# NLRP3 inflammasome as a key molecular target underlying cognitive resilience in amyotrophic lateral sclerosis

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**BRIEF REPORT**

# NLRP3 inflammasome as a key molecular target underlying cognitive resilience in amyotrophic lateral sclerosis

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## Abstract

**Up to 50% of amyotrophic lateral sclerosis patients present with cognitive deficits in addition to motor dysfunction, but the molecular mechanisms underlying diverse clinical and pathological presentations remain poorly understood. There is therefore an unmet need to identify molecular drivers of cognitive dysfunction to enable better therapeutic targeting and prognostication. To address this, we employed a non-biased approach to identify molecular targets using a deeply phenotyped, clinically stratified cohort of cognitively affected and unaffected brain regions from three brain regions of 13 amyotrophic lateral sclerosis patients with the same cognitive screening test performed during life. Using NanoString molecular barcoding as a sensitive mRNA sequencing technique on post-mortem tissue, we profiled a data-driven panel of 770 genes using the Neuropathology Panel, followed by region and cell type-specific validation using BaseScope *in situ* hybridisation and immunohistochemistry. We identified 50 significantly dysregulated genes that are distinct between cognitively affected and unaffected brain regions. Using BaseScope *in situ* hybridisation, we also demonstrate that macromolecular complex regulation, notably NLRP3 inflammasome modulation, is a potential, therapeutically targetable, pathological correlate of cognitive resilience in ALS.**



**Keywords:** cognition; amyotrophic lateral sclerosis; NLRP3 inflammasome; interleukin 6; interleukin 10; SIRT2

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## Introduction

Up to 50% of amyotrophic lateral sclerosis (ALS) patients present with cognitive deficits and 15% have frank frontotemporal dementia (FTD) in addition to motor dysfunction [1], but the molecular mechanisms underlying diverse clinical and pathological presentations remain poorly understood. In our recent work, we have shown that the Edinburgh Cognitive and Behavioural ALS Screen (ECAS), a multidomain brief and valid cognitive assessment tool, which includes assessment of functions typically affected in ALS (executive function, social cognition, language, and fluency), is a good clinical predictor of extramotor TDP-43 pathology with high sensitivity but low specificity [2–4]. Specifically, the ECAS subdomain scores correlate with the distribution of TDP-43 inclusions in brain regions corresponding to the affected cognitive domains. However, there is a subset of cases (22%) that are mismatch cases, in that they have a misfolded TDP-43 burden without the associated clinical manifestations of cognitive dysfunction, that appear to have cognitive resilience [4,5]. We propose that there may be other neuropathological correlates of cognitive involvement, with greater specificity, that remain to be identified, and hypothesise that additional pathological features may correlate more closely with domain-specific cognitive impairment. Identifying these correlates through neuropathological correlation performed on deeply phenotyped cohorts of post-mortem material could provide crucial therapeutic targets and/or targets for biomarker development to improve clinical prognostication.

Here, we employed a non-biased approach to identify such molecular targets using a clinically stratified cohort of affected and unaffected brain regions from ALS patients with the same cognitive screening test performed during life. We implemented NanoString sequencing to assess a data-driven panel of genes using the Neuropathology

Panel, followed by region and cell type-specific validation using BaseScope *in situ* hybridisation.

## Materials and methods

### Transcriptional analysis

To identify pathological correlates of cognitive resilience, transcriptional profiling was carried out on a deeply clinically phenotyped cohort of cognitively stratified ALS post-mortem cases. Transcriptional profiling was performed using the NanoString nCounter Neuropathology Panel (Cat. No. XT-CSO-HNROP1-12; NanoString, Amersham, UK). NanoString nCounter is a recently developed technology combining single-molecule fluorescence imaging with highly specific and sensitive nucleic acid binding probes providing an accurate quantitative analysis of the expression of multiple genes from one sample [6]. Crucially, NanoString molecular profiling works effectively on partially degraded and formalin-fixed, paraffin embedded (FFPE) post-mortem tissue and is robust to low-abundance RNAs, unlike other RNA sequencing technologies [7]. RNA was extracted from 2 × 10 µm curls of FFPE tissue using the RNAStorm™ FFPE RNA extraction kit (Cell Data Sciences, Fremont, CA, USA) according to the manufacturer's guidelines. Using the NanoString nCounter Neuropathology Panel, we profiled the expression of 770 genes across six pathways with relevance to neurodegenerative diseases in our stratified cohort. Bioinformatics analysis was performed using the freely available nSolver software (v4.0, https://www.nanostring.com/products/analysis-solutions/ncounter-advanced-analysis-software/) using predefined cut-offs for selection ($p < 0.05$ with a $\log_2$ fold-change in the same direction following Benjamini–Hochberg FDR correction for multiple testing). PANTHER GO term enrichment analysis was then implemented, comparing dysregulated genes with the entire neuropathology panel (with Bonferroni correction for multiple testing).

