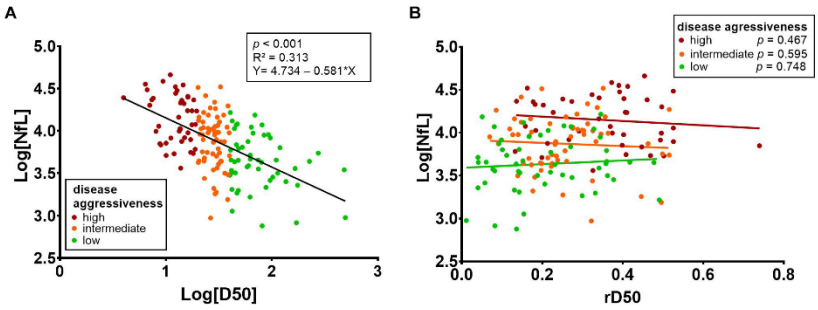


FIGURE 4 | (A) There was a negative correlation between the D50 parameter and CSF NfL ($p < 0.001$, $\rho = -0.553$). Linear regression analysis showed that 31.3% of the variation in CSF NfL can be explained by the D50 parameter ($R^2 = 0.313$, Log[NfL] = 4.734 - 0.581 × Log[D50]; $p < 0.001$). **(B)** Stratification of patients into the three aggressiveness subgroups (based on D50) reveals that there is no significant correlation of CSF NfL with rD50. CSF, cerebrospinal fluid; rD50, relative D50; NfL, neurofilament light chain.

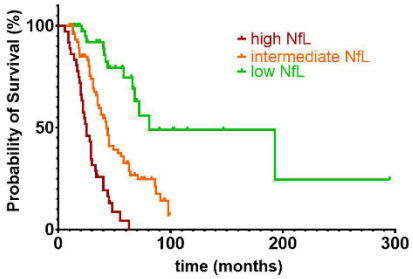


FIGURE 5 | Kaplan–Meier survival curves and log-rank test showed significant differences in survival [$\chi^2(2) = 56.505$, $p < 0.001$], when trichotomizing the ALS patients into groups with high ($n = 36$), intermediate ($n = 77$), and low ($n = 43$) CSF NfL concentrations. Estimated marginal means of the previously described analysis of covariance were used for the subdivision of ALS patients into groups with high (Log[NfL] > 4.149), intermediate (3.651 < Log[NfL] < 4.149), and low (Log[NfL] < 3.651) CSF NfL concentrations. Of the 156 ALS patients included in the survival analysis, 97 patients (13 with low, 51 with intermediate, and 33 with high CSF NfL levels) reached the endpoint death or tracheostomy, whereas the remaining 59 patients were censored. ALS, amyotrophic lateral sclerosis; CSF, cerebrospinal fluid; NfL, neurofilament light chain.

measurements from both laboratories ($r = 0.918$, $p < 0.001$) (**Supplementary Figure 2**).

DISCUSSION

In the present study we showed that CSF NfL levels in ALS patients significantly differ between patients according to their D50-derived disease aggressiveness. In addition to interlaboratory variation, significant effects for age and FTD on CSF NfL concentrations were also noted. However, the rD50 value and the derived disease phase did not influence NfL levels.

Associations between CSF NfL and the disease progression rate in ALS have been previously proposed (Tortelli et al., 2012; Lu et al., 2015; Menke et al., 2015; Steinacker et al., 2016, 2018b; Poesen et al., 2017; Andres-Benito et al., 2018; Gong et al., 2018; Scarafino et al., 2018; Schreiber et al., 2018; Abu-Rumeileh et al., 2020). However, the interpretation of these analyses remained constrained, because of the incomplete evaluation of confounding factors that influence NfL levels and/or the lack of longitudinal validation studies. Moreover, former results were limited to correlations with single disease metrics, such as the disease progression rate or the ALSFRS-R. This neglects the huge interindividual heterogeneity of disease courses in ALS, requiring a quantifiable framework within which to interpret patients' individualized disease trajectories and putative biomarkers.

We therefore applied the D50 model that provides quantifications for both measures of disease aggressiveness (D50), as well as the amount of disease covered (rD50, phase) at the time of CSF sampling to generate a large-scale pseudo–longitudinal analysis. This allowed us to demonstrate that CSF NfL is increased in patients with higher disease aggressiveness, even after adjustment for interlaboratory variation, age, gender, ALS phenotype, presence of FTD, and disease phase at the time of sampling. Former studies showed correlations between CSF NfL and linearly approximated progression rates. Most of these studies calculated the decline in ALSFRS-R from symptom onset until CSF sampling (Tortelli et al., 2012; Lu et al., 2015; Menke et al., 2015; Steinacker et al., 2016, 2018b; Poesen et al., 2017; Andres-Benito et al., 2018; Gong et al., 2018; Scarafino et al., 2018; Schreiber et al., 2018) or from symptom onset until disease diagnosis (Gaiani et al., 2017; Rossi et al., 2018). However, linear mixed-effects models using consecutively obtained ALSFRS-R scores have also been used to demonstrate associations with CSF NfL levels (Huang et al., 2020).

All these studies presume a linear decline of the ALSFRS-R score over time, despite prior observations that the rate of decline varies with disease progression and follows a curvilinear course (Gordon et al., 2010). Moreover, the calculation of progression rates based on a single score is highly susceptible to the known intrarater and interrater variability associated with ALSFRS-R assessments (Bakker et al., 2020). We therefore propose that the D50 model provides a more accurate representation of clinical progression, as it calculates an individualized sigmoidal curve of functional deterioration for each patient (Poesen et al., 2017; Prell et al., 2020; Steinbach et al., 2020).

The association between CSF NfL and survival in our ALS cohort further substantiates the ability of this biomarker to reflect prognosis in these patients and is in accordance with previous studies on CSF NfL and survival in ALS (Zetterberg et al., 2007; Pijnenburg et al., 2015; Gaiani et al., 2017; Gong et al., 2018; Illán-Gala et al., 2018; Rossi et al., 2018; Scarafino et al., 2018; Schreiber et al., 2018; Kasai et al., 2019).

The lack of a significant effect of the disease phase on NfL levels indicates that CSF concentrations remain longitudinally stable throughout the disease course. This suggests that any baseline NfL measurement is able to predict patients' disease aggressiveness, independent of the time point of CSF sampling. While longitudinal studies on CSF NfL concentrations in ALS would be best suited to support this observation, these are scarce and mostly comprise small numbers of patients. Some longitudinal studies reported rather stable levels throughout the disease course (Benatar et al., 2018; Huang et al., 2020), but slightly decreasing (Steinacker et al., 2016) and increasing concentrations in specific subpopulations of ALS patients (Lu et al., 2015; Poesen et al., 2017; Skillbäck et al., 2017) have been reported as well.

Several longitudinal studies following presymptomatic ALS-causing mutation carriers until the occurrence of manifest disease have aided in the understanding of the temporal profile of CSF NfL concentrations (Benatar et al., 2018, 2019). In these studies, while asymptomatic patients initially had CSF NfL concentrations similar to controls, increases were observed more than a year prior to phenoconversion (defining a presymptomatic stage) (Benatar et al., 2018, 2019). Recent findings also suggest that the duration of this presymptomatic stage may differ in accordance to the patient's survival (Benatar et al., 2019).

Studies have also reported correlations between CSF NfL and the ALSFRS-R at the time of sampling (Tortelli et al., 2012; Steinacker et al., 2016, 2018b; Gong et al., 2018; Scarafino et al., 2018), suggesting that NfL reflects cumulative neuroaxonal damage (disease accumulation) rather than the rate of neuronal breakdown (i.e., aggressiveness). We would like to emphasize that both aspects (disease accumulation and aggressiveness) are inherently interdependent in ALS cohorts, as patients with higher disease aggressiveness typically have reached a more advanced disease phase at the time of referral to ALS centers (sampling shift).

Moreover, most studies on neurofilaments performed univariate analyses to assess associations between clinical metrics and CSF NfL concentrations and neglected possible confounders. In one multivariate study by Gaiani et al., a repeated-measures ANCOVA was performed to investigate the effects of CSF NfL, ALS subtype, age, disease progression rate, gender, and cognitive impairment on longitudinal ALSFRS-R and MiToS scores. It was shown that all covariates, except cognitive impairment, exhibited significant effects on the functional-impairment scores (Gaiani et al., 2017). Another recent study investigated the effect of several clinical predictors of prognosis (including age, sex, C9ORF72 status, site of onset, baseline ALSFRS-R, and disease progression rate) on the ALSFRS-R slope in a multivariate model and demonstrated that serum NfL adds prognostic value to the model, but a comparable analysis on CSF NfL was lacking (Benatar et al., 2020). However, to the best of our knowledge, no former study has used multivariate analysis to probe the impact of several disease-specific variables on CSF NfL levels in ALS.

The present study provides strong evidence that CSF NfL reflects overall disease aggressiveness in ALS, independent of disease accumulation. This supports the concept that NfL and, more broadly, neurofilament proteins reflect disease activity. They might be directly linked to the pathophysiological process itself rather than being a collateral by-product of neuronal degeneration (Julien, 2001; Petzold, 2005). NfL may thus be used to directly monitor the therapeutic effects of neuroprotective or other disease-modifying drugs in clinical trials, where a positive therapeutic effect may be reflected by a reduction in the rate of release of NfL into the CSF. There is currently a growing momentum for the implementation of neurofilaments as secondary endpoints in such trials, with first promising findings in ALS (Miller et al., 2020), as well as spinal muscular atrophy patients (Olsson et al., 2019) under disease-modifying treatments. Our data suggest that CSF NfL represents a suitable monitoring biomarker for ALS that might be sensitive to therapeutic regimens aimed at decreasing disease aggressiveness. However, future longitudinal studies would be needed to assess its potential as an outcome measure for long-term treatment in ALS.

Besides disease aggressiveness, three covariates exhibited statistically significant effects on CSF NfL levels of ALS patients. In accordance with previous studies, age showed a positive association with CSF NfL (Vågberg et al., 2015; Gong et al., 2018; Steinacker et al., 2018b; Sun et al., 2020). This most likely reflects the degenerative process in the brain associated with normal aging, which leads to a slowly progressive rise of neurofilaments in the CSF. The ELISAs were performed in two different laboratories, and the site of analysis showed a statistically significant impact on NfL concentrations in the CSF. Stability issues of NfL measurements have been reported in previous multicentric studies on NfL and have been related, *inter alia*, to differences in perianalytical procedures (Petzold et al., 2010; Oeckl et al., 2016; Gray et al., 2020). This underlines the necessity for the implementation of standard operating procedures and round-robin tests. However, the coefficient of variation between measurements of both participating laboratories in this study was lower than previously reported for the same ELISA kit (Petzold et al., 2010; Miller et al., 2016; Gray et al., 2020), and the interlaboratory variations did not obscure the highly significant effect of disease aggressiveness on CSF NfL. Higher NfL levels in ALS patients with a concomitant diagnosis of FTD in our study are also in accordance with previous reports (Illán-Gala et al., 2018; Steinacker et al., 2018a).

We did not find a significant association between CSF NfL and the number of regions affected by UMN or LMN degeneration at the time of CSF collection. This further corroborates the notion that NfL levels are independent of disease accumulation. Previous studies, however, have reported conflicting results. CSF NfL was reported to increase with increasing number of regions affected by both UMN and LMN degeneration (Poesen et al., 2017); several studies also showed that NfL correlated with UMN burden (defined clinically or by neuroimaging) but not with the extent of LMN damage (Menke et al., 2015; Gong et al., 2018; Schreiber et al., 2018). Conversely, a recent study identified a significant association of NfL with the number of regions affected by LMN degeneration, but not UMN damage (Abu-Rumeileh et al., 2020).

This study is not without limitations. Comprehensive genetic profiles were not available for the entire ALS cohort. Given that CSF NfL levels are reported to be higher in patients with *C9orf72* mutations (Huang et al., 2020) and lower in those with *SOD1* mutations (Zetterberg et al., 2007), this may also represent a confounding factor. Further studies are needed to clarify if genotype-specific differences exist independent of disease aggressiveness, as, for example, *C9orf72* expansion carriers are known to have a worse prognosis relative to patients with sporadic ALS or other familial mutations (Miltenberger-Miltenyi et al., 2019; Rooney et al., 2019). The presence of clinically overt FTD was assessed, but this should be examined in more detail in future studies, as previous data have indicated links between cognitive deterioration and NfL levels (Illán-Gala et al., 2018; Delaby et al., 2020). Furthermore, this study is limited to the analysis of NfL concentrations in the CSF. Owing to recent technical advances, assessment of serum NfL is becoming increasingly available and holds promise as a prognostic biomarker for ALS (Benatar et al., 2020). However, future large-scale studies with matched assessments in both serum and CSF are necessary to adequately compare the prognostic potential of NfL in both biofluids. While serum and CSF levels of NfL are known to correlate well (Gille et al., 2019; Benatar et al., 2020), the considerably less invasive manner of collection speaks in favor of using blood biomarkers. However,

taking into consideration the proximity of CSF to the key pathological processes in ALS, we posit that CSF analyses should still play an important role in future research, as relevance has been demonstrated in this and other studies. Furthermore, a baseline lumbar puncture constitutes an essential step in the diagnostic workup of any patient with (suspected) ALS. Future studies should also incorporate pNfH and multicenter data, in order to fully explore the biomarker potential of neurofilaments.

Given the number of pseudolongitudinal CSF NfL data points analyzed in this study, our findings provide strong evidence for the ability of CSF NfL to reflect the rate of neuroaxonal degeneration in ALS and its potential to serve as a biomarker in future clinical trials. We show that the D50 progression model is an easily applicable and precise tool for investigating associations between biomarkers and clinical parameters in a heterogeneous ALS cohort. We recommend the use of this model for future ALS biomarker studies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Jena University Hospital Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MD, RS, NG, and JG contributed to conception and design of the study. JG developed the D50 model and the database. JG, BS, MD, RS, NG, and KM performed the data curation. NG and KM conducted the laboratory analyses. MD and RS performed the statistical analysis. MD wrote the first draft of the manuscript. MD, NG, and RS wrote sections of the manuscript. JG and OW provided the funding acquisition. JG conducted the Project administration and supervision. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2021.651651/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Tables

disease control (n = 56)		ALS mimic (n = 11)		non-neurological control (n = 15)	
4	parkinsonism	1	degenerative spondylopathy with radiculopathy	1	rheumatologic disorder
8	atypical parkinsonian syndrome	1	multifocal motor neuropathy	1	somatoform vertigo
1	parkinson and frontotemporal dementia	3	polyneuropathy	1	gait disorder due to accident
2	frontotemporal dementia	2	spinal stenosis	1	obstructive sleep apnea
2	dementia	1	ependymoma	9	benign fasciculations
3	mild cognitive impairment	1	proximal myotonic myopathy (PROMM)	1	schizophrenia
2	hereditary spastic paraplegia	1	adrenoleukodystrophy	1	psychic fatigue syndrome
3	gait disorder	1	spinal and bulbar muscle atrophy/ Kennedy disease		
2	stroke				
1	transient global amnesia				
1	leukoencephalopathy				
1	migraine				
3	pseudotumor cerebri				
8	normal pressure hydrocephalus				
1	obstructive hydrocephalus				
1	muscle hypertonia of unknown origin				
2	myopathy				
3	muscle fatigue of unknown origin				
1	diabetic amyotrophy				
2	chronic inflammatory demyelinating polyneuropathy				
1	pain and dysesthesia of lower limbs of unknown origin				
1	dermatomyositis				
1	spinal disc herniation				
1	vitamin B12 deficiency				
1	chronic pain syndrome				

Supplementary Table 1 diagnoses of patients included in the study.

Supplementary Material: Cerebrospinal Neurofilaments predict disease aggressiveness in ALS

		Disease aggressiveness			*p*
		high (D50 < 20)	**intermediate** (20 ≤ D50 < 40)	**low** (D50 ≥ 40)	
n		37	37	37	
Neurofilament light chain measurements					
NfL (pg/ml)		17180.10 (7906.10 – 24843.00)	8959.67 (4529.00 – 12157.00)	4511.00 (2717.22 – 8967.00)	<0.001*
laboratory: Germany/ Belgium		25 / 12 (67.6% / 32.4%)	24/13 (64.9% / 35.1%)	26 / 11 (70.3% / 29.7%)	0.939
demographics					
age at lumbar puncture		64.46 ± 10.02	62.56 ± 10.22	62.85 ± 9.83	0.679
male/female		21 / 16 (56.8% / 43.2%)	22 / 15 (59.5% / 40.5%)	22 / 15 (59.5% / 40.5%)	0.964
D50 disease progression model parameters					
D50 $		13.62 (8.87 – 16.16)	28.06 (22.62 – 30.42)	62.52 (46.55 – 99.42)	<0.001*
rD50		0.34 ± 0.14	0.27 ± 0.10	0.24 ± 0.13	<0.001*
Phase	I (rD50 < 0.25)	11 (29.7%)	15 (40.5%)	18 (48.6%)	0.284
	II (0.25 ≤ rD50 < 0.5)	23 (62.2%)	20 (54.1%)	19 (51.4%)	
	III/IV (rD50 ≥ 0.5)	3 (8.1%)	2 (5.4%)	0 (0%)	
Traditional disease metrics					
ALSFRS-R at lumbar puncture $		36 (32 – 40.50)	40 (37 – 42.50)	42 (36 – 44.50)	0.003*
disease progression rate $		1.44 (0.99 – 2.37)	0.60 (0.49 – 0.80)	0.21 (0.15 – 0.33)	<0.001*
disease duration at lumbar puncture (months) $		8 (5.5 - 10.5)	15 (10.5 - 17.5)	25 (16.5 - 45.5)	<0.001*
Kings' stage	I	8 (21.6%)	12 (32.4%)	16 (43.2%)	0.082
	II	10 (27%)	13 (35.1%)	15 (40.5%)	
	III	14 (37.8%)	8 (21.6%)	6 (16.2%)	
	IV a	3 (8.1%)	1 (2.7%)	0 (0%)	
	IV b	2 (5.4%)	3 (8.1%)	0 (0%)	
	V	0 (0%)	0 (0%)	0 (0%)	
MiToS stage	0	18 (48.6%)	31 (83.8%)	31 (83.8%)	<0.001*
	I	15 (40.5%)	4 (10.8%)	6 (16.2%)	
	II	4 (10.8%)	2 (5.4%)	0 (0%)	
	III-V	0 (0%)	0 (0%)	0 (0%)	
ALS phenotype	classic	17 (45.9%)	21 (56.8%)	22 (59.5%)	0.279
	bulbar	16 (43.2%)	13 (35.1%)	9 (24.3%)	
	pyramidal	3 (8.1%)	3 (8.1%)	2 (5.4%)	
	respiratory	1 (2.7%)	0 (0%)	0 (0%)	
	flail arm	0 (0%)	0 (0%)	3 (8.1%)	
	flail leg	0 (0%)	0 (0%)	0 (0%)	
	pure LMN	0 (0%)	0 (0%)	1 (2.1%)	
Revised El Escorial	Definitive	8 (21.6%)	2 (5.4%)	0 (0%)	<0.001*
	Probable	20 (54.1%)	22 (59.5%)	13 (35.1%)	
	Laboratory supported probable	7 (18.9%)	12 (32.4%)	14 (37.8%)	
	possible	2 (5.4%)	1 (2.7%)	6 (16.2%)	
	suspected	0 (0%)	0 (0%)	4 (10.8%)	
presence of FTD: yes / no		2 (5.4%)	2 (5.4%)	1 (2.7%)	1.000
Riluzole treatment: yes/ no		36 (97.3%)	36 (97.3%)	35 (94.6%)	0.912

Supplementary Table 2 demographic and clinical data for patients with ALS in the filtered cohort for the ANCOVA (n = 111). Continuous variables with normal distribution are expressed as mean with standard deviation, non-parametric nominal variables are marked with $ and represented as median and interquartile range. Categorical variables are expressed as number and percentage. For the comparison of demographic and

clinical variables amongst the three aggressiveness subgroups, analyses of covariance, Kruskal Wallis tests, chi square tests or Fisher-Freeman-Halton Exact tests were applied where appropriate. Asterisks * mark statistical significance at $p < 0.05$. *Abbreviations*: *ALS:* Amyotrophic Lateral Sclerosis, *ALSFRS-R:* revised ALS functional rating scale, *FTD:* frontotemporal dementia, *LMN*: lower motor neuron, *MiToS:* Milano Torino staging system, *NfL*: Neurofilament Light chain, *rD50*: relative D50.

