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# Shared and subtype-specific proteomic signatures in C9ORF72 and sporadic ALS indicate two molecularly distinct ALS subtypes

**Bas C.R. Lahaije**
KU Leuven
**Anne Wiedmer**
KU Leuven
**Jolien Schaeverbeke**
KU Leuven
**Pegah Masrori**
VIB-KU Leuven Center for Brain & Disease Research
**Geethika Arekatla**
VIB-KU Leuven Center for Brain & Disease Research
**Alicja Ronisz**
KU Leuven
**Lieselot Dedeene**
KU Leuven
**Evelien Van Schoor**
KU Leuven
**Pedro Magalhães**
VIB-KU Leuven Center for Brain & Disease Research
**Albert Ludolph**
Universität Ulm
**Jochen Weishaupt**
University Hospital Ulm
**Ludo Van Den Bosch**
VIB-KU Leuven Center for Brain & Disease Research
**Philip Van Damme**
VIB-KU Leuven Center for Brain & Disease Research
**Dietmar Rudolf Thal**
Dietmar.Thal@kuleuven.be

KU Leuven

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# Abstract

Amyotrophic lateral sclerosis (ALS) is characterized by progressive loss of motor neurons in the motor cortex, brain stem, and spinal cord. Sporadic ALS (sALS) and *C9ORF72* ALS are the most prevalent forms of the disease. However, the extent to which the pathogenic mechanisms underlying *C9ORF72* ALS overlap with those of sALS remains incompletely understood. We therefore compared protein abundance and solubility profiles between *C9ORF72* ALS, sALS, and healthy controls to identify shared and distinct disease-associated pathways.

Postmortem primary motor cortex and/or spinal cord tissue of 11 *C9ORF72* ALS cases, one *C9ORF72* frontotemporal lobar degeneration case, 14 sALS, and 14 healthy control cases were studied. To determine proteomic changes, the proteome was separated into four physiochemical fractions representing different grades of solubility. Data-independent acquisition mass spectrometry-based proteomics and subsequent bioinformatic analysis were performed to compare the proteomic profiles of *C9ORF72* ALS and sALS. Furthermore, the relative distribution of proteins across fractions was compared to identify disease-associated changes in protein solubility, which may reflect alterations in protein structure and function.

In spinal cord, 110 proteins of interest were distinct to *C9ORF72* ALS, 17 to sALS, with 24 proteins shared between both forms when comparing with healthy controls. Gene ontology analysis revealed enrichment of, among other mechanisms, the transcription-translation axis and metabolic processes in both *C9ORF72* ALS and sALS compared to controls. In contrast, proteins associated with protein degradation, reactive oxygen species homeostasis, and pro-inflammatory signaling were specific to *C9ORF72* ALS. Furthermore, several proteins implicated in cytoskeleton modification, intracellular transport, and protein degradation exhibited solubility changes in *C9ORF72* ALS spinal cord tissue when compared to controls. In primary motor cortex, only two differentially abundant proteins were observed in *C9ORF72* ALS cases compared to controls, whereas no significant changes were detected in sALS.

These findings demonstrate that *C9ORF72* ALS and sALS share key disrupted biological pathways while exhibiting largely distinct underlying molecular alterations. The observed differences in protein abundance and solubility highlight subtype-specific mechanisms that may contribute to ALS pathogenesis and disease progression, i.e., the molecular distinction between the sporadic and *C9ORF72*-related subtypes of ALS.


# Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder, that primarily affects the motor system, involving degeneration of the upper motor neurons in the motor cortex and lower motor neurons in the brainstem and spinal cord. This causes muscle wasting and weakness, ultimately leading to respiratory failure, typically within 2–5 years after disease onset [78, 88].

The neurodegenerative process of ALS involves the formation of neuronal inclusions composed of protein aggregates. Nuclear depletion and subsequent intracellular aggregation of transactive response DNA-binding protein 43 (TDP-43), has been identified as a common pathological feature in ALS, representing a loss of physiological TDP-43 function and a simultaneous gain of toxic cytoplasmic function, respectively [78, 88, 97]. Vital cellular functions are hypothesized to be impaired during TDP-43 aggregation, as TDP-43 is involved in transcription, RNA splicing, transport, and translation [78, 88].

The underlying genetic architecture of ALS is heterogeneous. Over 50 disease-associated genes have been implicated in the development of ALS [79]. Patients diagnosed with familial ALS usually inherit disease-causing genes in an autosomal dominant manner. However, about 90% of ALS patients have no known family history of the disease and are classified as sporadic ALS (sALS) [78, 88]. Heterozygous GGGGCC hexanucleotide repeat expansions (HREs) in the *chromosome 9 open reading frame 72* (*C9ORF72*) gene are the most common genetic variant of ALS [31, 79, 102]. It has been shown that 38 HREs repeats are sufficient to cause ALS [29]. The *C9ORF72* gene product is abundantly expressed in the human brain and spinal cord and has many known functions, including membrane trafficking and immune homeostasis [11, 115]. Although *C9ORF72* RNA and protein levels are reduced in *C9ORF72* ALS, both mouse models and ALS patient–derived induced pluripotent stem cell neurons tolerate *C9ORF72* knockdown [35, 63, 67, 126]. Accordingly, loss of *C9ORF72* function alone is presumably not sufficient for developing ALS [11]. Transcription of HREs can form highly stable DNA and RNA structures, leading to the accumulation of aborted transcripts and sequestration of local RNA-binding proteins [41, 50, 101]. These so-called RNA foci impair post-transcriptional processing [7, 41, 63, 64, 81, 115]. Furthermore, transcripts containing HREs can interact with ribosomes, enabling repeat associated non-AUG translation of dipeptide repeat proteins (DPRs) that can form intracellular aggregates [5, 43, 73, 83]. These DPRs have been found in affected brain regions and other organs of *C9ORF72* ALS patients [24, 28–30, 71, 84].

Both loss of physiological *C9ORF72* function and a toxic gain of function caused by the RNA foci and aggregation of DPRs, could be possible mechanisms leading to ALS [89]. Previous studies of the *C9ORF72* ALS proteome showed an increase in microglial and astrocytic proteins in the frontal cortex compared to sALS cases [124]. The *C9ORF72* ALS transcriptome showed modulated phagocytosis and lysosomal function in microglia [77]. These studies highlight potential *C9ORF72* ALS-specific transcriptomic and proteomic alterations, linked to the HREs [124]. Understanding these potential subform-specific alterations in the ALS proteome, and their contribution to the disease phenotype, may be crucial for identifying new therapeutic targets.

