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# Theme 06 - TISSUE BIOMARKERS

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# THEME 6 TISSUE BIOMARKERS

## BIO-01 Not just STMN2 – hunting for TDP-43 cryptic biomarkers in ALS/FTD neuronal tissue

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TDP-43 has a crucial role in the repression of cryptic splicing, however, its targets are species and even tissue specific. Here, by performing CRISPRi knockdown of TDP-43 in human iPSC cortical neurons and cross referencing them with the NYGC ALS RNA-seq data set, consisting of 1349 tissues across 377 patients, we increase the compendium of splice events occurring selectively in patients affected by TDP-43 proteinopathy. We uncover 66 genes with cryptic splicing that are specific to and highly expressed in patient tissue affected by TDP-43 proteinopathy. These genes are enriched for genes involved in synaptic vesicle priming as well as genes involved in endocytic vesicle membranes. These genes include previously described TDP-43 regulated cryptic exons such as STMN2 and PFKP, as well as novel events such as cryptic exon in the ALS/FTD risk gene UNC13A, and a novel exon skipping event in potassium voltage-gated channel protein KCNQ2 which plays a crucial role in regulation of neuronal excitability. This analysis could pave the way to biomarker panels for TDP-43 loss of function in patient tissue and uncover the critical disease affectors of TDP-43 loss of function in ALS/FTD.

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## BIO-02 GSR and SOD1: oxidative stress biomarkers in ALS and FTD patients

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**Background:** There is a growing need to identify specific biomarkers that facilitate the diagnosis and prognosis of neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). Previous studies performed on muscle biopsies from mice SOD1G93A suggested that this animal model presents an alteration in the expression of several proteins (MEF2C, GSR, COL19A1, CALM1, SOD1 and SNX10) (1–4). In this point, two of the previous proteins (GSR and SOD1) are involved as an important part of defense against that stress cellular metabolism in ALS as in other neurodegenerative diseases.
The aim of this study is to determinate the diagnostic capacity of these inflammatory genes as potential biomarkers both on ALS and FTD, and their clinical predictors of prognosis.
**Methods:** Total protein from lymphocytes of 39 ALS patients, 43 FTD patients and 27 healthy controls were subjected to Western Blot in order to quantify expression levels of GSR and SOD1. These levels were related to the main clinical parameters like days since symptoms onset, ALSFRS-r, ALSFRS-r slope, diagnostic delay, age of onset of symptoms, and others. Statistical analysis was performed using SPSS Statistics version 24 (IBM, Spain).
**Results:** We found a significant down expression of GSR in FTD patients and overexpression of SOD1 in ALS patients with respect to control group. Furthermore, an increased SOD1 protein expression and decreased GSR expression levels are risk factors for survival in ALS patients.

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**Discussion:** Expression levels of GSR and SOD1 seem like good diagnostic biomarkers of ALS and FTD, but the expression of these proteins could not be considered as a diagnostic biomarker due to the low sensitivity and specificity values shown in the present study. Protein levels expression is associated with survival in ALS patients. Unfortunately, the progression study does not yield concordant data based on the prognostic capacity of these genes as biomarkers in FTD.

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# BIO-03 Parkin level in plasma of ALS patients

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**Background:** Accumulation of misfolded and damaged proteins are known to be associated with the pathogenesis of many neurodegenerative diseases such as Parkinson's Disease, Alzheimer Disease, ALS (1–4). Parkin, an E3 ubiquitin-protein ligase, is an important component of ubiquitin-proteasome system (5). It is known that Parkin is important for especially genetic Parkinsonism. There is limited study on Parkin for ALS disease.
**Methods:** In this study, Parkin Protein level was measured in blood plasma from 207 volunteerstotally in order to test Parkin Protein during ALS disease progression. Method of Enzyme Linked Immunosorbent Assay was used to measure Parkin level in blood plasma. Blood plasma was collected from ALS Patients, healthy individuals, Parkinson Patients and other neurological patients except ALS and Parkinson. Progression of disease clinically were divided as fast, slow and stable/recovering patient.
**Results:** Parkin protein amount was changed significantly amongst healthy control, neurological control and ALS patients. Mean of Parkin value is $3.3901 \pm 0.35188$ for ALS, $2.1183 \pm 0.28761$for healthy control, $1.0305 \pm 1.4956$ for Parkinson's group and $2.1253 \pm 0.34370$ for neurological control. Difference among their mean of Parkin level is statistically significant ($p < 0.05$).
**Discussion:** In the present study, for the first time, plasma Parkin concentration was found significantly elevated in ALS patients. Diagnostic and prognostic utility of this data must be validated by further investigation to use parkin protein in clinical practice.

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# BIO-04 miRNA biomarkers for diagnosis of ALS and FTD, developed by a nonlinear machine learning approach

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The neurodegenerative disorders amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) reside on a clinical and pathological continuum. Heterogeneity in clinical presentation too often delays clinical diagnosis and calls for molecular biomarkers to assist diagnosis, including cell free microRNAs (miRNA). However, nonlinearity in the relationship of miRNAs to clinical states and underpowered cohorts has limited research in this domain. Here, we prospectively enrolled a large cohort of 495 subjects with ALS ($n = 202$) and FTD ($n = 168$), or non-neurodegenerative controls ($n = 125$). Based on cell-free plasma miRNA profiling by next generation sequencing and machine learning approaches, we develop nonlinear prediction models that accurately distinguish ALS and FTD from non-neurodegenerative controls in ~90% of cases. Among the miRNAs that contribute to classifying disease, we identified miRNAs shared between conditions as well as disease-specific miRNAs. The fascinating potential of diagnostic miRNA biomarkers might enable early-stage detection and a cost-effective screening approach for clinical trials that can facilitate drug development.

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## BIO-05 Plasma derived small and large extracellular vesicles showed different immune phenotypes in patients with amyotrophic lateral sclerosis

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**Background:** Extracellular vesicles (EVs) play central role in inflammatory processes and they could be plausible targets in ALS related to immunological reaction to motor neuron loss (1). We have previously demonstrated that leukocyte-derived EVs are upregulated in ALS patients and they can be considered markers of disease progression (2).
**Objectives:** The aim of this study was to investigate specific immunological surface markers on Large and Small EVs (LEVs and SEVs) from plasma of sporadic ALS (sALS) patients and healthy donors (CTRLs).
**Methods:** EVs were isolated from plasma of 50 sALS and 30 CTRLs by differential centrifugation and characterized by Nanoparticle Tracking Analysis (NTA), Atomic Force Microscopy (AFM), and COlorimetric NANoplasmonic method (CONAN). For a simultaneous identification of 37 surface protein markers in each sample, we used a multiplex bead-based flow cytometric assay (MACSPlex Exosome Kit).
**Results:** Endosome-specific tetraspanins (CD63 and CD9), leucocyte, platelet and endothelial cell adhesion molecules (leukocyte common antigen (CD45), platelet endothelial cell adhesion molecule (PECAM-1, CD31), human Integrin alpha 5 (CD49e), E-selectin (CD69), B-lymphocyte antigen (CD20) were more expressed in SEVs derived from CTRLs than in sALS patients ($p < 0.05$). LEVs derived from CTRLs were enriched in platelet surface expression of CD62P (P-selectin) ($p < 0.05$) and CD63 ($p < 0.001$) compared to sALS patients.
**Discussion:** Endosome-specific tetraspanins decrease in both LEVs and SEVs in accordance with the autophagy-endolysosomal system dysregulation described in ALS (3). LEVs from sALS patients also had fewer platelet activation markers in line with the literature as suggested by the platelet variation in blood of ALS patients (4). These data suggest that LEVs and SEVs carry different surface markers which might discriminate their role in ALS pathogenesis.

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## BIO-06 Biomarkers for patient stratification and target engagement in ALS patients with TDP-43 pathology

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Patient identification and stratification as well as early identification of target engagement can be crucial to the development of effective therapies in ALS. Multiple prior studies have shown that Stathmin-2 is depleted in the spinal cords of ALS patients. However, it was only recently discovered that TDP-43 mislocalization leads to alternative splicing of a number of genes including Stathmin-2. Stathmin-2, previously named SCG10, is known to play an important role in axonal growth and maintenance, and restoration of Stathmin-2 expression in neurons with TDP-43 nuclear depletion is capable of rescuing axonal health. Intriguingly, in the case of Stathmin-2, the splicing changes seen in patients and human cell models are not recapitulated in lower species including rodents and non-human primates. Given this dichotomy, there is no animal model that is appropriate to assist in human dose prediction. Thus, a clinical dose will be based on efficacy in cell-based assays. With this paradigm it is crucial to be able to evaluate target engagement rapidly in clinical studies. We have developed Stathmin-2 based biomarker assays in human biofluids and characterized these markers in ALS patients and healthy individuals. These assays could be useful to identify patients with TDP-43 proteinopathy and future targeting of these patients for precision medicine-based therapeutics.