### Cohort and region selection

All clinical and demographic data relating to the cohort are included in supplementary material, Table S1. NanoString transcriptional profiling was performed on the following representative brain regions for which we have detailed extramotor clinical correlates (ECAS subdomain scores): BA46 (executive function), BA44 (fluency), and BA39 (language) [4,8–10]. These brain regions were analysed together for sequencing analysis (i.e. they were analysed as cognitively affected versus unaffected) and then were analysed separately for validation (to show region-specific differences). For each brain region, we profiled RNA (60 ng/µl) from three ALS patients with cognitive dysfunction (defined by impairment in one of these domains as measured by an ECAS subdomain score below the published cut-off), three patients without cognitive dysfunction, and three non-neurological controls (no cognitive dysfunction; cohort demographics detailed in supplementary material, Table S1), noting that all the ALS patients profiled had comparable TDP-43 pathology in those brain regions (as quantified and reported previously [4]).

### Immunohistochemistry and BaseScope *in situ* hybridisation

BaseScope™ probes (Advanced Cell Diagnostics, Abingdon, UK) were designed for two transcripts of interest: *SIRT2* and *NLRP3*. Both were designed to amplify all transcript variants to allow extensive coverage of expression. BaseScope™ RED Reagent Kits were used to identify mRNA transcripts and assays were run according to the manufacturer's protocol. In brief, sections were dewaxed and rehydrated, blocked for endogenous peroxidases, and then antigen retrieval was carried out using the ACD pretreatment reagent. Protease III was used (30 min; 40 °C) before incubation with probe (2 h; 40 °C). Each Amp reagent was used as per kit instructions; incubations at 40 °C were conducted using the HybEZ II Oven. Following final amplification and detection with Fast Red (Fast Red incubation time of 10 min), slides were counterstained in haematoxylin and then left to dry prior to being cleared in xylene and coverslipped. Immunohistochemical staining was performed using the Novolink Polymer detection system (Leica Biosystems, Buffalo Grove, IL, USA). Antigen retrieval was performed in a pressure cooker using Tris–EDTA, pH 9, and anti-NLRP3 antibody (19771-1-AP; Proteintech, Manchester, UK) was used at a 1 in 200 dilution. DAB was used as a chromogen and counterstaining was performed with haematoxylin. Histological analysis was performed by a clinical pathologist (JMG), blinded to all demographic and clinical information. Motor neurons were identified based on anatomical location (layer V for neurons and white matter for glia) and according to established neuropathological criteria including size and morphology, nuclear chromatin pattern, and nuclear morphology. Non-parametric statistical comparisons were performed on grouped count data ($n = 3$ patients per group with 10 ROIs assessed per region; Mann–Whitney $U$ with Bonferroni correction for multiple testing).

## Results

### Distinct transcriptional signatures differentiate between cognitively involved and uninvolved brain regions

Using the NanoString nCounter Neuropathology Panel, we profiled the expression of 770 genes across six pathways with relevance to neurodegenerative diseases in our stratified cohort. Fifty genes were found to be statistically significantly different using predefined cut-offs for selection ($p < 0.05$ with a $\log_2$ fold-change in the same direction) between cognitively affected and unaffected individuals in all three brain regions examined (Figure 1A and supplementary material, Table S1). PANTHER GO term enrichment analysis comparing dysregulated genes with the entire neuropathology panel (with Bonferroni correction for multiple testing) demonstrated that dysregulated genes

included those involved in inflammatory pathways, with higher expression of pro-inflammatory markers in cognitively affected individuals compared with cognitively resilient (supplementary material, Table S1). Indeed, *IL10* (considered to be an anti-inflammatory cytokine) and *IL6* (pro-inflammatory cytokine) were reciprocally dysregulated between cognitively affected and unaffected individuals (Figure 1B), with cognitively affected individuals demonstrating a pro-inflammatory phenotype and cognitively resilient an anti-inflammatory phenotype. *IL6* was one of the mRNAs noted to be lower in all three brain regions of cognitively resilient individuals ($p = 0.0363$ and $\log_2$ fold-change $= -0.465$; supplementary material, Table S1). *LIF*, a marker of inflammatory stress, was also lower in all three brain regions in cognitively resilient individuals ($p = 0.0197$ and $\log_2$ fold-change $= -0.701$; supplementary material, Table S1). Given this notable inflammatory signature, we also calculated, from the raw count data, a ratio comparing a marker of microglial homeostasis (*P2RY12*) to a marker of microglial activation (*TLR2*), demonstrating a more activated phenotype in cognitively affected individuals (Figure 1C).