Furthermore, given the formation of TDP-43 aggregates in ALS, and DPRs, and RNA foci specifically in *C9ORF72* ALS, one can speculate that changes in proteome solubility may play a critical role in this context. Advances in fractionation protocols enable the determination of changes in protein abundance across distinct solubility-defined fractions [66]. Proteins observed in one solubility-defined pool in the healthy proteome may move to another solubility pool during disease. This process may be associated with conformational and subsequent functional changes of the respective protein [18]. Therefore,

identifying changes in protein solubility in ALS will increase our insights into the pathogenesis of ALS [66].

Accordingly, the primary objective of this study is to compare the proteomic abundance and solubility patterns in *C9ORF72* ALS and sALS cases with those of healthy controls to identify relevant changes for the respective ALS phenotypes. We aim to characterize shared and differing proteomic alterations and identify associated biological processes. Our results point to the impairment and/or dysfunction of shared and subform-specific pathways in *C9ORF72* ALS and sALS.

# Material and methods

Cohort description

This study included 25 ALS cases and one *C9ORF72* case with the clinical picture of frontotemporal dementia (12 *C9ORF72* ALS or FTD and 14 sALS) and 14 non-neurodegenerative controls (sTable 1, sTable 2) for which human spinal cord and/or primary motor cortex (Brodmann area 4 (BA4)) tissue was collected at autopsy, in accordance with Belgian and German law. Recruitment protocols were approved by the ethical committees of UZ Leuven (Belgium, Approval no. S-55312, S-59292, S-65097) and the University of Ulm (Germany, Approval no. 54/08). Furthermore, this study was approved by the ethical committee of UZ Leuven (Approval no. S-60803). Tissue collection was performed with a mean post-mortem interval (PMI) of 31.2 h (+/- 23.4 h). Spinal cord was dissected in the transversal plane. Tissue from the respective right hemispheres was frozen at -80°C. The left hemisphere was fixed in 4% phosphate-buffered formaldehyde.

Genotyping

HREs in the *C9ORF72* gene were detected using DNA extracted from peripheral blood and/or cerebellum. Fragment-length analysis by polymerase chain reaction (PCR) and repeat-primed PCR was used to determine HREs length in intron 1 of the *C9ORF72* gene, as previously described [27]. In addition, the frontal cortex was immunohistochemically stained to identify the presence of poly GA (MABN889, Merck Millipore) in ALS cases. In the absence of *C9ORF72*, *SOD1*, *FUS* or *TARDBP* mutations, and of a family history of ALS, cases were considered as sALS.

Clinical and Neuropathological Diagnosis

ALS and/or frontotemporal dementia (FTD) diagnosis was based on clinical assessment, according to the consensus criteria for ALS [16, 20, 21] and FTD [48, 100]. The clinical diagnosis was confirmed post-mortem by assessing: TDP-43 pathology according to Brettschneider et al. [15], Braak neurofibrillary tangle (NFT) stage [10], amyloid phase [118], and the Consortium to Establish a Registry for Alzheimer's disease (CERAD) score [80]. For this purpose, immunohistochemical staining with antibodies against phosphorylated (p)TDP-43 (phosphorylated at serine 409/410), pTau (phosphorylated at serine 202 and threonine 205), and amyloid-β peptide (Aβ), was conducted (sTable 3).

## Sample preparation for LC-MS/MS

### Protein extraction and fractionation

Fresh-frozen tissue samples were homogenized and prepared for liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a serial centrifugation protocol (sFigure 1A). By doing so, water-soluble (SOL), water-insoluble dispersible (DISP), sodium dodecyl sulfate-soluble (SDS), and formic acid-soluble (FA) proteins were extracted from each tissue sample.

In detail, 100 mg of tissue were homogenized using a micro pestle in 500 µL of Tris-buffered saline (TBS) with protease/phosphatase inhibitors (Thermo Scientific). Samples were sonicated six times for 20s with a probe sonicator, while being kept on ice between sonication rounds. After sonication, samples were centrifuged at 14000 g for 30 min at 4°C. Supernatant I was collected and subjected to ultracentrifugation at 121656 g for 30 min at 4°C. The resulting supernatant was further separated into the SOL fraction (supernatant II) and pellet II. Pellet II was resuspended in 500 µL of TBS with protease/phosphatase inhibitor and sonicated for 20 sec (DISP fraction). Pellet I was resuspended in 500 µL TBS with 2% sodium dodecyl sulfate and sonicated five times for 20s. After sonication, samples were centrifuged at 14000 g for 30 min at room temperature (RT). Supernatant III (SDS fraction) was collected, and pellet III was resuspended in 100 µL of 70% formic acid. Pellet III was shaken overnight at 450 rpm at RT and then centrifuged at 14000 g for 30 min. Supernatant IV (FA) was collected, and residual formic acid was evaporated in the Concentrator plus SpeedVac (Eppendorf). Lyophilized samples were resuspended in 50 µL of 1x lithium dodecyl sulfate sample buffer. All fractions were stored at -80°C until further use.

### Chloroform-methanol precipitation

SDS and FA fractions were subjected to chloroform-methanol precipitation to remove chemicals incompatible with future sample preparation and/or data acquisition. Prior to this, SDS and FA samples were centrifuged at 14000 g for 20 min at RT. Approximately 40 µL of SDS and 50 µL of FA fraction were diluted in Milli-Q water (MQ) up to a total volume of 100 µL. To each sample, 400 µL of methanol, 100 µL of chloroform, and 300 µL of MQ were added, then the samples were incubated on ice for 2 min. Samples were centrifuged at 14000 g for 5 min at 4°C. The upper aqueous layer was removed, and an additional 400 µL of methanol was added. After centrifugation at 14000 g for 5 min at RT, supernatants were discarded, 400 µL of methanol was added, and samples were gently inverted twice. Samples were centrifuged at 14000 g for 5 min at RT, before discarding the remaining supernatants. Protein pellets were air-dried under a fume hood to evaporate residual methanol. Dry pellets were resuspended in 49 µL of rapigest-ammonium bicarbonate solution (ABS) (10 mg rapigest dissolved in 500 µL of 50 mM ABS). Precipitated SDS and FA samples were shaken ON at 450 rpm at RT and stored at -80°C until further use.