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## BIO-07 RNS60 increases mitochondrial oxidative metabolism in skeletal muscle of healthy volunteers

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RNS60 is an electrokinetically altered aqueous fluid composed of saline and oxygen. The electrokinetic processing is hypothesized to produce oxygen nanobubbles consisting of an oxygen core surrounded by layers of positive and negative electrical charges. In vitro and in vivo studies in rodent models of ALS and other neurodegenerative diseases have demonstrated that RNS60 has neuroprotective and anti-inflammatory effects and augments mitochondrial biogenesis and function. RNS60 has been well tolerated in Phase I and Phase II clinical studies, including open label pilot studies in people with amyotrophic lateral sclerosis (ALS) and multiple sclerosis. RNS60 is in Phase II of clinical development as a treatment for ALS, where it is administered by IV infusion and inhalation (nebulization).

The overall aim of this project was to measure the effect of RNS60 on mitochondrial energy metabolism in human volunteers. To accomplish this, we used a phosphorus magnetic resonance spectroscopy (MRS) measurement of skeletal muscle in healthy human subjects before and after the administration of RNS60. Phosphorus MRS is a well-established technique that uniquely measures oxidative phosphorylation in vivo and can be used to quantify the metabolic rate non-invasively in tissue. The phosphorus MRS data were collected using a 7T MRI instrument. MRS data were acquired every 1.2 s for 450 s using a radiofrequency surface coil placed over the tibialis anterior muscle. The MRS protocol included a rest period (120 s), an isometric exercise period (90 s) at 30% maximum voluntary contraction, and a recovery period (240 s). The rate constant of phosphocreatine recovery post-exercise was the primary outcome measure and was extracted by fitting the integrated signal intensity of phosphocreatine peak to a single exponential function during the recovery period. Sixteen healthy men, ages 21–64 y (43 ± 14 y) with self-reports of mild-moderate sedentary lifestyle were enrolled. RNS60 was investigated using two routes of administration, inhalation (nebulization) and IV infusion, using a multi-visit study design. Normal saline inhalation and infusion were used as control interventions.

All subjects tolerated the procedures and treatments well. The exercise-induced phosphocreatine depletion average was 22% (SD, 4%) with no substantial change in pH and no difference between control and RNS60 conditions ($p > 0.4$; $N = 15$). Both inhalation and infusion routes of RNS60 administration increased the rate of phosphocreatine recovery post-exercise. Averaging across inhalation and infusion interventions, RNS60 was found to increase phosphocreatine recovery kinetics from 0.0332 (0.0014) s-1 to 0.0396 (0.0028) s-1, demonstrating a 19% increase ($p < 0.02$; $N = 15$) compared to controls. These results indicate that RNS60 increases tissue oxidative capacity in healthy men <65 years of age with mild-moderate sedentary lifestyles.

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## BIO-08 Rescue of ALS relevant biomarkers by VRG50304 in rNLS8 mouse model

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**Background:** TAR DNA binding protein 43 (TARDBP, TDP43) is an RNA/DNA binding protein and is involved in CNS development and function. Mutations in this gene accounts for 5–10% of genetic causes for familial ALS. Moreover, TDP43 mutations result in cytoplasmic mis-localization and aggregation with inclusion bodies containing TDP43 in the brain and spinal cord. This is also present in most ALS patients, not limited to patients with TDP43 mutations. The rNLS8 model is a transgenic mouse model created by the Lee lab to mimic ALS disease with a TDP43 genetic defect. In this model expression of a truncated TDP43 missing nuclear localization signal is controlled by a doxycycline-off system. When doxycycline (dox) is removed from diet, this truncated TDP43 form will express at high levels and accumulate in cytoplasm, forming protein aggregates, and showing behavioral impairments and early death (1,2).

**Objectives:** To evaluate the efficacy of VRG50304 in the rNLS8 ALS mouse model – novel Verge Genomics compound for treating ALS.

**Methods:** rNLS8 transgenic mice were generated by breeding NEFH-tTA mice (Jackson Laboratory stock (JAXs) #025397) to tetO-TARDBP mice (JAXs #014650). The double transgenic animals (TG) were kept on dox until five weeks of age; the dox was removed once the administration of VRG50304 started. TG were dosed once a day, orally for 28 days with vehicle, VRG50304 10 mg/kg or 40 mg/kg; wild type littermates were treated with vehicles on the same schedule. At the end of the study plasma, cerebrospinal fluid (CSF) and caudal cortex were collected. Neurofilament light chain (NF-L) measurements in plasma and CSF were conducted by Quanterix (Billerica, MA); acid sphingomyelinase (ASM) activity in caudal cortex was measured internally at Verge Genomics.

**Results:** Comparing to TG vehicle group, at 10 mg/kg VRG50304 showed significant 15% reduction in NF-L; at 40 mg/kg – significant 15% reduction in plasma NF-L and 24% reduction in CSF NF-L, as well as significant 67% reduction in ASM activity.

**Discussion:** This study evaluated the efficacy of VRG50304 using two disease-associated measures: NF-L – a clinical biomarker in ALS used in diagnosis and progression of neurodegeneration; ASM – critical enzyme in sphingolipids metabolism, accumulating evidence of contribution and upregulation of which was shown in the pathology of multiple neurodegenerative diseases and aging (3). VRG50304 showed rescue at 40 mg/kg in all these measures; at 10 mg/kg – in plasma NF-L. Obtained results make VRG50304 a promising new treatment for ALS.

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# BIO-09 Lipidomic analysis of mutant TDP-43 and C9orf72 mice reveals a lipid biomarker panel for disease progression


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Metabolic dysfunction, particularly affecting lipid metabolism, is a common and early feature of Amyotrophic Lateral sclerosis (ALS). For example, low body mass index and low body fat is associated with ALS prognosis. In contrast, high body fat and being overweight may delay disease progression in ALS. This indicates a highly important role of whole-body lipid metabolism in both sporadic and familial ALS. To date, preclinical investigations of lipid metabolism have been performed on familial ALS mouse models. Results from these studies highlight an important role for dysregulation of glycosphingolipid (GSL) metabolism. Here, we show for the first time distinct lipidomic changes in the spinal cord and skeletal muscle of transgenic TDP43Q331K mice, a model that resembles sporadic ALS. Specifically, we used untargeted and targeted lipidomics, showing significant upregulation of gangliosides, sphingomyelins and ceramides in spinal cord, while significant downregulation of acylcarnitines was detected in muscle, compared to WT mice ($n = 5$ mice per group). When treated with ambroxol, a selective glucocerebrosidase 2 inhibitor which improves GSL metabolism, TDP43Q331K mice revealed improved motor functioning, motor neuron survival and neuromuscular junction integrity. Lipidomic assessment of ambroxol treated TDP43Q331K mouse tissue showed correction of sphingomyelin subspecies in spinal cord and hexaceramide and ganglioside subspecies in muscle. Next, we sought to address the biomarker potential of a panel GSL-related species in the blood of familial mouse models SOD1G93A and C9ORF72-500 as well as the sporadic TDP43Q331K mouse. Notably, blood lipidomic analysis of C9ORF72-500 mice revealed significant accumulation of ganglioside GM3 and acylcarnitine, as well as a reduction of dihydroceramide. Together these results reveal a distinct tissue and circulating lipidomic signature reflected by GSL dysregulation throughout disease progression relevant to both sporadic and familial ALS mouse models. This discovery may lead to the development of an ALS specific lipid biomarker panel with diagnostic, prognostic as well as pharmacodynamic potential.

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# BIO-10 Neutrophil-to-lymphocyte ratio in sporadic amyotrophic lateral sclerosis


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**Background:** The neutrophil-to-lymphocyte ratio (NLR) is considered a robust prognostic biomarker for predicting patient survival outcomes in many diseases. However, it remains unclear whether it can be used as a biomarker for amyotrophic lateral sclerosis (ALS).
**Methods:** To correlate NLR with disease progression and survival in sporadic ALS, 1030 patients with ALS between January 2012 and December 2018 were included in this study.
**Results:** These patients were assigned into three groups according to their NLR values: Group 1 (NLR $<2$, $n = 544$ [52.8%]), Group 2 (NLR $= 2$–3, $n = 314$ [30.5%]), and Group 3 (NLR $>3$, $n = 172$ [16.7%]). All patients were followed up until April 2020. Patients in Group 3 had a significantly older onset age, a lower score on the Revised ALS Functional Rating Scale, and rapidly progressing disease conditions. Furthermore, faster disease progression rates were associated with higher NLR values (odds ratio $=1.211$, 95% confidence interval [CI]: 1.090–1.346, $p < 0.001$) after adjusting for other risk factors. Compared with Groups 1 and 2, the survival time in Group 3 was significantly shorter (log-rank $p = 0.002$). The NLR value was considered an independent parameter for the prediction of survival in ALS patients after normalizing for all other potential parameters (hazard ratio [HR] $= 1.079$, 95% CI: 1.016–1.146, $p = 0.014$). The effects on ALS survival remained significant when adjusted for treatment (HR $=1.074$, 95% CI: 1.012–1.141, Ptrend $= 0.019$) or when considering the stratified NLR value (HR $= 1.115$, 95% CI: 1.009–1.232, Ptrend $= 0.033$).
**Conclusion:** The NLR may help to predict the rate of disease progression and survival in patients with sporadic ALS.


