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Theme 6 Biomarkers

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Theme 6 Biomarkers

P162 ANSWER ALS: ESTABLISHING A CLINICAL AND COMPREHENSIVE MULTI-OMICS SIGNATURE FOR ALS EMPLOYING INDUCED PLURIPOTENT STEM CELL DERIVED MOTOR NEURONS FROM 1000 SPORADIC AND FAMILIAL ALS PATIENTS NATIONWIDE

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Keywords: big data, informatics, iPS cells, individualized medicine

Background: ALS, like many other neurodegenerative diseases, likely represents a collection of different subtypes of patient populations and molecular etiologies. Over a dozen different genetic mutations cause familial ALS (fALS) and fALS is clinically indistinguishable from the far more common sporadic ALS. Disappointingly, no new drug treatments have been found to be reproducibly successful in large clinical trials since the first and only FDA approved drug, more than 20 years ago.

Methods: Using approaches gleaned from personalized medicine approaches in cancer, Answer ALS was conceived and organized as a comprehensive multi-omics approach to ALS to ascertain, at a population level, the various clinical molecular- biochemical subtypes of ALS. The overall organization was built on the collaborative NIH initiated NeuroLinc's consortium. Specifically, ALS patients nationwide are being enrolled at 6 University clinics distributed throughout the USA and longitudinally followed with deep clinical data collection. In addition, patients wear a personal health monitoring device with a linked Android/iOS app collecting 24/7 data on motor activity, sleep activity, heart rate, motor performance and learning "games", voice and pulmonary function. The iPS-derived neurons are centrally generated from a novel, rapid and highly reproducible specific differentiation protocol. Whole genome sequencing, transcriptomics, epigenomics, proteomics, metabolomics, lipomics, high content imaging and longitudinal high throughput single cell analysis are collected on the patients iPS motor neurons also employing standardized and parallel cultures.

Results: Integrated clinical and biological signatures are being generated using bioinformatics, statistics and computational biology to establish patterns that may lead to a better understanding of the underlying mechanisms of disease. The data acquired in this consortium effort is open source and freely available online to academic and commercial researchers along with the library of patient derived iPS cells, all without IP restrictions. The data is being analyzed using deep machine learning algorithms performed in collaboration with partner organizations.

Discussion: The overall goal of this comprehensive individualized clinical and biological national effort will be to identify biological subsets of ALS which will inform future clinical trials, help develop therapies targeting the proper molecular pathway for the right patient subgroup, provide a platform of human patient derived authentic neurons for use in patient subgroup drug discovery and appropriate biomarker and/or pharmacodynamic markers for use in clinical trials.

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P163 NEUROFILAMENT IN CSF AS A DIAGNOSTIC BIOMARKER IN MOTOR NEURON DISEASE: A META-ANALYSIS

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Keywords: CSF, neurofilament, diagnostic value, meta-analysis

Objective: Neurofilaments in CSF are a promising biomarker which might help in the diagnosis of motor neuron disease (MND). We aim to assess the diagnostic value of neurofilaments in CSF for MND.

Methods: Pubmed, Embase and Web of Science were searched for relevant studies systematically. Articles in English that evaluated the utility of neurofilaments in CSF in the diagnosis of MND were included. Data were extracted by two independent investigators. Diagnostic indexes for neurofilament light chain (NfL) and phosphorylated neurofilament heavy chain (pNfH) were calculated separately. Stata 12.0 software with a bivariate mixed-effects model was used to summarize the diagnostic indexes from eligible studies.

Results: Five studies on NfL and six studies on pNfH met inclusion criteria. For NfL, the pooled sensitivity and specificity were 80% (95% confidence interval [CI], 70%–88%) and 86% (95%CI, 76%–92%), respectively; the positive likelihood ratio (PLR) and negative likelihood

ratio (NLR) were 5.8 (95%CI,3.1–10.7) and 0.23 (95%CI, 0.14–0.38), respectively; the summary diagnostic odds ratio (DOR) was 25 (95%CI, 9–71), and the area under summary receiver operator characteristic curve (sROC) was 0.9 (95%CI, 0.87–0.92). For pNfH, the pooled sensitivity, specificity, PLR and NLR were 83% (95% CI, 79%–87%), 86% (95%CI, 78%–91%), 5.9 (95%CI, 3.6–9.6) and 0.19 (95%CI, 0.15–0.25) respectively; the summary DOR was 31 (95%CI, 16–59), and the area under sROC was 0.89 (95%CI, 0.86–0.92).

Conclusion: Neurofilaments in CSF have a high value in the diagnosis of MND, though the optimal cut off value remains to be further investigated.

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P164 COVARIANCE PATTERNS BETWEEN DISTINCT BIOMARKERS AND THEIR IMPACT ON CLINICAL OUTCOME IN ALS

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Keywords: biomarkers, covariance, survival

Background: There is a need to establish prognostic biomarkers for amyotrophic lateral sclerosis (ALS) allowing for timely interventions and improved stratification of patients within clinical trials.

Objectives: ALS is a clinically heterogeneous disease with potential alterations of several biomarkers. We aimed to understand how distinct biomarker modalities co-vary or differ from one another to define broader biomarker profiles. We hypothesized that profiles would exist that display robust properties to predict longitudinal clinical performance and survival in ALS.