### Trypsin digestion and sample desalting

Protein concentration of each sample was determined using the Pierce™ BCA Protein Assay Kit according to the manufacturer's instructions. Prior to trypsin digestion, 2 µg of protein were diluted using 8M Urea/50 mM ABS to a final volume of 50 µL. Samples were further diluted to a final volume of 200 µL using 50 mM ABS. 1 mM dithiothreitol was added to the samples prior to incubating for 45 min at 56°C. Following reduction with dithiothreitol, proteins were alkylated with 20 µL of 0.1 M iodoacetamide for 45 min at RT. Protein digestion was achieved by adding 10 µL of trypsin (1 µg/µL dissolved in 50mM acetic acid, diluted 200x in 50 mM ABS), and overnight incubation on a shaker at 350 rpm and 37 $^{O}$C. After incubation, digestion was halted using 2 µL of trifluoroacetic acid. Samples were dried using a SpeedVac and stored at -80°C until further use.

Pierce™ C18 Spin Columns were used according to the manufacturer's instructions, in order to desalt the peptide samples. Desalted peptide samples were lyophilized using a SpeedVac and stored at -80°C until further use.

LC-MS/MS data acquisition

Prior to mass spectrometry analysis, samples were resuspended in 80 µL of 0.1% formic acid in water (LC-MS grade). Following Evosep's guidelines, 20 µL (500 ng) was loaded onto Evotips, using the Bravo Automated Liquid Handling Platform. Samples were analyzed using an Evosep One LC system (Evosep) coupled to a ZenoTOF 7600 mass spectrometer (Sciex), utilizing Sciex OS (version 3.3 or higher). Peptides were separated employing the "30 samples per day" method with a 44-minute gradient, using the EV1137 Performance column (ReproSil Saphir C18, 1.5 µm beads (Dr Maisch, Ammerbuch-Entringen, Germany), 15 cm x 150 µm) connected to the low micro electrode for flow rates of 1–10 µL/min. Mobile phases included 0.1% formic acid in water (LS-MS/MS grade) and acetonitrile in 0.1% formic acid. The ZenoTOF mass spectrometer operated in SWATH mode with an OptiFlow Turbo V ion source. Source conditions included a spray voltage of 4500 V, gas 1 at 20 psi, gas 2 at 10 psi, a curtain gas at 40 psi, and a temperature range of 100°C. The Zeno SWATH data independent acquisition (DIA) method consisted of 85 variable-width windows enabling the selection of precursors from 350–1250 m/z. TOF-MS scans ranged from 350–1250 m/z with an accumulation time of 25 ms, and TOF-MS/MS scans covered 230–1400 m/z with accumulation times of 20 ms with dynamic collision energy turned on, a charge state of 2, and Zeno pulsing enabled.

Raw data from the ZenoTOF 7600 system was processed using Spectronaut (version 20, directDIA+ deep mode). Spectra were compared against the Human reference proteome database (https://www.uniprot.org/proteomes/UP000005640) using default Spectronaut settings with trypsin specificity, up to two missed cleavages, precursor- and protein-level q-value cutoffs of 0.01, MS2 peak area–based quantification, 'automatic' normalization, and default mass tolerances. Protein N-terminus acetylation and oxidation of methionine residues were considered variable modifications, while carbamidomethylation of cysteine was considered a fixed modification. The pivot protein quantification reports were exported for subsequent downstream processing and analysis.

Bioinformatic analysis

Data pre-processing

Downstream analysis (sFigure 1B) was done in R (version 4.4.1) and R Studio (version 2024.04.2 + 764). Identified proteins with ≥ 30% missing values across samples per fraction were removed, in order to limit impact of missing data on downstream analyses. Furthermore, keratin proteins were identified as contaminants and were therefore excluded from the dataset. Protein abundance values were $Log_2$-transformed. For each individual fraction (SOL, DISP, SDS and FA), outliers were assessed for each case by conducting principal component analysis and by assessing protein coverage in Spectronaut. Based on these measures, 2 samples (FA fraction of cases No. 16 (control) and No. 37 (sALS)) were excluded due to insufficient protein coverage.

Differential abundance analysis

Differential abundance analysis was performed based on pairwise contrasts between disease states (Control, *C9ORF72* ALS and sALS) using equal variance *T*-Tests with significance at $p < 0.05$ (Benjamini-Hochberg corrected). A threshold of |Log2 FC| > 0.1 was used to select for biological relevant differentially abundant proteins (DAPs). Data was visualized using the EnhancedVolcano package in R.

Weighted gene co-expression network analysis

Weighted gene co-expression network analysis (WGCNA) was conducted using the WGCNA package in R (version 1.73), following previously described procedures [119]. First, the protein abundance matrix was subjected to a soft threshold power analysis by evaluating the scale-free topology fit index and mean connectivity across a range of power values to optimize the variance. The soft threshold value with the most optimal variance was 7 for the SOL fraction, 8 for the DISP and SDS fractions and 18 for the FA fraction, to ensure an optimal balance between biological relevance and network connectivity. The WGCNA “blockwiseModules()” function, using power β as mentioned per fraction, “TOMType” = "unsigned", “deepSplit” = 2, minimum module size of 30, merge cut height of 0.25 and “reassign Threshold” = 0, was used to generate protein modules. Given the large scale of the datasets, “deepSplit” = 2 was selected to increase the sensitivity of module detection. Module eigengenes (MEs) were calculated for all proteins. Subsequently, MEs were correlated to individual clinical parameters using Pearson correlation. These parameters included: age, sex, PMI, and disease state (Control, *C9ORF72* ALS, sALS and ALS) coded as a binary variable (no = 0 or yes = 1). Modules with significant correlations ($p < 0.05$) to clinical parameters of interest were selected for subsequent analysis. For all proteins within selected modules, protein significance and module membership were calculated. Using VerboseScatterplot, the relation between protein significance and module membership was visualized for proteins within selected modules. Modules with a significant ($p < 0.05$), positive Pearson's correlation coefficient (r), were designated as modules of interest. Using the enrichGO() function in R, the top associated gene ontology (GO) term (BP) was identified for all modules of interest. The human reference proteome database was used as background for the functional enrichment analysis.