Tests of Between-Subjects Effects			
Dependent Variable: Log[NfL]			
Factor	**df**	**F**	***p***
disease aggressiveness (D50 subgroups)	2	23.050	0.000
rD50-derived disease Phase	1	0.156	0.693
age at LP	1	4.557	0.035
Laboratory	1	9.118	0.003
gender	1	0.210	0.648
FTD	1	3.534	0.063
ALS Phenotype	1	0.008	0.931

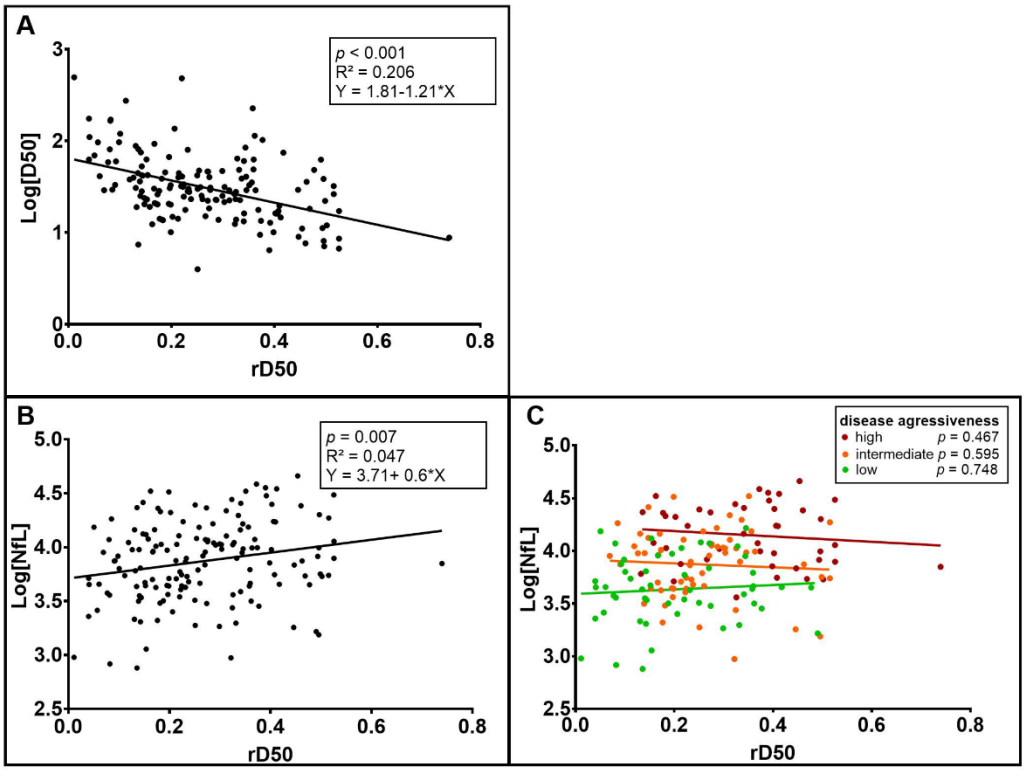
Supplementary Table 3 ANCOVA results of the filtered cohort
Abbreviations: *ALS:* Amyotrophic Lateral Sclerosis, *ANCOVA:* Analysis of covariance, *FTD:* frontotemporal dementia, *LP:* lumbar puncture, *NfL*: Neurofilament Light chain.

		disease aggressiveness						Association with CSF NfL (ANOVA)
		high		**intermediate**		**low**		
		n	%	n	%	n	%	
number of regions with UMN/LMN involvement (clinical)	1	1	2.3	4	6.6	8	15.4	$p = 0.059$
	2	14	32.6	22	36.1	19	36.5	
	3	28	65.1	35	57.4	25	48.1	
number of regions with LMN involvement (clinical)	1	5	11.6	12	19.7	15	28.8	$p = 0.210$
	2	17	39.5	20	32.8	20	38.5	
	3	21	48.8	29	47.5	17	32.7	
number of regions with UMN involvement (clinical)	1	11	25.6	22	36.1	27	51.9	$p = 0.060$
	2	17	39.5	26	42.6	17	32.7	
	3	15	34.9	13	21.3	8	15.4	
number of regions with LMN involvement (clinical + EMG)	1	1	2.3	3	4.9	6	11.5	$p = 0.519$
	2	6	14.0	14	23.0	16	30.8	
	3	36	83.7	44	72.1	30	57.7	
number of regions with UMN/LMN involvement (clinical+ EMG)	1	0	0	0	0	1	1.9	$p = 0.249$
	2	6	14.0	11	18.0	16	30.8	
	3	37	86.0	50	82.0	35	67.3	
number of regions with LMN involvement (EMG)	1	9	20.9	11	18.0	12	23.1	$p = 0.218$
	2	21	48.8	25	41.0	22	42.3	
	3	13	30.2	25	41.0	18	34.6	

Supplementary Table 4 number of regions affected at the time of sampling
Abbreviations: CSF: cerebrospinal fluid, *EMG:* Electromyography, *LMN:* lower motor neuron, *NfL:* Neurofilament light chain, *UMN:* upper motor neuron.

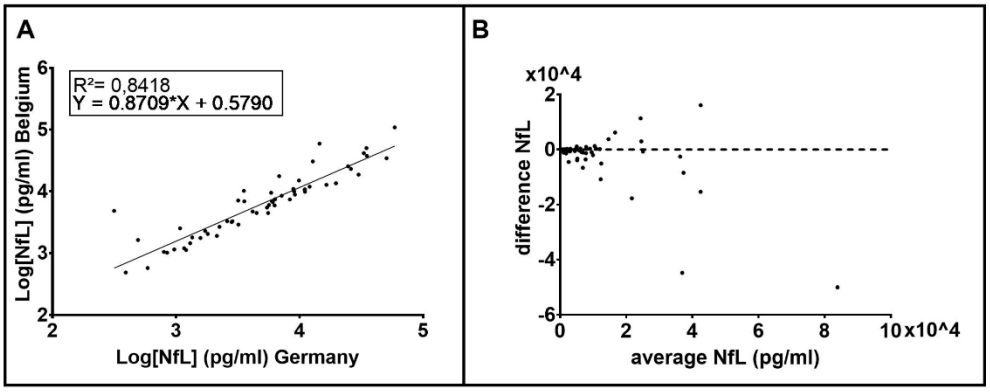
Supplementary Material: Cerebrospinal Neurofilaments predict disease aggressiveness in ALS

Supplementary Figures



Supplementary Figure 1 Sampling shift in aggressive disease causes apparent correlations of rD50 with NfL.
(A) Patients with low and intermediate disease aggressiveness were still in the earlier Phases of the disease at the time of sampling, while patients with highly aggressive disease had already reached later Phases by the time, they were referred to our center and lumbar puncture was performed. This is reflected by a negative correlation between rD50 and D50 in our ALS patient cohort ($p < 0.001$, $\rho = -0.432$). **(B)** For the entire ALS patient cohort, an apparent correlation between CSF NfL and rD50 could be calculated ($p = 0.005$, $\rho = 0.224$). **(C)** There was no significant correlation when stratifying patients into the three D50 subgroups (high in red: $p = 0.467$, intermediate in orange: $p = 0.595$, low disease aggressiveness in green: $p = 0.748$). This confirms that the aforementioned NfL-rD50 correlation (B) can be attributed to the sampling shift.
Abbreviations: *ALS:* Amyotrophic Lateral Sclerosis, *CSF:* cerebrospinal fluid, *NfL*: Neurofilament Light chain, *rD50*: relative D50.

Supplementary Material: Cerebrospinal Neurofilaments predict disease aggressiveness in ALS



Supplementary Figure 2. Paired sample comparison of ELISA measurements in the two laboratories. (A) measurements from Germany and Belgium highly correlate. (B) Altman-Bland figure, indicating that CSF samples with higher NfL concentrations tended to have higher inter-laboratory variations. *Abbreviations*: *CSF:* Cerebrospinal fluid, *ELISA:* Enzyme linked immunosorbent assay, *NfL*: Neurofilament Light chain.

6.3 Monocyte-Derived Macrophages Contribute to Chitinase Dysregulation in Amyotrophic Lateral Sclerosis: A Pilot Study **(Frontiers in Neurology, May 2021)**

This pilot study aimed at assessing potential *in vivo* sources of chitinase dysregulation in ALS. We used standard protocols to generate non-polarized macrophages from primary monocytes (MoMas) in a clinically characterized cohort of ALS patients and appropriately matched healthy controls (HCs). Macrophages were selected as the population of interest because **a)** myeloid lineage cells are functionally altered in ALS and **b)** they are an established physiological source of chitinases. We demonstrated that while CHIT1 and CHI3L1 displayed similar temporal expression dynamics in both groups, profound between-group differences were noted for these targets at later time-points i.e., when cells were fully differentiated. CHIT1 and CHI3L1 expression were significantly higher in MoMas from ALS patients at both the transcriptomic and protein level, with CHI3L1 levels also being influenced by age. In summary, our manuscript provided the first proof-of-principle of a dysregulated chitinase profile in peripheral innate immune cells from ALS patients.

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BRIEF RESEARCH REPORT
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Monocyte-Derived Macrophages Contribute to Chitinase Dysregulation in Amyotrophic Lateral Sclerosis: A Pilot Study

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Neuroinflammation significantly contributes to Amyotrophic Lateral Sclerosis (ALS) pathology. In lieu of this, reports of elevated chitinase levels in ALS are interesting, as they are established surrogate markers of a chronic inflammatory response. While post-mortem studies have indicated glial expression, the cellular sources for these moieties remain to be fully understood. Therefore, the objective of this pilot study was to examine whether the peripheral immune system also contributes to chitinase dysregulation in ALS. The temporal expression of CHIT1, CHI3L1, and CHI3L2 in non-polarized monocyte-derived macrophages (MoMas) from ALS patients and healthy controls (HCs) was examined. We demonstrate that while CHIT1 and CHI3L1 display similar temporal expression dynamics in both groups, profound between-group differences were noted for these targets at later time-points i.e., when cells were fully differentiated. CHIT1 and CHI3L1 expression were significantly higher in MoMas from ALS patients at both the transcriptomic and protein level, with CHI3L1 levels also being influenced by age. Conversely, CHI3L2 expression was not influenced by disease state, culture duration, or age. Here, we demonstrate for the first time, that in ALS, circulating immune cells have an intrinsically augmented potential for chitinase production that may propagate chronic neuroinflammation, and how the ageing immune system itself contributes to neurodegeneration.

Keywords: neuroinflammation, chitinases, macrophages, neurodegeneration, ageing

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INTRODUCTION

Amyotrophic Lateral Sclerosis (ALS) is a fatal and relentlessly progressive neurodegenerative disorder. Although, clinically characterized by the loss of both upper and lower motor neurons, it is a multi-systemic condition driven by several cell non-autonomous processes. Glial dysregulation in particular can exacerbate disease progression and is necessary for motor neuronal death to occur (1–3). Multiple lines of evidence have shown that this dysregulation extends to the peripheral innate immune system. Patient monocytes have a pro-inflammatory transcriptomic profile (4), secrete increased levels of pro-inflammatory cytokines (5), and can infiltrate the central nervous system (CNS) (6); furthermore, these alterations can influence disease progression. Crucially, monocytes

can be readily sampled and differentiated to macrophages *ex vivo* and although ontogenetically different, monocyte-derived macrophages (MoMas) and microglia functionally complement each other (7). Studying macrophages may therefore help understand disease-associated inflammatory sequelae in the CNS.

In lieu of this, reports of elevated cerebrospinal fluid (CSF) levels of chitinases in multiple neurodegenerative conditions are particularly intriguing, as they are considered markers of chronic gliosis (8–10). The chitinases, including CHIT1, CHI3L1, and CHI3L2, belong to the family 18 glycosyl hydrolases and bind to chitin, a natural polysaccharide found in the coating of various pathogens with high affinity. Dysregulated chitinase levels have been noted in a range of non-infectious diseases, including asthma, chronic obstructive pulmonary disease, multiple sclerosis, and even Alzheimer's disease. Multiple lines of evidence suggest that chitinases aren't merely markers of disease status, but are active components of the immunological response in pathological conditions characterized by chronic inflammation.

In ALS, these moieties exacerbate neuroinflammation and directly affect neuronal viability (11–13). While studies using post-mortem motor cortex and spinal cord tissue from ALS patients have reported micro-and astroglial expression of CHIT1 and CHI3L1, respectively, the cellular origins of these targets remain to be fully understood. *In vitro* studies using healthy controls (HCs) have shown that the chitinases are produced by mature macrophages, wherein they display distinct temporal expression patterns (14–16). Therefore, the objective of this pilot study was to examine whether these cells also contribute to chitinase dysregulation in ALS. To do so, we examined the "baseline" expression of CHIT1, CHI3L1 and, CHI3L2 in non-polarized monocyte-derived macrophages (MoMas) in patients with ALS relative to HCs.

MATERIALS AND METHODS

Participant Recruitment

All experimental procedures were approved by the local Ethics committee of the Jena University Hospital (Jena, Germany, Nr. 3633-11/12) and conducted in accordance with the Helsinki Declaration; written informed consent was obtained from all participants prior to enrollment. Patients with a diagnosis of either definite or probable ALS (as per the revised El-Escorial criteria) and HCs were consecutively recruited between January and July 2020 from the Departments of Neurology and Transfusion Medicine at the Jena University Hospital, respectively. Cerebrospinal fluid (CSF) was only available for ALS patients (7 of 8) as these patients underwent lumbar punctures (LPs) as part of their clinical examinations. Therefore, to enable between-group (healthy vs. disease) comparisons of CSF and plasma chitinase levels, we enlisted a second independent cohort of individuals termed non-neurological disease controls (NDCs) who were also undergoing lumbar punctures as part of their consultations at the Department of Neurology. Physical impairment was assessed using the Amyotrophic Lateral Sclerosis revised ALS Functional Rating Scale (ALSFRS-R) and calculated Progression Rate [PR; (48-current score)/disease duration in months]. The novel D50 progression model was used to ascertain disease aggressiveness and relative disease phase (17). Briefly, D50 is a summative descriptor for overall disease aggressiveness and refers to the time taken in months for a patient to lose 50% of functionality (ALSFRS-R score of 24 from a possible maximum of 48). It is calculated using iterative least-square fitting of available ALSFRS-R scores. Relative D50 (rD50) is an open-ended reference point that describes the individual disease course covered in reference to D50, wherein 0 signifies disease onset and 0.5 indicates halved functionality. Using rD50 allows the categorization of patients into contiguous disease phases: an early semi-stable Phase I ($0 \leq rD50 < 0.25$), an early progressive Phase II ($0.25 \leq rD50 < 0.5$), and late progressive and late stable Phases III/IV ($rD50 \geq 0.5$).

Participants receiving immunomodulatory medication and/or suffering from an acute infection were excluded. All participants were also screened for HIV, Hepatitis B and C, and SARS-CoV-2 infection at the time of blood collection. Detailed genetic testing was not performed.

Primary Human Monocyte Isolation, Culture, and Differentiation

Peripheral venous blood was collected from all participants in EDTA-K vacuum tubes (Sarstedt, Germany). Monocytes were isolated from 7.5 ml of freshly drawn blood *via* positive immunomagnetic selection using StraightFrom® WholeBlood CD14 MicroBeads (Miltenyi Biotec, Germany) as per the manufacturer's instructions. Eluted monocytes were re-suspended in simple RPMI-1640 Glutamax medium, counted, and seeded at a density of 5×10^5 cells/well of a 24-well plate and allowed to adhere for 2 h. Cells were gently washed with warm DPBS (Gibco) to remove unbound cells. From thereon, cells were cultured in "differentiation medium" supplemented with 20% v/v human serum (Sigma Aldrich), 1% v/v penicillin-streptomycin and 20 ng/ml of recombinant human M-CSF (BioLegend). Cells were cultured under standard conditions (5% CO_2, 37°C) for 9 days with media changes performed every 2 days; cell lysates and supernatants were harvested for qRT-PCR and ELISA experiments, respectively, at days 1, 3, 6, and 9. Cell health and morphology were continuously tracked using brightfield microscopy.

RNA Isolation and qRT-PCR

Cells were homogenized in QIAzol lysis reagent (Qiagen) at the specified time points and total RNA was isolated using the phenol/chloroform method. RNA quantitation and purity were

Abbreviations: ALS, Amyotrophic Lateral Sclerosis; ALSFRS-R, Amyotrophic Lateral Sclerosis Functional Rating Scale Revised; cDNA, complementary DNA; CHIT1, Chitotriosidase-1; CHI3L1, Chitinase 3-Like 1; CHI3L2, Chitinase 3-Like 2; CI, confidence interval; CNS, central nervous system; CSF, Cerebrospinal fluid; ELISA, Enzyme-linked immunoabsorbent assay; HCs, Healthy Controls; HPRT1, hypoxanthine phosphoribosyltransferase 1; IFN-γ, Interferon gamma; LPs, lumbar punctures; MCP-1, Monocyte Chemoattractant Protein 1; MoMas, monocyte-derived macrophages; NDC, non-neurological disease controls; PR, Progression Rate; qRT-PCR, quantitative reverse transcription polymerase chain reaction; rD50, relative D50; RSP18, ribosomal protein S18; SD, standard deviation; TNF-α, Tumor Necrosis Factor alpha.

spectrophotometrically assessed (ND-1000, Nanodrop, USA). RNA integrity was assessed on the QIAxcel Advanced System using the Qiaxcel RNA QC kit V2.0 (both QIAGEN). Samples with an RNA integrity number 6 ≥ were included for further analyses. An equal amount of RNA (200 ng) was reverse transcribed from each sample using the RevertAid First Strand cDNA Synthesis Kit in a final reaction volume of 20 µl. All qRT-PCR reactions were performed using the Brilliant III SYBR Green qPCR Master Mix (Agilent Technologies) on the Rotor-Gene 6,000 instrument (Corbett Research) with the following cycling parameters: 3 min of polymerase activation at 95°C followed by 40 amplification cycles (95°C for 10 s, 60°C for 60 s).

All primer pairs were designed to be exon-spanning and are detailed in **Supplementary Table 2**. Primer specificity was verified in preliminary experiments using melt-curve analysis and capillary electrophoresis to verify the presence of single PCR products at the correct size. The Pfaffl equation was used for relative quantification of gene expression; expression was calculated relative to the housekeeping genes *HPRT1* and *RSP18* and to HC samples at day 1.

ELISA Analyses

Cell culture supernatants were harvested at the specified time points, centrifuged to eliminate cellular debris (400 × g for 10 min), and frozen at −20°C until further analyses. CSF and plasma were prepared by centrifugation (1000 × g, 15 min) within a maximum of 1 h from collection, aliquoted and stored at −80°C until use. All CSF samples were inspected for evidence of a traumatic puncture. CHIT1, CHI3L1, and CHI3L2 levels in were determined using commercially validated kits in accordance with the manufacturer's instructions. The kits used were as follows: CHIT1 and CHI3L2 from MBL Life Science and CHI3L1 from R&D Systems. All samples and standards were assayed in duplicate with intra- and inter-assay variation ≤10 and 15%, respectively. Absorbance was measured at 450/540 nm. Sample concentrations were extrapolated *via* 4 parameter logistic regression fitting of the standard curve.

Statistical Analyses

Statistical analyses were performed using the SPSS (version 25.0) and GraphPad Prism software packages. The Shapiro-Wilk test was used to check for normal distribution. Correlations between continuous variables were assessed using the Spearman's test. Between-group comparisons were performed using either the Student's *t*-test or Mann-Whitney *U*-test. Mixed two-way ANOVAs were performed to assess the effect of group (ALS vs. HC) on chitinase expression over time. Assumptions for sphericity and homogeneity of variances and co-variances were met unless stated otherwise. The Greenhouse-Geisser adjustment was used to correct for violations of sphericity where necessary. All outliers were retained for analyses. Summary data are reported as the mean with either 95% confidence intervals (CI) or the standard deviation.

For gene expression comparisons: Analyses were performed on the $\log_2$-transformed fold-change ratios calculated using the Pfaffl equation.

For secreted protein comparisons: Analyses were performed on rank-transformed data (data were transformed for normalization). Studentized residuals for outliers noted prior to transformation were as follows: CHIT1 dataset 3.07 and 3.5 and CHI3L1 dataset 3.22. Two-tailed statistical significance was set at $p < 0.05$.

RESULTS

The final cohort from which MoMas were generated included 8 patients with ALS and 8 HCs. ALS patients were significantly older than HCs (ALS = 60.5 ± 7.7 years vs. HC = 51 ± 7.9 years, $t(14) = 2.403$, $p = 0.03$) and both groups had a greater proportion of males (ALS = 6, HC = 7) than females. Four patients were receiving riluzole for ≥2 months at the time of sampling. None of the ALS patients had active cancer or manifest diabetes. The additional NDC cohort recruited to allow CSF chitinase analyses was representative of the HC cohort both in terms of age and sex distribution. Further demographic details are outlined in **Table 1**. Additional diagnostic information for the NDC cohort is provided in **Supplementary Table 1**.

As seen in **Figure 1**, CHIT1, CHI3L1, and CHI3L2 were detectable in MoMas from both ALS patients and HCs. However, they displayed distinct regulatory profiles. Temporal expression patterns were similar for CHIT1 (**Figures 1A,D**) and CHI3L1 (**Figures 1B,E**) in both ALS and HCs: relative gene and protein expression for both targets were minimal at earlier time-points, increased over time, and peaked on Day 9. However, no such temporal regulation was observed for CHI3L2 at either the transcriptomic (**Figure 1C**) or the protein level (**Figure 1F**) in either group. To illustrate, in HCs, mean secreted CHIT1 and CHI3L1 levels increased by 42.6 and 625%, respectively, from Day 1 to 9; conversely, for CHI3L2 only a 9% increase was observed. Indeed, secreted CHIT1 and CHI3L1 levels on D9 correlated significantly with each other but not with CHI3L2 ($r_s = 0.69$, $p = 0.003$).

Profound between-group differences were observed for CHIT1 and CHI3L1 at later time-points. Relative *CHIT1* expression was significantly higher in ALS MoMas on day 6 ($F(2, 12) = 17.93$, $p = 0.001$, partial $\eta^2 = 0.6$) and day 9 ($F(2, 12) = 15.42$, $p = 0.002$, partial $\eta^2 = 0.56$). This effect was recapitulated at the protein level, wherein a statistically significant *time* × *group* interaction was observed despite the inclusion of age as a covariate, thus underscoring the effect of group on CHIT1 levels over time ($F(3, 39) = 4.97$, $p = 0.005$, partial $\eta^2 = 0.27$). ALS MoMas secreted significantly higher CHIT1 levels than HC MoMas on Day 9 (ALS = 39.4 ng/ml, [15.3, 53.5] vs. HC = 6.5 ng/ml, [−17.5, 30.6]) ($F(1, 13) = 15.7$, $p = 0.002$, partial $\eta^2 = 0.55$), despite having lower levels on Day 1 (ALS = 3.6 ng/ml [3.1, 4.2] vs. HC = 4.6 ng/ml [4.1,5.2] ($F(1, 13) = 5.77$, $p = 0.032$, partial $\eta^2 = 0.31$). An analogous trend was noted for CHI3LI: as seen in **Figure 1B**, relative *CHI3L1* expression was higher in the ALS group at all time-points and particularly so at Day 6. However, this effect did not reach statistical significance ($F(1.6, 19.2) = 1.59$, $p = 0.23$, partial $\eta^2 = 0.12$). At the protein level however (**Figure 1E**),

TABLE 1 | Participant demographics and clinical data.

	ALS patients	Healthy Controls	NDCs
n	8	8	7
Age (years) mean ± SD	60.5 ± 7.7	51 ± 7.9	53.8 ± 14.6
Males	6	7	5
Females	2	1	2
ALSFRS-R mean ± SD	38.1 ± 7.5	-	
PR mean ± SD	0.6 ± 0.4	-	
Disease duration (months) mean ± SD, range	18.1 ± 17.8, 7–60	-	
D50 mean ± SD	34.7 ± 19.6	-	
rD50 ± SD	0.25 ± 0.12	-	
rD50-derived Disease Phase I/II/III	4/4/-	-	
Bulbar onset	2	-	
Limb onset	6	-	

ALSFRS-R, Amyotrophic Lateral Sclerosis Functional Rating Scale Revised; ALS, Amyotrophic Lateral Sclerosis; NDCs, non-neurological disease controls; PR, progression rate; rD50, relative D50.