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# BIO-11 Senescent blood lymphocytes and disease progression in amyotrophic lateral sclerosis


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**Background:** We have shown the activation of cell senescence pathways in the proteome of peripheral blood mononuclear cells (PBMCs) (1), the systemic increase of pro-inflammatory cytokines and of autoantibodies against brain antigens in amyotrophic laterals sclerosis (ALS), a fatal neurodegenerative disorder (2,3).
**Objectives:** We further appraise the development of a senescence phenotype, including loss of CD27/CD28 and acquisition of CD57 and of KLRG1 natural killer markers in lymphocytes from ALS patients at baseline (V1) and in 2 consecutive time points (V2 and V3).
**Methods:** We perform immunophenotyping of PBMCs from ALS patients (*n*:60) and healthy controls (HC; 40 individuals) using unsupervised multi-dimensional time-of-flight mass cytometry (CyTOF) and targeted flow cytometry (FC) in two separate subsets of participants. Data are analysed using manual gating and unsupervised clustering analysis by Phenograph. Treatment of healthy PBMCs with ALS and HC sera is undertaken to look at the potential senescence-inducing effect of biological fluids.
**Results:** Unbiased PBMCs profiling by CyTOF and unsupervised clustering shows higher frequencies of senescent (CD4 + CD28-CD57+) T cells in ALS patients ($n = 20$) compared to healthy controls (HC, n:10) ($p < 0.05$). Flow cytometry (FC) of PBMCs from a second ALS cohort (*n*: 40) demonstrates an increase of naïve ($p = 0.0092$), memory ($p = 0.0052$), memory cytotoxic T cells (M-Tc; $p = 0.0006$) frequencies while late memory B cells (LM-B; CD19 + CD27-IgD-) are also elevated predominantly in bulbar-onset and fast progressing ALS ($p = 0.0059$) compared to HC (*n*: 30). M-Tc levels separate bulbar from limb onset ALS (AUC: 0.887; $p < 0.0001$) while higher LM-B and memory B cells predict a shorter survival in ALS (above median =30 and 27 months respectively, below median =109 and 52 respectively). Stable CD4 and rising CD3 lymphocytes levels from V1 to V3 are in contrast with a steep decline of senescent KLRG1-expressing CD4 + T cells. Treatment of healthy PBMCs with ALS and not with HC sera generates an excess of CD4 + CD28-CD57+ expressing senescent CD4+ cells.
**Discussion:** Peripheral lymphocytes in ALS acquire distinctive features of ageing, chronic inflammation and autoimmunity. Further characterization of this unreported aspect of immune dysregulation in ALS may lead to novel biomarkers and therapeutic strategies for this incurable disorder.

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# BIO-12 Decoding distinctive features of plasma extracellular vesicles in amyotrophic lateral sclerosis

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**Background:** Amyotrophic lateral sclerosis (ALS) is a multifactorial, multisystem motor neuron disease for which currently there is no effective treatment. There is an urgent need to identify biomarkers to tackle the disease's complexity and help in early diagnosis, prognosis, and therapy. Extracellular vesicles (EVs) are nanostructures released by any cell type into body fluids. Their biophysical and biochemical characteristics vary with the parent cell's physiological and pathological state and make them an attractive source of multidimensional data for patient classification and stratification (1).

**Objectives:** To define whether plasma EVs can be used for prognosis and/or diagnosis in ALS.
**Methods:** We analyzed plasma-derived EVs of ALS patients ($n = 106$) and controls ($n = 96$), and SOD1G93A and TDP-43Q331K mouse models of ALS. We purified plasma EVs by nickel-based isolation, characterized their EV size distribution and morphology respectively by nanotracking analysis and transmission electron microscopy, and analyzed EV markers and protein cargos by Western blot and proteomics. We used machine learning techniques to predict diagnosis and prognosis.
**Results:** Our procedure resulted in high-yield isolation of intact and polydisperse plasma EVs, with minimal lipoprotein contamination. There were more particles in the plasma of ALS patients and the two mouse models of ALS while their average diameter was smaller. HSP90 was differentially represented in ALS patients and mice compared to the controls. In terms of disease progression, the levels of cyclophilin A, with the EV size distribution, distinguished fast and slow disease progressors, suggesting a new means for patient stratification. We also measured the levels of phosphorylated TDP-43 and showed that is not an intravesicular cargo of plasma-derived EVs.
**Discussion:** Our analysis unmasked features in plasma EVs of ALS patients with potential straightforward clinical application. We conceived an innovative mathematical model based on machine learning which, by integrating EV size distribution data with protein cargoes, gave very high prediction rates for disease diagnosis and prognosis.

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# BIO-13 Urinary N-terminal titin fragment is a candidate lower motor neuron biomarker in ALS

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**Background:** Markers of degradation of skeletal muscle proteins may have biomarker potential in ALS. The transcription levels of certain skeletal muscle proteins were shown to be downregulated in ALS compared to healthy controls (1), one of which was titin. Titin is an essential sarcomere protein aiding in the contraction of muscle and urinary N-terminal titin fragment was recently proposed as a potential diagnostic biomarker in ALS (2).
**Objectives:** To assess urinary N-terminal titin fragment normalised to urinary creatinine (titinCr) in combination with plasma neurofilament light chain (NfL) levels in a cohort of ALS patients compared with control samples.
**Methods:** Biofluid samples from the 'AMBRoSIA' biorepository were studied according to Research Ethics Committee approval. Urine from 49 ALS patients, 9 PLS patients and 39 age-matched healthy controls (HC) was assessed by highly sensitive sandwich ELISA. Plasma NfL was assessed in-house by Meso Scale Discovery R-plex assay (ALS $n = 44$, HC $n = 29$), and creatinine by commercial Jaffe reaction assay. Creatinine kinase (CK) was performed by the Oxford University Hospitals NHS Foundation Trust laboratory. Correlational analyses were undertaken with ALS clinical parameters.
**Results:** Urinary titinCr was elevated in ALS patients compared to HC, (ALS $27.3 \pm 24.5$ nmol/L, HC $3.1 \pm 3$ nmol/L, $p < 0.0001$), also after adjusting for sex and age. Low mean levels were noted in the PLS patients ($14 \pm 9$ nmol/L, NS $p > 0.05$). A trend of higher mean levels of titinCr were seen in bulbar, upper limb and lower limb-onset sub-groups respectively. TitinCr was correlated with disease duration ($r = 0.39$, $p = 0.006$) and the respiratory sub-score of the ALSFRS-R ($r = -0.5$, $p < 0.001$). TitinCr showed a limited and inverse correlation with serum NfL (ALS: $r = -0.3$, $p = 0.04$), with a stronger correlation to CK in both ALS patients and HCs (ALS: $r = 0.56$, $p < 0.0001$, HC: $r = 0.75$, $p < 0.0001$). TitinCr ROC analysis gave an AUC of 0.971 (95% CI 0.944–0.998) when discriminating ALS patients from HC, with a cut-off of 7.9 nmol/L and accuracy of 0.92. In combination with serum pNfL, ROC AUC was improved to 0.995.
**Conclusion:** Urine N-terminal titin fragment is an easily acquired marker which may have diagnostic potential in ALS by reflecting lower motor neuron pathology. There is potential for the earlier discrimination of PLS from upper motor neuron predominant ALS and combining urinary titinCr with serum pNfL may increase the ability to discriminate ALS patients from other disorders. Prospective studies in an ALS clinic diagnostic referral population are underway to evaluate any diagnostic role more definitively. Further analysis is also required to understand longitudinal variation in ALS (data to be shown), which will influence any wider potential as a therapeutic outcome measure.

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## BIO-14 Integrated proteomic analysis of fractionated motor cortex, spinal cord and paired cerebrospinal fluid samples in ALS

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**Background:** Region-specific intracellular aggregation of TDP-43 is the hallmark neuropathological feature of ALS. We sought to use proteomic analysis of insoluble and soluble protein fractions from pathology-affected brain tissue to identify proteins and pathways specifically related to TDP-43 proteinopathy to enhance understanding of underlying disease mechanisms. We also aimed to explore the relationship of motor cortex and spinal cord pathology with findings in the cerebrospinal fluid (CSF) proteome, thereby enhancing understanding of the relevance of biofluid alterations to the underlying disease process in ALS.
**Methods:** Human cortex (CTX) and spinal cord (SPC) tissue from 16 ALS (8 with overlapping cerebrospinal fluid samples), 8 PD, 8 AD and 8 CTL patients were microscopically staged for the presence of pTDP-43, p-tau, alpha-synuclein and beta-amyloid pathology. Samples were biochemically fractionated, trypsin digested and analysed by LC-MS/MS with TimsTOF (tissue) and Orbitrap (CSF).
**Results:** Increased abundance of TDP-43, alpha-synuclein and Tau in the insoluble fraction of ALS, PD and AD tissue respectively confirmed disease-specific protein enrichment by serial fractionation. Principal component analysis of soluble proteins from CTX and SPC discriminated ALS from AD, PD and CTL, driven by enrichment of mitochondrial pathways in CTX and monocyte chemotaxis and dendritic cell migration pathways in SPC. Gene set enrichment analysis indicated enrichment of TDP-43 interacting proteins (1) in the set of proteins distinguishing ALS from other conditions. Weighted correlation network analysis revealed downregulation of cell adhesion and tRNA processing modules (ALS vs AD $p < 0.05$) and upregulation of mitochondrial modules (ALS vs AD $p > 0.01$) in CTX, and upregulation of leukocyte activation, extracellular vesicles (ALS vs PD $p < 0.05$) and ribosomal modules (ALS vs PD $p < 0.001$) in SPC. Late endosomal proteins were downregulated in SPC and CTX (ALS vs PD $p < 0.01$, ALS vs CTL $p < 0.05$). Analysis of correlation between protein abundance in paired ALS CSF (2) and tissue samples suggested a TDP-43 specific pathway (e.g. GSTO1 ($r = 0.087$)). Negative correlation for CSF:tissue was observed for TDP-43 interactors (PGK1 and PEBP1 (both $r = -0.4$)), suggesting TDP-43 specific regulatory alterations.
**Conclusion:** This study reveals TDP-43-interacting tissue-based proteins and pathways as a potential source of TDP-43 pathology-specific biomarkers and outlines the relationship with the CSF proteome.