Linear regression analysis was conducted for all proteins in modules of interest with $Log_2$ protein abundance as dependent and disease state as independent variable. Proteins whose abundance were not associated ($p > 0.05$) with the clinical parameters of interest were excluded from analysis. A similar method was used to assess collinearity between relevant clinical parameters and covariates. Proteins whose abundance was correlated ($p < 0.05$) to age, sex and/or PMI were excluded from further analysis.

### Identifying module-relevant differentially abundant proteins

Proteins of interest were generated, per fraction, by intersecting DAPs with the selected proteins within WGCNA modules of interest. As no modules of interest were obtained for sALS across all fractions, DAPs specific to sALS were used as proteins of interest.

### Functional enrichment analysis

Using the enrichGO() function in R, the top associated gene ontology (GO) term (BP) was identified for all proteins of interest. The human reference proteome database was used as the background for the functional enrichment analysis. Many of the featured GO terms correlated with mechanisms known to be dysfunctional in neurodegenerative diseases and were subdivided accordingly for downstream interpretation. Furthermore, protein-protein interaction (PPI) networks were identified for proteins of interest using STRING (version 12.0) with an interaction score > 0.4 and medium confidence and visualized in Cytoscape (version 3.10.2). Proteins of interest with no interactions to other proteins of interest were excluded from PPI network analysis.

### Implication of affected pathways

Pathways implicated in *C9ORF72* ALS and sALS were identified by complementing the detected abundance values, GO terms, and protein-protein interactions of the proteins of interest with a targeted literature search. Protein names, in combination with neurodegeneration and/or ALS if applicable, were used as keywords. By doing so, relevant biochemical pathways were identified, in which the proteins of interest play specific roles.

### Identifying solubility changes in ALS

Protein solubility changes were determined by generating “fraction1-abundance/fraction2-abundance-ratios” per case and comparing the mean ratios between control and *C9ORF72* ALS/sALS (equal variance *T*-Tests with significance at $p < 0.05$ (Benjamini-Hochberg corrected)). Proteins not represented in both fractions were excluded from the comparison. Due to relatively low coverage in the FA fraction, an additional threshold of at least 72% coverage was required to achieve the power for statistics. Protein abundance ratios generated using the FA fraction, with ≥ 28% missing values across cases were removed, before continuing with statistical comparisons. Proteins that showed a significant difference in ratio between *C9ORF72* ALS/sALS compared to control were qualified as a subform-specific solubility change. Solubility changes, with a significantly increased *C9ORF72* ALS/sALS “fraction1-abundance/fraction2-abundance-ratio” when compared to control, was characterized as a solubility

change from fraction 2 to fraction 1 and vice versa. A targeted literature search, as described in the previous section, was conducted to investigate the potential impact of solubility changes in *C9ORF72* ALS and sALS.

## Results

# Differentially abundance analysis reveals specific proteins for *C9ORF72* ALS and sALS

To identify proteome signatures in *C9ORF72* ALS and sALS fresh frozen spinal cord and/or primary motor cortex (Brodmann area 4) (BA4) tissue of control (n=14), *C9ORF72* ALS (n=12), and sALS (n=14) cases was processed to obtain four protein fractions with different grades of solubility and prepared for LC-MS/MS. In our dataset of *C9ORF72* ALS, sALS and controls, between 3904 and 5339 total proteins were detected per fraction (SOL, DISP, SDS and FA). After data pre-processing, the total number of proteins detected per fraction ranged between 2300 and 5121 (sFigure 2).

Differential abundance analysis was used to understand which proteins drive *C9ORF72* ALS and sALS, and whether they overlap or differ among ALS subforms and healthy controls. Analysis of spinal cord tissue revealed a total of 275 differential abundant proteins (DAPs) when comparing *C9ORF72* ALS to control, across all fractions, e.g. significant reduction in SULT4A ($p$=0.001) and significant increases in TAP1, and APOE ($p$= 0.01 and $p$= 0.001 respectively). Of these DAPs, 224 were considered specific to *C9ORF72* ALS, as they were not differentially abundant between sALS and controls (Figure 1A). When comparing sALS to controls, 68 DAPs were found across all fractions, e.g. significant reductions in MAP1A, and RAB4B ($p$= 0.0009 and $p$= 0.04 respectively) and significant increase in RHOA ($p$=0.01). Seventeen of these DAPs were specific to sALS (Figure 1B). During analysis, 51 DAPs were found to be differentially abundant in both ALS subforms (Figure 1C). Protein levels for these DAPs were not different among *C9ORF72* ALS and sALS, indicating that the abundance changes are similar for both subforms when compared to healthy controls (sFigure 3).

Differential abundance analysis in BA4 identified 2 DAPs when comparing *C9ORF72* ALS to controls, across all fractions. No DAPs were revealed at BH correction when comparing sALS to control (Figure 2).

# Weighted gene co-expression network analysis (WGCNA) highlights DAPs involved in sALS and *C9ORF72* ALS

Next, we focused on the proteins that contribute to distinct cellular processes/pathways using WGCNA. WGCNA modules group genes/proteins with correlated expression/abundance levels and are typically enriched for shared biological functions or pathways [19, 46]. In spinal cord, 12 protein modules correlated with *C9ORF72* ALS (Figure 3 and sFigure 4-sFigure 7). A total of 5747 proteins were covered by these modules. To further focus on disease-relevant proteins, proteins within these modules that

were not significantly associated with disease state (Control, *C9ORF72* ALS, sALS or ALS) were excluded. Likewise, proteins showing significant associations with potential confounding variables, including age, sex, or postmortem interval (PMI), were also excluded to ensure that the final protein set primarily reflected ALS-related changes. After applying exclusion criteria on the *C9ORF72* ALS associated modules and proteins, 641 proteins remained in the biological relevant modules. By intersecting these proteins with the 224 DAPs specific to *C9ORF72* ALS, we focused on disease-relevant pathways driven by abundance change. This yielded 110 proteins of interest, i.e., DAPs that were included in relevant modules. None of the protein modules showed correlations with sALS (Figure 3). Here, the 17 DAPs specific to sALS were used for further analysis of this ALS subform. The presence of ALS (*C9ORF72* ALS and sALS) was correlated with 17 protein modules in spinal cord (Figure 3 and sFigure 8A-sFigure 10A). The biologically relevant modules contained 1810 proteins after applying exclusion criteria. These proteins were intersected with 51 DAPs found to be differentially expressed in both ALS subforms. This yielded 24 proteins of interest implicated in both subforms of ALS. Overall, 110 proteins from the *C9ORF72* ALS, 17 from the sALS, and 24 shared between both groups were selected for subsequent pathway analysis.

