**Transcriptomic profiling of cerebrospinal fluid identifies ALS pathway enrichment and RNA biomarkers in MND individuals**

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**Running title:** CSF transcriptomic profiling in ALS individuals

**Abstract**

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder and the most common form of motor neurone disease (MND) which is characterised by the damage and death of motor neurons in the brain and spinal cord of affected individuals. Due to the heterogeneity of the disease, a better understanding of the interaction between genetics and biochemistry with the identification of biomarkers is crucial for therapy development. In this study, we used cerebrospinal fluid (CSF) RNA sequencing data from the New York Genome Center (NYGC) ALS Consortium and analysed differential gene expression between 47 MND individuals and 29 healthy controls. Pathway analysis showed that the affected genes are enriched in many pathways associated with ALS, including nucleocytoplasmic transport, autophagy, and apoptosis. Moreover, we assessed differential expression on both gene- and transcript-based levels and demonstrate that the expression of previously identified potential biomarkers, including *CAPG*, *CCL3* and *MAP2*, was significantly higher in MND individuals. Ultimately this study highlights the transcriptomic composition of CSF which enables insights into changes in the brain in ALS and therefore increase the confidence in the use of CSF for biomarker development.

**Keywords**

Cerebrospinal fluid, amyotrophic lateral sclerosis, motor neurone disease, transcriptome, RNA-Seq, biomarker

**Impact Statement**

In the present study, we describe significant expression profile changes and enrichment of key ALS pathways in MND individuals compared to healthy controls using RNA sequencing data. We demonstrate that protein biomarkers in CSF are reflected by their corresponding increased expression in CSF RNA sequencing profiles highlighting CSF RNA data as a valuable resource for biomarker development. We also suggest another potential biomarker in form of the COPA gene which plays important roles in protein transport between endoplasmic reticulum and Golgi apparatus, a key pathway in ALS pathology. Overall, this study highlights the importance of studying RNA sequencing data for both biomarker development and complementation of CSF proteomic analysis.

**Introduction**

Motor Neurone Disease (MND), the most common form being amyotrophic lateral sclerosis (ALS), is a rare progressive neurodegenerative disease which results in the damage and death of motor neurons in the brain and spinal cord of affected individuals. This degeneration of neurons leads to the weakening and stiffening of muscles, which eventually results in individuals losing the ability to walk and breathe with sufferers dying between 3 and 5 years following symptom onset 1,2. Starting with the discovery of pathogenic variants in *SOD1*, *FUS*, *TARDBP* and *C9ORF72*, there are now over 100 genes associated with the disease which reflects the heterogeneity of ALS 3–6. Several pathological mechanisms, such as oxidative stress, inflammation, protein aggregation, impairment of autophagy or RNA processing and aberrant retrotransposon function have been hypothesised to be involved in disease pathogenesis 7–9. Therefore, a better understanding of the interaction between genetics and biochemistry including the identification of biomarkers is crucial to progress therapy development and effective clinical trial design by allowing stratification of cohorts into more homogenous groups. The focus on biomarker development has shifted to the use of cerebrospinal fluid (CSF) due to its contact with the borders/extracellular space of the brain and neuroglia cell enriched composition which facilitates diagnosis and monitoring of diseases, as previously shown by the increased application in diagnosis of Alzheimer’s disease (AD) 10,11. ALS-specific biomarkers are urgently needed for the early confirmation of ALS; to date several proteomic approaches and immunoassays having been developed and several molecules have been identified with the potential to act as progression and prognosis biomarkers 12. These include, for example, neurofilaments, proteins implicated in neuroinflammation, cytokines, chemokines and cytoplasmic protein indicators, including TDP-43 which is a hallmark of ALS pathology 3,12. However, large-scale studies are still needed to validate the use of these potential biomarkers and to correlate corresponding biomarker concentrations with medical status and progression of ALS. Furthermore, as yet, CSF RNA sequencing analysis has not been explored as a source for identification of novel biomarkers in ALS/MND, we hypothesise it will provide both transcriptomic information that overlaps with the previous proteomic approaches employed but also provide further unique insights and potential biomarkers.