Methods: Our multicenter study included 124 patients with sporadic ALS (mean age 61 a, 65% male, 67% classic, 18% LMND, 8% UMND, 7%PLS; all FTLD negative), with multimodal biomarker baseline data: motor cortex thickness; whole-brain gray matter atrophy (quantified using the number of suprathreshold voxels derived from MPRAGE 3T MRI W-score maps); sonographic ulnar nerve cross sectional area (CSA); ulnar nerve motor amplitudes; cerebrospinal fluid (CSF) levels of total protein, progranulin (PGRN), neurofilament light-chain (NfL) and total tau (t-tau). These biomarkers were included in a principal component analysis (PCA, FactoMineR v1.27, missing values were imputed using missMDA v1.10). Over a mean [SD] observation period of 20.2 [16] months, follow-up ALSFRS-R scores were assessed at least once; 40% of the cases had died within 48 months and surviving patients were censored at their most recent follow-up. Mixed effects linear models and Cox-proportional hazard models (all adjusted for age, gender, height, weight) were conducted each simultaneously including all biomarker components extracted from PCA.

Results: Extracted biomarker components (n=4, 85% variance explained) were named based on the individual biomarker variables that expressed the highest component loading score for the respective component: 1 – "ulnar nerve atrophy, high PGRN, high NfL, high t-tau"; 2 – "preserved motor cortex thickness, preserved motor amplitudes, low t-tau"; 3 – "preserved whole-brain gray matter volume"; and 4 – "small motor amplitudes, high total protein". Patients scoring high on component 1 or scoring low on component 3, each compared to their lower or higher scoring counterparts, declined faster on longitudinal ALSFRS-R (significant time-interaction effects derived from mixed effects linear models). Considering a total illness duration of less than 36 months (until death), subjects scoring high compared to cases scoring low on component 2 revealed decreased hazard of death and increased survival times. Considering an illness duration of >66 months, results were the same when comparing patients scoring high against cases scoring low on component 3.

Discussion and conclusions: In ALS, we identified constructs of biomarker variables showing meaningful covariance across distinct biomarker modalities. Components giving information about cerebral gray matter pathology were superior to predict the patients' survival compared to components which do not include that information.

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P165 WHOLE CSF PROTEOME ANALYSIS FOR THE IDENTIFICATION OF SPECIFIC BIOMARKERS IN ALS

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Keywords: CSF, biomarker, proteomics

Background: The disease course of ALS is gradual but progression is variable. Up to one third of patients survive for either more than 48 months, or die before 18 months

after symptom onset. A successful therapy is one which slows disease progression, yet it is unknown which factors protect patients from a rapid disease course. Therefore specific biomarkers are required for early diagnosis of ALS and for monitoring and evaluation of the efficacy of new treatments.

Objectives: A comprehensive proteomic search strategy was applied for the search of new specific biomarkers in CSF of patients with ALS. CSF was acquired from rapidly and slowly progressive ALS patients and controls. The proteomes were analyzed and compared to identify potential specific biomarker candidates discriminating these 3 groups. Proposed biomarker candidates were evaluated.

Methods: Pooled CSF samples of each group were used. CSF-pools were concentrated and 2D-fractionated (SEC, AEC). For each pool we got 1560 2D-fractions. Fractions with protein conc. >0.03 mg/ml were analyzed by LC-MS/MS; data were processed by Proteome Discoverer and Sieve. The validation of the protein concentration of proposed candidates in individual samples was performed by ELISA.

Results: We identified 1824 protein groups supported by 1 peptide and 676 with 2 peptides. Control CSF vs. CSF of ALS patients showed significantly increased or decreased proteins, some of which are strongly associated with brain injury and neuronal death. ELISA evaluation in a new cohort showed statistically relevant alterations in a subgroup of the identified proteins.

Conclusions: We identified and validated significantly increased or decreased proteins in CSF of ALS patients. These candidates further require evaluation in larger patient cohorts, in CSF as well as in blood. They could be used for early diagnosis of ALS, as prognostic indicators and/or for monitoring and evaluation of the efficacy of new treatments. They can also contribute to a better understanding of the complex mechanisms of ALS.

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P166 THE CSF EXTRACELLULAR VESICLE PROTEOME AS A NOVEL BIOMARKER SOURCE

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Keywords: biomarker, extracellular vesicles, method

Background: Biomarker development is a priority in ALS and CSF markers are a leading neurochemical source. Extracellular vesicles (EVs) have been shown to contain proteins specifically relevant to neurodegenerative disorders, and are implicated in the interneuronal propagation of pathology (1). They may therefore represent a more targeted pool from which to search for novel markers of disease activity and propagation. The utility of cerebrospinal fluid (CSF) EVs has been limited by a lack of reproducible, high-purity, high-yield extraction techniques compatible with proteomic analysis methods such as mass spectrometry.

Objectives: To characterize the CSF EV proteome using an optimized method of EV extraction, ultrafiltration liquid chromatography, and compare with the proteome of whole CSF.

Methods: CSF (8ml) was pooled from small extra volumes taken from consenting patients undergoing routine ward lumbar puncture as part of their investigation for a range of neurological symptoms. EVs were purified using an in-house optimized ultrafiltration liquid chromatography method and characterized using nanotracking analysis, Western blotting for known EV proteins and transmission electron microscopy. Proteomic analysis of CSF and CSF-derived EVs was performed using LC-MS/MS.

Results: CSF EVs isolated demonstrated characteristic size distribution and morphology, and expressed typical EV markers. Over 1300 individual proteins were identified in proteomic analysis. Of these, over 1100 proteins were enriched in the EV proteome, unexpectedly (in this random cohort) including several proteins of potential relevance to ALS such as TUBA4A and PABP. An analysis comparing results from CSF EV fraction with the whole CSF proteome will be presented.

Conclusions: Extraction and analysis of the EV proteome is feasible as a platform for novel CSF biomarker development, and studies using ALS patient CSF samples and relevant controls are now planned.