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## BIO-15 Cardiac Troponin T is elevated and increases longitudinally in ALS patients

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**Objective:** To test whether high-sensitivity cardiac troponin T (hs-cTnT) could act as a diagnostic or prognostic biomarker in ALS, comparing hs-cTnT to neurofilament light (NfL).
**Methods:** We performed a case-control study, including 150 ALS patients, 28 ALS mimics, and 108 healthy controls, and a follow-up study of the ALS patients, during 2014–2020 in Stockholm, Sweden. We compared concentrations of hs-cTnT in plasma and NfL in the cerebrospinal fluid between cases and controls. To evaluate the diagnostic performance, we calculated the area under the curve (AUC). Hazard ratios (HRs) were estimated from Cox models to assess associations between hs-cTnT and NfL at ALS diagnosis and risk of death. The longitudinal analysis measured changes of hs-cTnT and NfL since ALS diagnosis.
**Results:** We noted higher levels of hs-cTnT in ALS patients (median: 16.5 ng/L) than in ALS mimics (11 ng/L) and healthy controls (6 ng/L). Both hs-cTnT and NfL could distinguish ALS patients from ALS mimics, with higher AUC noted for NfL (AUC 0.88; 95%CI 0.79–0.97). Disease progression correlated weakly with hs-cTnT (Pearson's $r = 0.18$, $p = 0.04$) and moderately with NfL (Pearson's $r = 0.41$, $p < 0.001$). Shorter survival was associated with higher levels of NfL at diagnosis (HR 1.08, 95%CI 1.04–1.11), but not hs-cTnT. hs-cTnT increased (12.61 ng/L per year, 95%CI 7.14–18.06) whereas NfL decreased longitudinally since ALS diagnosis.
**Conclusions:** NfL is a stronger diagnostic and prognostic biomarker than hs-cTnT for ALS. However, hs-cTnT might

constitute a disease progression biomarker as it increases longitudinally. The underlying causes for this increase need to be investigated.

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## BIO-16 Investigation of neuroinflammatory responses at single-cell resolution reveals novel insights into the therapeutic potential of GPNMB (glycoprotein non melanoma protein B) in ALS

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**Background:** Despite motor neurons (MNs) are the primary cell type affected in ALS, accumulating evidence suggests that neuroinflammation actively participates in the pathology and has an impact on neurodegeneration. Glial cell responses in ALS are heterogeneous, since they reflect not only the extent of neuronal demise in different CNS districts, but also variable engagement in the attempts to cope with the neuronal damage. Understanding this heterogeneity could allow to identify critical pathways or cell subpopulations amenable to be targeted to improve ALS therapy.
**Methods:** We used the SOD1.G93A (TG) rat model of ALS to investigate microglia heterogeneity in six brain and spinal cord regions characterized by different extent of neuronal demise at two disease stages (onset versus late-symptomatic). Microglia cells were isolated by sorting and submitted to single-cell RNAseq analysis. Relevant gene-expression signatures were identified by UMAP analysis and validated by RNAscope in-situ hybridization combined with immunohistochemistry on spinal cord samples from ALS rats and human post-mortem samples. In parallel, peripheral blood biomarkers were explored by ELISA on samples retrieved from TG rats at different disease stages and cross-validated in blood from ALS patients.
**Results:** Gpnmb was the top-ranked upregulated gene in two microglia clusters specifically enriched in the spinal cord of TG animals. Pathway enrichment analysis revealed that one Gpnmb + cluster (that was present only in late symptomatic animals) displayed a typical anti-inflammatory neurosupportive signature whereas the second Gpnmb + cluster (detected only at the early symptomatic stage) displayed a pro-inflammatory signature. RNAscope and IHC on rat spinal cord highlighted the expression of Gpnmb mainly in MNs in healthy conditions. However, in TG animals both mRNA and protein levels decrease in MNs and are upregulated in reactive glia cells as the disease progresses. Gpnmb is a transmembrane protein that can be cleaved to release a soluble ectodomain. By combining RNAscope with antibodies specific for the membrane-bound versus the extracellular domain of Gpnmb we highlighted a critical role for glial cells in the synthesis and release of Gpnmb ectodomain at the late stages of the disease. In parallel, serum analysis in TG rats as well as in ALS patients spotted a significant increase of Gpnmb ectodomain when the pathology is more severe.
**Discussion:** Gpnmb ectodomain has been previously proposed as potential neuroprotective factor in ALS rodent models. However, it has been recently observed that increased CSF and blood levels of Gpnmb correlate with poor prognosis in patients. Indeed, our in-depth analyses highlighted that Gpnmb might be part of an endogenous, though insufficient and delayed, attempt of reactive glia to counteract the neurodegeneration. Currently, we are exploring novel strategies aimed at enhancing the therapeutic potential of Gpnmb in the early stages of the disease.

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## BIO-17 Peripheral inflammatory markers in amyotrophic lateral sclerosis: a pilot analysis

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**Introduction:** Over the last decades, increasing evidence has emerged regarding the role of neuroinflammation in Amyotrophic Lateral Sclerosis (ALS). Several leucocyte alterations have been reported in ALS patients, but the precise contribution in ALS pathogenesis is still unclear. Among the peripheral inflammatory markers, the cellular microvesicles (cMVs) have received growing interest, due to their essential role in brain homeostasis and crosstalk between neural cells and the periphery.
**Aim:** The study aims to identify potential diagnostic and prognostic ALS markers investigating the balance between neuroprotection and neurotoxicity through the changes in peripheral immunity, including the lymphocyte profile and the cMVs (endothelial, leukocyte, and platelet MV).
**Methods:** We enrolled 42 ALS patients and 25 healthy controls who underwent a single collection of plasma samples for flow cytometry. All patients' samples were collected

within 24 months from symptoms onset. The results of the analyzes were integrated with the clinical patient's data (phenotype, age at onset of symptoms, sex, comorbidity, functional and cognitive status, and any known genetic mutation).
**Results:** Immunophenotypic analysis. Significant differences emerged when comparing patients and controls and different groups of patients classified according to specific clinical features. Lower expression of T helper lymphocytes (CD4+ and CD4 memory) was evident in the patients' group compared with controls ($p$-value = 0.02). ALS patients with concomitant cognitive impairment showed much lower CD4+ values than patients with normal cognition ($p$-value =0.04). Higher levels of naïve B cells in fast progressors than in slow progressors ($p$-value =0.04). We observed a higher level of CTL + cells and a lower level of Th17 in bulbar patients than spinal ones. ALS patients with early-onset of symptoms (age <45 years) had a reduction in CD4+ and CD8+ lymphocytes compared with late-onset ALS. Lower levels of plasma cells were found in C9Orf72 patients compared with sporadic ones. By correlating lymphocyte profile characteristics and clinical data, a positive correlation between NK levels and ALSFRS-R at baseline was observed in the ALS patients' group ($p$-value =0.001). Microvesicles. No statistically significant differences emerged between the patients and the control group.
**Discussion:** The immunophenotypic analysis showed several changes of peripheral inflammatory markers in ALS patients. Specifically, we found a dysregulation between anti-inflammatory agents (e.g. CD4+), globally lower in patients, and proinflammatory agents (e.g. CD8+ and Th17), higher in more aggressive forms. These findings are in line with previous literature data. Conversely, no statistical differences between patients and controls emerged regarding the cMVs profile, likely due to the small number of participants of this study.