In this study, we made use of transcriptomic data from CSF of 47 MND and 29 healthy control individuals from the New York Genome Center (NYGC) ALS Consortium (**Fig. 1**). The aim was to assess differential expression of genes encoding previously determined ALS biomarkers (extensively reviewed in 12) between individuals with MND and controls, and to integrate that data into pathway analysis which has not been commonly studied within neurological conditions such as MND (**Fig. 1**). We demonstrated that the differentially expressed genes were enriched in many pathways associated with ALS, including nucleocytoplasmic transport, autophagy, and apoptosis. In addition, the expression of previously identified potential CSF biomarkers, including *CAPG*, *CCL3* and *MAP2* among others 12, was significantly higher in MND individuals. The results of this study demonstrate that protein or immunological biomarkers in CSF are often correlated with a corresponding increased expression in CSF RNA sequencing data highlighting CSF RNA data as a valuable resource for biomarker development and potentially expanding or complementing those identified from proteomic studies.

**Materials and Methods**

**Overview of study**

In this study, we used transcriptomic data from the New York Genome Center ALS Consortium cohort (<https://www.nygenome.org/als-consortium/>). We analysed cerebrospinal fluid data from 47 MND and 29 age-matched healthy control individuals. MND individuals include 45 classic/typical amyotrophic lateral sclerosis (ALS) subjects complemented by one case of primary lateral sclerosis and one case of progressive muscular atrophy. CSF from MND and neurologically normal controls was collected at Stony Brook University Hospital. Healthy control individuals were recruited to participate in the study as part of a hip or knee arthroplasty, whereby patients gave consent to collect CSF.

**Differential transcriptome and pathway analysis**

To evaluate the changes in the transcriptome and transcriptional involvement in disease pathways, we performed differential gene expression analysis by comparing the MND data to controls. Quantification of transcriptomic data from the NYGC ALS consortium cohort on a gene- and transcript-based level was performed by using the *Salmon* tool (https://salmon.readthedocs.io). The *tximport* function from the *tximport* package 13 was used to import salmon generated quantification files into *R*. Raw counts were extracted with the *DESeqDataSetFromTximport* function and normalised using the median-of-ratios method, specifically by dividing the raw counts by sample-specific size factors which represent the median ratio of gene counts to the geometric mean per gene. Differential gene expression analysis was performed between MND and healthy control individuals by using the *DESeq2* package in *R* 14. Resulting *P* values for differentially expressed genes and transcripts were adjusted by Benjamini and Hochberg False Discovery Rate (FDR) and only FDR-adjusted values <0.05 were considered significant. After formal statistical comparison, KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis was performed using the *clusterProfiler* package in *R* 15 and only *P*-adjusted values ≤0.05 were considered as significant.

**Results**

**Pathway analysis shows enrichment for ALS disease pathway**

We used transcriptomic data from CSF and compared expression changes between MND and healthy control individuals (**Fig. 1**). Following this analysis, we performed KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis which utilises a database with integrated genomic, chemical, and systemic functional information 16 to identify pathways associated with gene expression changes in the individuals with MND. This analysis revealed the enrichment of 37 pathways including multiple nervous system-associated pathways (**Table 1**). Interestingly, one of these pathways represents amyotrophic lateral sclerosis (ALS) with a gene ratio of 310/6292 (*P*-adj.=9.35E-05). We expanded the analysis by generating an UpSet plot visualising the association between affected genes and overlapping gene sets associated with certain pathways/diseases (**Fig. 2**). Increased gene overlapping was detected between the neurodegenerative diseases: ALS, Parkinson’s disease and Huntington’s disease indicating common disease related pathways were affected (**Fig. 2**). However, 75 genes were solely associated with ALS pathway demonstrating the over-representation of affected genes in the pathway of interest (**Fig. 2**). To further this analysis, we specifically looked at the effect of this differential gene expression data on the ALS pathway (**Supplementary Data 4**). This identified several genes involved in key disease pathways including nucleocytoplasmic transport, autophagy, apoptosis, regulation of actin cytoskeleton or protein processing in endoplasmic reticulum which were up- or down-regulated in MND individuals compared to healthy controls (**Supplementary Data 4**). Interestingly, increased *TARDBP* expression encoding TDP-43, a hallmark of ALS pathology, was detected in MND individuals, as previously described (**Supplementary Data 4**). 12,17