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P167 EVIDENCE OF DEFECTIVE CHOLESTEROL METABOLISM IN ALS

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Keywords: metabolomics, cholesterol, diagnosis

Background: For the development of effective disease-modifying therapies for ALS, reliable markers of disease activity are a research priority. High plasma levels of cholesterol have been suggested to be neuroprotective for ALS, associated with an increased survival time (1). The gene CYP27A1, encoding cholesterol (25R) 26-hydroxylase, the enzyme initiating extra-hepatic metabolism of cholesterol through bile acid biosynthesis, was recently identified as a susceptibility gene for sporadic ALS (2). In addition, statins, cholesterol lowering drugs which inhibit HMG-CoA reductase, have been suggested to accelerate functional decline in ALS patients (3). Cholesterol is also a major component of neurons; hence this sterol and its metabolites are candidate biomarkers of neurodegeneration in ALS on several levels.

Objectives: To quantify sterols and their metabolites in CSF from ALS patients.

Methods: CSF from 20 ALS patients from the Oxford Study for Biomarkers in MND (BioMOx) and 15 controls were assayed for >50 different sterols using quantitative liquid chromatography – mass spectrometry (LC-MS) (4).

Results: The CSF concentration of cholesterol was elevated in ALS compared with controls ($p < 0.01$). When metabolite levels were normalised to cholesterol, the metabolite 3β,7α-dihydroxycholest-5-en-26-oic acid ($p < 0.05$) along with its precursor 3β-hydroxycholest-5-en-26-oic acid ($p < 0.01$) and product 7α-hydroxy-3-oxocholest-4-en-26-oic acid ($p < 0.01$) were reduced in concentration. These three acids are members of the acidic pathway for bile acid biosynthesis.

Discussion and conclusions: Levels of cholesterol are tightly regulated in brain by a balance between synthesis and metabolism. The blood brain barrier prevents direct import and export of cholesterol from and to the circulation. When neurons die, cholesterol is released. In the healthy state cholesterol is metabolized to hydroxycholesterols which enter the bile acid biosynthesis pathway, members of which can cross the blood brain barrier and be exported. Our results indicate that the acidic branch of bile acid biosynthesis may be defective in ALS, leading to a failure of the CNS to remove excess cholesterol. Importantly, neither elevated CSF cholesterol levels nor reduced metabolite levels were found in Alzheimer's disease, vascular dementia or multiple sclerosis patients (5). Our ALS data may be important on two fronts, (i) excess cholesterol is known to be toxic to neuronal cells, and (ii) 3β,7α-dihydroxycholest-5-en-26-oic acid has been shown to be neuroprotective towards motor neurons (6).

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P168 CHANGES IN CEREBROSPINAL FLUID CYTOKINE LEVELS ACROSS DIFFERENT CLINICAL DISEASE MILESTONES IN AMYOTROPHIC LATERAL SCLEROSIS

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Keywords: ALS, cytokine, clinical staging

Objectives: We aimed to investigate the correlation between alterations in cytokine and chemokine levels and clinical milestones in amyotrophic lateral sclerosis (ALS).

Methods: The study population consisted of 146 patients with sporadic ALS (sALS). In addition, 135 healthy control individuals were included in the study. All sALS patients were classified according to the clinical staging system for ALS proposed by (1). Considering that no patient at onset or with respiratory support was enrolled, we defined three subsets of patients in stages 2, 3, and 4, each including 48, 50, and 48 patients, respectively. Cerebrospinal fluid (CSF) samples were collected at different clinical stages and levels of interleukin 2 (IL-2), IL-4, IL-6, IL-8, IL-10, IL-17, IL-21, IL-22, IL-23, IFN-γ, TNF-α, RANTES, macrophage inflammatory protein 1α (MIP-1α), monocyte chemotactic protein 1 (MCP-1), vascular endothelial growth factor (VEGF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) were measured using Luminex multiplex cytokine analysis (Bioplex kits, Bio-Rad Laboratories).

Results: Among the cytokines/chemokines measured, RANTES, VEGF, TNF-α, IFN-γ, MCP-1, IL-4, IL-6, IL-8, IL-10, and IL-17 levels were significantly higher in stage 2, 3, and 4 in all 146 ALS patients than in the healthy controls. CSF from ALS patients demonstrated significantly increased intrathecal production of soluble IFN-γ in clinical stage 2 (p=0.0112), 3 (p<0.0001), and 4 (p<0.0001) compared with that from healthy controls. Comparing ALS patients in clinical stage 2, we observed increased IFN-γ levels in clinical stage 3 (p=0.0319) or in clinical stage 4 (p=0.0161). IL-4 similarly demonstrated increased expression in CSF from ALS patients in clinical stage 2 (p=0.0062) and 3 (p=0.0313) compared with healthy controls, but not in stage 4 (p=0.1319). There were no significant differences between stage 2 and 3, stage 2 and 4, or stage 3 and 4 in the levels of IL-4, IL-10, and IL-17. ALS patients in clinical stage 2 (p<0.0001), 3 (p<0.0001), and 4 (p<0.0001) also exhibited significantly elevated levels of IL-10 compared with healthy controls. Compared with healthy controls, IL-17 levels in the CSF from ALS patients were higher in clinical stage 2 (p=0.0388), 3 (p=0.0003), and 4 (p=0.0011). These data suggest a robust Th1-polarized response with evidence of active inflammatory activation within the intrathecal compartment, especially in clinical stages 3 and 4.

Conclusions: These results may provide a rational basis for future studies of immune modulation in ALS treatment.