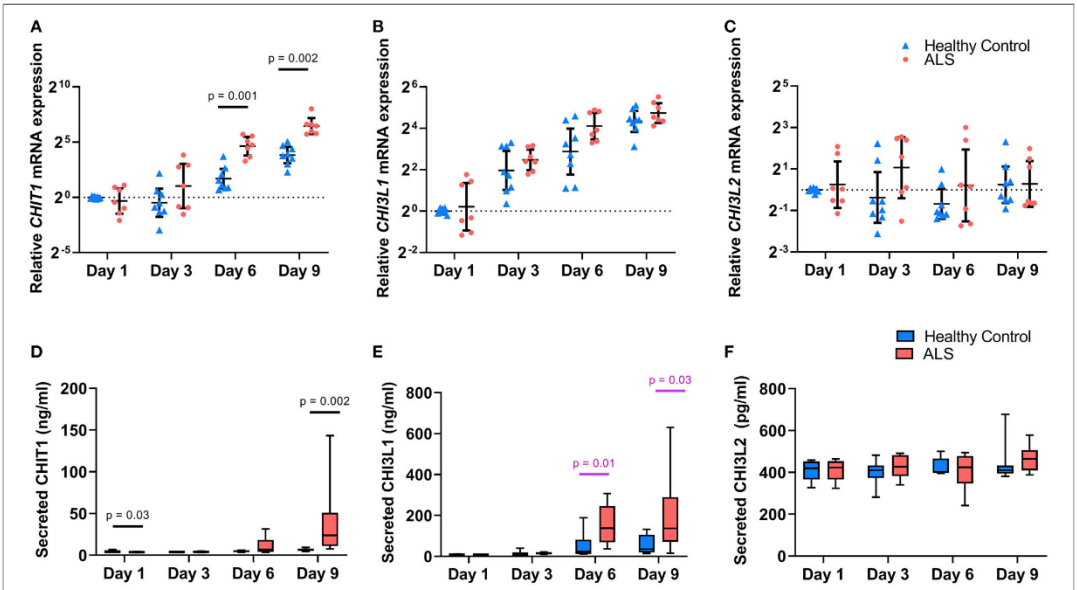

FIGURE 1 | Chitinase expression in ALS and healthy control monocyte-derived macrophages. Relative expression of *CHIT1* **(A)**, *CHI3L1* **(B)**, and *CHI3L2* **(C)** in cell lysates from ALS patients ($n = 8$) and controls ($n = 8$) over time. Data are presented as individual scatterplots with the geometric mean and 95% confidence intervals. Dashed line at y = 1 corresponds to the relative expression of the calibrator samples (controls at Day 1). Protein levels of CHIT1 **(D)**, CHI3L1 **(E)**, and CHI3L2 **(F)** secreted by monocyte-derived macrophages from ALS patients ($n = 8$) and controls ($n = 8$) in culture over time. Data are presented as boxplots with whiskers indicating 95% confidence intervals. The effect of group and time on chitinase expression was assessed using a 2-way mixed ANOVA with significance set at $p < 0.05$. *P*-values are reported for statistically significant results; values reported in pink did not retain statistical significance after the inclusion of age as a covariate. Y axes for **(A–C)** are displayed in $\log_2$ scale.

a statistically significant *time* × *group* interaction was noted ($F(1.96, 27.43) = 7.09$, $p = 0.003$, partial $\eta^2 = .34$, Greenhouse-Geisser correction $\chi^2(5) = 13.04$, $p = 0.02$). Further, univariate analyses showed that ALS MoMas secreted significantly higher CHI3L1 levels than HC MoMas on Day 6 (ALS = 153.18 ng/ml, [91.6, 214.7] vs. HC = 51.2 ng/ml [−10.2, 112.7]) ($F(1, 14) = 8.92$, $p = 0.01$, partial $\eta^2 = 0.39$), and Day 9 (ALS = 199.7 ng/ml [91.2, 308.2] vs. HC = 57.3 ng/ml [-51.2, 165.8]) ($F(1, 14) = 5.83$, $p = 0.03$, partial $\eta^2 = 0.29$). Crucially, this group effect did not retain significance after the inclusion of age as a covariate ($F(2.08, 26.99) = 2.60$, $p = 0.09$, partial $\eta^2 = 0.17$).

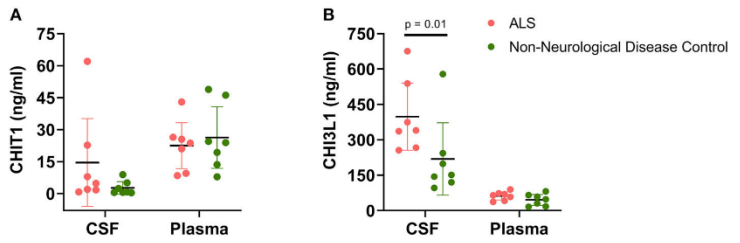

FIGURE 2 | Chitinase levels in cerebrospinal fluid and plasma. Levels of **CHIT1 (A)** and **CHI3L1 (B)** were measured in the CSF and plasma of ALS patients ($n = 7$) and non-neurological disease controls ($n = 7$). Between-group comparisons were performed using the Mann-Whitney U-test with significance set at $p < 0.05$.

As seen in **Figure 2**, between-group biofluid analyses revealed that a disease-associated chitinase upregulation was only evident in CSF rather than plasma. Both CHIT1 and CHI3L1 plasma levels were largely similar between ALS patients and NDCs. Conversely, CSF CHI3L1 levels were significantly upregulated within the ALS group relative to the NDC group (ALS = 398.4 ng/ml, [256, 540.8] vs. NDC = 218.9 ng/ml [65.25, 372.5], $U = 6$, $p = 0.017$). While a considerable upregulation was also noted for CSF CHIT1 levels, the effect did not reach significance (ALS = 14.56 ng/ml, [−6.02, 35.14] vs. NDC = 2.65 ng/ml [−0.31, 5.62], $U = 6$, $p = 0.017$).

Finally, within the ALS group, no significant correlations were observed between secreted chitinase levels on Day 9 (this time-point was selected as this is when transcriptomic and protein expression peaked) and the total ALSFRS-R score, calculated PR, D50 and rD50 (*data not shown*).

DISCUSSION

To our knowledge, this pilot study is the first to report a dysregulated chitinase profile in peripheral innate immune cells from ALS patients. By studying the transcriptomic and protein expression of key chitinases in non-polarized MoMas, we show here that macrophages in ALS have an intrinsically augmented capacity to secrete chitinases. To begin with, the temporal regulation patterns observed here are in keeping with previous studies; CHIT1 and CHI3L1 are minimally expressed in monocytes and highly upregulated during later stages of macrophage differentiation (14, 15). Conversely, CHI3L2 expression remains minimal across the differentiation process and is only upregulated as a result of stimulation (16). Here, the static and minimal CHI3L2 expression in both groups also serves to reinforce that the cells were at "baseline" and not stimulated as a result of the differentiation process itself. This, coupled with the absence of a group-associated effect, suggests that CNS rather than systemic immune cells likely contribute to the CHI3L2 elevations reported in the CSF of ALS patients. It is therefore unsurprising that the profound elevations we observed in CHIT1 and CHI3L1 expression in the ALS group were only evident at later time-points i.e. when cells were fully differentiated. Given the evidence that the chitinases are a feature of "M1-like" pro-inflammatory macrophages (18, 19), the upregulations observed here underscore how in ALS, peripheral myeloid cells are skewed toward a pro-inflammatory phenotype (4, 6). Indeed, monocytes from ALS patients are more readily differentiated toward an M1-like phenotype, wherein they produce higher levels of pro-inflammatory cytokines, including TNF-α and IL-6, than macrophages differentiated from HC monocytes (5). The data reported here are also interesting given that the chitinases themselves are active immune-modulators; for instance, stimulating monocytes with either CHI3L1 or CHIT1 resulted in the release of IL-8, MCP-1, and RANTES (20). Indeed, a "feed-forward" loop wherein the chitinases sustain neuroinflammation in ALS *via* their auto- and paracrine effects has already been postulated (12). For instance, Varghese et al. demonstrated that microglia appear to be the primary cellular source for CHIT1 in the CNS using murine cultures and that microglia themselves were susceptible to the effects of accumulated CHIT1, as they were chronically activated as a result of exposure (11). Another study also showed that conditioned medium from MoMas induced *CHI3L1* transcription and morphological changes in cultured human astrocytes (19). Crucially, chitinase exposure was shown to increase leukocytic migratory capacity across an *in vitro* blood-brain barrier (BBB) model (20). Therefore, one might hypothesize that neuronal death and aggregate deposition could trigger chitinase expression by glial cells, thus creating a chemotactic axis recruiting circulating monocytes. Finally, the monocytes, by virtue of their intrinsically augmented chitinase synthesis capacity, exacerbate the neuroinflammatory milieu upon differentiation. In keeping with this hypothesis, Steinacker et al. (8) reported that in post-mortem spinal cord tissue from ALS patients, CHIT1 immuno-staining was primarily observed in CD68+ve macrophages: no expression was noted in tissue from HCs.

The upregulations in CSF CHIT1 and CHI3L1 levels in ALS patients relative to NDCs are concordant with previous studies (8, 21). Indeed, CSF CHIT1 in particular is now considered a surrogate marker of microglial activity and recommended for the differential diagnosis of ALS (22). As also previously reported in the literature, we noted no significant between-group differences in plasma levels, which suggests that the chitinase dysregulation observed in ALS MoMas is more reflective of the inflammatory microenvironment in the CNS than the periphery. This is reinforced by our observation that monocytic expression of chitinases in both ALS and HCs was almost negligible.

Undoubtedly, the effect of age must be considered; the results observed here are to be expected, given that chitinase levels, particularly those of CHI3L1, increase with age and are potentially indicative of the wider "*inflammaging*" process (23). While further studies with age-matched cohorts are warranted, we posit that the effects of age and disease on chitinase expression are not mutually exclusive and should not be studied as such, as the contribution of "*immunosenescence*" to neurodegenerative conditions has been extensively reported (24).

The present study is not without its limitations, with the restricted sample size being foremost. While it sufficed for demonstrating proof-of-principle, these results warrant validation within a more sizeable cohort. We believe this also explains why no correlations were observed with clinical indices. Detailed information on existing chronic comorbidities like diabetes was only available for some individuals. However, these have also been reported to influence chitinase levels (25).

Next, the present study did not assess enzymatic CHIT1 activity as genetic information for *CHIT1* polymorphisms was not available for the cohort. The 24 bp duplication in exon 10 of the gene directly affects activity; heterozygous carriers display reduced activity and homozygous carriers display none at all (26). Therefore, the interpretation of these results would have been constrained, especially given that the prevalence of this polymorphism is almost 50% in European populations (27). However, given the observation that CHIT1 activity and protein levels are highly correlated, i.e., "elevated CHIT1 levels do not constitute inactive enzyme" (28), we posit that the results reported here are indeed evidence of a disease-associated CHIT1 upregulation in MoMas. Nevertheless, we recommend that future studies should include an assessment of CHIT1 activity.

Further studies with larger, age-matched and more representative cohorts can (1) help dissect the cumulative effect of age and disease on chitinase expression, (2) examine the implications for overall disease aggressiveness and acute activity, and (3) account for the dynamicity of the immune response by tracking chitinase expression across different disease phases.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics committee of the Jena University Hospital (Jena, Germany, No. 3633-11/12). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

NG and JGr: study conceptualization and design. NG, EH, JGu, and AS: data acquisition, experimental execution, and troubleshooting. NG and TP: data analysis and visualization. NG: manuscript draft preparation. JGr, OW, RS and TP: revising the work for intellectual content. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.629332/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Information

Supplementary Table 1: Diagnostic Information for independent Non-Neurological Disease Control Cohort

Non-Neurological Disease Controls	n
Idiopathic Intracranial Hypertension	3
Benign Fasciculations	1
Normal Pressure Hydrocephalus	1
Neurologically Healthy	1
Suspected pseudodementia	1

Supplementary Table 2: Primer Pair Details

Target	Forward Primer 5'-3'	Reverse Primer 5'-3'	Product length
CHIT1	CGCTTCACAACCCTGGTACA	AGCATCCACATAGGTCTGCC	120
CHI3L1	CCAGGAAAGCGTCAAAAGCAA	TTGATGGCATTGGTGAGAGGG	141
CHI3L2	GGAACCATAACTTTGATGGACTTG	GAGAAGCCTTTCCTTGGTGGA	140
HPRT1	GACCAGTCAACAGGGGACATA	GCTTGCGACCTTGACCATCT	165
RSP18	CCACGCCAGTACAAGATCCC	AAGTGACGCAGCCCTCTATG	158

6.4 Chitinase Dysregulation predicts Disease Aggressiveness in ALS: Novel insights from a Clinical Cohort and Murine Models **(Manuscript in Preparation)**

This "hybrid" study combined a well-characterized clinical cohort and relevant preclinical mouse models of ALS to assess the diagnostic and prognostic utility of key chitinases and identify their cellular sources. CHIT1, CHI3L1, and CHI3L2 were measured in matched CSF and plasma samples from ALS patients, controls with other neurodegenerative diseases (NDegs), and controls with non-neurodegenerative diseases (NDCs). The D50 model was used a validation framework and we also measured neurofilament levels as these are established biomarkers for ALS. Significant between-group differences were only noted in CSF and not plasma. We confirmed that although chitinase upregulation is a feature of ALS, it is not exclusive to it. While CHIT1 and CHI3L2 could significantly distinguish between ALS vs. Non-ALS individuals, they did not diagnostically outperform the neurofilaments. D50 was used to stratify the ALS cohort into high vs. low aggressiveness sub-cohorts and we observed that CHIT1 and CHI3L1 associated with and predicted individual disease aggressiveness. Crucially, this effect was independent of factors like onset-type, age, and accumulated disease as measured by rD50 and significantly enhanced the prognostic utility of the neurofilaments alone. The most parsimonious model included NfL and CHI3L1, as this combination explained the highest amount of variation in D50. Quantitative immunostaining in the GA-CFP mouse model showed that microglia, astrocytes, and surprisingly, neurons are physiological sources for CHIT1 and CHI3L1. Additionally, they are vulnerable to dysregulation in ALS, as symptomatic GA-CFP+ mice displayed upregulated chitinase levels in these populations. Qualitative inspection of the SOD1-G93A and rNSL8-hTDP-43 models supported the link between chitinase upregulation and disease aggressiveness seen in human patients: dysregulation was much more pronounced in these models than the GA-CFP model, possibly because they present with a much more aggressive phenotype and substantial neuronal loss.

Title: Chitinase Dysregulation predicts Disease Aggressiveness in ALS: Novel insights from a Clinical Cohort and Murine Models

Running Head: Chitinases predict disease aggressiveness in ALS

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List of Abbreviations

AUC, Area under the Curve

ALS, Amyotrophic Lateral Sclerosis

ALSFRS-R, Amyotrophic Lateral Sclerosis Functional Rating Scale (Revised)

a.u., Arbitrary Units

ΔFRS, delta-FRS or Disease Progression Rate

CHIT1, Chitotriosidase 1

CHI3L1, Chitinase 3 like 1

CHI3L2, Chitinase 3 like 2

CSF, Cerebrospinal Fluid

DAPI, 4′,6-diamidino-2-phenylindole

GA-CFP, Thy1 (GA149)-Cyan Fluorescent Protein

GFAP, Glial fibrillary acidic protein

Iba-1, Ionized calcium binding adaptor molecule 1

MND, Motor Neuron Disease

NDCs, Non-Neurodegenerative Disease Controls

NDeg, Neurodegenerative Disease Controls

NeuN, Neuronal Nuclei

NfL, Neurofilament light chain

PBS, Phosphate Buffered Saline

PCR, Polymerase Chain Reaction

pNfH, Phosphorylated neurofilament heavy chain

rD50, Relative D50

ROC, Receiver operating characteristic

ROI, Region of Interest

SOD1, Superoxide dismutase 1

TDP-43, TAR DNA-binding protein 43

wt, Wild-Type

Structured Abstract

Objective: Although the chitinases are considered biomarkers of neuroinflammation in amyotrophic lateral sclerosis (ALS), reports on their prognostic utility are contradictory. Additionally, the cellular sources that contribute to dysregulation in ALS remain to be identified as studies reporting glial expression of CHIT1 and CHI3L1 are primarily based on post-mortem material.

Methods: CHIT1, CHI3L1, CHI3L2 were immunoassayed in cerebrospinal fluid (CSF) and plasma from ALS patients, neurodegenerative disease controls (NDegs), and non-neurodegenerative disease controls (NDCs). CSF neurofilament levels were also assayed as these are established neuroaxonal damage markers. The D50 model was used as a validation framework to assess links with disease aggressiveness and/or accumulated disease. Quantitative immunostaining on spinal cord sections from symptomatic *C9orf72* GA-CFP mice was performed to identify populations expressing CHIT1 and CHI3L1.

Results: While CSF levels of all three chitinases were elevated in the CSF of ALS patients relative to NDCs, only CHIT1 and CHI3L2 were elevated relative to NDegs. No significant differences were noted in plasma. Chitinases strongly correlated with neurofilament levels but did not diagnostically outperform them. CHIT1 and CHI3L1 were associated with increased disease aggressiveness (stratified by D50 values) and significantly improved the prognostic power of the neurofilaments alone. This effect was independent of other prognostic factors, including disease phase. Chitinase levels were upregulated in Iba-1+ microglia and GFAP+ astrocytes in symptomatic GA-CFP+ mice. Surprisingly, robust neuronal expression was noted for CHIT1 and CHI3L1, suggesting that these are a major physiological source and vulnerable to dysregulation in ALS.

Interpretation: Key chitinase family members are dysregulated in ALS and multiple sources, including glia and neurons, contribute to this dysregulation. The D50 model provided compelling evidence that the extent of this dysregulation is predictive of overall disease aggressiveness, highlighting the prognostic utility of the chitinases.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is the most prevalent form of adult-onset motor neuron disease (MND) and clinically presents with the destruction of primarily upper and lower motor neurons. Median survival is 2-3 years from symptom onset, with most patients succumbing to respiratory complications. Limited disease-modifying therapies exist, as therapeutic development has been severely constrained by the disease's multi-factorial etiology and phenotypic heterogeneity. Precision biomarkers that reflect specific pathological processes can assist with patient stratification and provide readouts of treatment efficacy. Biomarkers of neuroinflammation are particularly relevant, given that non-cell autonomous mechanisms significantly exacerbate ALS pathology and are necessary for its manifestation[1, 2]. In lieu of this, several studies have reported the chitinases as novel inflammatory markers in ALS[3, 4]. The chitinases belong to the family 18 glycosyl hydrolases and cleave chitin, a natural polysaccharide found in the coating of various pathogens. However, their roles extend beyond innate immunity against chitin, as they have been implicated in various processes, including tissue remodeling, Th2 inflammatory responses, and chemotactic signaling[5, 6]. The family comprises enzymatically active chitinases (CHIT1 and AMCase) and several chitinase-like proteins (e.g., CHI3L1 and CHI3L2) which bind chitin with high affinity but have no catalytic ability. Chitinase dysregulation is a common feature of several chronic inflammatory disorders, both systemic (e.g., diabetes and several cancers) and neurodegenerative (e.g., frontotemporal dementia (FTD) and Alzheimer's disease (AD)).[7-9]

Although independent groups have reported that CHIT1, CHI3L1, and CHI3L2 are elevated in ALS, there is conflicting evidence regarding their prognostic relevance. While some studies have reported significant (albeit modest) links with either mortality, total ALSFRS-R score, or the calculated disease progression rate (ΔFRS), several others haven't [10-12]. In addition to cohort and methodological differences, this may stem from limitations associated with the Amyotrophic Lateral Sclerosis Functional Rating Scale (ALSFRS-R) itself. The ALSFRS-R, a 48-point questionnaire (a maximums score of 48 indicating full health), is the primary clinical tool used to assess physical deterioration; however, it presumes linearity in functional decline[13]. Moreover, the derived ΔFRS is entirely dependent on the sampling time point i.e., there is substantial intra-individual variation across the disease course. Genuine biological signals may therefore be occluded because of the inability to disentangle disease aggressiveness from accumulated degeneration. Progression type stratification is also entirely arbitrary and differs from study to study as there are no universally agreed on cut-offs. Therefore, this project aimed to use the novel D50 progression model[4,5] to analyze the prognostic utility of the chitinases by independently examining their relationship with **a)** overall disease aggressiveness and **b)** cumulative functional loss. Furthermore, despite the increasing focus on the chitinases' biomarker potential, detailed studies on their cellular sources are limited. Existing reports suggest that microglia/macrophages and astrocytes are exclusive sources for CHIT1 and CHI3L1, respectively[14, 15]. However, these are based primarily on post-mortem tissue and thus offer a very narrow glimpse into what is most likely a substantially advanced disease stage. Examining chitinase sources in the CNS under both physiological and pathological conditions is critical for understanding their contribution to disease and targeting them for therapeutic intervention. Therefore, we used established murine models of ALS to investigate **1)** which cell types are key *in vivo* sources

of chitinases, **2)** whether the chitinases dysregulation seen in human patients can be recapitulated, and **3)** whether expression patterns are influenced by the underlying pathology.

2. Materials and Methods

2.1 Participant Recruitment and Case Classification

All participants were consecutively recruited through the Department of Neurology at the University Hospital of Jena between 2015 and 2019. This study was performed in accordance with regulations stipulated by the local ethics committee and the Declaration of Helsinki. The final study cohort comprised 39 patients with either definite, probable, or laboratory-supported probable ALS (as per the revised El Escorial Criteria)[16], 13 individuals with alternate neurodegenerative diseases (Neurodegenerative Disease Controls, NDegs) and 11 individuals with non-neurodegenerative conditions (Non-Neurodegenerative Disease Controls, NDCs). Diagnoses for NDeg and NDC cohorts are provided in Supplementary Table 1. The primary exclusion criteria constituted current intake of any immunomodulatory therapy, recent surgical interventions, and the presence of comorbid FTD within the ALS cohort.

2.2 D50 modelling for patients with ALS

The D50 model was applied to the ALS cohort as previously described[17]. Briefly, the model uses iterative fitting of all ALSFRS-R scores available for an individual to generate a sigmoidal curve spanning the disease course from full health to functional loss. This yields the parameter D50 (time in months for the total ALSFRS-R score to drop to 24), a summative descriptor of disease aggressiveness. Normalizing absolute disease duration to D50 gives the metric relative D50 (rD50), an open-ended reference scale where 0 indicates disease onset and 0.5 the time-point of halved functionality. rD50 therefore provides a unit-less scale that reflects individual disease covered and enables comparison between individuals with different progression types. It can also be used to stratify patients into contiguous disease phases: early semi-stable Phase I ($0 \leq rD50 < 0.25$), early progressive Phase II ($0.25 \leq rD50 < 0.5$), and late progressive and late stable Phases III/IV ($rD50 \geq 0.5$).

2.3 Biofluid Collection and Laboratory Marker Analyses

CSF was obtained by lumbar puncture and venous blood was drawn in EDTA Monovettes immediately after. CSF and plasma were prepared by centrifugation (5000 × g, 10 mins, 4°C) within a maximum of 1 hour from collection and stored at –80°C until further use. All CSF samples were inspected for evidence of a traumatic puncture. Commercially validated sandwich ELISA kits were used to assay all analytes: CHIT1 (MBL), CHI3L1 (R and D Systems), CHI3L2 (MBL), pNfH (EuroImmun Diagnostics) and NfL (IBL International). All samples and standards were assayed in duplicate in accordance with manufacturer instructions and mean intra and inter-assay coefficients of variation for all assays were <10% and <20%, respectively. Absorbance was measured at 450 nm (wavelength correction at 540 nm or 635 nm (for pNfH) using a TECAN multi-mode plate reader. Sample concentrations were extrapolated using 4-parameter logistic regression analysis.