In BA4, 3 modules containing a total of 1931 proteins were correlated to *C9ORF72* ALS. After applying exclusion criteria and intersection of the remaining proteins with the DAPs specific to *C9ORF72* ALS in BA4, one protein of interest (HEXB) was identified.

Proteins of interest were subjected to GO analysis, which yielded biological processes that are associated with sALS, *C9ORF72* ALS, or both. Many of the featured GO terms were linked to the transcription–translational axis, intracellular transport, cytoskeleton modification, metabolic processes, and/ or synaptic modification (Table 1). The results shown highlight proteins which are likely to contribute to major disrupted pathways in spinal cord and BA4 of *C9ORF72* ALS and sALS. Many of these proteins contribute to similar mechanisms in both subforms, however the exact protein alterations are distinct to *C9ORF72* ALS or sALS. These proteins will be subjected to in-depth analyses aimed at elucidating subform-specific and/or overlapping molecular mechanisms that may contribute to ALS pathophysiology.

# Distinct proteins change in solubility in *C9ORF72* ALS

As previously shown in Alzheimer's disease, solubility changes in the proteome may be a distinct driver of neurodegenerative disease pathology [119]. We employed a fractionation protocol aimed at identifying solubility changes across the proteome. To determine changes in solubility, we compared "fraction1-abundance/fraction2-abundance" ratios across 14 non-neurodegenerative controls, 11 *C9ORF72* ALS, and 11 sALS cases (Figure 4 and sFigure 12, respectively). Changes in meanprotein abundance ratios between groups highlighted solubility changes of proteins, given that fractions represent different grades of solubility.

When comparing the SOL and DISP fractions between *C9ORF72* ALS and control spinal cord no solubility changes were found (Figure 4A). In contrast, comparing the SOL and SDS fractions of *C9ORF72* ALS and control cases identified 11 solubility shifting proteins. Based on the SOL/SDS ratios of the respective proteins in *C9ORF72* ALS and control, 8 proteins shifted from the SDS fraction to the SOL fraction in *C9ORF72* ALS, while 3 other proteins shifted from the SOL fraction to the SDS fraction (Figure 4B). SOL/FA ratios yielded no solubility changes between *C9ORF72* ALS and control cases (Figure 4C). When comparing DISP and SDS fractions, 5 proteins shifted from the SDS fraction to the DISP fraction in *C9ORF72* ALS cases (Figure 4D). A solubility shift in *C9ORF72* ALS from the FA fraction to the DISP fraction was observed for HSPB1 (Figure 4E). SDS/FA ratios comparisons did not identify any solubility shifting proteins between *C9ORF72* ALS and control (Figure 4F). Similar comparisons between sALS and control cases did not yield solubility changes (sFigure 12).

# Targeted pathway analysis results in *C9ORF72* ALS/sALS-specific and shared pathways

GO analysis on the proteins of interest was performed to better understand which biological processes may be influenced by the identified proteomic changes (sFigure 4B-sFigure 10B and sTable 4). Subsequently, a targeted literature search was carried out to pinpoint how protein abundance and solubility changes might disrupt pathways and cause dysfunction and disease.

By doing so, protein abundance changes in *C9ORF72* ALS and sALS pointed to mutually shared alterations in key pathways essential for normal cellular function. These pathway alterations were associated with subform-specific proteome changes in *C9ORF72* ALS or sALS. Specifically, the transcription–translational axis, intracellular transport, cytoskeleton modification, metabolic processes, and synaptic modification showed such alterations in *C9ORF72* ALS and sALS. In addition, protein degradation, reactive oxygen species homeostasis, and pro-inflammatory signaling showed alterations specifically in *C9ORF72* ALS and were not identified in sALS (Table 1A and sFigure 13). Furthermore, proteins related to intracellular transport, cytoskeletal modification and protein homeostasis underwent solubility changes in *C9ORF72* ALS (Table 1B and sFigure 13).

*Transcription-translation axis:* Key differences within the transcription-translation axis between ALS subforms were observed. In *C9ORF72* ALS, multiple proteins involved in RNA synthesis and post-transcriptional processing, such as 10 ribosomal subunits had elevated levels [2, 45, 96]. Furthermore, elevated rRNA synthesis and ribosome biogenesis were present in *C9ORF72* ALS [117]. Increased levels of ELP1, an important regulator of the elongation step of protein translation point to an enhanced protein translation [25]. Moreover, elevated HSP1B levels argue in favor of enhanced chaperone activity [104]. Elevated ELP1 levels in sALS indicate similar changes in translation as in *C9ORF72* ALS [25]. Furthermore, elevated WDR48 levels in sALS modulate post-translational ubiquitination of proteins [132]. Our analysis yielded no solubility shifting proteins that may affect this mechanism.

*Intracellular transport*: Altered intracellular transport routes in *C9ORF72* ALS were indicated by elevated levels of SAR1A and COPE, two vesicle formation mediators between the endoplasmic reticulum (ER) and Golgi system. This finding represents enhanced cargo trafficking between the ER and Golgi system and vice versa [12, 36, 60, 74, 125]. Moreover, the reduced levels of MYO5A, an organelle motor protein that traffics cargo over actin filaments, and DYNLL2, a dimeric light chain known to bind to MYO5A, argue for a potential reduction of intracellular transport along actin filaments in *C9ORF72* ALS [38, 51, 53]. Intracellular transport may also be dysregulated by the solubility shift of RAB18 and SAR1A from the SDS fraction to the DISP fraction. Given that membrane-specific proteins are usually identified within the SDS fraction, and RAB proteins and SAR1A are known to attach to vesicles and granules to facilitate transport, a shift towards the DISP fraction may indicate a decreased functionality of RAB18 and SAR1A [32, 95, 99, 107]. In sALS, a similar change in SAR1A abundance levels facilitates a change in ER to Golgi transport as hypothesized in *C9ORF72* ALS, while a decrease of RAB4B could impede trafficking from endosomes to the Golgi system [47].