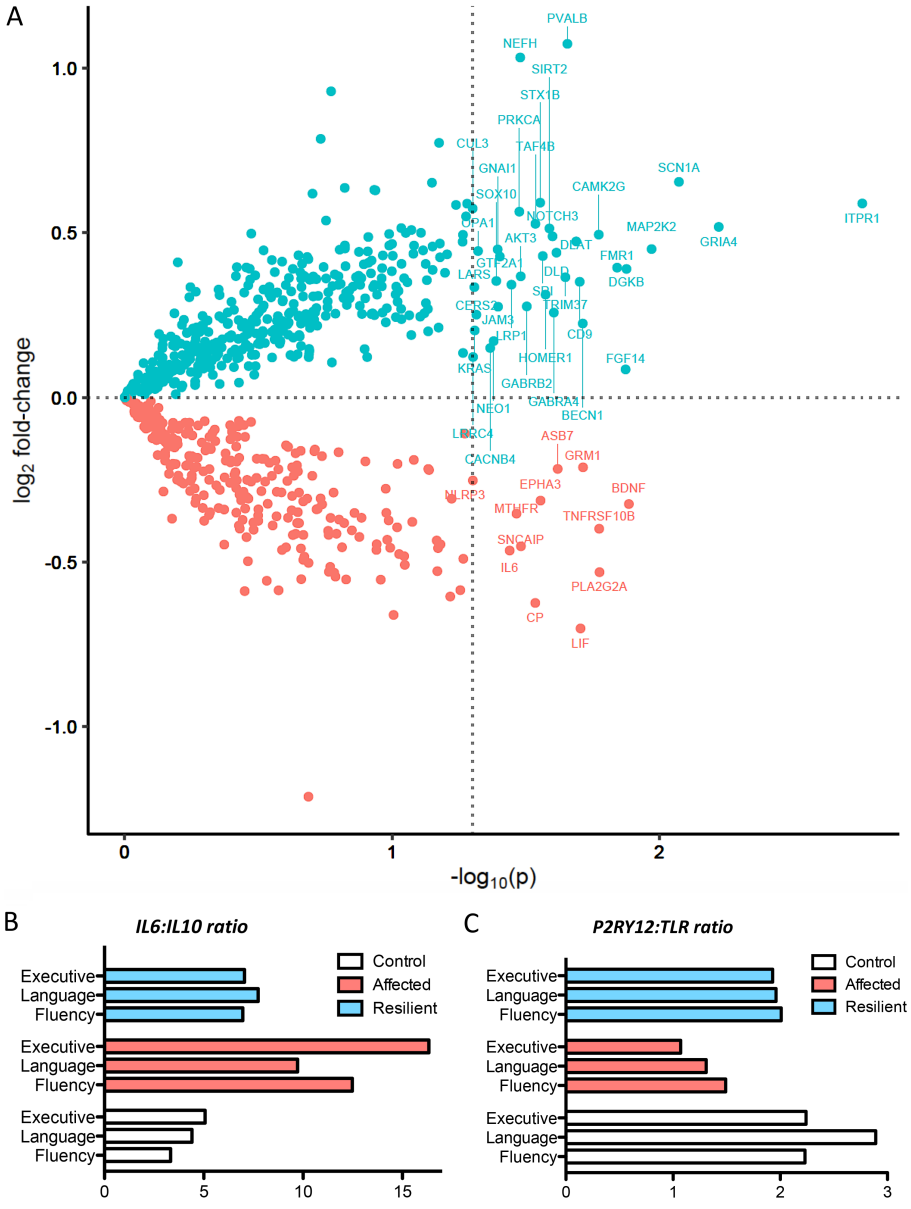


**Figure 1.** Distinct transcriptional signatures differentiate between cognitively involved and uninvolved brain regions. (A) Volcano plot demonstrating differentially expressed genes across three brain regions stratified by cognitive susceptibility ($n = 3$ patients per brain region per group; $n = 8$ patients profiled in total) and cognitive resilience ($n = 3$ patients per brain region per group; $n = 5$ patients profiled in total). See supplementary material, Table S1 for patient information. Analysis was performed using nSolver comparing $\log_2$ fold-change in gene expression ($y$-axis) and the adjusted $P$ value on the $x$-axis. Genes that lie above the horizontal dotted line are more highly expressed in cognitively resilient individuals and genes to the right of the vertical dotted line are statistically significant. All genes that are statistically significantly different between cognitively resilient and susceptible individuals are labelled (to the right of the vertical line). A full dataset is provided in supplementary material, Table S1. (B) *IL6:IL10* ratio calculated from mean count data. (C) *P2RY12:TLR2* ratio calculated from mean count data.

## Reciprocal changes in regulators of the inflammasome demonstrate distinct differences in coordinated responses to inflammation

Whilst inflammation is well documented in the ALS and FTD literature [11], other findings of note from this enrichment analysis, involving 20 of the 50 significantly dysregulated genes, were the downregulation of pathways involving the regulation of macromolecular complexes (supplementary material, Table S1). Dysregulation of this pathway has recently been implicated in ALS and is thought to drive the initial stages of phase