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P169 OXIDATION-REDUCTION POTENTIAL OF CEREBROSPINAL FLUID AS A POTENTIAL BIOMARKER FOR ALS PROGRESSION

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Keywords: cerebrospinal fluid, reduction potential, oxidative stress

Background: ALS is an oxidative stress-related fast-paced motor neuron disease for which there are no accurate biomarkers of progression. This largely hampers the development of therapeutics. Our aim was to test the applicability of oxidation-reduction potential (ORP) of cerebrospinal fluid (CSF) in ALS progression follow-up.

Methods: ORP was measured using RedoxSYS (Aytu BioScience, Inc.) in CSF of 49 ALS patients (mean age 63 ± 1.29 years; range 43-80 years; mean ALSFRSr score 37.47 ± 0.90, range 21-48; m/f = 35/14) and 15 controls (mean age 48.93 ± 3.68 years, range 21-67 years; m/f = 10/5).

Results: Pearson correlation coefficient (R) between ALSFRSr score and ORP was -0.27 ($p = 0.06$). R was higher (-0.45) when two outlier values were excluded from the calculus. Importantly, ALS patients had significantly higher mean ORP (ALS: 121.83 ± 2.76mV; controls: 111.14 ± 2.94 mV; $p = 0.033$). The difference was also significant for ORP normalized to age. Of note, ORP is reciprocally proportional to the pro-oxidative settings in biological samples.

Discussion: Increased ORP values in ALS patients further confirm the role of oxidative stress in this neurodegenerative disease. ORP might find application as a biomarker for ALS progression/prognosis but further measurements on a larger cohort is warranted.

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P170 MICROVESICLES AND EXOSOMES AS NEW BIOMARKERS OF DISEASE PROPAGATION IN AMYOTROPHIC LATERAL SCLEROSIS

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Keywords: extracellular vesicles, CD45, misfolded proteins

Background: The lack of biomarkers in Amyotrophic Lateral Sclerosis (ALS) makes it impossible to determine the stage of the illness in patients, delaying therapeutic trials. Blood contains extracellular vesicles (EVs), (mainly classified for size and biological function into microvesicles-MVs and exosomes-EXOs), pro-inflammatory vesicles that transfer mRNA, non-coding RNA or proteins among different cell types (1). MVs can initiate prion propagation from prion-infected neuronal cells to uninfected cells, underlying a new mechanism of the disease propagation (2). In fact mutated or "misfolded" proteins (SOD1, TDP-43 and FUS) are templates for the formation of protein oligomers that accumulate and interfere with neuronal function, eventually leading to cell death (3).

Objectives: "Misfolded" proteins have been found in plasma EVs of ALS patients highlighting a connection between motoneurons and peripheral blood (4). The aim of our study is to investigate MVs and EXOs in plasma of ALS patients, in order to discover a new mechanism in disease progression.

Methods: Microvesicles were isolated from plasma of 30 ALS, 30 healthy volunteers and 30 Alzheimer's Disease (AD) patients. Venous blood was centrifuged (1000xg for 10 min, 1600xg for 20 min) and the plasma was then centrifuged at 20,000xg for 1 hour for MVs; supernatant was then centrifuged at 100,000xg for 1 hour for EXOs. Markers for MVs of leukocyte (CD45), endothelial (CD31), platelet (CD61), erythrocyte (CD235a) derivation and the apoptotic marker, Annexin V were used for flow cytometry. Specific markers for MVs and EXOs (i.e. Alix, Annexin V, floatillin) were checked by WB. SOD1, TDP-43, FUS protein level was investigated by WB and normalized against annexin V and Alix, respectively in MVs and EXOs.

Results: We found two groups of ALS patients: one with high CD45 derived blood MVs (6-fold more the healthy control group, $p<0.0001$) and one with low CD45 MVs (0.5-fold less the healthy control group) (ANOVA test, $p<0.0001$). Our preliminary data showed higher SOD-1 and TDP43 level in plasma derived microvesicles of 22 patients compared to 22 controls (SOD1-mean two fold more the controls, ANOVA test, $p<0.0001$, TDP43-0,5 fold more than controls, ANOVA test, $p<0.05$). Our preliminary data also showed that some patients had an increased misfolded SOD-1 protein level in CD45 derived MVs.

Discussion and conclusion: EVs could have a relevant role in the disease propagation of ALS. Leukocyte derived MVs can be overexpressed in a group of ALS patients and they might be the "carriers" of misfolded proteins, a main cause of disease propagation.

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P171 USING BIOMARKERS IN BLOOD AND CSF FOR DIAGNOSIS AND PROGNOSIS IN ALS

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Keywords: diagnosis, biomarker

Background: The diagnosis of ALS is difficult and dependent upon clinical examination with signs of upper and lower motor neuron affection combined with clinical progression as hallmark of a correct decision. Further information about the patients' condition are provided with radiological examination, neurophysiology and biomarkers in blood and cerebrovascular fluid. Neurofilament is a reliable marker of brain damage in ALS, MS and cardiac arrest. At our clinic all patients are examined with blood tests and lumbar puncture routinely in the investigation.

Objectives: Compare the benefits of using biochemical markers in blood and cerebrospinal fluids with clinical examination, electromyography and radiological examination in the process of giving the diagnosis of ALS/MND.

Methods: Thirty-eight patients with clinical signs of ALS were followed from disease suspicion and further on. Patients were clinically examined by one of two physicians very familiar with the ALS diagnosis. CT and MRI scans were examined at the Neuroradiological department by experienced doctors. EMG was performed according to common routines at the Clinical Neurophysiological lab at our Hospital. Routine blood tests and the analysis of the CSF were done at the Clinical Chemical Laboratory at the hospital.