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## BIO-18 Chitinase dysregulation in ALS: translational insights on expression dynamics from a clinical cohort and murine models

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**Background:** The chitinases are considered biomarkers of glial activation in neurodegenerative conditions, including amyotrophic lateral sclerosis (ALS). However the implications of chitinase upregulation for disease severity and progression and their cellular origins are not well understood. Although studies have reported CHIT1 and CHI3L1 expression in microglia and astrocytes, respectively, the majority of these results are based on post-mortem patient material, thus limiting information on expression dynamics through the disease course.
**Objectives:** We analyzed the diagnostic and prognostic utility of key chitinases (CHIT1, CHI3L1 and CHI3L2) using the D50 disease progression model. Established murine models of ALS were then employed to address: (1) Which cell types are in vivo sources of key chitinases during disease progression (2) How chitinase expression relates to the disease course and (3) Whether expression is influenced by the underlying pathology.
**Methods:** Using immunoassays, we characterized CHIT1, CHI3L1 and CHI3L2 levels in matched CSF and plasma samples from ALS patients ($n = 39$), neurodegenerative disease controls (NDEGs, $n = 13$), and non-neurodegenerative disease controls (NDCs, $n = 11$). CSF neurofilament (NfL and pNfH) levels were also assayed. SOD1-G93A, C9orf72 GA-CFP, and rNLS8-hTDP43 mice were selected for further study at pre-symptomatic, disease-onset, and late-disease stagesthe symptomatic stage. Immunostaining on spinal cord and brain sections was performed to identify CHIT1 and CHI3L1 expressing cell types (astrocytes – GFAP, microglia – Iba1, oligodendrocytes – Olig2, neurons – NeuN).
**Results:** We confirmed substantial elevations of all three chitinases in the CSF of the ALS cohort relative to NDCs. CHIT1 and CHI3L2, but not CHI3L1, were significantly elevated in the ALS cohort relative to NDEGs. No differences were noted in plasma. Elevation of CSF chitinases levels appeared to be a feature of highly aggressive disease in ALS and correlated robustly with neurofilaments, a neuron-specific cytoskeletal component and an established prognostic biomarker. Surprisingly, we observed in wild-type mice that neurons (NeuN+), including lower motor neurons (ChAT+), express both CHIT1 and CHI3L1. Preliminary results in the SOD1-G93A model further suggest that advancing pathology skews chitinase expression towards the glia. Initial results from the C9orf72 mouse model indicate CHIT1 and CHI3L1 expression in neurons that contain pathogenic poly-GA aggregates.
**Discussion and outlook:** The work presented here is an important translational bridge between observations in clinical cohorts and the corresponding animal models. We confirm that in human patients, chitinase upregulation is a feature of the CNS rather than the periphery. Importantly, our preliminary results suggest that chitinase-mediated gliosis may actually originate in neurons i.e. the most vulnerable cell population in ALS. The shift to glial expression reiterates the duality of the neuroinflammatory response across neurodegenerative diseases.

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## BIO-19 Microsampling tools comparison in amyotrophic lateral sclerosis

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**Background/Introduction:** Care of neurological disorders (NDs) is an increasing burden to health services worldwide (1). The development of tools for early diagnosis of NDs allowing timely therapeutic interventions is one of the responses to this problem (2). Neurofilaments light chain (Nf-L), the product of axonal injury and degeneration, has emerged as a robust fluid biomarker for the fatal neurodegenerative disorder amyotrophic lateral sclerosis (ALS) (3). Remote micro-sampling tools could facilitate large-scale neurofilaments-based studies into diagnostics and clinical stratification of ALS, reducing samples collection costs, processing and cold-chain storage (4). We have recently tested Dry Blood Spots (DBS) and Dry Plasma Spots (DPS) for the measurement of blood neurofilaments. Here we would like to evaluate the potential use of recently developed blood cards that minimize the confounding effect of haematocrit (Hct) and haemoglobin (Hb) (5), testing the feasibility of the so-called quantitative DBS (qDBS) microsampling.
**Methods:** We have measured Nf-L in blood fractions obtained from different commercially available micro-sampling tools, including DBS, qDBS, Noviplex DPS and Combas DPS compared to the same measurement in plasma obtained from standard blood processing. We have analysed neurofilaments concentrations in blood from ALS patients compared to healthy controls, as this represents an important application in clinical practice. The highly sensitive immunoassay (SIMOA) is used for the sensitive determination of Nf-L in all products of elution from the microsampling cards.
**Results:** Our preliminary data demonstrate that the use of an ultra-sensitive immunoassay technology alongside the DPS and the new microfluidic Hct and Hb-controlled devices (qDBS) provide improved reading of neurofilaments blood concentrations reducing considerably the effect of haematocrit and the interference of blood by-products produced by erythrocytes lysis, improving sensitivity and specificity compared to standard DBS.
**Discussion:** Our preliminary data from an ongoing investigation provide evidence that an improved micro-sampling tool can be used for remote collection and for blood neurofilaments determinations in biomarkers studies for the clinical stratification of ALS, an important pre-requisite in the development of clinical trials.

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# BIO-20 Red cell distribution width (RDW) in ALS

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**Background:** The rate of amyotrophic lateral sclerosis (ALS) progression is very variable between subjects, the identification of an easily accessible biomarker predictive of disease progression and survival is very important. This would permit an appropriate prognostic stratification in clinical trials. The red cell distribution width (RDW) is a measure of the range of variation of red blood cell volume, and it has been reported to be positively correlated inflammation and to be associated with a shorter survival in patients with lung disorders.
**Objectives:** We aimed to explore RDW as a potential biomarker in ALS.
**Methods:** We included data from 65 ALS patients followed in our centre. Patients were clinically evaluated at the time of blood sampling and evaluated longitudinally by applying the revised ALS Functional Rating Scale (ALSFRS-R). RDW was quantified from a standard complete blood count. In the same period and using the same methodology we studied 80 healthy controls. We compared results between ALS patients and controls (Mann-Whitney U test). In the ALS group, and RDW values were correlated with ALSFRS-R, respiratory subscore of ALSFRS-R, and forced vital capacity (FVC).
**Results:** The sample of ALS patients consisted of 32 men (49.25%) and 33 women (50.75%), with a mean age of $62.4 \pm 9.05$ years (mean ± SD). The control group consisted of 45 men (56.1%) and 35 women (43.9%), with a mean age of $47.3 \pm 11.6$ years (mean ± SD). Gender distribution was similar across groups ($p > 0.30$), but controls were significantly younger ($p < 0.001$). The RDW value was significantly higher in ALS patients ($12.23 \pm 1.34$%, mean ± SD) vs controls ($11.32 \pm 1.30$ %; mean ± SD; $p < 0.001$). In the ALS group, RDW was not influenced age and gender, it was not correlated to ALSFRS-R and its respiratory subscore, but was significantly correlated with FVC ($p = 0.01$).
**Discussion:** Our preliminary findings suggest that RDW is a marker of respiratory function. Both can be associated with a higher inflammatory state. Its value on survival and functional decay is being analysed. This simple measurement deserve a more extensive investigation in ALS.

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## BIO-21 HERV-K (HML-2) levels in serum correlate with disease duration in patients with ALS

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**Background:** Human Endogenous Retrovirus K (HERV-K/HML-2) expression in neurons of patients with ALS may contribute to neurodegeneration and disease pathogenesis. Currently, it is not well-understood what factors correlate with elevated HERV-K levels in these patients.
**Methods:** We analyzed HERV-K levels in patients with ALS from two demographically similar cohorts from the National Institutes of Health and the Healey Center for ALS. Information was collected for patients that had off-site records, which included demographics and ALS disease characteristics. HERV-K was measured in serum as a ratio to RPP30, a housekeeping gene, using digital droplet PCR. HERV-K ratios were used in analysis as a binary outcome (greater than or less than 13, a cut-off established based on prior analyses that included healthy volunteer samples) and as a continuous measurement. Parametric and non-parametric statistical tests were chosen based on variable distribution.
**Results:** Patients in these two cohorts were 34% female, 95% White, and 3% Hispanic, with a median disease duration of 24 months (IQR 14, 63 [$n = 130$]) and a mean ALSFRS-R of 34.60 (SD 8.16 [$n = 110$]). The overall median HERV-K ratio in serum among ALS patients ($n = 147$) was 13.60 (IQR 10.30, 16.00). Using the cut-off of 13, 61 participants (41%) fell into the low HERV-K group, with a median of 9.70 (IQR 8.05, 11.48), and 86 participants (59%) fell into the high HERV-K group, with a median of 15.43 (IQR =14.00, 17.64). HERV-K was not associated with patient demographics (i.e. age, sex, race), disease duration, or ALSFRS-R. There was a modest positive correlation between disease duration and HERV-K ratio ($R = 0.216$, $p = 0.019$) in patients with symptoms for less than five years ($n = 119$).
**Conclusions:** The HERV-K ratio is elevated in over half of patients with ALS in these two cohorts, similar to previous reports. In patients less than five years from disease onset, HERV-K correlates with disease duration. In ALS intervention studies that target HERV-K, this association with disease duration should be considered in study design and interpretation of results. Our analyses are limited by retrospective data collection, however the large sample size and inclusion of two different cohorts allows for generalizability of these results to many patients with ALS.