2.4 Mouse lines and Animal husbandry

Mice of both sexes were included in all experiments. Animals were housed in individually ventilated cages with ad libitum access to food and water; experimental animals were housed with littermates. All animal experiments were performed with the approval of the responsible local authorities of the Government of Upper Bavaria and in accordance with institutional guidelines.

2.5 Experimental mice and genotyping

Transgenic SOD1G93A[18] mice were obtained from Jackson Laboratory (Tg(SOD1)2Gur/J], *SOD^{G93A}*, #002726), and heterozygous mice were crossed to generate littermate controls. *Thy1*-GA-CFP[19] and rNSL8-hTDP-43[20] mice were kindly provided by the Edbauer group. Genomic DNA was extracted from tail biopsy lysis (lysis buffer in mM: 67 Tris pH 8.8, 16.6 $(NH_4)_2SO_4$, 6.5 $MgCl_2$, 5 β-mercaptoethanol, 10% Triton and 50 µg/ml Proteinase K; incubation at 55°C for 5 hours, followed by 5 min inactivation at 95°C). PCR was performed with GoTaq Green Master Mix (Promega, #M7121) following a standard protocol. PCR products were separated on 1% agarose gel. Genotyping primers and expected products are provided in Supplementary Table 2.

2.6 Tissue Processing, Immunofluorescent Staining and Image Analysis

Animals were anaesthetized and transcardially perfused; brains and spinal cords were collected in 4% PFA in PBS and post-fixed at 4ºC for maximum for 48 hours. Serial free-floating sections were cut at a thickness of 50 µm using the VT1200S vibratome (Leica). Antigen retrieval was performed for 15 mins at 90ºC in citrate buffer (pH 6). Sections were incubated for a maximum of 2 nights at 4ºC with appropriate primary antibodies prepared in Block/Perm Buffer (0.5% Triton, 5% donkey serum and 5% BSA in PBS). Following washes in PBS-T (PBS with 0.05% Tween-20), sections were incubated with corresponding secondary antibodies coupled to either Alexa 488, 594, or 647 for 1.5 hours at room temperature, counterstained with DAPI and mounted using Fluromount-G (Southern Biotech). Antibody details and dilutions are provided in Supplementary Table 3. Staining fidelity was confirmed by the omission of primary antibodies (Supplementary Figure 2)

2.7 Image Processing and Analyses

Z-stack images were acquired using a confocal laser scanning microscope (LSM 710, Zeiss) at magnifications of either 40x or 63x with all acquisition parameters held constant. Mean gray values for CHIT1 and CHI3L1 immunoreactivity were quantified within marker-positive (NeuN, Iba-1, GFAP) regions of interest (ROIs) using the ImageJ software suite. ROIs were defined using in-house macros and then superimposed on the corresponding chitinase channel (additional details in Supplementary Information).

2.8 Statistical Analyses

All analyses were performed using the SPSS Statistics, GraphPad Prism, and SciStat® software packages. Normality was assessed using the Shapiro-Wilk test and all bio-analyte levels were log-transformed. Differences in age and gender between diagnostic cohorts and ALS sub-cohorts were assessed using a one-way ANOVA and Fisher's exact test. Correlations between variables were assessed using Pearson's r. Between-group differences were assessed using a one-way ANCOVA and the Bonferroni correction for follow-up post-hoc tests. Linear regression analyses were performed using D50 as the "outcome" variable. Receiver operating characteristic (ROC) curve analysis was used to assess classifier performance when distinguishing between a) ALS vs. Non-ALS or b) aggressiveness types. Optimal cut-off values were determined using the Youden index. Finally, between-group differences in immunostaining experiments were assessed using the Mann Whitney-U test. Individual data points were normalized to the mean of the wild-type (wt) group to enable visualization as relative fold-changes. Two-tailed statistical significance was set at $p < 0.05$.

3. Results

3.1 Demographic Details

A detailed overview of demographic and clinical characteristics is provided in Table 1. No significant differences in age were noted between ALS patients and NDegs; however, both these cohorts were significantly older than the NDC cohort. No significant differences in gender distribution were noted between the cohorts. The ALS cohort was sub-stratified into sub-groups based on the median D50 value of 30 months: high aggressiveness = D50 ≤ 30 vs. low aggressiveness D50 > 30. Significant differences in age, disease duration, onset-type, total ALSFRS-score, PR, D50, and rD50 at sampling were noted between patients with high vs. low aggressiveness; no differences were noted in gender distribution (all results in Table 1). *C9orf72* mutations were confirmed in 3 ALS patients and 37/39 patients were receiving Riluzole at the time of sampling.

3.2 CSF chitinase levels are substantially increased in ALS and correlate with the neurofilament proteins but do not diagnostically outperform them

Cross-sectional analyses of biofluid immunoassays were performed with age as a co-variate. In keeping with previous reports, we confirmed substantial elevations of CSF CHIT1, CHI3L1, and CHI3L2 levels in ALS patients relative to NDCs (Fig.1A-C). Of note, CHIT1 and CHI3L2, but not CHI3L1, were also significantly elevated in the ALS sub-cohort relative to NDegs. While no significant between-group differences were noted for plasma chitinase levels (Table 1), significant correlations between plasma and CSF levels were noted for CHIT1 ($r = .345$, $p < 0.001$) and CHI3L1 ($r = .417$, $p < 0.001$). Additionally, plasma CHIT1 and CHI3L1 levels significantly correlated with each other ($r = .410$, $p < 0.001$). Within the ALS cohort, significant differences between onset-types were only noted for CSF CHI3L1 (bulbar mean = 597.46 ± 358.4 ng/ml vs. limb mean = 410.06 ± 205.2 ng/ml; ($t(37) = 2.417$, $p = .02$). We then assessed CSF levels of pNfH and NfL as these are established markers of neuronal damage. As seen in Fig. 1D-E, neurofilament levels were significantly higher in the ALS cohort relative to both the NDeg and NDC cohorts and significantly correlated with levels of all three chitinases (Fig. 1F-G, $p < 0.001$ for all). Diagnostic power was further assessed by combining the NDeg and NDC groups and then using ROC curve analysis to distinguish between "ALS" vs. "Non-ALS" (Fig.1H). The strongest classifier performance was noted for the two neurofilaments, with pNfH having the largest area under the curve (AUC). Both CHIT1 and CHI3L2 were able to discriminate fairly well between groups; CHI3L1 however, performed poorly and showed no significant discriminatory power.

3.3 Glial chitinase levels are strongly elevated in symptomatic disease in the GA-CFP model of ALS

Having confirmed **1)** detectable chitinase levels in controls and **2)** an ALS-associated upregulation, we next identified *in vivo* cellular sources for chitinases using relevant pre-clinical models: GA-CFP, TDP-43 and SOD1 mice. We used lumbar spinal cord sections of 5-month-old symptomatic mice (n = 5, GA-CFP+) and their wt littermates (n = 5, GA-CFP-) and first examined microglia and astrocytes as these have been previously reported as chitinase sources[14, 15]. Iba-1+ object counts were significantly higher in GA-CFP+ mice than in control animals (median GA-CFP+ = 365 vs. GA-CFP- = 241.5, $U = 0$, $p = 0.01$), while no difference was noted for GFAP+ object counts (median GA-CFP+ = 1854 vs. GA-CFP- = 1739, $U = 10$, $p = 0.69$). Thus, GA-CFP+ mice displayed explicit microgliosis but no astrogliosis, as originally reported by Schludi et al[19].

3.3.1 Microglia

In keeping with earlier reports, we confirmed CHIT1 staining in Iba-1+ microglia[14] (Fig. 2A). Surprisingly, we also noted CHI3L1 staining in this cell population, contradicting previous findings suggesting exclusive astrocytic expression of CHI3L1 in ALS[15] (Fig. 2E). Staining for both chitinases was visible primarily in the cell bodies and the processes. Between-group comparisons of mean grey values in Iba-1+ ROIs showed that microglial levels of CHIT1 were significantly higher in GA-CFP+ mice (median GA-CFP+ = 19,764 vs. GA-CFP- = 14,977, $U = 1, p = 0.01$, Fig. 2B). This upregulation was also observed for CHI3L1 (median GA-CFP+ = 33,862 vs. GA-CFP- = 20,879, $U = 1$, $p = 0.01$, Fig. 2F). Elevated microglial expression of CHIT1 and CHI3L1 was also visually evident in the TDP-43 (Fig. 2C, G) and SOD1 models (Fig. 2D, H).

3.3.2 Astrocytes

In contrast, we found no distinct overlap between CHIT1 signal and GFAP+ ROIs in any of the 3 examined models in either diseased or wt animals. (Supplementary Fig. 1). CHI3L1 staining was noted in GFAP+ astrocytes with staining localized around the perinuclear cytoplasm and extending to long processes, as previously reported[21] (Fig. 2I). CHI3L1 immunoreactivity in GFAP+ ROIs was significantly higher in GA-CFP+ animals (median GA-CFP+ = 16, 835 vs. GA-CFP- = 12,294, $U = 2$, $p = 0.03$, Fig. 2J). This disease-associated trend was also confirmed in the TDP-43 (Fig. 2K) and SOD1 models (Fig. 2L). Additionally, since micro- and astroglial populations were co-stained within the same sections, we were able to compare cell populations and infer that microglia express significantly more CHI3L1 than astrocytes (GA-CFP- microglial CHI3L1 median = 20,879 vs. astrocytic = 12,294, $U = 1, p = 0.0079$). This difference was even more obvious in GA-CFP+ mice (microglial CHI3L1 median = 33,862 vs. astrocytic = 16,835, $U = 0, p = 0.0079$).

To summarize, we confirmed physiological microglial expression of both CHIT1 and CHI3L1 and astrocytic expression of CHI3L1 only. Further, each of these populations displayed a disease-associated chitinase upregulation, suggesting that they are all vulnerable in ALS.

3.4 Neurons are an unreported physiological source of CHIT1 and CHI3L1 and vulnerable to pathological dysregulation in the GA-CFP model of ALS

We then examined neurons and surprisingly found robust somatic staining for both chitinases in all NeuN+ objects in the ventral horn of both GA-CFP- and + mice (Fig, 3A, E). Overall, CHIT1 staining was granular and "speckly", while CHI3L1 staining appeared smooth and diffused in the cytoplasm. NeuN+ object counts did not differ between groups, confirming no overt neuronal loss in GA-CFP+ mice[19] (median GA-CFP+ = 125 vs. GA-CFP- = 124, $U = 2$, $p = 0.9$). Mean gray value quantification in NeuN+ objects indicated that CHIT1 intensity was significantly higher in GA-CFP+ mice (median = 4949 vs. GA-CFP- = 3776, $U = 2, p = 0.03$, Fig. 3B). Representative stainings showed the same disease-associated neuronal CHIT1 upregulation in the TDP-43 and SOD1 models (Fig. 3C, D). This effect was particularly pronounced in the TDP-43 model, which showed substantial neuronal and extracellular staining in diseased tissue. Neuronal CHI3L1 levels did not significantly differ between GA-CFP+ and – mice, (median = 10,284 vs. GA-CFP- = 8426, $U = 12, p > 0.05$, Fig. 3E, F). However, the TDP-43 and SOD1 models visually indicated a possible disease-associated elevation, and this effect was again especially obvious in the TDP-43 animals (Fig. 3G). Given the almost ubiquitous neuronal chitinase expression in the spinal cord, we examined representative brain sections from a wt animal. We noted pan-neuronal CHIT1 and CHI3L1 expression across different brain regions, including

the motor cortex, hippocampus, and cerebellum (Fig. 4). Taken together, we show that neurons are a major physiological source of both CHIT1 and CHI3L1, with CHIT1 elevated in GA-CFP+ mice. Conversely, a disease-associated increase in CHI3L1 may only be evident in models with more severe phenotypes.

3.5 Higher CSF chitinase levels are associated with increased disease aggressiveness

We observed more pronounced disease-associated chitinase upregulation across all examined cell populations in the SOD1 and TDP-43 models, both of which have a more aggressive clinical phenotype than the GA-CFP model. Accordingly, we speculated that increased neuroinflammation and concomitant chitinase upregulation are associated with higher disease aggressiveness. To examine this in humans, we used the D50 model to stratify the ALS cohort into individuals with high vs. low disease aggressiveness. ANCOVA analyses were performed to determine if bio-analyte concentrations differ between disease aggressiveness sub-groups; rD50 at sampling, gender, age at- and site-of onset were included as co-variates (Fig. 5A-E). As expected, patients with increased disease aggressiveness had significantly higher CSF levels of both pNfH ($F(1, 35) = 15.662$, $p < 0.0005$, partial $\eta^2 = .309$) and NfL ($F(1, 35) = 23.27$, $p < 0.0005$, partial $\eta^2 = .399$). Interestingly, CSF CHIT1 ($F(1, 35) = 7.476$, $p = .01$, partial $\eta^2 = .176$) and CHI3L1 ($F(1, 35) = 8.997$, $p = .005$, partial $\eta^2 = .204$) levels were significantly higher in patients with increased disease aggressiveness. Crucially, this effect persisted even after controlling for rD50 as a surrogate for accumulated disease, suggesting that CSF CHIT1 and CHI3L1, like the neurofilament proteins, associate exclusively with disease aggressiveness independent of disease phase. As no main effect of disease aggressiveness was noted for CSF CHI3L2 ($F(1, 35) = 3.593$, $p = .0566$, partial $\eta^2 = .093$), it was excluded from further analyses.

3.6 CSF chitinase levels can predict overall disease aggressiveness in ALS

ROC curve analysis suggested that like the neurofilament proteins, both CHIT1 and CHI3L1 could significantly distinguish between aggressiveness types (Fig. 5F). Follow-up multiple linear regression analysis confirmed that a model comprising CHIT1 and CHI3L1 significantly and independently predicted D50 and accounted for 60.9% of its variation (Fig. 5G). We then performed hierarchical regression to determine if the addition of CHIT1 and CHI3L1 significantly contributed to the prediction of D50 above the neurofilaments alone. The model comprising only NfL and pNfH as predictors accounted for 67.6% of the variation in D50 (Block 1); however, only NfL contributed a significant predictive effect. Upon adding CHIT1 and CHI3L1, the total variance explained increased to 75.8%. Notably, the inclusion of the chitinases in the model led to a statistically significant increase in R^2 of .090 ($F(2, 34) = 7.045$) (Fig. 5H). However, of the 4 combined analytes, only NfL and CHI3L1 contributed a significant predictive effect. Indeed, a final model combining only NfL and CHI3L1 accounted for 75.9% of the variation in D50, confirming that of the 4 analytes, these had the most predictive utility for disease aggressiveness (Fig. 5I). Results of all regression analyses are detailed in Table 2. Finally, plasma chitinase levels did not significantly differ between disease aggressiveness sub-groups (*data not shown*).

3.7 Chitinase levels are stable across the functional ALS disease course

ANCOVA analyses were performed to assess differences across rD50-derived disease phases. Phases II and III were combined as the latter had only four individuals. D50, gender, age-at and site-of onset were included as co-variates. As seen in Supplementary Table 4, no significant between-phase differences were noted in

either CSF or plasma levels for any of the analytes. Interestingly, plasma CHI3L1 levels were strongly influenced by the co-variate age ($F(1, 34) = 18.04, p < 0.001, \eta^2 = .340$).

4. Discussion

Despite several studies describing chitinase dysregulation in ALS, implications for disease remain unclear. This study used a hybrid approach combining an independent clinical cohort and relevant preclinical models to examine the *in vivo* sources, diagnostic and prognostic utility of the chitinases. In keeping with the literature, we observed that CHIT1 and CHI3L2 are significantly elevated in the CSF of ALS patients relative to both individuals with other neurodegenerative diseases (NDegs) and neurologically healthy controls (NDCs)[11, 15, 22]. Contrary to these studies, CSF CHI3L1 levels did not significantly differ between ALS patients and NDegs and a significant increase was only noted relative to NDCs. This is unsurprising, as CHI3L1 is substantially elevated in several other neurodegenerative conditions, including AD[23] and Parkinson's disease[24], both of which were represented in our NDeg cohort, and has also previously shown poor classifier performance[11]. This aligns with our ROC analysis which indicated that CHI3L1, unlike CHIT1 and CHI3L2, had no discriminatory power when distinguishing between ALS vs. Non-ALS. Interestingly, of the three chitinases, CHI3L2 had the highest AUC and correlated the most strongly with pNfH, which showed the best diagnostic power overall. The differing diagnostic potential between chitinases supports the idea of varying expression reflecting subtle differences in the underlying pathology. This is particularly evident across the MND spectrum; despite the considerable overlap, ALS and FTD display specific chitinase patterns. Higher CHIT1 and CHI3L2 levels are associated with an increased motoric component: CSF CHIT1 and CHI3L2 levels are higher in ALS relative to mimicking diseases, PLS and FTD[11, 25, 26]. Further, CHIT1 and CHI3L2 levels could distinguish between *C9orf72*-ALS and *C9orf72*-FTD patients[27]. Conversely, CHI3L1 is associated with increasing cognitive deficits; CSF levels are higher in FTD relative to ALS[25], correlate with worsening performance on cognitive tests[11], and can predict the risk of developing cognitive impairment in preclinical AD[28]. Therefore, our results indicate that while the chitinases may not diagnostically outperform neurofilaments, they can assist with subtle distinctions between different neurodegenerative conditions and increase diagnostic certainty. Indeed, CHIT1 levels could predict progression to El Escorial diagnostic categories[29]. In keeping with the literature, no significant differences between either diagnostic or aggressiveness groups were noted for CHIT1, CHI3L1 or CHI3l2 in plasma, showing that chitinase upregulation is predominantly a feature of the CNS rather than the periphery. This makes the use of a blood-based marker unlikely, especially since several systemic conditions can substantially influence peripheral chitinase levels[8, 30]. Of note, plasma-CSF level correlations were only noted for CHIT1 and CHI3L1. This concurs with a recent finding that circulating macrophages in ALS had an intrinsically augmented potential for CHIT1 and CHI3L1 production. Since no such effect was observed for CHI3L2, it further supports the idea that individual chitinases have distinct expression profiles[31].

To the best of our knowledge, this study is the first to use relevant preclinical models to examine *in vivo* chitinase sources and quantitatively recapitulate the upregulation seen in ALS patients. Using a cohort of GA-CFP+ mice and their wt littermates, we confirmed that Iba-1+ microglia are a source of CHIT1, corroborating both post-mortem and cell-culture findings[14, 32]. Additionally, symptomatic GA-CFP mice displayed upregulated CHIT1 immunoreactivity within this population. Interestingly, the same phenomenon was noted

for CHI3L1: here microglial expression was noted in both GA-CFP- and GA-CFP+ mice, with a significant upregulation in the latter. Reports on CHI3L1 in ALS have suggested that it is predominantly astrocytic[15], though pan-glial expression of CHI3L1 has been reported in other neurodegenerative conditions. In MS, positive immunostaining was detected in GFAP+ astrocytes and CD68+ microglia in the vicinity of highly inflammatory lesions[33]. While we did observe a disease-associated upregulation of CHI3L1 in astrocytes, it was less pronounced than that observed in microglia, suggesting that chitinase elevation in ALS indicates proportionally more microgliosis. Indeed, a recent study using both total and single nucleus RNA-sequencing reported that major transcriptomic changes in the motor cortex of ALS patients, including substantial CHI3L1 and CHI3L2 upregulation, were likely driven by an overrepresentation of microglial cells[34]. By combining our cross-sectional biomarker results and observations in mice, we posit that spatially differing immunoreactive patterns drive the varying chitinase profiles between different neurodegenerative diseases i.e., proportionally more microgliosis in ALS and astrogliosis in FTD[3, 35-37]. Oeckl et al. proposed a similar concept wherein elevated CSF GFAP levels were only observed in FTD patients, despite the upregulation in CSF CHI3L1 levels being similar to that in ALS patients, possibly indicating a higher degree of astrogliosis in FTD[25].

Surprisingly, we found that neurons, including motor neurons, were a major chitinase source in both the brain and spinal cord, which to the best of our knowledge has not been reported in the context of ALS. Ubiquitous staining was noted for both CHIT1 and CHI3L1 in the CNS of wt mice across all three models. We cross-checked the Allen Mouse Atlas and found ISH data supporting our observation of pan-neuronal cytoplasmic staining in the ventral horn. Data from the Human Protein Atlas also corroborates our findings, with evidence at both the transcriptomic and protein level for neuronal expression of all three chitinases. We speculate that technical difficulties with immunostaining owing to the susceptibility of CNS tissue to delayed post-mortem processing may have obscured this finding in existing studies. Interestingly, we noted that simultaneous staining with a specific antibody combination led to a cross-reaction between CHI3L1 and GFAP, creating the impression of perfect co-localization (Supplementary Fig. 2). Discrepancies between *in vivo* and *vitro* chitinase sources have also been previously reported: Bonneh-Barkay et al. showed that macrophages, despite being a major *in vitro* source of CHI3L1, displayed minimal CHI3L1 expression *in vivo* in neuroinflammatory conditions. Additionally, while conditioned macrophage media induced astrocytic CHI3L1 expression, direct co-culturing with macrophages did not, leading the authors to speculate that regulatory pathways in the CNS environment and neuronal contact *in vivo* modulate expression[38]. This re-iterates the importance of assessing sources *in vivo* and the results reported here.

Neuronal chitinase expression is also conceivable from a disease mechanism perspective, as these are the most heavily compromised cell population in ALS. It is plausible that mounting cell death is accompanied by elevated intra-neuronal chitinase levels and increased extracellular secretion, possibly as an acute inflammatory response. Indeed, quantitative immunostaining revealed significantly increased CHIT1 neuronal levels in symptomatic GA-CFP+ mice, with a similar disease-associated upregulation visually evident in the TDP-43 and SOD1 models as well. Secreted chitinases can then act on neighboring glia, which as demonstrated previously and here, are themselves chitinase sources [14, 32, 39]. An analogous "neuronal-glial" inflammatory shift has also been reported in the context of AD. Neuron-derived IL-6 and MCP-1 led to the recruitment and activation of neighboring microglia[40]; crucially, these cytokines were only evident in neurons and not glia in

the earliest stages of disease suggesting that neurons were the "primary proinflammatory agent". Degenerating neurons may therefore create a damage-associated chemotactic axis, thus recruiting and triggering neighboring glia, as suggested by our observation of several CHI3L1+ astrocytes positioned directly adjacent to CHI3L1+ neurons in SOD+ mice (Fig. 3H, white arrows). Similarly, CHI3L1 expression was noted in both GFAP+ astrocytes and interstitial white matter neurons in post-mortem frontal cortex samples from AD patients[28].