Results: Thirty-seven patients were given the ALS/MND diagnosis according to El Escorial criteria. The 38th patient was shown to have another diagnosis. Age range was 35 to 83. 18 were women and 19 were men. EMG gave support to the diagnosis in all cases. MRI scans showed normal conditions or discrete white matter except for three cases with sclerosis of the pyramid tractus, suggestive for ALS. Concerning the findings of the CSF analysis, no cellular abnormalities were found in any case. Furthermore, cytology and blood brain barrier were normal. We found nothing on the examination of the immune electrophoresis. All cases except for one, had high levels of NFL, range 250 - 16 000 ng/L. Cases with a fast progression had higher values. Five cases had high levels of Tau, mostly in cases with high NFL levels. All levels of GFAP were normal. 12 cases had a second lumbar puncture performed. Low values were found in cases with discrete progression of the disease. Analysis of ordinary blood samples did not add anything to diagnosis.

Discussion and conclusions: It is well known that NFL is a valid biomarker of ALS. Our analysis showed that it proved to be more important than other analysis of the CSF. High values of NFL strongly support the accuracy of diagnosis. NFL is a marker of neuronal damage and the absence of changes on cytology; immune electrophoresis and blood brain barrier do not support the theory that ALS is an inflammatory disease.

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P172 PERIPHERAL NERVE ATROPHY AND PROGRANULIN INTERACT TO PREDICT LONGITUDINAL CLINICAL PERFORMANCE AND SURVIVAL IN ALS

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Keywords: peripheral nerve sonography, progranulin, survival

Background: In amyotrophic lateral sclerosis (ALS), high-resolution ultrasound became a promising biomarker to quantify peripheral nerve atrophy. Thus far, however, it

has failed to show robust associations with clinical data and other biomarkers in ALS, such as revised ALS functional rating scale (ALSFRS-R) and electrophysiology. In a rodent model, peripheral nerve axonal pathology seems to be related to increased progranulin (PGRN) levels, which, in turn, are found in the cerebrospinal fluid (CSF) of ALS patients.

Objectives: Hypothesizing a link between peripheral nerve atrophy and altered CSF PGRN concentrations, we examined whether the two biomarkers in combination (compared to sonographic measures alone) have added value to aid in the prediction of clinical performance and survival in ALS.

Methods: The study included 49 ALS patients (mean age 58y, 61% male; 43% classic, 27% LMND, 14% UMND, 12% bulbar, 4% PLS; n=1 with accompanying FTLD) with baseline data available for forearm ulnar and median nerve cross sectional area (CSA, in mm^2), CSF PGRN normalized against CSF/serum albumin ratio (PGRN/Qalb) to account for the relationship between PGRN and blood-CSF barrier integrity and CSF neurofilament light-chain (NfL, marker of neuroaxonal damage). Over a mean [SD] observation period of 17.8 [13] months, 61% of the subjects came for at least one follow-up visit to undergo electrophysiological and clinical measures; considering a total illness duration of less than 48 months, 54% had died and surviving patients were censored at their most recent follow-up. General/mixed effects linear models and Cox proportional hazard regression models were conducted to examine the interaction between CSA' PGRN/Qalb on distinct outcome variables. All models were adjusted for baseline age, gender, height and baseline weight. To follow significant interaction effects patients were split into terciles for CSA and PGRN/Qalb.

Results: There was an inverse relationship between CSA and PGRN/Qalb (for ulnar nerve: $r = -0.4$, $p = 0.008$, for median nerve: $r = -0.3$, $p = 0.039$; bivariate correlations adjusted for age, gender, height, weight, disease duration). ALS-patients revealing both, small nerve CSA and high PGRN/Qalb levels compared to subjects displaying large nerve CSA and low PGRN/Qalb values, (i) displayed higher CSF NfL concentrations, (ii) performed worse on ALSFRS-R (for scores collapsed across all available time points), (iii) showed smaller motor amplitudes and slower motor conduction velocities (for values collapsed across all follow-up visits), and (iv) had a 2.8-fold higher mortality rate within 48 months.

Discussion and conclusions: Elevated CSF PGRN may indicate progressive peripheral axonal damage, and nerve atrophy and high CSF PGRN levels, in turn, have a synergistic effect on outcome in ALS. Although the specific function of PGRN has not fully been characterized, it appears to have significant potential as a CSF-based biomarker in ALS, particularly when combined with peripheral nerve ultrasound.

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P173 BRAIN DERIVED PROTEINS IN PLASMA FROM ALS ENDOPHENOTYPES

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Keywords: TMT® 10plex, LC-MS/MS; biomarkers

Introduction: Discovery proteomics in body fluids such as plasma has relatively poor sensitivity and many tissue-derived proteins are not surveyed. Consequently, most biomarker panels comprise acute-phase reactant proteins, chemokines and cytokines that often lack specificity.

Objective: We have developed a novel MS3 workflow using isobaric tagging and including multi-point reference peptide mixture (TMTcalibratorTM MS3) that allows a sensitive quantification of proteins in plasma samples from ALS patients with bulbar (B-ALS, n=15) and limb (L-ALS, n=15) onset in an early stage of disease.