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## BIO-22 Age-dependent increase of cytoskeletal components in peripheral sensory nerve fibers in human skin

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**Background:** Neurons are polarized cells, which receive input on dendrites, while signal transmission occurs at synapses located along axons. Synaptic structures require large amounts of energy and hence, highly depend on energetic supply in form of mitochondria generated at the soma. To this end, the neuronal cytoskeleton provides not only physical support but also forms the basis for axonal transport and electrical pulse velocity (1). Aging is a complex process, with various changes concerning gene expression affecting fundamental processes like inflammation and senescence (2). We strived for studying cytoskeletal alterations in healthy axons during aging. Cytoskeletal misbalances could contribute to axonal pathology in MNDs for several reasons: neurological disorders predominantly manifest with higher age and axonal transport deficits are associated with ALS pathology (3).
**Objectives:** We used sensory axons as a model, being easily accessible by skin biopsies. Alike motor neurons, their cell bodies are located in ganglia close to the spinal cord with one axon branch bridging all the way towards distant parts of the body. Thus, axons innervating the proximal (thigh) and distal (ankle) leg differ in absolute length, which enables us to quantify protein levels and diameter by immunostainings in a length-dependent manner.
**Methods:** Skin biopsies of 27 healthy male individuals between 24 and 79 years were immunostained for non-phosphorylated neurofilament heavy chain (npNfH), betaIII-tubulin, and actin. Confocal image stacks were analyzed for mean gray values and diameter using ImageJ software. Statistical analyses included intergroup comparisons using one-way analysis of variance, ANOVA, followed by Tukey's multiple-comparisons test. This study was approved by the local ethics committee. Participants gave informed written consent.
**Results:** The expression levels of the cytoskeletal components tested in proximal biopsies drastically increased until age 65, but then plateaued or even declined. NpNfH increased 2.7-fold and betaIII-tubulin 3.2-fold with a maximum at the 75-year-old individuals. Similarly, actin expression peaked at the 62-year-old controls with a 3.8-fold change. In contrast, sensory axon caliber increased 2.2-fold in a linear fashion, reaching a maximum at age 78/79. We obtained similar data sets for the distal skin biopsies.
**Discussion:** We discovered age-driven alterations in the cytoskeleton of peripheral sensory nerve endings regarding expression and caliber. While the diameter steadily increased, cytoskeletal protein content stagnated with higher age. This might result in a destabilizing imbalance serving as potential risk factor regarding MNDs. Longer axons are not different in

immunostaining intensity at their distal branches than shorter axons, which could indicate that the "dying-back phenomenon" is restricted to MND pathology.

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# BIO-23 Aberrant enteric neuromuscular system and microbiome in amyotrophic lateral sclerosis

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**Background:** Amyotrophic Lateral Sclerosis is a neuromuscular disease characterized by the progressive death of motor neurons and muscle atrophy. Early diagnosis of ALS has been a long-standing challenge in the field. There are case reports that celiac disease with neurologic manifestations was misdiagnosed as ALS (1). The gastrointestinal symptoms in patients were largely ignored or underestimated, especially before the diagnosis of ALS. Emerging evidence has demonstrated that microbiota directly affects the enteric neuron system (ENS) and smooth muscle cell functions via metabolic products or endogenous bacterial components. The relationship between ENS and microbiome in ALS progression is unknown. We are the first to report the elevated intestinal inflammation, reduced beneficial bacteria, and shift of microbiome profile in ALS (2–4). Later, there are studies from several groups reporting the dysbiosis in human ALS and experimental animal models.
**Methods:** Here, we performed longitudinal studies on the ENS and microbiome in the ALS human-SOD1G93A transgenic mice. We treated age-matched wild-type and ALS mice with bacterial product butyrate or antibiotics to investigate microbiome and neuromuscular functions. Intestinal motility, microbiome, an ENS marker GFAP, a smooth muscle marker (SMMHC), and human colonoids have been examined. The distribution of human-G93A-SOD1 was tested as an indicator of ALS progression.
**Results:** At 2-month-old before ALS onset, G93A mice had significant lower intestinal motility, decreased grip strength, and reduced time in the rotarod ($n = 6$ per group). We observed increased GFAP and decreased SMMHC expression. These changes correlated with consistent increased aggregation of mutated SOD1G93A in the colon, small intestine, and spinal cord. Butyrate and antibiotic treatment showed a significantly longer latency to fall in the rotarod test, reduced SOD1G93A aggregation, and enhanced ENS and muscle function. Feces from 2-month-old SOD1G93A mice significantly enhanced SOD1G93A aggregation in human colonoids transfected with a SOD1G93A-GFP plasmid. Longitudinal studies of microbiome data further showed the altered bacterial community related with autoimmunity (e.g. Clostridium sp. ASF502, Lachnospiraceae bacterium A4), inflammation (e.g. Enterohabdus Muris,), and metabolism (e.g. Desulfovibrio fairfieldensis) at 1- and 2- month-old SOD1G93A mice, suggesting the early microbial contribution to the pathological changes.
**Conclusions:** We have demonstrated a novel link between microbiome, hSOD1G93A aggregation, and intestinal mobility. Dysbiosis occurred at the early stage of the ALS mice before observed mutated-SOD1 aggregation, slow intestinal motility, and ENS dysfunction. Manipulating the microbiome improves the muscle performance of SOD1G93A mice. We provide insights into fundamentals of intestinal neuromuscular structure/function and microbiome in ALS, suggesting the potential to use microbial biomarkers for the diagnosis and to manipulate the microbiome for the treatment.

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# BIO-24 Longitudinal analysis and validation of CSF biomarkers that distinguish fast versus slow disease progression

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**Background:** ALS is a heterogeneous disease with a complex etiology, which has contributed to the failure of many late stage clinical trials. Biomarkers can assist ALS clinical trial efforts to stratify the patient population and identify those that may best respond to a particular drug treatment. Most studies define the rate of ALS disease progression based on the change in the ALS functional rating scale revised (ALSFRS-r) to segregate patients into fast progressing (FP) and slow progressing (SP) disease. Many prior research studies have explored candidate biomarkers that can distinguish between FP and SP using a cross-sectional study design, and therefore the temporal response of many potential biomarkers has not been explored or validated in a separate set of samples.

**Objectives:** Utilize mass spectrometry to identify candidate biomarkers in the cerebrospinal fluid (CSF) that distinguish FP from SP and assess how these biomarkers change over time.
**Methods:** We employed shotgun proteomics on longitudinal CSF samples collected from 6 FP and 5 SP ALS patients to identify biomarker candidates that distinguish FP from SP. We further validate top candidates using longitudinal CSF from a separate cohort of 5 FP and 6 SP ALS patients using immunoassays specific for each candidate.
**Results:** Overall, we identified 1148 proteins in the CSF of all ALS patients in this study. Pathway analysis determined enrichment of pathways related to complement and coagulation cascades in FPs and synaptogenesis and glucose metabolism in SPs. Longitudinal analysis revealed a panel of 59 candidate markers that could segregate FP and SP ALS. Based on multi-variate analysis, we identified three biomarkers, F12, RBP4, and SERPINA4, as top candidates that segregate ALS patients based on rate of disease progression. These proteins were validated by immunoassays in both the discovery cohort and a separate validation cohort.
**Conclusions:** Overall, we identified pathways and candidate biomarkers that separate the patient population based on the rate of disease progression. These biomarkers also demonstrate distinct pathways that are modulated in FP versus SP patients and represent new potential therapeutic targets.

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## BIO-25 Urinary neopterin: a novel biomarker of disease progression in motor neuron disease/ amyotrophic lateral sclerosis

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**Background:** Biomarkers that are fit for purpose hold great potential to accelerate therapy development for people with motor neuron disease/ amyotrophic lateral sclerosis (MND/ALS) in clinical trials. Building upon our discovery that urinary p75ECD is a prognostic and disease progression biomarker and has potential as a pharmacodynamic marker (1–3), here we describe the potential of urinary neopterin as an MND/ALS biomarker of pro-inflammatory response.

**Methods:** Urine levels of neopterin were evaluated in 21 healthy individuals and 42 people with MND/ALS, 19 of whom donated samples longitudinally. Baseline and longitudinal changes in clinical measures, neopterin and p75ECD were examined using both mixed model and survival analyses.
**Results:** Baseline urinary neopterin was higher in MND/ALS compared to healthy individuals ($178 \pm 80$ μmol/mol creatinine vs. $122.3 \pm 59.4$ μmol/mol creatinine, $p = 0.003$, Welch's t-test) and correlated with baseline ALSFRS-R ($r = -0.42$, $p = 0.006$). Combining previously published urinary p75ECD results with a further 21 people with MND/ALS, baseline urinary p75ECD was also higher compared to healthy individuals ($6.3 \pm 2.6$ versus $3.2 \pm 1.2$ ng/mg creatinine, $p < 0.0001$) and correlated with the ALSFRS-R ($r = -0.36$, $p = 0.027$). Urinary p75ECD and neopterin correlated with each other at baseline ($r = 0.49$, $p = 0.0008$). In longitudinal analysis from diagnosis, average (±SE) urinary neopterin increased by $6.5 \pm 1.1$ μmol/mol creatinine/month ($p < 0.0001$) and p75ECD by $0.22 \pm 0.03$ ng/mg creatinine/month ($p < 0.0001$). Baseline urinary neopterin did not predict the future course of disease.
**Conclusions:** Urinary neopterin holds promise as biomarker of disease progression in MND/ALS, but not as a prognostic marker. Its potential utility as a predictive marker of response to anti-inflammatory therapies warrants future evaluation.

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## BIO-26 Diagnostic value of plasma neurofilament light: a multicentre validation study

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Increased cerebrospinal fluid neurofilament light (NfL) is a recognized biomarker for neurodegeneration that can also be assessed in blood. Here, we investigate plasma NfL as a marker of neurodegeneration in 13 neurodegenerative disorders, Down syndrome, depression and cognitively unimpaired controls from two multicenter cohorts: King's College London ($n = 805$) and the Swedish BioFINDER study ($n = 1464$). Plasma NfL was significantly increased in all cortical neurodegenerative disorders, amyotrophic lateral sclerosis and atypical parkinsonian disorders. We demonstrate that plasma NfL is clinically useful in identifying atypical parkinsonian disorders in patients with parkinsonism, dementia in individuals with Down syndrome, dementia among

psychiatric disorders, and frontotemporal dementia in patients with cognitive impairment. Data-driven cut-offs highlighted the fundamental importance of age-related clinical cut-offs for disorders with a younger age of onset. Finally, plasma NfL performs best when applied to indicate no underlying neurodegeneration, with low false positives, in all age-related cut-offs.