This, combined with the autocrine and paracrine effects of the chitinases, can set up a feed-forward pathological cascade, ultimately leading to the chronic neuroinflammatory milieu characteristic of ALS. This hypothetical cascade also aligns with observations from longitudinal studies showing that chitinase elevation is a feature of the late pre-symptomatic disease phase: levels rise sharply in mutation carriers at the time of pheno-conversion, possibly in response to neuronal loss, and then stay relatively stable over time [41]. Here too, no significant differences in chitinase levels were noted between rD50-derived functional disease phases. Although our cohort included only 4 patients in late disease Phases III/IV, it confirms reports that chitinase levels are stable over time[10, 11, 15] and underscores the ability of the D50 model to approximate longitudinal results from cross-sectional data.

The prognostic relevance of the chitinases has remained unclear to date: studies have used outcomes ranging from the ΔFRS to survival and respiratory function and reported discrepant results. For instance, Abu-Rumeileh et al noted no differences in chitinase levels between fast, intermediate or slow progressors stratified using the ΔFRS[42], while conversely Gille et al reported significant correlations with ΔFRS for CHIT1 and CHI3L1[12]. In a similar vein, one study used multi-variate modelling to show that CHIT1 associated with survival[11], while another reported no correlation[14], and yet another reported an association with CHI3L1 rather than CHIT1[12]. These discrepancies stem from not only cohort differences, but also the aforementioned limitations of the ΔFRS: in particular that it only reflects the rate of decline at a circumscribed time point. Survival is also a direct function of the quality of end-of-life care patients receive, which can vary greatly across institutions. The D50 model provides a more reliable framework as it distinguishes between disease aggressiveness and accumulated degeneration, thus links between potential biomarkers and either of these outcomes can then be examined without the confounding influence of the other. Stratification based on D50 values showed that CHIT1 and CHI3L1 levels were significantly elevated in individuals with higher disease aggressiveness. Crucially, by correcting for rD50, we were able to show that this effect is independent of the functional disease phase i.e., levels are singularly influenced by aggressiveness. This is an important finding as stability over the disease course is critical when using a prognostic marker for cohort enrichment for clinical trials. By using D50 as an outcome metric, we could demonstrate that the chitinases not only associate with disease aggressiveness but can also predict it. Hierarchical regression analysis further demonstrated that including the chitinases significantly improved the predictive utility of the neurofilaments alone, which, to the best of our knowledge, has not been reported thus far. Interestingly, however, when combining all 4 predictors (pNfH, NfL, CHIT1, CHI3L1), only NfL and CHI3L1 contributed a significant effect. A final model comprising only NfL and CHI3L1 was actually the most parsimonious and accounted for the most variation in D50. Indeed, NfL and CHI3L1 also had the highest AUCs in ROC analysis when discriminating between aggressiveness types, with CHI3L1 even having a negative predictive value of 100%. NfL has previously been reported as a superior prognostic marker in ALS and could best predict both survival and ΔFRS[42], and while

conflicting results have been reported for CHI3L1's prognostic ability in ALS, it is indicative of disease severity in several conditions. For instance, in MS, it distinguishes between progressive and relapsing MS, and correlates with spinal cord atrophy and the number of active lesions[43, 44]. The link between chitinases and aggressiveness is also supported by our observations in mice. Immunostainings in both the TDP-43 and SOD1 models, although qualitative, indicated a more pronounced disease-associated upregulation relative to the GA-CFP model. This may be because both these models present with more aggressive phenotypes, including the key pathological hallmark of neuronal loss, which is not a feature of the GA-CFP model.

In addition to demonstrating the auxiliary prognostic and diagnostic utility of the chitinases, our results reiterate the value of biomarker panels for the prognostication of heterogeneous conditions, as single biomarkers are unlikely to capture the multiple pathogenic mechanisms at play. In the case of ALS, these mechanisms are also highly inter-connected, as evidenced by the highly significant correlations between the neurofilament proteins and chitinases and the neuronal expression of CHIT1 and CHI3L1.

Our study is limited by the cohort size, and further analysis with more patients in later disease phases, would enable true longitudinal analyses and validate our findings. Adequate coverage of familial vs. sporadic ALS cases, as well as larger control groups with true ALS mimicking diseases would enable an assessment of whether chitinase profiles reflect the underlying pathology and concomitant neuroinflammatory reaction. Further quantitative and longitudinal studies with other genetic models of ALS could provide insight into how the underlying genetic component can influence disease aggressiveness. An obvious parallel would be comparing the chitinase profile between patients with different familial mutations and assessing whether differences translate to specific clinical outcomes. Next, we did not measure *CHIT1* activity as genetic information on the 24 bp duplication in exon 10 polymorphism was unavailable for this cohort. This polymorphism has a prevalence of almost 50% in European populations and directly affects activity, with homozygous carriers displaying no *CHIT1* activity at all[45, 46]. Future studies could therefore also measure CHIT1 activity as combining this with CHIT1 protein levels may improve the diagnostic predictive value for ALS [39]. Studies should also address the gap between immunostaining and sequencing methods although we speculate that experimental paradigms may have influenced results. For instance, Schneider et al.[44] used two independently published single-cell RNA sequencing datasets (Jäkel et. al and Masuda et. al) to investigate CHI3L1 sources in healthy and MS brains. However, Jäkel et. al [47] restricted analysis to only white matter while Masuda et. al [48] used fluorescence-activated cell sorting to pre-select for CD45+ cells, thus excluding neurons. Future studies should therefore employ multiple methods to confirm our finding of neuronal chitinase expression and verify this *in vivo* in human tissue. This would also identify cellular sources for CHI3L2 for which there is no murine homolog.

To conclude, the present study combined clinical and pre-clinical experimental paradigms to study the cellular sources underlying chitinase upregulation in ALS. Using the D50 model, we provide compelling and novel evidence for the link between chitinases and disease aggressiveness and their utility as prognostic biomarkers. The chitinases may also hold potential as pharmacodynamic biomarkers, as studies in conditions like MS have shown that levels are modulated by immunosuppressant treatment [49].

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6. Author Contributions

Study conception and design: NG, MW, JG, MLB; Data acquisition and analysis: NG, MW, HR. Manuscript and figure preparation: NG, MW, RS, HR, DE, OW, MLB, JG

7. Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

8. Data Availability

The data supporting the conclusions of this article will be made available by the authors upon reasonable request.

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10. Figure Captions

Figure 1. Cross-sectional analyses confirm chitinases are elevated in ALS and correlate with neurofilament proteins

CHIT1 (**A**), CHI3L1 (**B**), CHI3L2 (**C**), pNfH (**D**), and NfL (**E**) levels in the CSF of ALS patients (n = 39), Neurodegenerative Disease Controls (NDegs, n = 13) and Non-Neurological Disease controls (NDCs, n = 11). Between-group differences were assessed using a one-way ANCOVA with age included as a co-variate. Data are presented as box plots with whiskers demarcating 95% confidence intervals. Correlation analyses show that all 3 chitinases significantly correlate with pNfH (**F**) and NfL (**G**) levels; data are displayed as scatter plots with individual *rho* values reported. NDeg and NDC cohorts were combined to create a "Non-ALS" cohort to assess if analytes can distinguish between ALS vs. non-ALS. ROC Curve analysis results indicate that the neurofilament proteins had the best classifier performance (**H**). Sensitivities, specificities, and predictive values are provided in the accompanying table. Statistical significance for all analyses was set at $p < 0.05$ with * < 0.05 and ** < 0.01. Axes for (**A–G**) are displayed in $\log_{10}$ scale.

Figure 2. Glial Chitinase levels are upregulated in preclinical models of ALS

(**A, C, D**) Confocal images of spinal cord ventral horn in 5-month GA-CFP (**A**), TDP-43 (**C**) and SOD1 (**D**) mice and littermate controls, immunostained for Iba-1 (green), CHIT1 (red) and DAPI (blue). Insets in (**A**) show enlarged microglial processes. (**B**) Quantification of CHIT1 intensity normalized to the mean intensity of GA-CFP- littermate controls (n = 5 mice, ≥ 100 Iba-1+ ROIs per animal). (**E, G, H**) Confocal images of spinal cord ventral horn in 5-month GA-CFP (**E**), TDP-43 (**G**) and SOD1 (**H**) mice and littermate controls, immunostained for Iba-1 (green), CHI3L1 (red) and DAPI (blue). Insets in (**E**) show zoomed-in microglial processes.(**F**) Quantification of CHI3L1 intensity normalized to the mean intensity of GA-CFP- littermate controls (n = 5 mice, ≥ 100 Iba-1+ ROIs per animal). (**I**) Confocal images of spinal cord ventral horn in 5-month GA-CFP (**I**), TDP-43 (**K**) and SOD1 (**L**) mice and littermate controls, immunostained for GFAP (green), CHI3L1 (red) and DAPI (blue). Insets in (**I**) show zoomed-in astroglial processes. (**J**) Quantification of CHI3L1 intensity normalized to the mean intensity of GA-CFP- littermate controls (n = 5 mice, ≥ 100 GFAP+ ROIs per animal). Quantitative comparisons were performed using the Mann-Whitney U test. Statistical significance for all analyses was set at $p < 0.05$ with * < 0.05 and ** < 0.01 Data are presented as scatter dot plots with the mean and SD. Scale bars, 5 μm in (**A**), (**E**) and (**I**) insets, the rest 10 μm.

Figure 3. Neurons are an unreported source of chitinases

(**A, C, D**) Confocal images of spinal cord ventral horn in 5-month GA-CFP (**A**), TDP-43 (**C**) and SOD1 (**D**) mice and littermate controls, immunostained for NeuN (green), CHIT1 (red) and DAPI (blue). (**B**) Quantification of CHIT1 intensity normalized to the mean intensity of GA-CFP- littermate controls (n = 5 mice, ≥ 100 NeuN+ ROIs per animal). (**E, G, H**) Confocal images of spinal cord ventral horn in 5-month GA-CFP (**E**), TDP-43 (**G**) and SOD1 (**H**) mice and littermate controls, immunostained for NeuN (green), CHI3L1 (red) and DAPI (blue). White arrows in (**H**) indicate CHI3L1+ glia positioned directly adjacent to atrophying CHI3L1+ neurons (**F**) Quantification of CHI3L1 intensity normalized to the mean intensity of GA-CFP- littermate controls (n = 5 mice, ≥ 100 NeuN+ ROIs per animal). Quantitative comparisons were performed

using the Mann-Whitney U test. Statistical significance for all analyses was set at $p < 0.05$ with * < 0.05. Data are presented as scatter dot plots with the mean and SD. Scale bars, 10 μm.

Figure 4. Chitinase expression is ubiquitous in the brain

Confocal images of the motor cortex, cerebellum and CA1 region of the hippocampus in 5-month wild-type control mice, immunostained for **(A)** CHIT1 (red), **(B)** CHI3L1 (red) and DAPI (blue). Scale bars, 10 μm.

Figure 5. CSF Chitinase levels are associated with and can predict disease aggressiveness

CSF levels of CHIT1 **(A)**, CHI3L1 **(B)**, CHI3L2 **(C)**, pNfH **(D)**, and NfL **(E)** were compared between ALS patients with high vs. low disease aggressiveness. Disease aggressiveness stratification was performed using the group median of D50 = 30 months. Analysis was performed using a one-way ANCOVA with age, gender, onset-type, and rD50 at sampling included as co-variates. Data are presented as box plots with whiskers demarcating 95% confidence intervals. Dashed grey line indicates the optimal cut-off value as indicated by the Youden index. **(F)** ROC curve analysis was performed to assess classifier performance when distinguishing between high vs. low aggressiveness with NfL and CHI3L1 displaying the largest AUC. Sensitivities, specificities, and predictive values are provided in the accompanying table. **(G-I)** A series of regression analyses were performed to establish which combination of analytes shows the most predictive utility for disease aggressiveness (as reflected by individual D50 values). Simple linear regression analysis showed that both CHIT1 and CHI3L1 could significantly predict disease aggressiveness **(G). (H)** Follow-up hierarchical regression analysis showed that the chitinases significantly add to the predictive utility of the neurofilaments alone, while a final linear regression analysis showed that the most parsimonious model included NfL and CHI3L1 **(I)** as the combination of these two explained the highest amount of variation in D50. Data are presented as scatter plots showing actual D50 values vs. those predicted by each model with dotted red line indicating the line of best fit. R values give the correlation between observed and predicted D50 values. Statistical significance for all analyses was set at $p < 0.05$ with * < 0.05 and ** < 0.01. Axes for **(A–E)** are displayed in $\log_{10}$ scale.

Supplementary Figure 1. CHIT1 expression is not evident in GFAP+ astrocytes

Confocal images of spinal cord ventral horn in 5-month GA-CFP **(A)**, TDP-43 **(B)** and SOD1 **(C)** mice and littermate controls, immunostained for GFAP (green), CHIT1 (red) and DAPI (blue) show no clear overlap between CHIT1 and GFAP in either wild type or diseased animals in any of the 3 models. Scale bars, 10 μm.

Supplementary Figure 2. Immunostaining controls

Confocal images of spinal cord ventral horn in 5-month wild-type animals from immunostaining control experiments. Distinct immunoreactivity patterns are observed when sections were stained either **(A)** sequentially or **(B)** simultaneously for CHI3L1 (red, Abcam AB180569 and GFAP (green, ENCOR, CPCA-GFAP). Sequential staining resulted in both neuronal and glial immunoreactivity confirming results obtained with the CHI3L1 antibody from ThermoFisher. However, simultaneous staining resulted in almost perfect co-

localization between CHI3L1 and GFAP, suggesting a possible cross-reaction. **(C)** Omission of primary antibodies (CHIT1/CHI3L1 and population markers) confirms staining fidelity. Scale bars, 10 μm.

Table 1: Cohort Demographics and cross-sectional analyses

	ALS Patients			NDegs	NDCs	Significance
	All	High aggressiveness	Low aggressiveness			
Demographics						
n	39	20	19	13	11	-
Sex [m:f]	20:19	8:12	12:7	6:7	8:3	$p = 0.36^{S}$ $p = 0.14^{SS}$
Age at Sampling† [years]	67.4 (61.3-75.4)	70.5 (66.8-76.06)	61.3 (54-75.25)	69.8 (61.7-76.9)	56.92 (44.5-70)	$p = 0.015^{\$}$ $p = 0.006^{SS}$
Disease Metrics†						
ALSFRS-R at Sampling	39 (30-44)	34 (27.2-39.75)	44 (39-46)	-	-	$p < 0.001^{SS}$
Progression Rate at Sampling	0.8 (0.18-1.44)	1.4 (1.22-2.56)	0.18 (0.11-0.34)	-	-	$p < 0.001^{SS}$
Disease Duration at Sampling	11 (8-22)	8 (7-9)	22 (13-37)	-	-	$p < 0.001^{SS}$
Onset Type [bulbar:limb]	15:29	12:8	3:16	-	-	$p < 0.0079^{SS}$
D50 Model Parameters						
D50†	24.2 (13.7-62)	13.7 (7.7-18)	62.18 (45.2-102)	-	-	$p < 0.001^{SS}$
rD50 at Sampling†	0.24 (0.14-0.44)	0.3 (0.22-0.46)	0.15 (0.09-0.26)	-	-	$p = 0.002^{SS}$
Disease Phase I/II/III-IV	20/15/4	6/11/3	14/4/1	-	-	
CSF Levels (ng/ml)†						
CHIT1	10.12 (4.7-17.6)	15.38 (10.3-28.9)	6.79 (2.2-8.5)	1.94 (0.98-5.3)	1.83 (0.5-8.8)	$p = 0.004^{NDEG}$ $p = 0.019^{NDC}$ $p = 0.01^{\$\$}$
CHI3L1	383.5 (283-674.4)	577 (383.6-749.8)	283 (223-383.5)	457.2 (298.9-748.4)	144.1 (96.2-243.7)	$p = 0.1^{NDEG}$ $p = 0.02^{NDC}$ $p = 0.005^{\$\$}$
CHI3L2	18.8 (12.4-24.8)	22.2 (17.9-26.6)	14.05 (6.9-19.9)	8.7 (5.4-14.7)	6.5 (4.5-12.7)	$p = 0.001^{NDEG}$ $p = 0.01^{NDC}$ $p = 0.05^{\$\$}$
pNfH	3.15 (1.74-5.7)	5.42 (3.28-8.83)	2.33 (1.08-3.04)	1.005 (0.52-1.56)	0.53 (0.34-0.92)	$p < 0.0005^{NDEG.\ NDC}$ $p < 0.0005^{\$\$}$
NfL	4.23 (2.74-9.34)	8.9 (4.5-15.06)	2.83 (1.64-3.7)	0.96 (0.55-3.14)	0.42 (0.24-0.63)	$p < 0.0005^{NDEG.\ NDC}$ $p < 0.0005^{\$\$}$
Plasma Levels (ng/ml)†						
CHIT1	24.4 (17.1-35.7)	26.3 (19.1-36.8)	23.7 (13.2-35.7)	31.7 (21.5-56.8)	24.5 (13.5-48.8)	p > 0.05 for all
CHI3L1	62.86 (29.3-181.7)	99.3 (47.2-176.3)	45.4 (27.08-181.7)	80.98 (40.5-111.6)	58.65 (30.9-80.7)	p > 0.05 for all
CHI3L2	1.4 (0.7-2.42)	1.23 (0.7-1.9)	1.5 (0.7-2.52)	1.69 (0.7-2.3)	1.5 (0.3-5.5)	p > 0.05 for all

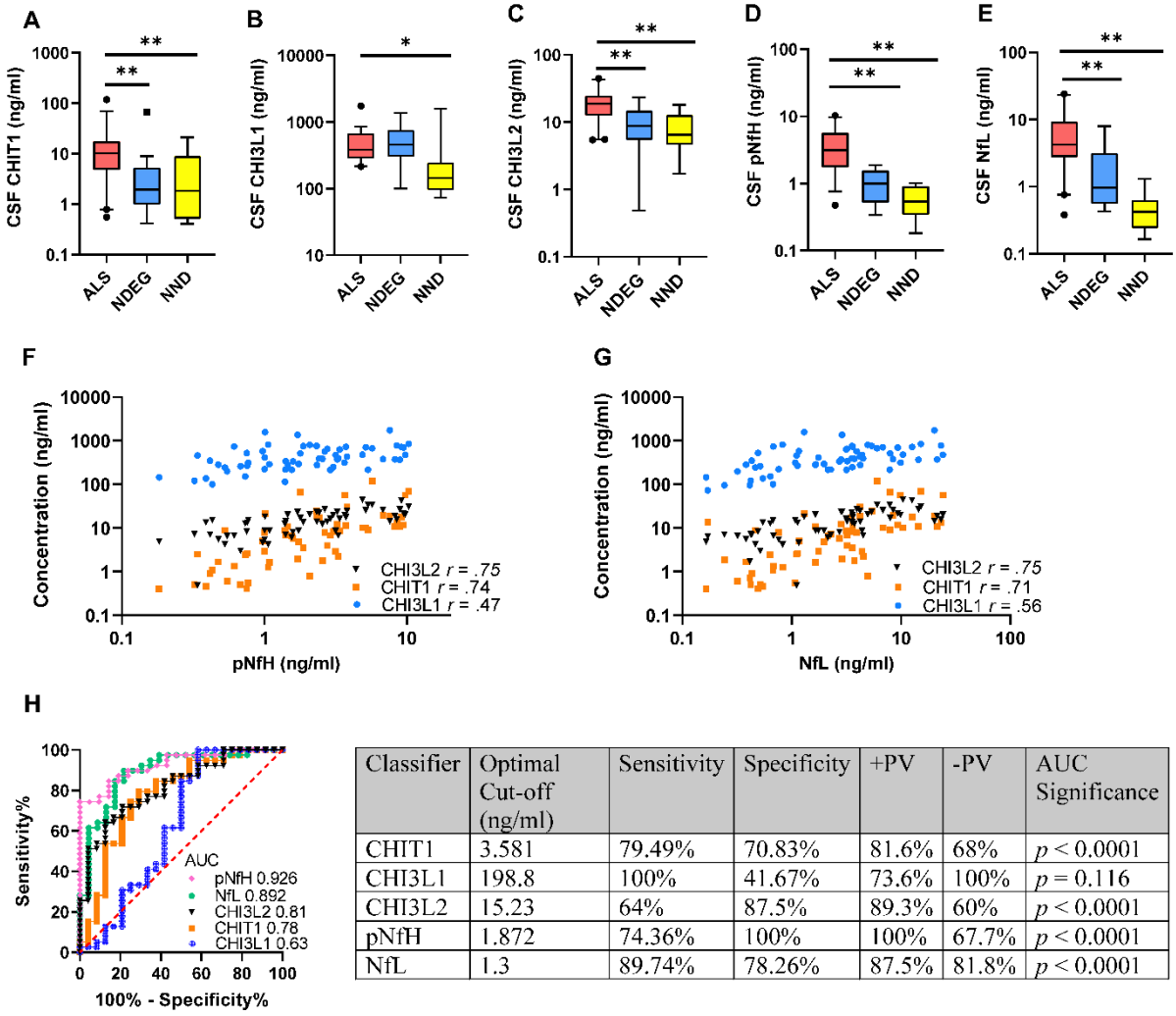
ALS, Amyotrophic Lateral Sclerosis; ALSFRS-R, Amyotrophic Lateral Sclerosis Functional Rating Scale Revised; ANCOVA, Analysis of covariance; CHIT1, Chitotriosidase 1; CHI3L1, Chitinase 3-like-1; CHI3L2, Chitinase 3-like-2; CSF, cerebrospinal fluid; NDeg, Neurodegenerative Disease Control; NDC, Non-neurodegenerative disease control; NfL, neurofilament light chain; pNfH, phosphorylated neurofilament heavy chain; rD50, relative D50

† Data presented as median with Interquartile range
$ Comparing ALS, NDeg and NDC cohorts
$$ Comparing high vs. low ALS aggressiveness cohorts

Table 2: Linear Regression Analysis Results

Model A: Multiple Linear Regression						
Variable	B	SE B	β	Significance	R, R^2 ($^{adj}R^2$)	df, F
Log CHIT1	-3.24	0.090	-.383	$p < 0.001$	.794, .630 (.609) $p < 0.001$	(2, 36), 30.64
Log CHI3L1	-1.19	0.212	-.596	$p < 0.001$		
Model B: Hierarchical Linear Regression						
Variable	B	SE B	β	Significance	R, R^2 ($^{adj}R^2$)	df, F
Block 1: pNfH + NfL						
Log pNfH	-.238	.338	-.190	$p = .486$	.833, .693 (.676) *$p < 0.001$*	(2, 36), 40.66
Log NfL	-.670	.278	-.651	$p = 0.021$		
Block 2: pNfH + NfL + CHIT1 + CHI3L1						
Log pNfH	.011	.313	.009	$p = .971$	.885, .783 (.758) *$p = 0.003$*	(4, 34), 30.68
Log NfL	-.580	.243	-.564	$p = .023$		
Log CHIT1	-.111	.088	-.131	$p = .219$		
Log CHI3L1	-.716	.196	-.356	$p < .001$		
Model C: Multiple Linear Regression						
Variable	B	SE B	β	Significance	R, R^2 ($^{adj}R^2$)	df, F
Log NfL	-6.54	0.099	-.635	$p < 0.001$	.879, .772, (.759) $p < 0.001$	(2, 36), 60.93
Log CHI3L1	-6.99	.193	-.348	$p = 0.001$		