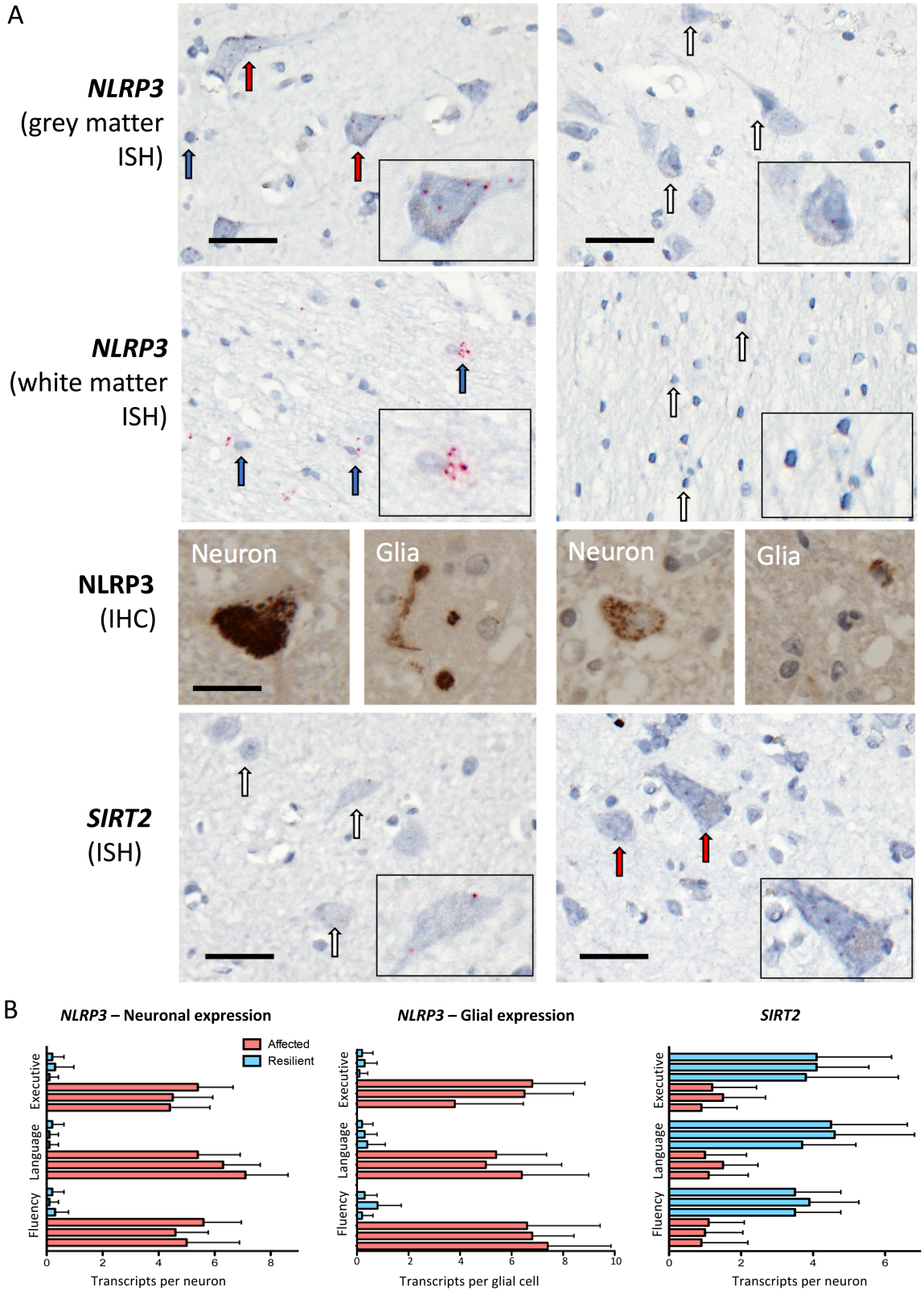


Figure 2 Legend on next page.

shift of aggregation-prone RNA-binding proteins like TDP-43, with low complexity prion-like domains, contributing to their accumulation [12,13]. Two of the genes in this list are reciprocally involved in the regulation of the inflammasome, a macromolecular complex that drives inflammation [14,15]: *NLRP3* and *SIRT2* (Figure 2 and supplementary material, Table S1). Using BaseScope *in situ* hybridisation and immunohistochemistry, we demonstrated that *NLRP3* was upregulated (activation of the inflammasome) in neurons and glial cells of cognitively affected individuals but it was found to be identical to controls in cognitively resilient individuals (Figure 2A, quantified in B). This was further supported by reciprocal neuronal expression of *SIRT2*, a neuron-specific suppressor of *NLRP3* activation (Figure 2). *SIRT2* levels were equivalent to control individuals in the cognitively affected group but were upregulated in the cognitively resilient group. Notably this reciprocal expression of *NLRP3* and *SIRT2* was in the same cell types (large layer V cortical neurons in the same region in serial sections; Figure 2A). Post-mortem tissue can also be subject to substantial degradation bias, especially as ALS patients often die in an acidotic state (due to respiratory arrest) and thus are more prone to autolysis at post-mortem. Crucially, NanoString molecular profiling works effectively on partially degraded and formalin-fixed, paraffin embedded (FFPE) post-mortem tissue and is robust to low-abundance genes, unlike other RNA sequencing technologies [7].

## Discussion

### Study limitations

Sample size is an issue with post-mortem studies of this nature, especially in a disease with such clinical and genetic heterogeneity. Indeed, experimental approaches are often limited by the cases available. Our approach in this study was to use only deeply phenotyped cases in a well-stratified cohort. Using this approach, we hoped to resolve differences between clinically diverse patient populations. However, only 13 ALS