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## BIO-27 Mixed modeling blood biomarkers-based to predict progression rate in motor neuron disease

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**Background:** Motor neuron diseases (MNDs) are lethal and heterogeneous neurodegenerative disorders characterized by different symptoms and progression rate (PR) that make the disease prognosis extremely complicated. Previous studies have proposed to use blood data to support the clinical differential diagnosis and prognosis of MNDs (1). However, a clear and rigorous relationship between the disease PR and blood-derived biomarkers is still far to be clearly understood.
**Objectives:** In this study, we aim to model the progression rate of MNDs as a function of blood analyte biomarkers, exclusively.
**Methods:** For this purpose, we analyzed our recently introduced dataset including 692 observations from 55 patients that combine 108 blood analytes and clinical information collected approximately every 3 months over the past 10 years. We applied linear mixed models to each analyte aiming to model the PR of the disease. To consider the interdependence of our observations related to repeated measures and the subject variability, we applied the random intercept and random slope mixed model using each blood analyte as a single predictor of the model. To test the null hypothesis that each blood analyte has no correlation with the progression rate of the disease, we applied the likelihood ratio test.
**Results:** Among all tested analytes, 10 of them ($\alpha_1$ and $\alpha_2$ globulins, erythrocyte sedimentation rate, monocytes, eosinophils (absolute number and percentage), immunoglobulin M, CD45RA, E-selectin, and β-interferon) contributed significantly to the regression model ($p < 0.05$). Although the variance explained by each of the 10 complete models (i.e. including the random effect) was very high (>90%), the marginal variance explained by 9 out of 10 significant analytes was less than 1%. Contrarily, the β-interferon accounted for 17% of the variance explained by the analyte solely.
**Discussion:** Our findings support the hypothesis of the presence of an immunological component in the pathogenesis and in the pathophysiology of MNDs, and in general reconfirm the importance of blood analytes as prognostic disease biomarkers.

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## BIO-28 Comparison of markers of inflammation and neurodegeneration in cerebrospinal fluid as predictors of survival in patients with amyotrophic lateral sclerosis

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**Objective:** To compare markers of neurodegeneration [neurofilament light chain (NfL) and phosphorylated neurofilament heavy chain (pNfH)] and markers of inflammation [chitotriosidase-1 (CHIT1), chitinase-3-like protein 1 (YKL-40) and monocyte chemoattractant protein-1 (MCP-1)] as biomarkers of survival in cerebrospinal fluid (CSF) of patients with amyotrophic lateral sclerosis (ALS).
**Methods:** Protein biomarkers were determined in CSF of 94 patients with ALS using enzyme-linked immunoassays. Univariate survival analyses were performed using the Kaplan-Meier analysis, and the log-rank test was conducted to determine differences between the survival curves ($n = 94$, censored: 14%). A Cox regression survival model was calculated including 8 established clinical predictors of survival in ALS ($n = 84$, censored: 13%). For this purpose patients were stratified as low and high biomarker level based upon the median concentration of the biomarker in the total ALS cohort.
**Results:** Univariate survival analyses revealed a significantly shorter survival in patients with ALS having high levels of pNfH ($\chi^2 = 12.69$, $p < 0.001$), NfL ($\chi^2 = 12.34$, $p < 0.001$), CHIT1 ($\chi^2 = 7.62$, $p < 0.01$), YKL-40 ($\chi^2 = 14.05$, $p < 0.001$) and MCP-1 ($\chi^2 = 8.45$, $p < 0.01$). In a multivariate Cox regression model, high levels of pNfH (HR: 3.36, 95% CI: 1.87 – 6.04, $p < 0.001$), NfL (HR: 2.06, 95% CI: 1.23–3.46, $p < 0.01$), CHIT1 (HR: 2.13, 95% CI: 1.22–3.73, $p < p < 0.01$), YKL-40 (HR: 2.17, 95% CI: 1.21–3.90, $p = 0.01$) and MCP-1 (HR: 2.49, 95% CI: 1.47–4.21, $p = 0.001$) were independently associated with a shorter survival. Patients with both high NfL and YKL-40 levels harbored a significantly shorter survival (median: 15.3 months, range: 1.43–49.0 months) compared to those patients with both low NfL and YKL-40 levels (median: 45.5 months, range: 2.03–74.5 months; $p < 0.0001$), but did not when compared to those patients with low NfL yet high YKL-40 levels (median: 20.2 months, range: 0.47–77.3 months; $p = 0.74$).
**Conclusion:** This study highlights the importance of CSF biomarkers to predict survival in patients with ALS. Furthermore, it demonstrates that patients with ALS without pronounced

neurodegeneration have a short survival in the presence of neuroinflammation, e.g. reflected by low NfL and high YKL-40 levels. These findings may have implications for future stratification of patients in clinical trials.

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## BIO-29 Role of the neurovascular unit and of the redox state in amyotrophic lateral sclerosis pathogenesis: modulatory effects elicited by acetyl-L-carnitine

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**Background:** Despite many previous and ongoing research studies, Amyotrophic Lateral Sclerosis (ALS) is still uncurable and its pathogenesis poorly understood. Oxidative stress and alteration of mitochondrial function and of the neurovascular unit (NVU) have been proposed as playing a crucial role in the physiopathology of ALS (1). Acetyl-L-carnitine (ALCAR), a substance with neurotrophic and protective effects, has been previously suggested as a potential treatment (2).
**Objectives:** To analyze the redox state in plasma of ALS patients at the time of diagnosis and during ALCAR treatment and to evaluate the effects of plasma on oxidative stress/mitochondrial function in human umbilical cord-derived endothelial vascular cells (HUVEC) and astrocytes.
**Methods:** The study was conducted on 25 patients with definite or probable ALS according to the El Escorial Criteria (Revised) recruited by the Tertiary ALS Centre in Novara (Italy) between September 2020–May 2021. They were compared with a control group of healthy subjects matched by sex and age. All subjects provided informed consent according with Declaration of Helsinki. Blood samples were taken from patients and controls to evaluate thiobarbituric-acid-reactive-substances (TBARS), glutathione (GSH) and nitric oxide (NO). In vitro, the effects of plasma on mitochondrial reactive oxygen species (mitoROS) release, NO release, cell viability and mitochondrial membrane potential were examined in HUVEC/astrocytes. The study provided for a clinical evaluation for each patient combined with blood samples used to analyze the aforementioned parameters at the time of diagnosis (T0). After the start of ALCAR (3 gr/day), the same investigations were repeated after three months (T1) and six months (T2).
**Results:** At T0 we found an increase in TBARS and a reduction of GSH and of NO in plasma of ALS patients. In vitro, an increase of mitoROS and a decrease of cell viability and mitochondrial membrane potential were found in both HUVEC and astrocytes treated with plasma of ALS patients. In HUVEC, NO release was reduced, as well. At T1, plasma TBARS levels were reduced whereas those of GSH were increased. In addition, an improvement of cell viability and mitochondrial membrane potential accompanied by a reduction of mitoROS in HUVEC and astrocytes was observed. The results have been confirmed at T2.
No statistically significant correlations were found with the genotype and phenotype characteristics of the patients.
**Discussion:** The results obtained confirm the central role exerted by oxidative stress in ALS and suggest that unknown plasma factors may cause mitochondrial dysfunction, which is, in turn, capable of inducing an increased oxidants release and reduction of cell viability of NVU members. Al those events can be modulated by ALCAR.

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## BIO-30 Circulating miR-181 is a prognostic biomarker for amyotrophic lateral sclerosis

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Amyotrophic lateral sclerosis (ALS) is a relentless neurodegenerative syndrome of the human motor neuron system, for which no effective treatment exists. Variability in the rate of disease progression limits the efficacy of ALS clinical trials, suggesting that developing of better biomarkers for prognosis will facilitate therapeutic progress. Here, we applied unbiased next-generation sequencing to investigate the potential of plasma cell-free microRNAs as biomarkers of ALS prognosis, in 252 patients with detailed clinical-phenotyping. First, we identified miRNAs, whose plasma levels remain stable over the course of disease in a longitudinal cohort of 22 patients. Next, we demonstrated that high levels of miR-181, a miRNA enriched in neurons of the brain and spinal cord, predicts a $>2$ fold risk of death in discovery cohort (126 patients) and an independent replication cohort (additional 122 patients). miR-181 performance is comparable with the established neurofilament light chain (NfL) biomarker and when combined together, miR-181 + NfL establish a novel RNA-protein biomarker pair with superior prediction capacity of ALS prognosis. Therefore, plasma miR-181 predicts ALS disease course, and a novel miRNA-protein biomarker approach, based on miR-181 + NfL, boosts precision of patient stratification and may greatly enhance the power of clinical trials.