B, unstandardized beta; β, standardized beta; $^{adj}R^2$, adjusted R^2; CHIT1, Chitotriosidase 1; CHI3L1, Chitinase 3-like-1; CHI3L2, Chitinase 3-like-2; df, degrees of freedom; NfL, neurofilament light chain; pNfH, phosphorylated neurofilament heavy chain, SE B, standard error for B



Classifier	Optimal Cut-off (ng/ml)	Sensitivity	Specificity	+PV	-PV	AUC Significance
CHIT1	3.581	79.49%	70.83%	81.6%	68%	$p < 0.0001$
CHI3L1	198.8	100%	41.67%	73.6%	100%	$p = 0.116$
CHI3L2	15.23	64%	87.5%	89.3%	60%	$p < 0.0001$
pNfH	1.872	74.36%	100%	100%	67.7%	$p < 0.0001$
NfL	1.3	89.74%	78.26%	87.5%	81.8%	$p < 0.0001$

Figure 1. Cross-sectional analyses confirm chitinases are elevated in ALS and correlate with neurofilament proteins

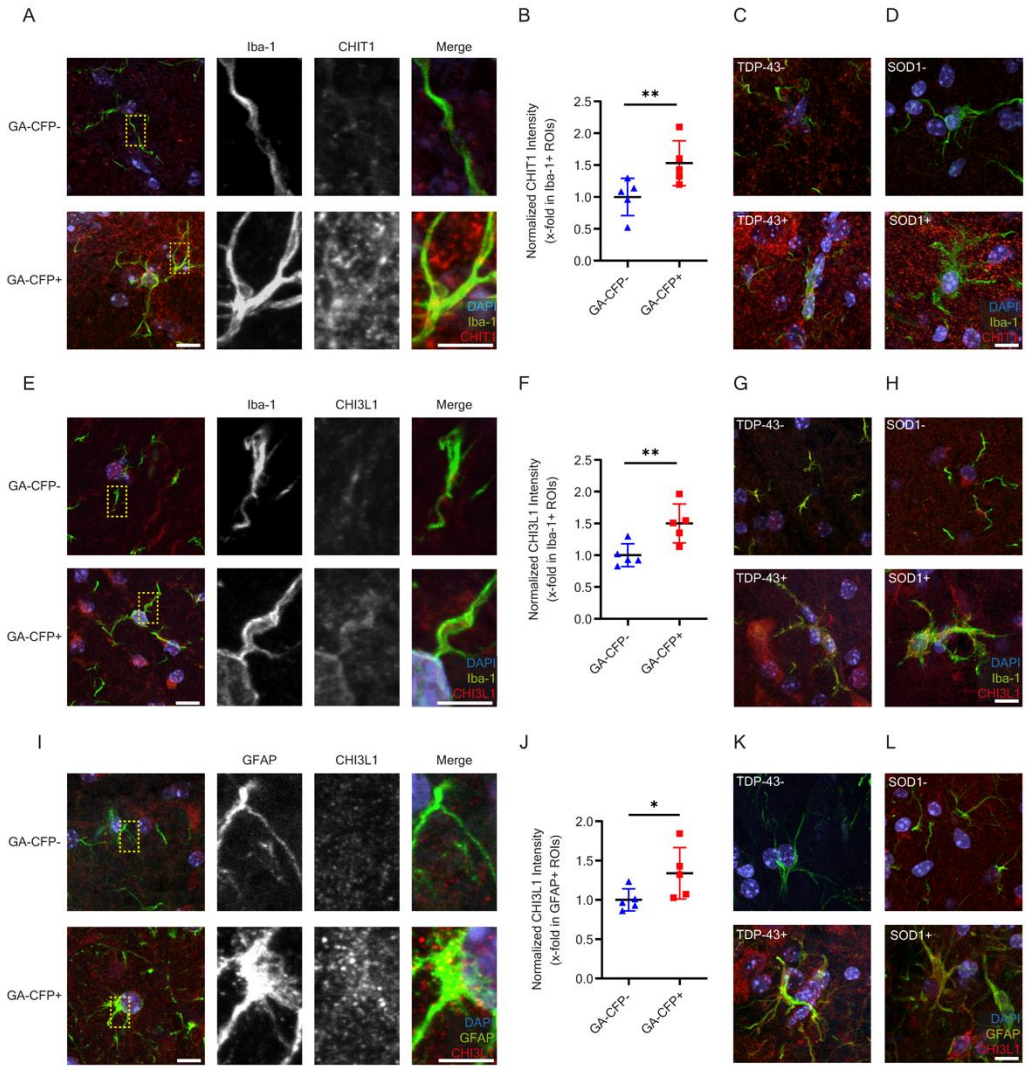


Figure 2. Glial chitinase levels are upregulated in preclinical models of ALS

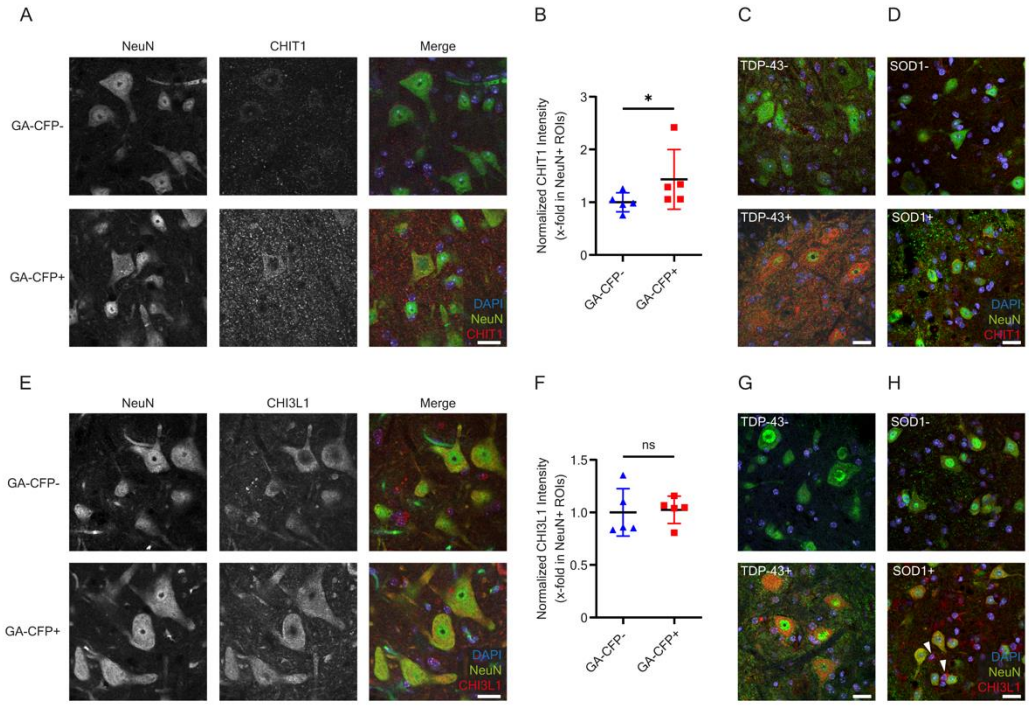


Figure 3. Neurons are an unreported source of chitinases

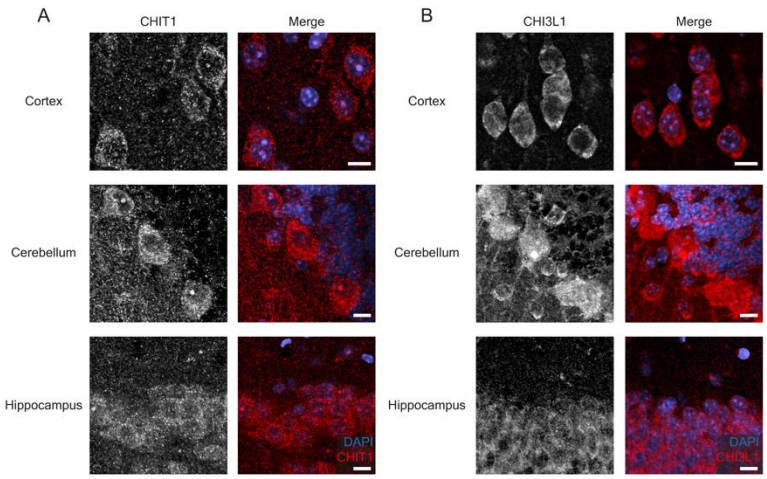
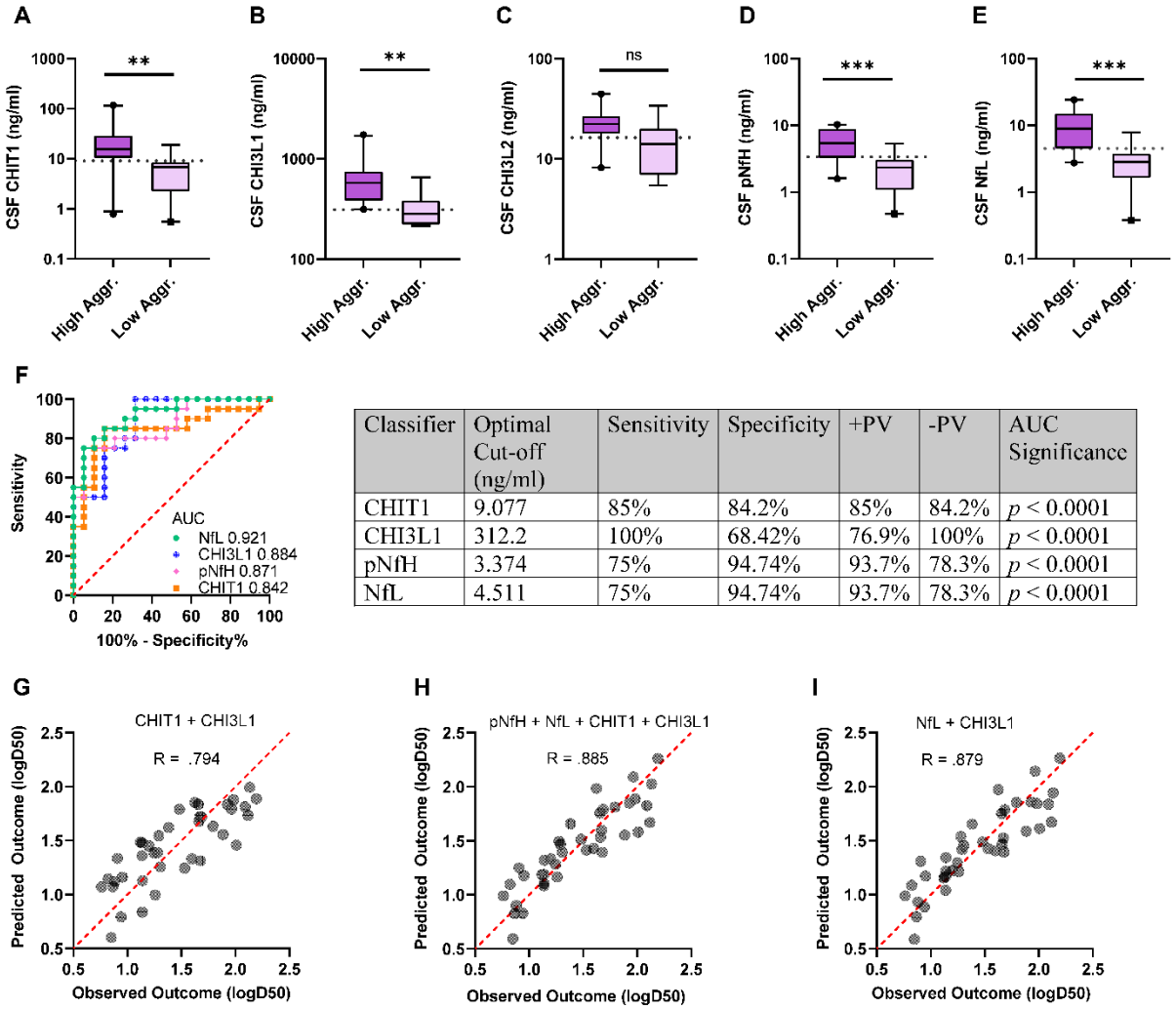
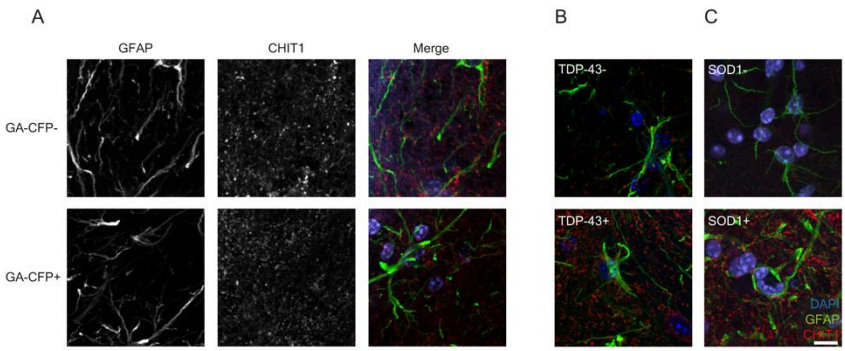


Figure 4. Chitinase expression is ubiquitous in the brain

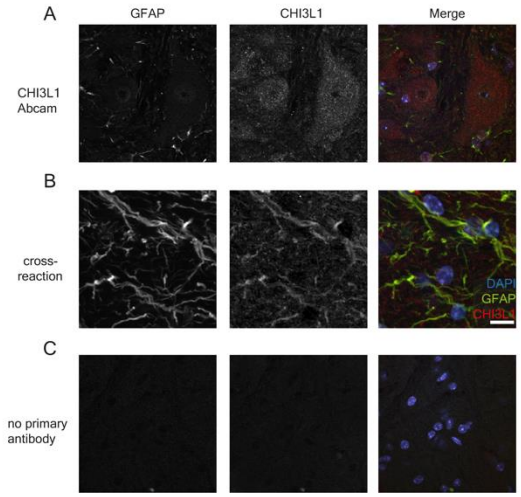


Classifier	Optimal Cut-off (ng/ml)	Sensitivity	Specificity	+PV	-PV	AUC Significance
CHIT1	9.077	85%	84.2%	85%	84.2%	$p < 0.0001$
CHI3L1	312.2	100%	68.42%	76.9%	100%	$p < 0.0001$
pNfH	3.374	75%	94.74%	93.7%	78.3%	$p < 0.0001$
NfL	4.511	75%	94.74%	93.7%	78.3%	$p < 0.0001$

Figure 5. CSF chitinase levels are associated with and can predict disease aggressiveness



Supplementary Figure 1. CHIT1 expression is not evident in GFAP+ astrocytes



Supplementary Figure 2. Immunostaining Controls

Supplementary Information

Table S1: Diagnostic Information for Control Cohorts

Neurodegenerative Disease Controls	n
Alzheimer's Disease	2
Atypical Parkinsonian Syndrome (1/3 individuals with potential overlap with Frontotemporal Dementia	3
Vascular Dementia	1
Gait Disorder	1
Parkinsonism	4
Multifocal Motor Neuropathy	1
Primary Lateral Sclerosis	1
Non-Neurodegenerative Disease Controls	
Idiopathic Intracranial Hypertension	3
Neurologically Healthy	1
Normal Pressure Hydrocephalus	3
Traumatic Gait Disturbance	1
Suspected Pseudodementia	1
Benign Fasciculation Syndrome	2

Table S2: Transgenic Mouse Lines and Genotyping Primers

mouse-line	in text	primer name	primer sequence (5'-3')	amplicon size
Tg(SOD1)2Gur/J], *SOD* ^{G93A}	SOD1-G93A	SOD-F	CATCAGCCCTAATCCATCTGA	236 bp
		SOD-R	CGCGACTAACAATCAAAGTGA	
Thy1 $(GA)_{149}$-CFP	GA-CFP	-F	TCCAGGAGCGTACCATCTTC	331 bp
		-R	GTGCTCAGGTAGTGGTTGTC	
B6;C3-Tg(NEFH-tTA)8Vle/J (JAX Stock No: 025397)	NEFH-tTA(B6C3)*	NEFH-tTA-TG-JAX-fw	CTCGCGCACCTGCTGAAT	151 bp (transgene)
		NEFH-tTA-TG-JAX-rv	CAGTACAGGGTAGGCTGCTC	
		NEFH-tTA-IC-JAX-fw	CTAGGCCACAGAATTGAAAGATCT	324 bp (internal positive control)
		NEFH-tTA-IC-JAX-rv	GTAGGTGGAAATTCTAGCATCATCC	
B6;C3-Tg(tetO-TARDBP*)4Vle/J (JAX Stock No: 014650)	TDP-43	tetO-TARDBP*-TG-JAX-fw	TTGCGTGACTCTTTAGTATTGGTTTGATGA	480 bp (transgene)
		tetO-TARDBP*-TG-JAX-rv	CTCATCCATTGCTGCTGCG	
		tetO-TARDBP*-IC-JAX-fw	CAAATGTTGCTTGTCTGGTG	200 bp (internal positive control)
		tetO-TARDBP*-TG-JAX-rv	GTCAGTCGAGTGCACAGTTT	

***rNLS8-hTDP-43 mice used in the study are an inter-cross of** NEFH-tTA(B6C3) transgenic and hTDP-43deltaNLS4 (B6C3) line

Table S3: Antibody information for Immunostaining Experiments

Target	Primary Antibody (Catalog number, dilution, duration)	Secondary Antibody (Catalog number, dilution, duration)
CHIT1	PA5-76778, 1:400, 48 hours at 4°C	A21246, 1:500, 2 hours at RT
CHI3L1	PA5-95897, 1:400, 48 hours at 4°C	A21246, 1:500, 2 hours at RT
NeuN	MAB377, 1:500, 48 hours at 4°C	A11005, 1:500, 2 hours at RT
Iba-1	015-28011, 1:500, 24 hours at 4°C	pre-conjugated to SPICA-568 dye
GFAP	A21282, 1:500, 48 hours at 4°C	A11005, 1:500, 2 hours at RT

CHIT1, Chitotriosidase 1; CHI3L1, Chitinase 3-like-1; GFAP, Glial fibrillary acidic protein; Iba-1, Ionized calcium binding adaptor molecule 1; NeuN, Neuronal Nuclei; RT, room temperature

Table S4: Demographic information and cross-sectional analysis results for rD50-derived disease phases

Demographic Information for rD50-derived disease phases					
		Phase 1	Phases II + III		Significance
n		20	19		-
Sex [m:f]		13:7	7:12		$p = .07$
Age at Sampling* [years]		61.5 (54-67.6)	72.58 (67.2-77.2)		$p < .001$
ALSFRS-R at Sampling†		44 (40-46)	30 (26-35)		$p < .001$
Progression Rate at Sampling†		0.32 (0.1-1.2)	1.44 (0.47-2.6)		$p < .001$
Disease Duration at Sampling*		12 (7.3-22.7)	9 (8-21)		$p = .97$
Onset Type [bulbar:limb]		6:14	9:10		$p = .26$
D50*		45.8 (19.6-95.9)	13.8 (8.1-39.5)		$p < .001$
rD50 at Sampling†		0.15 (0.09-0.2)	0.44 (0.3-0.5)		$p < .001$
ANCOVA results for between-phase comparisons					
Analyte	Phase 1	Phases II + III	F	Effect Size η^2	Significance
CSF levels (ng/ml)†					
pNfH	2.97 (1.16-4.94)	3.65 (2.32-8.93)	.211	.006	$p = .649$
NfL	3.67 (1.7-5.65)	5.4 (3.2-12.6)	.001	.000	$p = .974$
CHIT1	7.69 (2.34-12.6)	11.4 (7.8-18.7)	.256	.007	$p = .616$
CHI3L1	301 (229.2-423)	563.6 (367-722)	.776	.022	$p = .384$
CHI3L2	14.6 (7.3-19.6)	21.7 (17.6-26.7)	.586	.016	$p = .449$
Plasma levels (ng/ml)†					
CHIT1	21.9 (13.8-28.6)	31.5 (19.2-46.6)	.259	.007	$p = .614$
CHI3L1	38.8 (27.7-82.2)	119.8 (57.4-195.8)	.394	.011	$p = .534$
CHI3L2	1.375 (0.75-2.4)	1.29 (0.86-2.42)	.073	.002	$p = .788$

ALSFRS-R, Amyotrophic Lateral Sclerosis Functional Rating Scale Revised; ANCOVA, Analysis of covariance; CHIT1, Chitotriosidase 1; CHI3L1, Chitinase 3-like-1; CHI3L2, Chitinase 3-like-2; CSF, cerebrospinal fluid; NfL, neurofilament light chain; pNfH, phosphorylated neurofilament heavy chain; rD50, relative D50

† Data presented as median with Interquartile range

Image Processing and Workflow in Fiji for quantitative immunostainings

Custom in-house macros were used to define marker-positive (NeuN+, Iba-1+, GFAP+) regions of interest (ROIs). Images were pre-processed to develop an automated mask that could be applied to the original image for accurate segmentation.

- NeuN: Pre-processing included the application of a pseudo-flat field correction, rolling ball background subtraction, the "Median" filter and finally the "Huang 2" auto-threshold.
- Iba-1: Pre-processing included the application of a rolling ball background subtraction, the "Maximum" and "Minimum" filters sequentially, the "Li" auto-threshold, and exclusion of all objects < 500 pixels using the "Analyse Particles" function.
- GFAP: Pre-processing included the application of a rolling ball background subtraction, "Minimum" and "Maximum" filters sequentially, "Unsharp" mask and finally the "Huang 2" auto-threshold.

These ROIs were then superimposed on the corresponding chitinase channel thus enabling mean grey value measurement within specific cell populations.

7. Closing Discussion

The incidence of neurodegenerative disorders is confirmed to rise with a steadily ageing global population, making the need for early detection and effective disease-modifying therapies pressing. In the case of ALS, therapeutic translation has been constrained by several factors, most notably the involvement of multiple pathogenic mechanisms. Biomarkers that reflect specific mechanisms can help create "endotypes" (patient subgroups that share functional and/or pathological traits) (Agache and Akdis 2019), link these with phenotypes, improve trial stratification and the testing of mechanism-specific interventions. Accordingly, this doctoral thesis aimed to evaluate the utility of the chitinases as biomarkers of neuroinflammation in ALS. We performed this evaluation using the novel D50 disease progression model to circumvent limitations associated with traditional outcome metrics like the ALSFRS-R and ΔFRS. Our literature review indicated that this work was necessary as despite existing reports on chitinase upregulation in ALS, the clinical utility and contributing sources remained unclear.