patients in total were examined and whilst we do see statistically significant, biologically plausible differences in these groups, clearly other differences exist that we do not have the power to detect in this small sample size. Furthermore, with small sample sizes, cases with particularly divergent expression patterns can more easily cause an artefactual drive in fold-change of expression seen in pooled bulk sequencing data, which is why we also employed BaseScope ISH to further resolve and confirm these differences using a complementary technique applied to cases individually.

### Neuroprotective genes identified as important factors in cognitive susceptibility

Using this approach, a striking difference between cases with differential cognitive deterioration was genes in pathways already shown to be neuroprotective in animal and cell studies. Two such examples include the TGF-beta-regulated *MAP2K2* and growth factor *AKT3*. These two mRNAs were upregulated in cognitively resilient ALS cases and their increased expression has been shown to be neuroprotective in ALS preclinical studies [16] as well as other neurological disorders (such as multiple sclerosis, stroke, and Alzheimer's disease [17]). Additionally, *BDNF* (brain-derived neurotrophic factor) was downregulated in cognitively resilient individuals. BDNF signalling with its receptor, tropomyosin-related kinase B (TrkB), has broadly been shown to be neuroprotective [18]. However, stimulation of BDNF/TrkB signalling has also been shown to render motor neurons more vulnerable to excitotoxic insult, and the truncated isoform of TrkB, TrkB-T, appears to negatively affect motor neurons [19]. These studies, taken together with our data, highlight the hormetic nature of BDNF levels and the need for a more targeted approach in the event of therapeutically altering BDNF signalling [20]. It must be noted that most of these studies involve SOD1 mouse models, in which increased BDNF–TrkB is observed; the bias of preclinical studies towards SOD1 models may be why clinical trials of BDNF administration have not been successful [21].