*Cytoskeleton modifications:* Similarly, 7 proteins involved in actin filament remodeling were shown to be increased in this *C9ORF72* ALS [13, 22, 39, 54, 57, 68, 91, 105, 109, 113, 136]. Furthermore, the solubility shift of TUBB from the SDS fraction towards the SOL fraction in *C9ORF72* ALS indicates modified microtubule depolymerization, as studies showed that unpolymerized TUBB fragments are known to be more soluble than polymerized counterparts [9, 55]. In addition, as important regulators of the actin cytoskeleton, solubility changes of MARCKS, SEPTIN7 and SEPTIN9 could drive further cytoskeletal changes [8, 23, 108]. Protein abundance alterations in sALS were limited to elevated levels of 3 protein related to actin remodeling [39, 57, 68, 113].

*Metabolic processes*: Aberrations in energy production could also be found in both ALS subforms. Diminished PFKM levels, a key protein involved in glycolysis, may impair energy production in *C9ORF72* ALS [40, 42]. The increase in G6PD, which performs the rate-limiting step of the pentose-phosphate pathway, allows the generation of metabolites downstream of PFKM [75, 116]. Our data identified additional dysregulations of glucose metabolism in *C9ORF72* ALS, including reduced abundance of BCAT1, a major regulator of the TCA cycle, and elevated levels of PYGL, involved in glycogen catabolism, and ITIH1, which is known to exacerbate insulin resistance [58, 92, 134]. Energy production aberrations in sALS were limited to the decreased abundance of one core subunit and two complementary subunits of complex I of the electron transport chain [49]. No solubility shifting proteins that may affect this mechanism were identified during our analysis.

*ROS homeostasis*: Elevated ROS production and the subsequent reinforced response to ROS were mechanisms specific to *C9ORF72* ALS in our data. CYBC1 is crucial for the respiratory burst in phagocytes, whereas HVCN1 has been shown to facilitate this process specifically in leukocytes [86, 120, 135]. This potential elevation in ROS production was mirrored by increased abundance of GPX3 and G6PD, which are involved in the cellular response to oxidative stress [75, 127]. No ROS homeostasis related solubility changes were observed.

*Synaptic modifications*: Evidence for altered synaptic modifications was also found in *C9ORF72* ALS. Various proteins that regulate synaptic vesicle transport, synaptic strength, and neurotransmitter release had decreased levels in *C9ORF72* ALS [1, 33, 62, 114]. In addition, our data identified depleted β-synuclein (SNCB) levels in sALS spinal cord. This indicates an increased sensitivity to SNCA pathology, as SNCB is able to inhibit SNCA aggregation in neurodegenerative diseases [82, 103]. No synaptic related solubility changes were observed.

*Pro-inflammatory signaling*: Several proteins implicated in NLRP3 signaling showed elevated abundance in *C9ORF72* ALS. During pyroptosis, a form of regulated cell death implicated in ALS, NLRP3 activation results in GSDMD cleavage by CASP1 [70, 111]. In turn, GSDMD-N oligomerizes and creates a pore in the cell membrane. This pore releases the CASP1-activated, pro-inflammatory cytokines IL-1β and IL-18 [70, 111]. Excessive activation of this process leads to cell death [70, 111]. When in the cytosol, CTSB and CTSZ can induce the NLRP3 inflammasome [17, 130]. Our data showed an increased abundance of both proteins in the SOL fraction, which pools cytosolic proteins. In addition, C5, C6 and C8G were shown to have increased abundance in *C9ORF72* ALS. These proteins are part of the membrane attack complex (MAC), which can induce the NLRP3 inflammasome by facilitating K+ ion efflux [56, 112, 128, 133]. Furthermore, increased CASP1 levels indicate NLRP3 activation in *C9ORF72* ALS. Solubility changes with pro-inflammatory effects were not identified.

*Protein degradation*: Dysfunctional protein degradation in *C9ORF72* ALS was also highlighted by our study. While multiple proteins involved with lysosomal function had increased abundance, ATG4B, an important initiator of autophagy, had reduced abundance [69, 122]. Furthermore, a slight decrease of HEXB, a lysosomal enzyme, was observed in BA4 of *C9ORF72* ALS [52].

The solubility shift by TPP1, a lysosomal protease, from the SDS fraction to the SOL fraction could indicate a failure to incorporate the enzyme into the lysosome. The targeting and transport of lysosomal enzymes is well known [14]. The MP6-dependent pathway, which facilitates TPP1 transport to the lysosome, is known to secrete enzymes into the extracellular space during a variety of known pathway defects [14, 93]. When TPP1 is contained in vesicles, including lysosomes, it is expected to appear in the SDS fraction. The observed shift towards the SOL fraction in *C9ORF72* ALS could be explained by its secretion into the extracellular space.

The data generated in combination with the targeted literature revealed how biological processes identified during the GO analysis were disrupted in *C9ORF72* ALS and/or sALS. Furthermore, it contextualizes how the solubility changes may interact with these mechanisms. Notably, while the affected biological processes in *C9ORF72* ALS and sALS showed substantial overlap, the proteome signatures identified in our data were largely specific to either subform, arguing for subform-specific dysfunction in *C9ORF72* ALS and sALS.

# Discussion

In this study, we characterized the proteome profiles of the two most common forms of ALS: sALS and *C9ORF72* ALS in comparison to healthy controls. Our results showed that both exhibit alterations linked to GO-terms regarding transcription–translational axis, intracellular transport, cytoskeleton modification, metabolic processes, and synaptic modification. Importantly, although these broad biological processes overlapped between the two ALS subforms, the individual proteins driving these alterations were largely distinct to either *C9ORF72* ALS or sALS. This suggests convergence at the pathway level despite substantial molecular divergence at the protein level. Furthermore, groups of proteins linked to GO-terms regarding protein degradation, ROS homeostasis, and pro-inflammatory signaling were altered only in *C9ORF72* ALS. This points to a broader disturbance of proteostasis and inflammatory signaling in *C9ORF72* ALS compared with sALS in our dataset.

In addition, 16 proteins exhibited a shift in solubility in the spinal cord of *C9ORF72* ALS cases compared to healthy controls. Solubility changes were defined in this study as changes in the abundance ratio of a distinct protein among two different fractions when comparing control with *C9ORF72* ALS or sALS. These differences indicate altered relative protein distribution between physiochemical fractions, potentially reflecting increased/decreased protein solubility, as determined by analyzing healthy controls. Solubility changes are often driven by structural modifications of the tertiary or quaternary structures of a given protein, which could result in a modified protein function [3, 44]. GO-terms related to cytoskeleton modification, intracellular transport, and protein degradation were associated with the solubility shifting proteins in *C9ORF72* ALS. In sALS cases, solubility changes of distinct proteins compared to controls were not seen.