Methods: A pooled tryptic digest of brain (precentral gyrus) taken from two ALS patients was labelled with four different TMT® tags and mixed to form a four point calibration curve. This was mixed with a tryptic digest from three B-ALS and three L-ALS plasma samples individually labelled with the remaining six TMT® tags from a TMT® 10-plex kit. In total, five TMT® 10 sets were subsequently fractionated using strong cation exchange chromatography. Fractions were analysed using an Orbitrap Velos Pro™ Mass Spectrometer (Thermo Scientific). The ten most intense peaks in the MS scan were selected for CID MS2 fragmentation in the ion trap. To obtain highly accurate quantitative data several MS2 fragments were selected for HCD fragmentation (MS3) and quantified in the Orbitrap. Data were processed through Proteome Discoverer™. In-house bio-informatics pipelines were used to process the datasets, which allowed evaluation of consistency across TMT10 plex sets and identification of significantly regulated features.

Results: In total, we identified approximately 6500 proteins in each data set. The number of peptides and proteins was consistent between repeats. Significantly regulated proteins and molecular pathways have been identified for the first time in plasma samples from ALS endophenotypes using a sensitive, robust and high-throughput approach.

Discussion and conclusions: The consistency of our results suggests that the method is highly reproducible and reliable. Some of the regulated proteins mapped to a molecular pathway previously identified as early impaired component in ALS dynamic. Brain derived proteins were also found significantly regulated between the two groups. The sensitivity provided by TMTcalibrator and the use of

SCX fractionation and MS3 is considerable and provides broad and deep coverage of the plasma proteome.

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P174 ABERRATION OF MIRNAS EXPRESSION IN LEUKOCYTES FROM SPORADIC AMYOTROPHIC LATERAL SCLEROSIS

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Keywords: amyotrophic lateral sclerosis; miRNAs; microarray; biomarker

Background: Accumulating evidence indicates that miRNAs play an important role in the development of amyotrophic lateral sclerosis (ALS). Most previous studies on miRNA dysregulation in ALS focused on the alterative expression in ALS animal model or in limited samples from European patients with ALS. In the present study, the miRNA expression profiles were investigated in Chinese ALS patients to explore leukocyte miRNAs as a potential biomarker for the diagnosing of ALS.

Methods: We analyzed the expression profiles of 1733 human mature miRNAs using microarray technology in leukocytes obtained from 5 patients with sporadic ALS (SALS) and 5 healthy controls. An independent group of 83 SALS patients and 61 controls was used for validation by real-time polymerase chain reaction assay. Area under the receiver operating characteristic curve (AUC) was used to evaluate diagnostic accuracy.

Results: Eleven miRNAs, including four over-expressed and seven under-expressed miRNAs detected in SALS patients compared to healthy controls were selected for validation. Four under-expressed microRNAs, including hsa-miR-183, hsa-miR-193b, hsa-miR-451 and hsa-miR-3935, were validated in SALS. Moreover, we identified a miRNA panel (hsa-miR-183, hsa-miR-193b, hsa-miR-451 and hsa-miR-3935) having a high diagnostic accuracy of SALS (AUC 0.857 for the validation group).

Conclusion: This study provided evidence of abnormal miRNA expression patterns in the peripheral blood leukocytes of Chinese SALS patients. Leukocyte miRNAs provide a promising opportunity for detection of SALS.

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P175 SERUM AND LYMPHOCYTE MICRORNAS IN ALS: THEIR ROLE AS FUNCTIONAL BIOMARKERS OF THE DISEASE

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Keywords: microRNA, biomarkers, exosomes

Background: MicroRNA (miRNA)s have emerged as key players in regulating gene expression in many neurodegenerative diseases. This draws attention to the role of altered miRNA metabolism in Amyotrophic Lateral Sclerosis (ALS), and might represent the common mechanisms underlying different ALS genetics. Several miRNAs are deregulated in muscles, nervous system and blood of ALS patients (1). The main carriers of mRNA/miRNAs in the human blood are the extracellular vesicles (exosomes), involved in cell-cell communication: emerging evidence suggests that they are implicated in neurodegenerative diseases (2). Moreover, as peripheral blood lymphocytes (PBLs) appear to share common patterns of transcriptional activity with nerve cells, PBL miRNA assays might shed light on cell-cell interactions within the nervous system.

Methods: Forty clinically probable/definite ALS patients (according to the El Escorial criteria) were divided into groups, by taking into account of their symptoms at onset (bulbar vs. spinal), the predominant motor neuron involvement (upper vs. lower), the illness severity (ALSFRS scoring), and the rate of disease progression (slow vs. fast). Blood samples were collected from ALS patients and age- and sex-matched healthy controls. Routine analytes, PBL phenotypes, cytokines/growth factors, anti-oxidative markers, cell-adhesion molecules, and hormones were assayed. Extraction of free and exosome-bound miRNAs was performed in both sera and PBLs. RNA extracted were firstly assayed in a bioanalyser to test RNA quality; then a library carrying all RNA fragments was built, and reverse- transcripted to cDNA for PCR amplification. Western blot analysis was performed, to identify miRNA-targeted proteins. A comparison among the different expression profiles in different disease form/types and stages was accomplished.

Results: We obtained well-characterized serum and PBL profiles from ALS patients, we compared with those from age- and sex-matched healthy controls. Moreover, comparisons among subgroups of patients were done, according to their symptom onset, type, body localization and progression. Novel biomarkers, linked to disease clinical features, useful for diagnosis, prognosis and treatment have been investigated.

Discussion and conclusion: We assayed both free and exosome-bound miRNAs in sera and PBLs: to our knowledge, this is the first attempt to correlate miRNA

data from different blood components with biochemical and clinical data from different subgroups of ALS patients, synchronically and diachronically. Our findings might allow us to shed light on the complex relationships between deranged miRNA turnover and immune dysfunctioning which may be implicated in ALS progression. There is no successful treatment against ALS and the identification of novel signaling pathways, and molecular mechanisms are still the major task in the search for therapies. Our work provided such diagnostic and prognostic biomarkers. The identification of miRNA targets might allow to find potential targets for novel therapeutic approaches.