### Inflammasome and macromolecular complex regulation is key to cognitive resilience

Inappropriate or prolonged activation of the inflammasome is thought to be a driver of a diverse number of diseases and clinical syndromes including metabolic syndromes, cardiovascular diseases, inflammatory and autoimmune diseases, and neurological conditions

Figure 2. Reciprocal expression changes in regulators of the inflammasome demonstrate distinct differences in coordinated responses to inflammation. (A) Representative micrographs demonstrating spatially resolved expression of *NLRP3* and *SIRT2* in cognitively affected and resilient brain regions using BaseScope *in situ* hybridisation (ISH) and corresponding protein localisation using immunohistochemistry (IHC) for NLRP3. Red arrows indicate neurons with high expression; blue arrows indicate glial cells with high expression; and white arrows indicate no expression. The scale bar in ISH images is 100 μm and in IHC images 50 μm. (B) Graphs demonstrating the mean and standard error of mRNA transcripts in neurons and glia from ten randomly generated regions of interest (ROIs) within layer V of the cortical grey matter of each case. Each bar represents a single case. Non-parametric statistical comparisons were performed on grouped count data ($n = 3$ individuals per group and 10 ROIs per region; Mann–Whitney *U* with Bonferroni correction for multiple testing) demonstrating a statistically significant increase in the expression of *NLRP3* in neurons of cognitively affected individuals (BA46: $p = 0.003$; BA44: $p = 0.007$; BA39: $p = 0.005$) and glial cells of affected individuals (BA46: $p = 0.026$; BA44: $p = 0.005$; BA39: $p = 0.003$), and a statistically significant increase in the expression of *SIRT2* (BA46: $p = 0.003$; BA44: $p = 0.007$; BA39: $p = 0.001$) in cognitively resilient individuals.

including ALS [22–24]. Indeed, in combination with dysregulation of other macromolecular assemblies driving the aggregation of TDP-43, also seen in these same cells, this may well explain the differential phenotypic susceptibilities seen between resilient and affected individuals in our cohort. The increased expression of negative regulators of the inflammasome in our cohort of cognitively resilient individuals raises the possibility that drug therapies targeting inflammasome suppression could be a valuable therapeutic mechanism in cognitively affected individuals with ALS [15]. Indeed, two previous post-mortem studies demonstrated that *NLRP3* is elevated in sporadic ALS patients in both the spinal cord and the motor cortex [25,26], raising the possibility that the therapeutic application of inflammasome modulation may extend to improvement in motor symptoms as well as cognitive symptoms in ALS patients.

These results taken together highlight macromolecular complex regulation, exemplified by altered inflammasome modulation, as a potential therapeutic target in ALS patients presenting with cognitive deficits. However, they also demonstrate the utility of molecularly profiling deeply phenotyped post-mortem cohorts to allow us to progress towards a new approach to targeted therapeutics in ALS. For example, a clinical tool (e.g. the ECAS) is used here to stratify cohorts, leading to the identification of a perturbed pathway (e.g. the inflammasome) which, in turn, can be used as a therapeutic target. The evaluation of rescue of this pathway in a trial is therefore more relevant as we know that the ECAS can be used as an accurate surrogate/clinical correlate to stratify and monitor these patients, thus facilitating the identification of clinico-pathological correlations with high clinical value.

## Acknowledgements

All clinical data including the ECAS were collected as part of the Scottish Motor Neurone Disease Register (SMNDR) and Care Audit Research and Evaluation for Motor Neurone Disease (CARE-MND) platform (ethics approval from Scotland A Research Ethics Committee 10/MRE00/78 and 15/SS/0216) and all patients consented to the use of their data during life. Control brains were selected from the sudden death brain bank and therefore have not died of a chronic illness or neurological condition. All control cases are rigorously assessed by experts, both clinically and neuropathologically, to rule out any evidence of disease, including the use of an extensive standardised panel of immunohistochemistry, with pathology being assessed using well-defined and published grading systems. All post-mortem tissue was collected via the Edinburgh Brain Bank (ethics approval from East of Scotland Research Ethics Service, 16/ES/0084) in line with the Human Tissue (Scotland) Act. Use of human tissue for post-mortem studies has been reviewed and approved by the Edinburgh Brain Bank ethics committee and the Academic and Clinical Central Office for Research and Development (ACCORD) medical research ethics committee (AMREC). All clinical and demographic data are included in supplementary material, Table S1.

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## SUPPLEMENTARY MATERIAL ONLINE

**Table S1.** Data sheets compiling clinical demographic data of cases and brain regions sampled including full TDP-43 pathological grading by region, ECAS scores, full NanoString data analysis, and GO term enrichment analysis