**Expression of ALS biomarkers is elevated in MND individuals from CSF transcriptomic data**

Having demonstrated that differential gene expression in MND individuals is significantly associated with the enrichment of the ALS pathway (**Table 1**, **Fig. 3**), we next aimed to assess specific expression changes on a gene- and transcript-based level (**Tables 2, 3, Supplemental Data 1, 2**). The 20 most significant transcripts and genes are shown in **Tables 2** and **3**. We focused on changes in genes encoding previously characterised biomarkers, specifically capping actin protein, gelsolin like (*CAPG*) and C-C motif chemokine ligand 3 (*CCL3*) which showed significantly higher expression in MND individuals, with 2.02 (*P*-adj.=0.0098) and 1.32 (*P*-adj.=0.014) log2 fold changes (log2FC) obtained (**Fig. 3A**). In addition, we demonstrated transcript-based changes in gene expression of potential ALS biomarkers. One of these represented microtubule-associated protein 2 (*MAP2*); we found that three transcripts (MAP2-215, MAP2-206 and MAP2-213) were significantly more highly expressed in MND individuals (**Fig. 3B**), log2 fold changes of 2.43 (*P*-adj.=2.71E-05), 1.20 (*P*-adj.=0.033) and 1.07 (*P*-adj.=0.04) respectively were obtained. Other transcripts significantly elevated in MND individuals encoded for the cytokine interleukin 10 (IL-10), including IL10-204 (log2FC=1.74, *P*-adj.=0.00012), IL10-206 (log2FC=1.47, *P*-adj.=0.013), IL10-205 (log2FC=1.43, *P*-adj.=0.0062), the vascular endothelial growth factor (VEGF), including VEGF-206 (log2FC=1.38, *P*-adj.=0.0024) and the proteins chitinase 3 like 2/CHI3L2 (CHI3L2-214, log2FC=1.17, *P*-adj.=0.033) and glycoprotein nmb/GPNMB (GPNMB-206, log2FC=1.76, *P*-adj.=0.00079), involved in inflammatory processes (**Fig. 3B**). It should be noted that some of these transcripts (MAP2-215, MAP2-213, IL10-204 and GPNMB-206) are alternatively spliced transcripts of a protein coding gene for which the coding sequence has not been defined yet. However, these data confirm the validity of using CSF for biomarker discovery in ALS and illustrate the plethora of expression changes on a gene- and isoform-based level obtained from RNA sequencing data of CSF. In addition, the top hits obtained in this analysis, including the transcript ENST00000648280 (COPA-212, *P*-adj.=6.06E-13), have not been investigated as biomarkers to date.

**Discussion**

To date, there is no cure for ALS, highlighting the urgent need to understand the interaction between genetics and biochemistry including the identification of biomarkers. Advances in the development of biomarkers would help to deepen the knowledge both of the preclinical disease phase and to progress therapy development and design of effective clinical trials by stratification of patients into more homogenous groups.

Several CSF protein biomarkers have already been established for ALS such as neurofilaments, synucleins or tau 12,18,19. Recent studies including proteomic analyses have identified further novel biomarkers for ALS which include MAP2, CAPG and GPNMB plus others involved in neuroinflammation (CHI3L2) or with neuroprotective roles (VEGF) 12,20.

CHI3L2 is part of the chitinase-like proteins and secreted by astrocytes/microglia. This protein may lead to neuronal death in ALS as a direct correlation between its CSF concentration in ALS individuals and disease progression rate was found 21,22. CCL3, also termed macrophage inflammatory protein 1 alpha, is involved in the accumulation of microglia and has functions in inflammatory responses and therefore indicates neuroinflammation in ALS 12. CCL3 has been shown to inversely correlate with disease progression rate 23. Other proteins, including CAPG and GPNBM, have also be associated to inflammatory processes 20,24,25. More specifically, *GPNMB* expression has been linked to neurodegeneration by the observation that ALS patients were characterised by a shorter survival time with high GPNMB CSF levels and the correlation with the disease severity ALSFRS score 20. Tanaka *et al.* confirmed the increased GPNMB levels in the CSF 26. MAP2 is part of the family of microtubule-associated proteins which have crucial roles in modulating the microtubule network. Oeckl and colleagues showed a significant increase of MAP2 in the CSF of ALS individuals and the potential to act as a marker of motor neuron degeneration 20. It has been demonstrated that MAP2 can induce neurites 27, and the corresponding increased *MAP2* expression in the CSF may be an adjustment following axonal loss. Therefore, MAP2 could act as a marker of motor neuron loss. The cytokine VEGF has been associated with faster disease progression (and shorter survival) in patients with lower VEGF levels which may therefore represent a positive prognostic measure 23. Interestingly, VEGF CSF levels positively correlated with levels of PaO2 in ALS individuals, which suggests a hypoxia response dysfunction in ALS patients 28.