7.1 Chitinase Upregulation in ALS is primarily a feature of the Central Nervous System (CNS)

Several studies have used different methods, including mass spectrometry (Varghese, Sharma et al. 2013, Thompson, Gray et al. 2018), ELISA-based immunoassays (Pagliardini, Pagliardini et al. 2015, Steinacker, Feneberg et al. 2021), and immunohistochemistry (Steinacker, Verde et al. 2018, Vu, An et al. 2020) to report that the chitinases are upregulated in ALS relative to healthy controls. Therefore, our initial objective was to confirm this in an independent cohort. Indeed, we observed that CHIT1, CHI3L1 and CHI3L2 levels were significantly elevated in the CSF of ALS patients relative to neurologically healthy controls (NDCs) even after controlling for age and gender. This in itself is an important finding as ALS patients were significantly older than NDCs and because chitinase levels, particularly those of CHI3L1, significantly increase with age (Sanfilippo, Castrogiovanni et al. 2019). Here too, we noted significant correlations between age and CSF levels of CHI3L1 and CHI3L2. Conversely, and in keeping with the literature, no significant between-group differences were noted in plasma. Barring one study that observed significantly elevated CHIT1 activity in dried blood spots from ALS patients (Pagliardini, Pagliardini et al. 2015), there have been no reports of disease-associated upregulations in blood. This coupled with the fact that peripheral chitinase levels are influenced by multiple systemic conditions, including diabetes, atherosclerosis, and cancer (Kawada, Hachiya et al. 2007, Lee, Da Silva et al. 2011), makes the use of a blood-based biomarker unlikely. It also suggests that chitinase upregulation in ALS is primarily a feature of the CNS and is linked to accumulating neuronal damage, given that CSF levels of all three chitinases and both pNfH and NfL were robustly correlated. Studies aimed at characterizing

CNS sources have indeed reported that microglia/macrophages and astrocytes are exclusive sources for CHIT1 and CHI3L1, respectively (Bonneh-Barkay, Wang et al. 2010, Steinacker, Verde et al. 2018, Vu, An et al. 2020). However, these are based primarily on post-mortem patient tissue and offer a very narrow glimpse into what is most likely a substantially advanced disease stage.

In contrast, we used an established model of ALS to investigate **1)** which cell types are key *in vivo* sources of chitinases, and **2)** whether the dysregulation seen in human patients can be recapitulated. We focused on the GA-CFP model (Schludi, Becker et al. 2017), a transgenic mouse model that was developed to elucidate the role of *C9orf72*-mediated pathology. $(GGGGCC)_n$ hexanucleotide repeat expansions (HREs) in *C9orf72* are the most common genetic cause of ALS and FTD. While the exact disease mechanisms and pathways that culminate in neurotoxicity are still under debate, one mechanism is the gain-of-function toxicity associated with the translation of the HRE sense and anti-sense transcripts to aggregating dipeptide repeat (DPR) proteins. These DPRs, also known as c9RAN proteins, include poly-GA, -GR, -PA, -PR, -GP. Poly-GA inclusions are abundantly present in affected ALS/FTD brains wherein total burden correlates with disease onset (Mackenzie, Frick et al. 2015); their toxicity has also been demonstrated in cell culture systems (Zhang, Gendron et al. 2016). Transgenic GA-CFP+ mice express codon-modified $(GA)_{149}$ (conjugated to cyan fluorescent protein (CFP) at levels comparable to those seen in patients. Expression is driven by the *Thy1* vector and is therefore neuron-specific. Pathological hallmarks include substantial microgliosis, TDP-43 phosphorylation, and poly-GA inclusions, but no overt neuronal loss. Externally, gait and balance abnormalities become evident at 4 months homogenously across animals.

The SOD1-G93A and rNSL8-hTDP-43 models of ALS were included to enable an additional qualitative assessment of whether chitinase expression is modulated by the underlying genetic pathology. This is particularly relevant since each of these models manifests in a distinctive phenotype. A brief overview of these models is provided below:

- SOD1-G93A: This was the first model of ALS to be developed and remains the most commonly used (Tu, Raju et al. 1996). Transgenic mice express large amounts of human SOD1 with the causative G93A mutation and rapidly develop a neurodegenerative phenotype with paralysis onset at roughly 90 days. Pathology is comparable to that observed in humans and includes gliosis, axonal denervation, and motor neuronal loss. There have however been concerns that the model is overly aggressive and provides a limited window for intervention testing.
- rNSL8-hTDP-43: This is an inducible mouse model that expresses human TDP-43 with a defective nuclear localization signal (ΔNLS) under control of the neurofilament heavy chain

promoter (Walker, Spiller et al. 2015). Pathology can be reversibly induced in neurons and is doxycycline-dependent: expression is suppressed in the presence of doxycycline. Widespread hTDP-43 expression is evident in the brain and spinal cord after just 1 week off doxycycline, with motor symptom onset and decreased cortical thickness and neuromuscular junction denervation evident at 2 and 4 weeks off, respectively.

7.2 Chitinase Upregulation in ALS is propagated via a multi-source pro-inflammatory cascade

Quantitative immunostaining for CHIT1 and CHI3L1 was performed within specific CNS cell populations in the lumbar spinal cord of symptomatic GA-CFP+ mice and their wild-type (wt) littermates (GA-CFP-). To begin with, we noted CHIT1 staining in Iba-1+ microglia from both GA-CFP- and GA-CFP+ mice, thus corroborating findings from both post-mortem and cell culture studies (Varghese, Sharma et al. 2013); staining was primarily visible in the cell bodies and microglial processes. Additionally, GA-CFP+ mice displayed both microgliosis (absolute number of Iba-1+ objects) and significantly upregulated microglial CHIT1 levels. In keeping with the literature, no distinct CHIT1 signal was noted in GFAP+ astrocytes in either diseased or wt animals in any of the three models.

Interestingly, we also observed microglial expression of CHI3L1 in both GA-CFP- and GA-CFP+ mice, with significant upregulation in the latter. These results contradict reports that CHI3L1 is confined to astrocytes in ALS, although expression was confirmed in this population too. CHI3L1 staining was evident in the perinuclear cytoplasm and processes of GFAP+ astrocytes in both GA-CFP- and GA-CFP+ mice; levels were also significantly increased in GA-CFP+ mice. In keeping with the original phenotype described by Schludi et al (Schludi, Becker et al. 2017), no overt astrogliosis (absolute number of GFAP+ objects) was observed in GA-CFP+ mice, indicating that CHI3L1 upregulation in ALS can occur independent of astrocyte activation as has been previously reported for other conditions (Querol-Vilaseca, Colom-Cadena et al. 2017). Barschke et al. also reported that CSF levels of CHI3L1 were elevated in patients with both genetic and sporadic ALS while no changes were noted for GFAP levels (Barschke, Oeckl et al. 2020).

A direct comparison between micro- and astroglial populations (since we co-stained these in the same sections) revealed that microglia express significantly more CHI3L1 than astrocytes, with this difference being even more pronounced in GA-CFP+ mice. Therefore, we posit that chitinase elevation in ALS indicates proportionally more microgliosis. Indeed, a recent study using both total and single nucleus RNA-sequencing reported that major transcriptomic changes in the motor cortex of ALS patients, including substantial upregulation of CHI3L1 and CHI3L2, were likely driven by an overrepresentation of microglial cells (Dols-Icardo, Montal et al. 2020). Intriguingly, a similar

pattern was also noted in MS where microgliosis is also a key pathological hallmark: in a brain autopsy study by Cubas-Núñez et. al, CHI3L1 expression predominated in CD68+ macrophages/microglia at early disease stages but was more astrocytic in later stages featuring chronic active lesions (Cubas-Nunez, Gil-Perotin et al. 2021). These results are particularly interesting given that reactive neurotoxic astrocytes in ALS are induced by classically activated microglia via the secretion of inflammatory factors like IL-1α and TNF (Liddelow, Guttenplan et al. 2017). A parallel study showed that exposure to conditioned macrophage media led to astrocytic expression of CHI3L1 via mediators including IL-1β and TNF-α (Bonneh-Barkay, Bissel et al. 2012). It is therefore possible that upregulated astrocytic CHI3L1 expression in ALS follows as a sequela of neuronal damage and microgliosis. Our observation of neurons, including motor neurons, being a major chitinase source further supports the idea of a pro-inflammatory cascade. Ubiquitous staining was noted for both CHIT1 and CHI3L1 in neurons in both the brain and spinal cord of wt mice across all three genetic models. Neuronal CHIT1 levels were also significantly increased in symptomatic GA-CFP+ mice, with a similar disease-associated upregulation visually evident in the TDP-43 and SOD1 models as well. To the best of our knowledge, neuronal expression has not been reported in the context of ALS thus far. However, given that such a wide range of cell types have already been reported as chitinase sources (for e.g., eosinophils, epithelial cells, synoviocytes, chondrocytes, fibroblasts) (Kawada, Hachiya et al. 2007, Lee, Da Silva et al. 2011), neuronal expression is not that unusual. It is also conceivable from a disease mechanism perspective as motor neurons are the most heavily compromised cell population in ALS and an analogous "neuronal-glial" inflammatory shift has been reported in the context of AD. Neuronal IL-6 and MCP-1 led to the recruitment and activation of neighboring microglia; crucially, these cytokines were only evident in neurons and not glia in the earliest stages of disease suggesting that neurons were the "*primary proinflammatory agent*" (Welikovitch, Do Carmo et al. 2020).

We were nevertheless intrigued by the paucity of reports on neuronal chitinase expression and revisited the literature. Interestingly, both the Allen Mouse Atlas and Human Protein Atlas corroborated our observation of pan-neuronal cytoplasmic staining for all three chitinases as seen in the representative image below (Fig. 5). We can therefore only speculate as to why this has not been captured by existing post-mortem studies in ALS. An obvious reason could be that successful immunostaining is strongly dependent on tissue quality and can be severely impacted by post-mortem delay and the variable susceptibility of different CNS populations to poor preservation. For example, in one study that observed CHI3L1 expression in interstitial white matter neurons in frontal cortex samples from AD patients, the authors reported that staining was sparse, reiterating the difficulty of capturing signal in post-mortem tissue (Craig-Schapiro, Perrin et al. 2010).

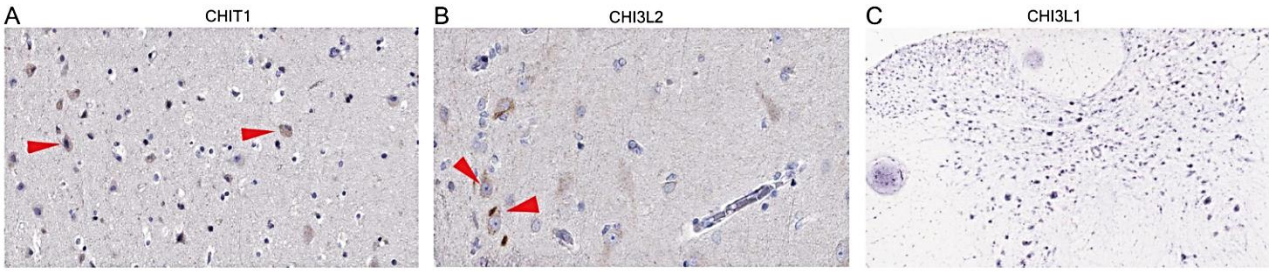


Figure 5: Representative images of the cortex from the Human Protein Atlas **(A, B)** and of the spinal cord from the Allen Mouse Atlas **(C).** Cytoplasmic neuronal localization is evident for CHIT1, CHI3L2, and CHI3L1; pan-neuronal expression is observed for CHI3L1 in particular**.** Staining for CHIT1 **(A)** and CHI3L2 **(B)** is much sparser compared to that for CHI3L1 **(C)** in mouse sections, indicating the difficulties of working with post-mortem human tissue. *(Images downloaded and reproduced from the public access data portals of www.proteinatlas.org and www.mousespinal.brain-map.org)*

In neurodegenerative conditions, this difficulty is potentially further compounded by the neuronal loss that might have already occurred till the point of death. A further possibility is technical peculiarities within the immunostaining procedure itself; we noted that simultaneous staining with a specific combination of antibodies led to an almost perfect co-localization between CHI3L1 and GFAP, consequently creating the impression that the former is exclusively astrocytic. Discrepancies between *in vivo* and *vitro* chitinase sources have also been previously reported: Bonneh-Barkay et al. showed that macrophages, despite being a major *in vitro* source of CHI3L1, displayed minimal CHI3L1 expression *in vivo* in neuroinflammatory conditions. Additionally, while conditioned macrophage media induced astrocytic CHI3L1 expression, direct co-culturing with macrophages didn't, leading the authors to speculate that regulatory pathways in the CNS environment and neuronal contact *in vivo* modulate expression (Bonneh-Barkay, Bissel et al. 2012). This re-iterates the importance of assessing sources *in vivo* and the results reported here.

Finally, although no significant differences were observed in plasma, we examined the chitinase profile of circulating MoMas from a second independent cohort of patients with confirmed ALS and appropriately matched healthy controls (HCs) because immune cells are major chitinase sources. We first showed that CHIT1 and CHI3L1 expression are temporally regulated in both HCs and patients; expression was minimal in monocytes and increased exponentially over time. In contrast, CHI3L2 expression remained minimal across the culture duration. This is concordant with the literature as both CHIT1 and CHI3L1 characteristically increase across the monocyte-macrophage differentiation process (Di Rosa, De Gregorio et al. 2013, Di Rosa, Malaguarnera et al. 2013), while CHI3L2 expression requires explicit stimulation (Rosa 2013). Next, profound between-group differences were observed for CHIT1 and CHI3L1 at both the transcriptomic and protein level at later time-points i.e., when cells were fully differentiated. ALS MoMas secreted

significantly higher amounts of CHIT1 and CHI3L1 than HC MoMas, indicating that macrophages in ALS have an intrinsically augmented capacity to produce chitinases at baseline. Our prior observation of no between-group differences in plasma chitinase levels also make sense, since expression is only evident in fully differentiated macrophages and not circulating monocytes. Of note, secreted CHI3L1 levels were significantly influenced by age, which aligns with the observed correlation between CSF CHI3L1 levels and age in our ELISA cohort. It is possible that climbing CHI3L1 levels are indicative of the wider "*inflammaging*" process that is a known risk factor for neurodegenerative conditions (Deleidi, Jaggle et al. 2015). Overall, these results supplement previous reports of peripheral myeloid cells being skewed towards an inflammatory state in ALS, since the chitinases are associated with an M1-like pro-inflammatory phenotype (Bonneh-Barkay, Bissel et al. 2012, Kunz, van't Wout et al. 2015) and expression can be induced via "classical" activation with IFN-γ and LPS (Bonneh-Barkay, Bissel et al. 2012). Indeed, patient monocytes secrete increased levels of pro-inflammatory cytokines (Du, Zhao et al. 2020), have a pro-inflammatory transcriptomic signature (Zhao, Beers et al. 2017) and display functional irregularities (Liu, Prell et al. 2016, McGill, Steyn et al. 2020).

Taken together, our results indicate that several sources contribute to chitinase dysregulation in ALS, thus creating a disease-exacerbating cascade:

- Mounting neuronal death is accompanied by elevated intra-neuronal chitinase levels and increased extracellular secretion, possibly as an acute inflammatory response to cell death and aggregate formation.
- Secreted chitinases act on neighboring microglia, which are themselves chitinase sources, and as discussed above, can induce the formation of reactive astrocytes and astrocytic chitinase upregulation.
- CHIT1 and CHI3L1 are also neurotoxic at higher concentrations and therefore unregulated secreted levels can directly damage remaining neurons (Matute-Blanch, Calvo-Barreiro et al. 2020, Varghese, Ghosh et al. 2020).
- Degenerating neurons may also create a damage-associated chemotactic axis, as supported by our observation of several CHI3L1+ astrocytes positioned directly adjacent to CHI3L1+ neurons in SOD1+ mice (white arrows, Fig. 3F, Manuscript 4).
- Accumulating extracellular and CSF chitinase levels may lead to the recruitment and invasion of circulating monocytes as chitinase exposure was shown to increase leukocytic migratory capacity across an *in vitro* blood-brain barrier (BBB) model (Correale and Fiol 2011).

- Upon differentiation, these monocytes contribute to chitinase upregulation, because as reported in Gaur et. al 2021, macrophages in ALS have an intrinsically augmented chitinase synthesis capacity (Gaur, Huss et al. 2021).

All of these steps, combined with the autocrine and paracrine effects of the chitinases, can set up a feed-forward pathological cascade, ultimately leading to the chronic neuroinflammatory milieu characteristic of ALS.

7.3 Chitinase Upregulation is not exclusive to ALS

Our cross-sectional analyses included a cohort of individuals with other neurodegenerative conditions (NDegs) to help assess the diagnostic utility of the chitinases. No significant between-group differences were noted in plasma, as previously observed in the ALS vs. NDC comparisons, reinforcing that chitinase upregulation in ALS is a feature of the CNS.

In contrast, CSF levels of CHIT1 and CHI3L2 were significantly elevated in ALS patients relative to NDegs, as previously reported (Thompson, Gray et al. 2018, Thompson, Gray et al. 2019, Vu, An et al. 2020). Contrary to these studies however, no significant differences were noted for CHI3L1. This is unsurprising, as CHI3L1 is substantially elevated in several other neurodegenerative conditions, including AD (Hellwig, Kvartsberg et al. 2015) and Parkinson's disease (Hall, Surova et al. 2016), both of which were represented in our NDeg cohort, and has also previously shown poor classifier performance (Thompson, Gray et al. 2019). This aligns with our ROC analysis which indicated that CHI3L1, unlike CHIT1 and CHI3L2, had no discriminatory power when distinguishing between ALS vs. Non-ALS. ROC analysis further indicated that of the 5 measured analytes, pNfH had the highest area under the curve (AUC) followed by NfL. This is in keeping with prior reports of pNfH's diagnostic primacy: Poesen et al. reported that CSF pNfH could better distinguish between ALS and mimicking diseases than NfL and showed a specificity of 88.2% for MND (Poesen, De Schaepdryver et al. 2017). Similarly, Feneberg et. al and Behzadi et. al reported that CSF pNfH showed higher specificity and had a higher AUC than NfL when discriminating between ALS and other neurological diseases and MND mimics (Feneberg, Oeckl et al. 2018, Behzadi, Pujol-Calderon et al. 2021).

Our results confirm that the chitinases do not diagnostically outperform the neurofilaments. This is not unexpected given that CHIT1 and CHI3L2 have also been implicated in several neurological disorders including MS and stroke (Sotgiu, Barone et al. 2005, Oldoni, Smets et al. 2020, Comabella, Sastre-Garriga et al. 2021). As also previously discussed, the chitinases participate in several cellular process and are a class of inflammatory mediators unto themselves, making it unlikely that their dysregulation is an exclusive feature of any single condition. Interestingly, of the

three chitinases, CHI3L2 had the highest AUC and correlated the most strongly with pNfH. The differing diagnostic potential between chitinases supports the idea of varying expression reflecting subtle differences in the underlying pathology. This is particularly evident across the MND spectrum; despite the considerable overlap, ALS and FTD display specific chitinase patterns. Higher CHIT1 and CHI3L2 levels are associated with an increased motoric component: CSF CHIT1 and CHI3L2 levels are higher in ALS relative to mimicking diseases, primary lateral sclerosis and FTD (Oeckl, Weydt et al. 2019, Thompson, Gray et al. 2019, Verde, Zaina et al. 2021). CHIT1 levels in ALS patients could also predict progression to El Escorial diagnostic categories (Steinacker, Feneberg et al. 2021). Further, CHIT1 and CHI3L2 levels could distinguish between *C9orf72*-ALS and *C9orf72*-FTD patients (Barschke, Oeckl et al. 2020). Conversely, CHI3L1 is associated with increasing cognitive deficits; CSF levels are higher in FTD relative to ALS (Oeckl, Weydt et al. 2019), correlate with worsening performance on cognitive tests (Thompson, Gray et al. 2019), and can predict the risk of developing cognitive impairment in preclinical AD (Craig-Schapiro, Perrin et al. 2010). Based on our observations in mice, we further posit that spatially differing immunoreactive patterns drive the varying chitinase profiles between different neurodegenerative diseases i.e., proportionally more microgliosis in ALS and astrogliosis in FTD (Rostalski, Leskela et al. 2019, Vucic 2019). Taken together, while the chitinases may not diagnostically outperform neurofilaments, specific combinations can assist with subtle distinctions between different neurodegenerative conditions, thereby increasing diagnostic certainty.

7.4 Chitinase upregulation in ALS is associated with disease aggressiveness but not the functional disease course

The prognostic relevance of the chitinases is still unclear because of conflicting results between the studies that have studied it. While cohort and methodological differences are indeed contributing factors, the outcome measures used are potentially the biggest source of these discrepancies; these range from the ΔFRS to survival and respiratory function. For instance, Abu-Rumeileh et al. noted no differences in chitinase levels between fast (ΔFRS >1.15), intermediate ($0.4 \leq \Delta FRS \leq 1.15$), or slow ($\Delta FRS \geq 0.24$) progressors stratified using the ΔFRS, while conversely Vu et al. noted that baseline CSF levels of CHIT1 and CHI3L1 were significantly higher in fast (ΔFRS >1) vs. slow ($\Delta FRS < 0.5$) progressors (Abu-Rumeileh, Vacchiano et al. 2020, Vu, An et al. 2020). Similarly, Gille et. al and Raju et. al reported significant correlations with the ΔFRS (Gille, De Schaepdryver et al. 2019, Varghese, Ghosh et al. 2020). These conflicting results reiterate the limitations of the ΔFRS: that **1)** cut-offs vary substantially across studies and **2)** it is not a cumulative descriptor of an individual's disease aggressiveness as it is dependent on the time of sampling.

In a similar vein, one study used multi-variate modelling to show that CHIT1 associated with survival (Thompson, Gray et al. 2019), while another reported no correlation (Steinacker, Verde et al. 2018), and yet another reported an association with CHI3L1 rather than CHIT1 (Gille, De Schaepdryver et al. 2019). Finally, Costa et. al showed that survival of patients stratified by CHI3L2, but not CHIT1 or CHI3L1, levels was significantly different (Costa, Gromicho et al. 2021). Survival, however, is a direct function of the quality of end-of-life care patients receive and this varies greatly across institutions. It is also a challenging outcome metric to use given the costs associated with building up a sizable cohort and tracking patients over time, particularly with a condition like ALS where drop-out rates are high. Therefore, we used the D50 model to assess how chitinase upregulation relates to:

- disease aggressiveness: patients were split into high vs. low aggressiveness groups using the cohort median of D50 = 30 months as the cut-off. This cut-off has been previously used to demonstrate that patients with increased aggressiveness display widespread white matter volume loss (as measured by voxel-based morphometry) (Steinbach, Batyrbekova et al. 2020).
- the functional disease course: rD50-derived functional disease phases were used to approximate the disease course. Phases II and III were combined since there were only 4 patients were in the latter.