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P176 GENE EXPRESSION BIOMARKERS IN LYMPHOCYTES OF SALS PATIENTS

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Keywords: biomarkers

Background: Previous studies performed on muscle biopsies from SOD1G93A mice suggested that this animal model presents an alteration in the expression of five genes (Mef2c, Gsr, Col19a1, Calm1 and Snx10). Therefore, with these previous results, the expression of those genes could behave like potential prognostic biomarkers of longevity (1). Even though the search of biomarkers in ALS is being carried out in a wide variety of patient samples, growing tendency relies on the study of new and less invasive tissues (2,3).

Objectives: Our aim was to study the expression level of MEF2C, GSR, COL19A1, IMPA1, NOGOA, SNX10, GSK3 and SOD1 in lymphocytes to obtain an association with disease progression and diagnosis.

Methods: cDNA serial samples from lymphocytes of 45 patients with sporadic ALS, were subjected to qPCR in order to study expression levels of MEF2C, GSR, COL19A1, IMPA1, NOGOA, SNX10, GSK3 and SOD1. The levels found in every sample were related to the main clinical parameters monitored on disease progression: ALSFRS-r, FVC and survival values. Patients were classified in two groups, short and long survival (less and more than 5 years from symptom onset) with statistically significant differences between them (Kaplan Meyer study). Statistical analysis and ROC curves were made with GraphPad Prism software.

Results: The decreased expression of the following genes: GSR, NOGOA, MEF2C and SOD1 were different ($p < 0.001$) compared with normal controls. The areas of their ROC curves were respectively: 0.9939; 0.8788; 0.8499 and 0.9251. COL19A1 expression was increased in patients with more than 5 years of survival with respect to normal controls ($p < 0.05$). The area of its ROC curve was 0.7035. Expressions of GSK3 and IMPA1 have no significant differences.

Conclusions: Abnormal expression of GSR, NOGOA, MEF2C and SOD1 can be used as diagnostic biomarkers. The increase of COL19A1 expression could be linked with a compensatory response in patients with longer survival, and could be useful as a prognostic biomarker. It is interesting to notice that GSK3 expression is not modified in patients. Then, the reported protein level increase (4,5,6), would be only due to a delicate interaction and to a cytoplasmic regulation.

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P177 MACROPHAGE MIGRATION INHIBITORY FACTOR LEVELS AS A BIOMARKER IN SYMPTOMATIC ALS

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Keywords: MIF, biomarkers, ALS

Background: Previously, in Amyotrophic Lateral Sclerosis (ALS) research, reasons for the disease were thought to be linked with unique mechanisms but now it is accepted that the disease has multifactorial etiopathogenesis (1). Factors causing ALS include: several gene mutations such as SOD1, TDP43, C9ORF72; excitotoxicity; neuroinflamation; and mitochondrial damage. In this study, ALS patients are evaluated in view of mitogen activated macrophage migration inhibitor factor (MIF) values. MIF a member of immune system molecules, is a multifunctional cytokine that has diverse immunological and neuroendocrine properties. In studies of familial ALS, especially with SOD mutated cases, it has been shown that

enzyme activity was not affected due to the mutation but instead protein aggregates were deposited in neuronal cells and organelles such as mitochondria and ER. Some studies have shown that MIF has chaperone properties besides its immunological role. Some studies have shown that MIF scavenges misfolded SOD1 by its chaperone function (2).

Objective: In this study we measured MIF levels in plasma of individuals with ALS, other neurology patients (multiple sclerosis MS, Parkinson's disease PD) and healthy volunteers.

Methods: ALS patients were diagnosed according to the El-Escorial criteria. Most ALS type of patients were diagnosed with upper motor neuron in this study. MIF level was measured by ELISA method.

Results: Thirty patients had ALS, 12 patients had other neurological diseases, (PD, MS). Twelve were healthy controls. There were 17 females and 13 males. MIF normal value: 5–12 ng/mL. Mean level of the patients was 9.67. Control group was 8.17. Neurological control was 3.5–10 ng/mL.

Discussion and conclusıon: Differences between MIF levels of control and ALS groups are statistically significant with a 95% confidence interval. MIF plasma levels of ALS patients have no difference between female and male.

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P178 MONOCYTES AS PROGNOSTIC BIOMARKERS OF LONGEVITY IN TRANSGENIC SOD1G93A MICE

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Keywords: monocytes, longevity, blood samples

Background: Monocytes are rapidly recruited from peripheral blood to the affected tissues at the beginning of tissue insult (1). In pathological conditions, monocytes enter into the damaged and inflamed tissues in a CCR2-dependent manner (2,3). In addition, inflammatory monocytes are more likely to differentiate to pro-inflammatory macrophages, which secrete pro-inflammatory cytokines able to combat pathogenic microorganisms, but which may be ultimately harmful in neurodegenerative diseases like ALS (4).

Objectives: The main aim of this study was to investigate the potential role of both populations of monocytes, inflammatory and non-inflammatory, in the survival of the transgenic SOD1G93A mice at the key stages of the disease.

Methods: Blood samples from transgenic mice (n=12 both sexes balanced) were collected to study the frequency of inflammatory and non-inflammatory monocytes by flow cytometry. The blood samples were first collected at the age of 50 days and then serial samples were obtained from each animal at 75 days and at the end-point, which ranged between 128 and 143 days, depending on the survival of the animals. Age-matched wild type litter mates were used as controls (n=12 both sexes balanced). Two-tailed t-Student tests were used to assess statistical significance between groups. Correlations between the cell percentage slopes and mice survival were calculated using Pearson's r.