Our findings extent earlier studies observing the proteome and transcriptome of sALS and *C9ORF72* ALS cases [34, 65, 98, 121] by identifying a) different underlying protein abundance patterns in sALS and *C9ORF72* ALS despite affecting similar GO-terms and b) proteins that exhibit changes in solubility, indicating a change in their biophysical properties with a potential modified function [3, 18, 44]. By doing so, we add novel insights into pathophysiology of ALS. We demonstrate that the same “overarching dysfunction“, due to the alteration of biological processes as represented by GO-terms, can be associated with abundance changes of different proteins across sALS and *C9ORF72* ALS. This distinction is important because pathway-level convergence does not necessarily imply a common upstream disease mechanism. Instead, different molecular alterations may ultimately affect similar cellular processes. For example, *C9ORF72* ALS is characterized by broad activation of the transcription-translation axis, including ribosomal biogenesis, whereas sALS only exhibits alterations in translation and post-translational modification. Likewise, dysregulated glucose metabolism in *C9ORF72* ALS contrasts heavily with the complex I impairments in sALS (sFigure 13). Another argument in favor of the different ALS forms actually representing pathogenetically different diseases is that ALS cases with *SOD1* and *FUS*-gene mutations show deposits of SOD1 or FUS, respectively, instead of TDP-43 [59, 87].

The differences in the proteomic patterns of *C9ORF72* ALS and sALS point to differences in the pathophysiological mechanisms of these two forms of ALS. The many disruptions in *C9ORF72* ALS when compared to sALS may be attributed to a higher TDP-43 burden in *C9ORF72* ALS [15, 131]. Higher levels

of TDP-43 aggregation induced by the HRE may facilitate additional disruptions downstream [72]. This hypothesis remains to be confirmed. However, evidence has been reported that contradict increased TDP-43-burdens in *C9ORF72* ALS [90].

The changes we identified are most predominant in the spinal cord and relatively mild in the primary motor cortex. This discrepancy when comparing spinal cord and motor cortex, might be due to the inclusion of one *C9ORF72* FTD case in the BA4 analysis. However, as our findings are in line with the discrepancies other groups identified between spinal cord and BA4, we believe this did not play a major role [6, 129]. One explanation for the difference in the proteomic patterns among these two regions is the fact that the spinal cord exhibits more severe degenerative changes, such as a severe loss of motor neurons and the related pyramidal fiber tracts compared to the precentral cortex where the loss of neurons is mainly restricted to motor neurons in layer V [6]. Given that many changes in the spinal cord are of presumably unspecific degenerative or reactive nature [37, 61, 106, 110], part of the proteome changes in the spinal cord may simply reflect the degeneration of motor neurons and related fiber tracts, and the accompanying reactive alterations. For example, pro-inflammatory changes, but also alterations in metabolism and proteostasis would be expected to occur as a result of degenerating/dying neurons with their axons [4]. Nonetheless, the observed solubility shifts in *C9ORF72* ALS that are lacking in sALS and the different protein abundance changes between both forms of ALS, argue that the findings are not purely reactive in nature. Future work may be needed to determine whether these differences reflect distinct disease mechanisms.

Our findings showed that several proteins related to cytoskeletal modification and intracellular transport change in solubility, which indicates an alteration of the respective pathways in *C9ORF72* ALS. As these proteins migrate out of their physiological fraction, they may induce changes critical for the development of an ALS phenotype. The proteomic and transcriptomic changes in these pathways as described by other authors, and the dynactin mutation causing a familiar form of ALS, support the critical role of these pathways in ALS [34, 85]. To what extent DPRs and/or RNA foci can impact or interfere with proteins undergoing *C9ORF72*-associated solubility or abundance changes in the ALS spinal cord is an important question that requires further research.

From a clinical perspective, our findings reinforce the molecular heterogeneity of ALS and suggest that genetic and sporadic ALS may converge on common cellular pathways through different molecular mechanisms. Different sets of proteins involved in *C9ORF72* ALS and sALS, although mostly encapsulated by the same pathways, may explain the differences in response to currently available therapies, the involvement of various metabolic imbalances in patient cohorts and differences in weight loss as seen in the end stage of ALS [26, 76, 123]. Proteins that contribute to either *C9ORF72* ALS or sALS highlight that specific dysfunction in ALS subforms may need to be targeted separately to ensure effective treatment. Whether the dysregulation of a given pathway may already allow therapeutic correction or whether causal events in the pathogenetic dysregulation require a multi-targeted approach will need to be addressed in future studies. Moreover, the molecular differences between sALS and *C9ORF72* ALS may argue in favor of the existence of distinct molecularly-defined subtypes of ALS. Given

that protein function may be modified by changes in protein solubility, our identification of proteins undergoing solubility changes in ALS may be a first step in better understanding the deficits in ALS central nervous tissue and for the development of novel therapeutic concepts for stopping ALS.

A limitation of this study is the small sample size of 40 cases. However, other studies focusing on ALS work with similar or even lower sample sizes as ALS is a rare disease [6]. Additionally, a bulk proteomics approach may bias analysis towards the most abundant signals. Furthermore, the lack of functional validation of the identified protein abundance changes and differentially regulated pathways is another general limitation of proteomics studies. To reduce the impact of lacking functional validation in this study, we performed targeted literature research to identify how DAPs or proteins shifting in solubility fit in the respective pathways and which other proteins are influenced in the respective pathways. By doing so, we could strengthen the plausibility of our findings.

# Conclusions

In conclusion, this study reveals distinct proteome patterns characterizing the two most prevalent ALS subforms. We showed convergence of *C9ORF72* ALS and sALS at the level of biological pathway alteration but substantial divergence in the proteins underlying these changes, and subform-specific pathways that were altered in *C9ORF72* ALS and sALS. The identified alterations in *C9ORF72* ALS protein solubility indicate severe changes in several cytoskeletal and transport-related proteins. These findings underscore the molecular heterogeneity of ALS and suggest that shared cellular dysfunction may arise through distinct molecular mechanisms across ALS subforms. Together, our findings pave the way towards a deeper understanding of the proteomic changes underlying ALS, how these may cause disease, and which different molecular subtypes of ALS exist.