ANCOVA analysis with correction for the co-variates age, gender, onset-type, rD50 (when comparing disease aggressiveness sub-groups) and D50 (when comparing disease phases) was performed. To begin with, no significant differences for either sub-group comparison (aggressiveness or disease phases) were noted in plasma, confirming again that chitinase elevation in ALS is a feature of the CNS. Plasma CHI3L1 levels were however significantly affected by age as was also previously noted for secreted CHI3L1 levels from MoMas. This reaffirms that physiological ageing substantially affects CHI3L1 in particular and potentially contributes to its limited diagnostic utility.

Next, as expected, both CSF pNfH and NfL levels were significantly elevated in the high aggressiveness group; this result was also observed for CHIT1 and CHI3L1 but not for CHI3L2. Crucially, this effect was entirely independent of the functional disease phase i.e., levels were singularly influenced by aggressiveness, which to the best of our knowledge has not been reported thus far. Corollary analyses using rD50-derived functional disease phases supported this: neither neurofilament nor chitinase levels significantly differed between early and late phases, suggesting that these are relatively stable over the disease course. Data from "true" longitudinal studies confirms this, further underscoring the utility of a pseudo-longitudinal metric like rD50. Thompson

et. al reported that chitinase levels in an ALS cohort remained stable for over 2 years from baseline (Thompson, Gray et al. 2019). Similarly, while Vu et. al noted that CSF CHIT1 and CHI3L1 significantly correlated with disease duration, follow-up modelling showed that levels were in fact stable over time (Vu, An et al. 2020). A longitudinal study tracking chitinase levels in at-risk mutation carriers and pheno-converters reported that upregulation was a feature of the late pre-symptomatic/early symptomatic phase, with levels subsequently staying relatively stable (Gray, Thompson et al. 2020). The longitudinal stability of the chitinases also hints at their potential to be used as pharmaco-dynamic biomarkers. There are already promising reports from other conditions like MS, where a decrease in CSF CHI3L1 levels was observed in response to immunosuppressive treatment (Malmestrom, Axelsson et al. 2014).

Finally, the link between chitinases and aggressiveness is also supported by our observations in mice. Immunostainings in both the TDP-43 and SOD1 models, although qualitative, indicated a more pronounced disease-associated upregulation relative to the GA-CFP model. We speculate that this may be because of the more aggressive phenotype associated with these mutations; substantial neuronal loss and astrogliosis are features of these mouse models, whereas the GA-CFP model predominantly presents with microgliosis.

7.5 CSF CHIT1 and CHI3L1 predict disease aggressiveness in ALS

By using D50 an outcome metric, we were able to test the hypothesis that the chitinases do not merely associate with disease aggressiveness but can predict it; only CHIT1 and CHI3L1 were analyzed since CHI3L2 levels did not differ between aggressiveness sub-groups. ROC curve analysis indicated that like the neurofilament proteins, both CHIT1 and CHI3L1 could significantly distinguish between aggressiveness types. Simple multiple linear regression analysis confirmed this: a model comprising both chitinases showed that each one significantly and independently associated with D50 and accounted for 60.9% of its variation. Next, hierarchical regression analysis was performed to assess whether the chitinases significantly contributed to the prediction of D50 above the neurofilaments alone. Indeed, the inclusion of the chitinases significantly increased the variation accounted for from 67.6% (pNfH + NfL) to 75.8% (pNfH + NfL + CHIT1 + CHI3L1). Curiously, however a significant effect was only noted for NfL and CHI3L1. A final model comprising only NfL and CHI3L1 accounted for 75.9% of the variation, indicating that the ability to predict individual disease aggressiveness was driven entirely by these two analytes. Indeed, a recent survival analysis by Masrori et. al. indicated that survival significantly differed between patients with high vs. low NfL and CHI3L1 levels (Masrori, De Schaepdryver et al. 2021). NfL has also previously been reported as a superior prognostic marker; in a comprehensive prospective

multi-center study with almost 230 ALS patients, baseline serum NfL but not pNfH could predict both survival and ΔFRS (Benatar, Zhang et al. 2020). Similarly, although reports on CHI3L1's prognostic potential in ALS have been conflicting, it is indicative of disease severity in other neurological conditions. For instance, in MS, it distinguishes between progressive and relapsing MS, and correlates with spinal cord atrophy and the number of active lesions (Burman, Raininko et al. 2016, Schneider, Bellenberg et al. 2021). Within ALS too, CHI3L1 but not CHIT1 or CHI3L2 levels, correlated with the extent of UMN and LMN burden (Thompson, Gray et al. 2019, Abu-Rumeileh, Vacchiano et al. 2020). It is therefore also relevant that within our cohort, only CHI3L1 differed by onset-type; CSF levels were significantly higher in patients with bulbar-onset, which is associated with a poorer prognosis.

7.6 The D50 model provides a suitable framework for biomarker assessment

The D50 model was developed to provide a framework for the discovery and validation of new biomarkers by addressing limitations associated with traditional metrics. An initial evaluation against the PRO-ACT database showed that D50 strongly correlates with outcomes including survival, ΔFRS, and the total ALSFRS-R score, indicating that it reflects the disease process and has construct validity. Additionally, by using D50 as an outcome metric, we showed that patients receiving Riluzole had a significantly higher mean D50 than those who weren't (Fig. 2C, Poster, Appendix II). In our own cohort, we used it to show that the chitinases have significant prognostic utility and can predict individual disease aggressiveness.

Unlike traditional metrics, D50 is a unified descriptor of overall disease aggressiveness that is independent of time. The use of a distinct metric i.e., rD50, to describe the functional disease course allows accumulated disease to be controlled for when assessing the link between potential biomarkers and disease aggressiveness. This is essential for rapidly progressive diseases like ALS, wherein significant "sampling shifts" occur. As described in our Dreger et. al manuscript, this refers to the phenomenon where patients with increased disease aggressiveness have typically progressed to a later disease phase by the time they are referred to a specialist or recruited for a trial. Of note, this phenomenon cannot be captured using a metric like disease duration i.e., absolute time as exemplified by the demographics of our cohort in Supplementary Table 4, Manuscript 4. Patients in early stable Phase I vs those in progressive Phases II/III did not have significantly different disease durations (Phase I = 12 months, Phase II/III = 9 months). However, patients in Phases II/III had a significantly lower total ALSFRS-R score, D50, and a ΔFRS almost three times higher than patients in Phase I. As previously discussed, this is because progression in ALS is curvilinear and differs vastly between individuals: in a given 3-month time period, one patient may experience a

10-point decline where another might have no change at all. The sampling shift also makes it difficult to interpret whether observed biomarker signals—for e.g., chitinase or neurofilament upregulation—are genuinely driven by disease aggressiveness or are merely reflecting patients being further along the disease course. Indeed, as seen in our own cohort almost all patients with high disease aggressiveness had progressed to Phases II/III at the time of sampling (Fig. 6A). In the case of CHIT1, we previously noted that it is associated with and can predict increased aggressiveness. As seen in Fig. 6B, in both Phases I and II/III the CHIT1 signal is predominantly driven by individuals with highly aggressive disease. Conversely, no unequivocal link with aggressiveness is evident for CHI3L2 (Fig. 6C), as levels also appear to slowly accumulate as individuals progress across the disease course. This aligns with our cross-sectional analyses where no significant differences in CHI3L2 levels were noted between either **a)** aggressiveness or **b)** disease phase sub-cohorts. Our results highlight the ability of the D50 model to distinguish between different domains of the disease process in ALS and why it is necessary to do so when evaluating new biomarkers.

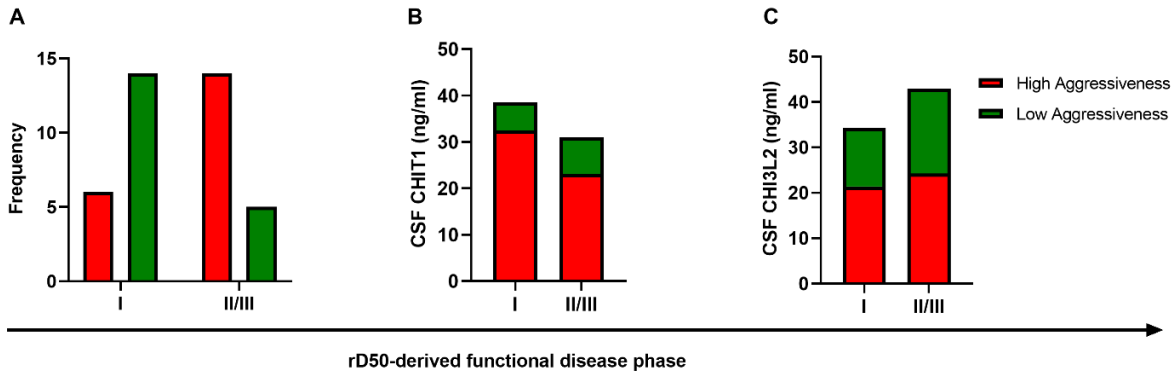


Figure 6: (A) The sampling shift in ALS shows how individuals with increased disease aggressiveness have typically already reached a more advanced disease phase at the time of referral/recruitment owing to the rapidly progressive nature of the disease. This can confound interpretation of whether biomarker signals are driven exclusively by disease aggressiveness as seen for CHIT1 **(B)** or are simultaneously influenced by accumulated disease as seen for CHI3L2 **(C)**.

The model also provides a way to describe and compare the composition of cohorts, something that is crucial in multi-center studies, where sampling times can differ substantially. This is also important for clinical trials where therapeutic efficacy is time sensitive. Researchers can also examine if a selected cohort is representative of a larger study population by comparing frequency distributions of parameters like D50 and rD50. Finally, using rD50, we were able to reproduce results from studies that have reported longitudinal stability of both the chitinases and the neurofilament proteins. No correlations with rD50 or differences across functional disease phases were noted for any of the analytes, reiterating the utility of pseudo-longitudinal metrics.

7.7 Study Limitations and Directions for Future Research

To summarize, by combining a clinical cohort and pre-clinical models, we demonstrated that key chitinase family members are dysregulated in ALS. Multiple sources, including several CNS cell populations and circulating macrophages can contribute to and sustain this dysregulation, thus exacerbating disease. Further, the D50 model provided compelling evidence that the extent of this dysregulation is predictive of overall disease aggressiveness, and that this is independent of the disease phase, thus validating the prognostic utility of the chitinases. Nevertheless, the present work is not without its limitations and has also raised questions that warrant further investigation:

- Owing to the cohort size, we had few patients in later disease phases and very few for whom complete survival data was available, thus precluding longitudinal analyses. There also wasn't adequate coverage of familial vs. sporadic ALS patients, which prevented assessment of differences in the chitinase profile. Future studies should prospectively recruit larger more representative cohorts that also include true ALS mimicking diseases. This would allow verification of our conclusion that neurodegenerative diseases present with individual chitinase profiles that reflect the underlying pathology.
- We were unable to assess CHIT1 enzymatic activity as genetic information on *CHIT1* polymorphisms was unavailable for the present cohort. Given the high prevalence (≈50%) in European populations (Malaguarnera, Simpore et al. 2003, Piras, Melis et al. 2007), cross-sectional analyses on CHIT1 activity were not possible. Nevertheless, we recommend that future studies measure CHIT1 activity as combining this with CHIT1 protein levels may improve the diagnostic predictive value for ALS (Varghese, Ghosh et al. 2020).
- Within our preclinical animal experiments, we focused on the symptomatic disease stage as a full longitudinal characterization was beyond the scope of this work. Extending this characterization to the entire disease course could help elucidate how and when the neuroinflammatory response vis-à-vis chitinase elevation develops and allow further mechanistic investigation of the proposed neuronal-glial shift. Given the dynamicity of the immune response, a longitudinal study would also help establish if the chitinases are involved in the neuroprotective-toxic "switch" in inflammation that occurs as the disease accelerates (Hooten, Beers et al. 2015).
- Here, quantitative immunostaining was only performed for the GA-CFP model. However, quantifying the chitinases in the TDP-43 and SOD1-G93A models would provide insight into how the underlying genetic component can influence disease aggressiveness and verify our observation of more aggressive phenotypes being associated with a heightened neuroinflammatory response. This is also necessary because the GA-CFP+ phenotype does

not include neuronal loss, which is a key pathological hallmark in ALS. An obvious parallel to this would be comparing the chitinase profile between patients carrying different familial mutations and whether differences translate to specific clinical outcomes. While some studies have already begun to examine this, they have the obvious caveat of small cohort sizes: Gray et. al noted no differences in chitinase levels between *C9orf72* vs. *SOD1* carriers and similarly, Masrori et. al. noted that median levels between sporadic ALS patients and *C9orf72* carriers were similar (Gray, Thompson et al. 2020, Masrori, De Schaepdryver et al. 2021). Comprehensive prospective studies with larger cohort sizes are therefore still needed.

- Additional methods should be used to confirm our finding of neuronal chitinase expression and verify this in human tissue. This would also help characterize the sources for CHI3L2 for which there is no murine homolog. Studies should address the current gap between immunostaining and sequencing methods although we speculate that here too, experimental paradigms and sample processing may have influenced results. For instance, Schneider et al. used two independently published single-cell RNA sequencing datasets (Jäkel et. al and Masuda et. al) to investigate cellular sources of CHI3L1 in healthy and MS brains (Schneider, Bellenberg et al. 2021). However, Jäkel et. al. restricted their analysis to only white matter (Jakel, Agirre et al. 2019) while Masuda et. Al (Masuda, Sankowski et al. 2019) used fluorescence-activated cell sorting to pre-select for CD45+ cells, thus excluding neurons.

7.8 Chitinases as Biomarkers in ALS: A Final Outlook

Our results indicate that despite being strongly inter-correlated, individual chitinases display distinct expression profiles, possibly because of being associated with different pathological domains. This is best exemplified by the expression profile of CHI3L2: despite CSF CHI3L2 levels showing the highest diagnostic AUC of the three chitinases, no link was observed with disease aggressiveness. Additionally, no temporal or disease driven regulation was observed in its MoMa secretion profile, suggesting that in ALS, CHI3L2 dysregulation is exclusive to the CNS. This is further supported by our observation that a correlation between plasma and CSF levels was only noted for CHIT1 and CHI3L1. A similar argument was put forth by Gray et. al who found that only CHIT1 levels sharply increased at the time of pheno-conversion (Gray, Thompson et al. 2020). They suggested that the chitinases display "*differing longitudinal profiles*" because of the "*underlying processes they represent*". These subtleties are crucial because they indicate that successful integration within a biomarker repertoire requires a nuanced approach instead of the

chitinases being used/interpreted interchangeably. When considered in the broader context of the biomarker literature in ALS, our results confirm that specific biomarker combinations show specific capabilities. Namely pNfH, CHIT1 and CHI3L2 are more reflective of ALS pathology and therefore better suited for diagnostic refinement while NfL and CHI3L1 are highly sensitive to disease aggressiveness and therefore apt for prognostic assessment. It is also worth considering that CHI3L2 may be the most appropriate chitinase candidate to include in diagnostic workups, given that it had the highest discriminatory power and unlike CHIT1, levels are not affected by any known polymorphisms. The sensitivities and specificities of these combinations can naturally be augmented as additional biomarkers are discovered and validated. Certainly, given the recognition of ALS as a "*phenotypically, etiologically, and biologically heterogeneous disease*" (van den Berg, Sorenson et al. 2019), identifying biomarker candidates that reflect the myriad pathological mechanisms involved and enable a "*precision medicine*" approach is necessary.

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9. Ehrenwörtliche Erklärung

Hiermit erkläre ich,
dass mir die Promotionsordnung der Medizinischen Fakultät der Friedrich-Schiller-Universität bekannt ist,

ich die Dissertation selbst angefertigt habe und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben sind,

dass die Personen, die mich bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts unterstützt haben, in den Autorenverzeichnissen der dieser Arbeit beigefügten Manuskripte vollständing als Co-Autoren gennant werden.

die Hilfe eines Promotionsberaters nicht in Anspruch genommen wurde und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen,

dass ich die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe und dass ich die gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung nicht bei einer anderen Hochschule als Dissertation eingereicht habe.

Teile dieser Dissertation wurden bereits publiziert.

_____ _____

Ort, Datum Unterschrift des Verfassers

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11. Appendices

11.1 Appendix 1. List of Tables and Figures in order of appearance

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Modelling individual Amyotrophic Lateral Sclerosis disease courses in the PRO-ACT data base using the D50 Progression Model

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Introduction: The routinely used Progression Rate (PR) index presumes that progression in ALS is linear and remains fixed over time. However progression in ALS is both curvilinear and vastly heterogeneous(**Fig. 1**).

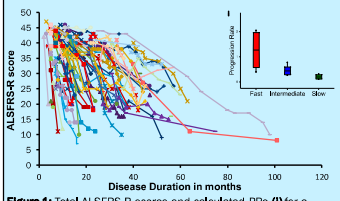


Figure 1: Total ALSFRS-R scores and calculated PRs **(I)** for a subset of individuals across the disease course clearly indicate the non-linearity of functional decline in ALS.

Objectives: To develop a model that uses regularly collected ALSFRS-R scores and reflects progression:
1) across the disease course
2) at the individual level and reduces noise associated with the ALSFRS-R

Methods: A sigmoidal decay function was used to describe the transition from full health to maximum disease for 4838 patients in the PRO-ACT database[1]. The model yields three key descriptive parameters.

1) D50: Time taken in months for ALSFRS-score to drop to 24

2) dx: Time constant of ALSFRS-R decay

3) Relative D50 (rD50): calculated open-ended value describing individual disease course covered in reference to D50. 0 = disease onset and 0.5 = time-point of halved functionality.

rD50 can also be used to mathematically derive disease phases (**Fig. 2B**)

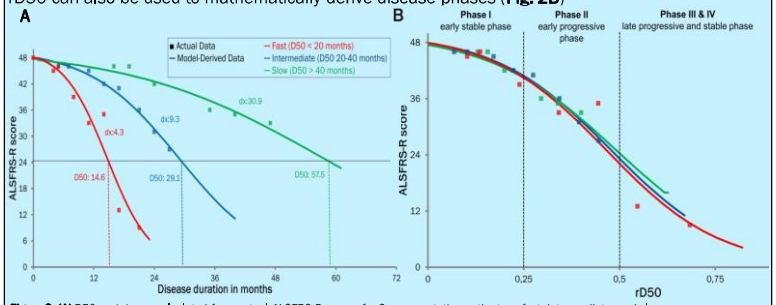


Figure 2: (A) D50 and dx are calculated from actual ALSFRS-R scores for 3 representative patients; a fast, intermediate, and slow progressor. **(B)** Normalization with rD50 allows for comparability between patients with vastly different disease time scales and shows that patients proceed through similar phases of functional decline independent of disease aggressiveness.

Results

i) Model application to PRO-ACT database and relation with traditional parameters

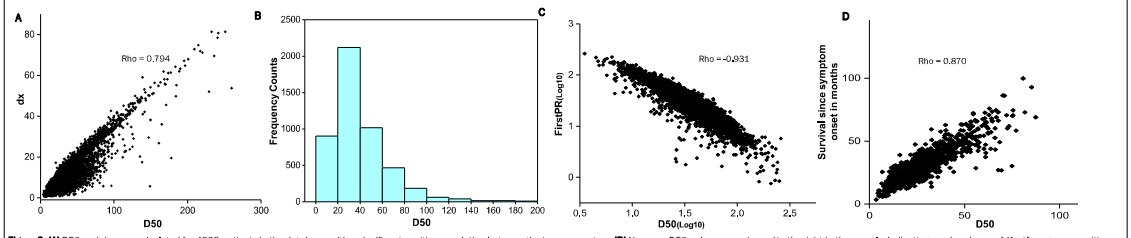
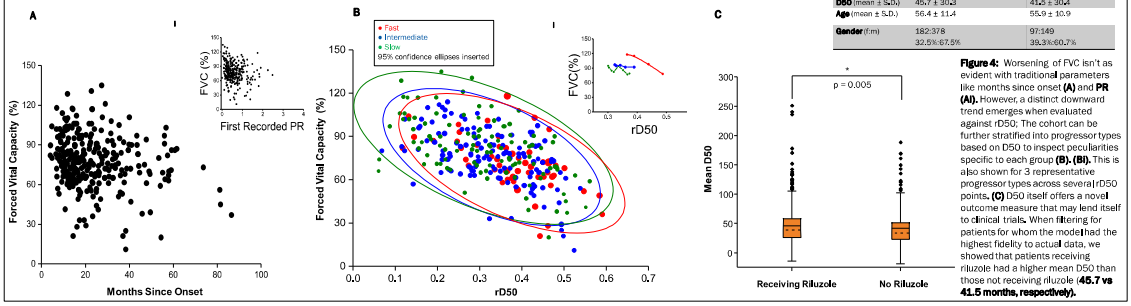


Figure 3: (A) D50 and dx were calculated for 4838 patients in the database, with a significant positive correlation between the two parameters. **(B)** However, D50 values were skewed to the right in the sample, indicating an abundance of "fast" progressors with a D50 < 30 months. **(C)** Significant negative correlations were also observed between D50 and both the first and last (data not shown here) recorded PRs (log transformed) for individual patients. The difference in correlations indicates how profoundly PR can vary over time and both within and between single patients. All scales were subjected to a log10 transformation. **(D)** Finally, a significant positive correlation was also observed between survival since symptom onset and D50. *Statistical significance was set at $p < 0.01$*

ii) Model parameters provide a new way to stage and interpret real-world clinical outcomes



	Receiving Riluzole (n = 560)	No Riluzole (n = 247)
D50 (mean ± S.D.)	45.7 ± 30.3	41.5 ± 30.4
Age (mean ± S.D.)	56.4 ± 11.4	55.9 ± 10.9
Gender (F:m)	182:378	97:149
	32.5%:67.5%	39.3%:60.7%

Figure 4: Worsening of FVC isn't as evident with traditional parameters like months since onset **(A)** and **PR (AI).** However, a distinct downward trend emerges when evaluated against rD50. The cohort can be further stratified into progressor types based on D50 to inspect peculiarities specific to each group **(B). (BI).** This is also shown for 3 representative progressor types across several rD50 points. **(C)** D50 itself offers a novel outcome measure that may lend itself to clinical trials. When filtering for patients for whom the model had the highest fidelity to actual data, we showed that patients receiving riluzole had a higher mean D50 than those not receiving riluzole (**45.7 vs 41.5 months, respectively**).

Discussion And Conclusions: The database itself did not include a representative spread of fast, intermediate, and slow progressors, with an abundance of the first type. However, the proposed parameters enable comparisons of different disease courses, enabling thus nullifying the obstacles associated with different sampling times across different centres, cohorts, and patients. This may aid the discovery of early prognostic markers and bolster understanding of pathomechanisms underlying ALS heterogeneity. Moreover, D50 and dx themselves represent meaningful clinical outcomes. rD50 offers an alternative reference point to survival, given that the latter is often a function of the quality of care the patient receives, rather than genuine disease progression. Future directions include continuing the validation of the model by applying it to larger and more varied cohorts.

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