Results: Our results showed a steady state upregulation of the inflammatory monocytes population, while the non-inflammatory monocytes population was found to be downregulated along disease progression, suggesting the presence of inflammatory processes in this animal model starting at a very early asymptomatic stage (p=0.02). Additionally, significant positive (r=0.625, p=0.03) and negative (r= −0.64, p=0.025) correlations with the longevity of the transgenic SOD1G93A mice were observed in the populations of non-inflammatory and inflammatory monocytes, respectively.

Discussion and conclusions: The alterations observed in the inflammatory and non-inflammatory monocytes populations in blood from transgenic SOD1G93A mice suggest that the inflammatory response in this animal model starts at an early and asymptomatic stage of the disease. Furthermore, the positive correlation observed between non-inflammatory monocytes suggests a positive effect of this monocyte population to compensate for the damage associated with disease progression. These findings could pave the way to translational studies in ALS patients, promoting the identification of new reliable biomarkers of disease progression at clinical level and consequently, new promising therapeutic strategies.

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P179 DISEASE PROGRESSION IN SBMA: IS SERUM CREATININE A RELIABLE BIOMARKER?

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Keywords: SBMA, biomarkers, creatinine

Background: Spinal and bulbar muscular atrophy (SBMA) is an adult-onset, X-linked, lower motor neuron disease caused by a CAG repeat expansion within the androgen receptor gene. No reliable index of disease progression has been established so far and, nevertheless, there is a critical need for biomarkers discovery and validation in order to improve the diagnostic process and organization of clinical trials (1).

Objective: We investigated if creatinine serum levels, a common used biomarker in neuromuscular diseases, could be a reliable index of disease progression in SBMA.

Methods: We studied 65 SBMA patients. They underwent biochemical analysis including creatinine and CK serum levels and completed a clinical protocol including 6-min walk test (6MWT), functional scale (ALSFRSr), ADL grade scale and respiratory evaluation (fVC) at baseline and after 1 year. Spearman's coefficient was used to assess correlations and a linear regression was used to fit the obtained results. Student t test was used to compare means.

Results: A significant decrease of 6MWT values ($p = 0.003$) and creatinine serum levels ($p = 0.0031$) was observed between baseline and 12 months evaluation. Creatinine serum levels at baseline did not correlate with age of the patients, disease duration or age of symptom onset. They correlated with 6MWT ($p = 0.0006$), total ALSFRS score ($p < 0.001$) and with upper and lower limbs subscores at baseline ($p < 0.001$). A correlation was found also with the ADL grade ($p < 0.001$) and with the muscular force megascore for upper and lower limbs ($p < 0.001$). Creatinine levels at baseline significantly correlated with ADL grade at 12 months ($r = -0.54$; $p < 0.001$), total ALSFRS score at 12 months ($r = 0.49$; $p = 0.001$), with the subscore for the lower limbs ($r = 0.58$; $p < 0.001$) and with muscle force for upper and lower limbs (respectively $r = 0.42$ with $p = 0.0006$ and $r = 0.57$ with $p > 0.0001$). We used a linear model to establish which proportion of the variation in the 6MWT and in the ALSFRS-r subscore for lower limbs at the 12 months examination was explicated by the creatinine serum levels at baseline. We found a R^2 value respectively of 0.20 and of 0.34.

Conclusions: Our study evidences that serum creatinine could be a good predictor of disease progression in SBMA patients and that it could be an early marker of clinical modifications over time. Our study could be a step forward filling the gap in the research of reliable biomarkers for SBMA.

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P180 THE NOGO-A PROTEIN IS NOT A BIOMARKER IN AMYOTROPHIC LATERAL SCLEROSIS (ALS)

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Keywords: biomarkers, disease progression, prognostic

Introduction: The lack of a sensitive and specific biomarker is a main concern in ALS. Skeletal muscle Nogo-A, a negative regulator of the axonal growth, has been proposed as a diagnostic and prognostic molecular marker but its sensibility/specificity in ALS is controversial. The aim of the study was to establish whether muscle Nogo-A can be considered as a reliable biomarker in ALS.

Materials and methods: Nogo-A protein expression was determined by both immunofluorescence and Western blot analyses in vastus lateralis ± biopsies of 16 patients diagnosed with clinically definite ALS, 12 patients with other neuromuscular diseases (4 patients with Spinal Muscular Atrophy (SMA) type III, 3 with Spinal-Bulbar Muscular Atrophy (SBMA), 3 with polymyositis (PM), 1 with Distal Hereditary Motor Neuropathy (HMN) type V and 1 with Hereditary Spastic Paraplegia (HSP) type 11. 6 healthy controls (no evidence of disease at biopsy) were also considered.

Results: All ALS cases, aged from 23 to 73 years (mean 56.9, SD 15), had had a spinal onset of the disease. Their disease duration at biopsy ranged from 11 to 13 months. In all cases the biopsied muscle was clinically affected. Mean age at biopsy of disease controls was 40.3 years, ranging from 25 to 68 (SD 14.2).We found that Nogo-A was significantly overexpressed in ALS patients muscle biopsies compared to healthy controls. However, increased levels of Nogo-A were also observed in all the neuromuscular controls. We did not observe any relationship between Nogo-A amount and/or subcellular distribution and disease severity.

Conclusions: Nogo-A cannot be considered either a diagnostic nor prognostic biomarker for ALS.

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