# Abbreviations

Amyotrophic lateral sclerosis (ALS)

Transactive response DNA-binding protein 43 (TDP-43)

Sporadic ALS (sALS)

Heterozygous GGGGCC hexanucleotide repeat expansions (HREs)

*Chromosome 9 open reading frame 72* (*C9ORF72*)

Dipeptide repeat proteins (DPRs)

Primary motor cortex (Brodmann area 4) (BA4)

Post-mortem interval (PMI)

Polymerase chain reaction (PCR)

Frontotemporal dementia (FTD)

Neurofibrillary tangle (NFT)

Consortium to Establish a Registry for Alzheimer’s disease (CERAD)

Phosphorylated (p)

Amyloid-β peptide (Aβ)

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Water-soluble (SOL)

Water-insoluble dispersible (DISP)

Sodium dodecyl sulfate-soluble (SDS)

Formic acid-soluble (FA)

Tris-buffered saline (TBS)

Room temperature (RT)

Milli-Q water (MQ)

Ammonium bicarbonate solution (ABS)

Data independent acquisition (DIA)

Differentially abundant proteins (DAPs)

Weighted gene co-expression network analysis (WGCNA)

Module eigengenes (MEs)

Gene ontology (GO)

Protein-protein interaction (PPI)

Endoplasmic reticulum (ER)

Membrane attack complex (MAC)

## Declarations

# Ethics approval and consent to participate

All autopsies were carried out according to local legislation with appropriate consent. Recruitment protocols were approved by the ethical committees of UZ Leuven (Belgium, Approval no. S-55312, S-59292, S-65097) and the University of Ulm (Germany, Approval no. 54/08). Furthermore, this study was approved by the ethical committee of UZ Leuven (Approval no. S-60803).

AI

OpenAI GPT-6 was used to optimize R coding and for language editing.

# Consent for publication

Not applicable for this study, which did not use person's data. Only anonymized or pseudonymized data were processed.

# Availability of data and materials

Data analyzed for this article have been deposited to the ProteomeXchange Consortium via the PRIDE [94] partner repository with the dataset identifier PXD085711. All files, including all LC-MS/MS raw files across four fractions and protein quantification results, have been uploaded. Further information can be requested from the corresponding author on reasonable request.

# Competing interests

DRT collaborated with Novartis Pharma AG (Switzerland), Fujirebio (Belgium), and GE-Healthcare (UK) and received consultant honoraria from Muna Therapeutics (Belgium). LVDB. is head of the Scientific Advisory Board of Augustine Therapeutics (Leuven, Belgium) and is part of the Investment Advisory Board of Droia Ventures (Meise, Belgium). PVD has served in advisory boards for Biogen, CSL Behring, Alexion Pharmaceuticals, Ferrer, QurAlis, Cytokinetics, argenx, UCB, Muna Therapeutics, Alector, Augustine Therapeutics, VectorY, Zambon, Amylyx, Novartis, Prilenia, Verge Genomics, Sapreme Technologies, Trace Neuroscience, NRG Therapeutics, Crucible Therapeutics, AcuraStem (paid to institution). PVD has received speaker fees from Biogen and Amylyx (paid to institution). DRT is member of the Acta Neuropathologica editorial board. He was not involved in handling and/or decision making for this manuscript.

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# Authors' contributions

BCRL: Optimization of the LC-MS/MS protocol, sample preparation, lead in bioinformatic analysis and manuscript preparation; AW: Contributions during protocol optimization, sample preparation, data analysis and manuscript preparation; JMS: Bioinformatic analysis, manuscript preparation and critical review of the manuscript draft; PMas: Bioinformatic analysis and critical review of the manuscript draft; GA: Contributions to the bioinformatic analysis and critical review of the manuscript draft; AR: Contributions to tissue sampling and critical review of the manuscript draft; LD: Additional neuropathology and critical review of the manuscript draft; EVS: Additional neuropathology and critical review of the manuscript draft; PMag: LC-MS/MS, processing of LC-MS/MS raw data and manuscript preparation; ACL: Clinical neurology and critical review of the manuscript draft; JW: Clinical neurology and critical review of the manuscript draft; LVDB: Study design and critical review of the manuscript draft; PVD: Clinical neurology, study design, and critical review of the manuscript draft; DRT: Study design and study supervision, neuropathology, and manuscript preparation & supervision.

All authors read and approved the final manuscript.

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# Table

**Table 1 Overview of disease mechanisms underlying ALS subforms**

While *C9ORF72* ALS and sALS spinal cord tissue reveals several overlapping mechanisms that may be affected, the proteins that drive dysfunction are mostly specific to each subform. There are also indications of specific pathways affected specifically in *C9ORF72* ALS. Proteins in bold are similarly changed in both ALS subforms (A). Protein solubility changes related to disrupted pathways were observed in *C9ORF72* ALS. Specifically intracellular transport, cytoskeleton modification and protein homeostasis are affected (B).

| *a Affected mechanism* | *C9ORF72 ALS* | *sALS* |
|---|---|---|
| *Transcription-Translation axis* | HNRNPR, HNRNPU, HNRNPDL, SNRPD1, NCL, RPS2, RPS3, RPS6, RPS7, RPS12, RPS15A, RPLP0, RPL9, RPL18, RPL18A, **ELP1**, HSPB1 | **ELP1**, WDR48 |
| *Intracellular transport* | **SAR1A**, COPE, ERGIC1, NDRG4, MYO5A, DYNLL2 | **SAR1A**, RAB4B |
| *Cytoskeleton modification* | **TJP1**, **CAPG**, **SCIN**, IQGAP1, ARPC1B, INF2, FLNA | **TJP1**, **CAPG**, **SCIN** |
| *Metabolic processes* | PFKM, G6PD, BCAT1, PYGL, ITIH1 | NDUFA9, NDUFA12, NDUFV1 |
| *Synaptic modifications* | STBXBP1, CAMK2B, DNM3 | SNCB |
| *ROS homeostasis* | CYBC1, HVCN1, GPX3 | |
| *Protein homeostasis* | ATG4B, TPP1, LAMP1, LAMTOR1 | |
| *Pro-inflammatory signaling* | CTSB, CTSZ, CASP1,<br>C5, C6, C8G | |

| *b* *Affected mechanism* | *C9ORF72 ALS* | *sALS* |
|---|---|---|
| *Intracellular transport* | RAB18, SAR1A | |
| *Cytoskeleton modification* | TUBB, MARCKS, SEPTIN7, SEPTIN9 | |
| *Protein homeostasis* | TPP1 | |