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# BIO-31 Affinity of antibodies to neurofilaments and dipeptide repeats in ALS

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**Background:** Neuronal degeneration in amyotrophic lateral sclerosis (ALS) is associated with increased concentration of neurofilament (Nf) proteins in body fluids, thus the release of Nf proteins may well trigger or boost specific antibody immune reactivity. We have observed increasing concentrations of plasma Nf antibodies and immnune complexes in a longitudinal study of fast progressing ALS and in ALS patients with a C9orf72 mutation (1). To qualitatively study the NfL antibody function, we aim to determine the affinity degree of antibodies. Affinity, as a measure of functional maturation of the humoral immune response, has been associated with effector functions and defective homeostatic pathways. In this study we will determine whether affinity of antibodies to Nf and dipeptide repeats may be contributors to disease progression.
**Objective:** To determine the affinity of blood antibodies to neurofilament and dipeptide-repeat proteins present in ALS patients compared to healthy controls.
**Methods:** Affinity of antibodies against neurofilament-light and heavy chains (anti-Nf) as well as poly-(GP)-(GR) dipeptide-repeats will be measured longitudinally in blood samples from ALS patients and healthy controls. Participants are stratified according to rate of disease progression estimated before and after baseline and C9orf72 genetic status. Affinity of anti-Nf and anti-poly-(GP)-(GR) dipeptide-repeats are being measured by competitive ELISA as previously reported (2). 96-well microtiter plates (standard MSD bind plate #L15XA-1) are coated with 0.1 μg/mL antigen, blocked and incubated with 25 μL plasma. Before transfer, plasma pools and/or individual plasma are pre-incubated for 1 h with the corresponding antigen at a range of concentrations: (10, 5, 1, 0.5, 0.1, 0.01, 0.01, 0.003 and 0.001 μM). MSD Sulfo Tag Goat anti-human IgG is used as a secondary antibody and the plates are read in MSD Buffer using an MSD Sector Imager 650 APC instrument.
**Results:** Preliminary results show specificity of binding of antibodies to neurofilaments. Inhibition curves performed in pooled plasma samples from 25 patients with ALS and 25 healthy controls showed different affinity profiles of Nf-L antibodies. Optimal concentrations will be tested in individual samples.

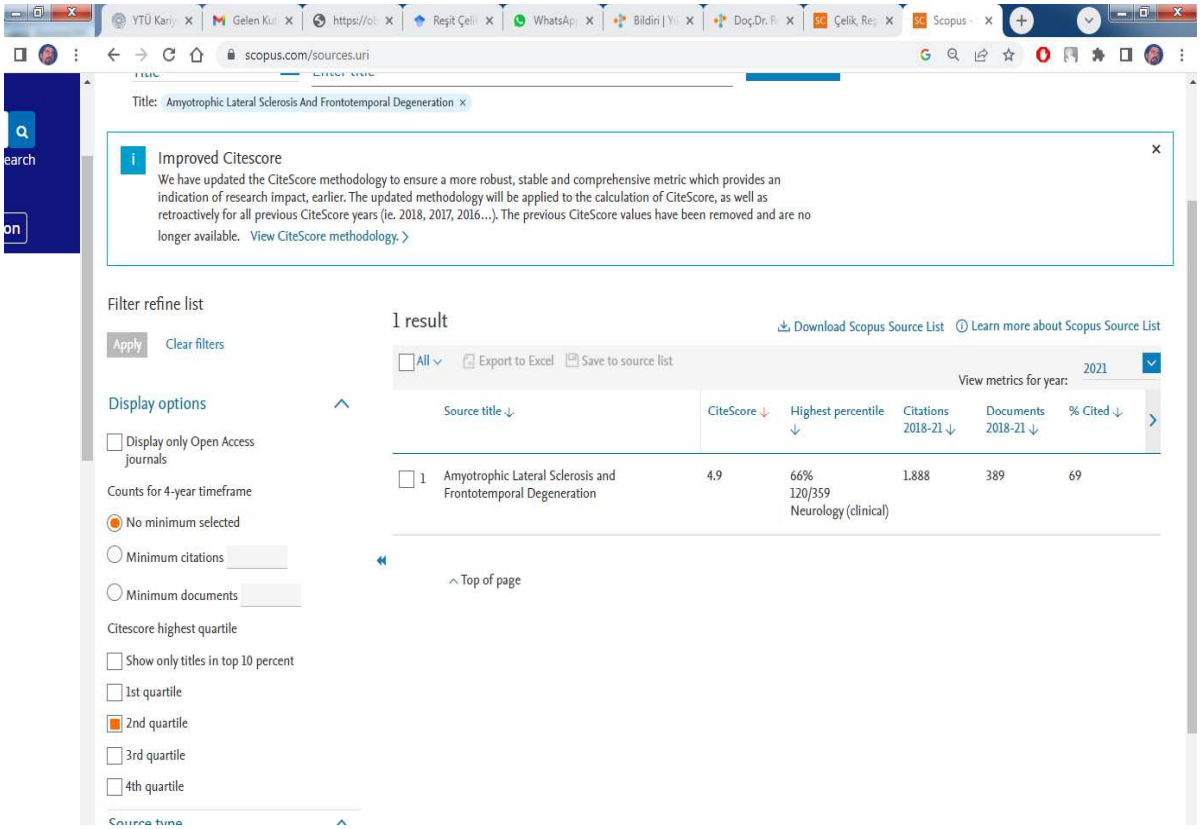
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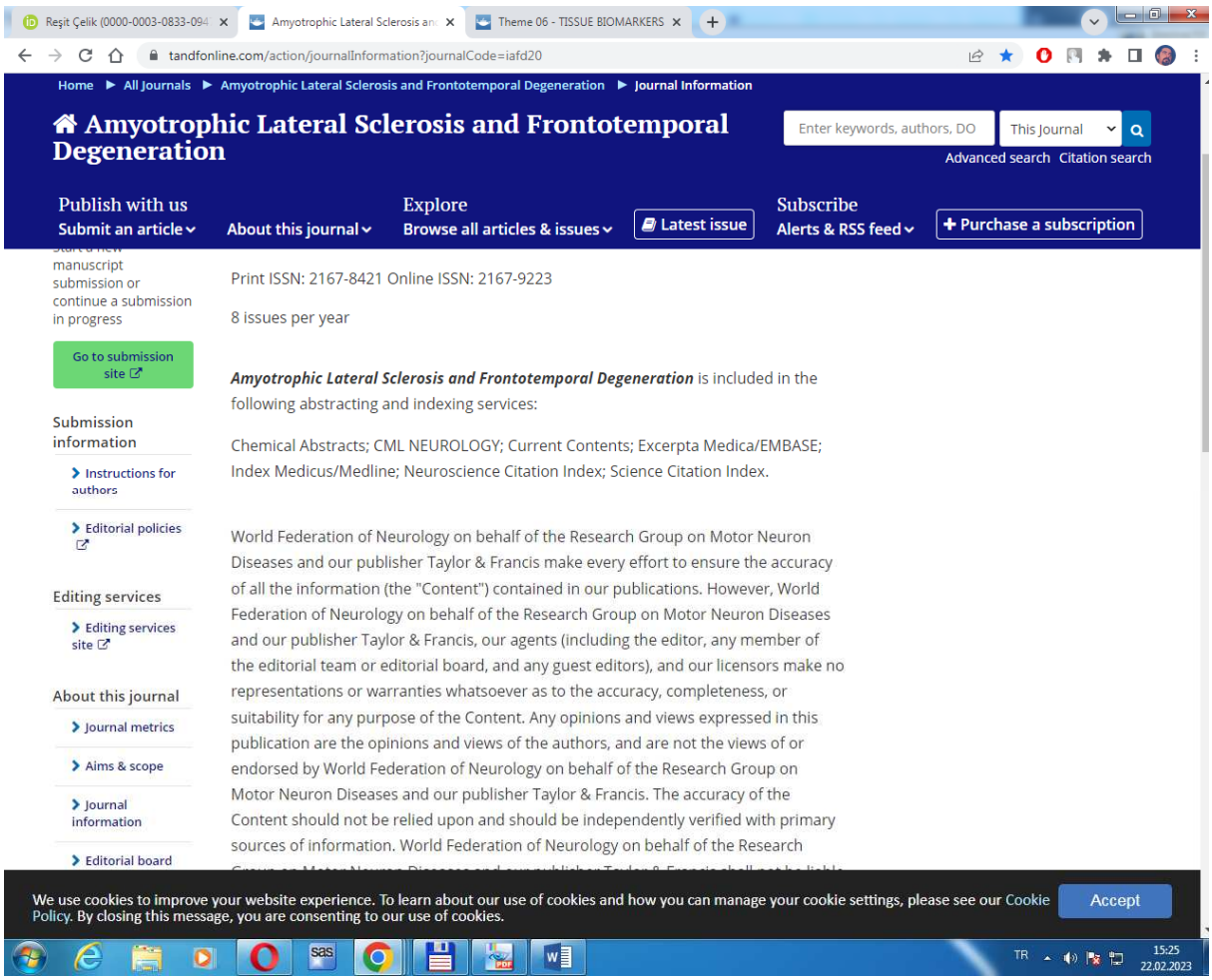
## Acknowledgements

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