Our data is consistent with previous CSF level studies, confirming the findings by transcriptomic data analysis focussed on the expression of the corresponding gene targets. By doing so it also conferred greater confidence in the other novel targets we identified thus highlighting the utility/validity of transcriptomic analysis in such studies.

Transcriptomic analysis of CSF is not commonly performed in neurological conditions and here we provide new data analysing RNA sequencing data from 47 MND and 29 healthy control individuals and assessing expression changes between the two groups. Utilising data derived from this biofluid, we demonstrated on the gene-based level that *CAPG* expression was significantly higher in MND individuals (log2FC=2.02, *P*-adj.=0.0098) (**Fig. 3A**). Furthermore, MND individuals showed a 1.32 log2FC increase (*P*-adj.=0.014) in *CCL3* which is line with previously reported elevated levels of the protein in ALS CSF 12,23,29. Moreover, our study highlighted transcript specific changes in isoform expression of ALS biomarkers, e.g., for MAP2; three transcripts (MAP2-215, MAP2-206 and MAP2-213) were significantly more highly expressed in MND individuals (**Fig. 3B**), log2 fold changes of 2.43 (*P*-adj.=2.71E-05), 1.20 (*P*-adj.=0.033) and 1.07 (*P*-adj.=0.04) were obtained. These results highlight the plethora and specificity of transcriptomic changes obtained from CSF transcriptomic datasets which can be a tool to confirm advances in biomarker development and their involvement in specific signalling pathways. In this study the gene demonstrating the greatest difference in cases versus controls wasa specific protein-coding transcript of the gene *COPA* (ENST00000648280) which was significantly more highly expressed in MND individuals (Log2FC=3.98, *P*-adj.= 6.06E-13) compared to controls. This gene encodes the COP1 protein which has been shown to be involved in protein transport between endoplasmic reticulum and Golgi apparatus, with gene mutations being associated with an inflammatory syndrome showing lung, renal, and joint involvement 30,31.

High sensitivity of measurement is crucial to detect small changes in a low concentration range, thus this sensitive RNAseq analysis may complement proteomic analysis to support biomarker development. This view is reinforced by the KEGG pathway enrichment analysis which demonstrated that the differentially expressed genes were enriched in the ALS pathway (hsa05014, gene ratio 310/6292, *P*-adj.=9.35E-05), with numerous pathways involved in ALS pathology affected, including nucleocytoplasmic transport, autophagy, apoptosis, regulation of actin cytoskeleton or protein processing in endoplasmic reticulum (**Supplementary Data 4**). We were able to detect significant changes in RNA expression for known CSF biomarker proteins which confirms CSF RNA-seq is a suitable method for this type of analysis. CSF analysis presents several challenges: CSF is challenging to collect, it is inconvenient and has risks as a procedure, however CSF contains cell free RNA, and this can be analysed by modern sensitive analytical tools. Future additional larger and longitudinal studies utilising CSF are needed to validate and reproduce these findings and to increase precision of biomarker development. Another limitation of the study is that the cut-off for the transcript-based level has not been identified yet and this limits the use of these as biomarkers.

**Authors’ Contributions**

Conceptualization, methodology and formal analysis, AF and SK; data interpretation, AF, ALP, VJB, JPQ, and SK; writing – original draft preparation, AF; writing – review and editing, AF, ALP, VJB, JPQ, and SK. All authors have read and agreed to the published version of the manuscript.

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**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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**Figure legends**

**Figure 1: Overview of study.** In this study, transcriptomic dataset from the New York Genome Center ALS Consortium was used. RNAseq data from cerebrospinal fluid of 47 MND and 29 healthy control individuals was available. The aim of the study was to perform differential gene expression analysis between MND and control individuals with a focus on biomarker expression and explore enriched pathways.

**Figure 2: UpSet plot to visualize intersections of differentially expressed genes.** The bar chart represents the size of the gene set. The number of differentially expressed genes for single (filled-in cells) and intersecting (filled-in cells with connecting lines) pathways/diseases is shown. A full list of genes affected in the corresponding disease/pathway is listed in Supplementary Data 3.

**Figure 3: Several genes encoding potential biomarker proteins are significantly upregulated in CSF from MND individuals.** Differential gene expression analysis was performed using transcriptomic datasets from 47 MND and 29 healthy control individuals. Expression changes were analysed on a gene (A)- and transcript (B)- based level. Several genes which have been previously identified to encode potential biomarker proteins were significantly more highly expressed in MND individuals. Log2 fold changes (log2FC) and *P* adjusted values are indicated. For transcript-based analysis, corresponding transcript IDs are shown.

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