

1 **Tissue-enhanced plasma proteomic analysis for disease stratification in**  
2 **amyotrophic lateral sclerosis**

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## 22 **ABSTRACT**

23

### 24 **Background:**

25 It is unclear to what extent pre-clinical studies in genetically homogeneous animal models of  
26 amyotrophic lateral sclerosis (ALS), an invariably fatal neurodegenerative disorder, can be  
27 informative of human pathology. The disease modifying effects in animal models of most  
28 therapeutic compounds have not been reproduced in patients. **To advance therapeutics in ALS,**  
29 **we need easily accessible disease biomarkers which can discriminate across the phenotypic**  
30 **variants observed in ALS patients and can bridge animal and human pathology. Peripheral**  
31 **blood mononuclear cells alterations reflect the rate of progression of the disease representing**  
32 **an ideal biological substrate for biomarkers discovery.**

33 **Methods:** We have applied TMTcalibrator™, a novel tissue-enhanced bio fluid mass  
34 spectrometry **technique**, to study the plasma proteome in **ALS**, using peripheral blood  
35 mononuclear cells as tissue calibrator. We have tested slow and fast progressing SOD1G93A  
36 mouse models of ALS at a pre-symptomatic and symptomatic stage in parallel with fast and  
37 slow progressing ALS patients at an early and late stage of the disease. **Immunoassays were**  
38 **used to retest the expression of relevant protein candidates.**

39 **Results:** The biological features differentiating fast from slow progressing mouse model  
40 plasma proteomes were different from those identified in human pathology, with only  
41 processes encompassing membrane trafficking with translocation of GLUT4, innate immunity,  
42 acute phase response and cytoskeleton organization showing enrichment in both species.  
43 Biological processes associated with senescence, RNA processing, cell stress and metabolism,  
44 major histocompatibility complex-II linked immune-reactivity and apoptosis (early stage) were  
45 enriched specifically in fast progressing ALS patients. Immunodetection confirmed regulation  
46 of the immunosenescence markers Galectin-3, Integrin beta 3 and Transforming growth factor  
47 beta-1 in plasma from pre-symptomatic and symptomatic transgenic animals while  
48 Apolipoprotein E differential plasma expression provided a good separation between fast and  
49 slow progressing ALS patients.

50 **Conclusions:** These findings implicate immunosenescence and metabolism as novel targets  
51 for biomarkers and therapeutic discovery and suggest immunomodulation as an early  
52 intervention. The variance observed in the plasma proteomes may depend on different  
53 biological patterns of disease progression in human and animal model.

54  
55 **Key Words**

56 Amyotrophic lateral sclerosis, proteomics, biomarkers, TMTcalibrator™, SOD1G93A animal  
57 models.

58 **BACKGROUND**

59 Amyotrophic lateral sclerosis (ALS), a fatal neurodegenerative disorder, is a clinically  
60 heterogeneous condition where survival can be less than a year or more than a decade from  
61 symptoms onset, making any assessment of treatment response in clinical trials difficult in  
62 absence of reliable estimates of prognosis [1]. A significant diagnostic delay in ALS prevents

63 early treatment reducing the prospect of therapeutic success [2]. Mutant superoxide dismutase  
64 1 (SOD1G93A) transgenic mice models of ALS are widely used as surrogates of human  
65 pathology in pre-clinical research. A relatively uniform genetic background and the strictly  
66 controlled environmental and breeding conditions make SOD1G93A transgenic animals ideal  
67 models to investigate the disease pathobiology and the pre-symptomatic disease stage [3, 4].

68

69 Experimental evidence suggests that the rate of disease progression in ALS may be linked to  
70 the immunological response to neuronal degeneration, which is reproduced systemically by  
71 altered blood levels of cytokines, acute phase reactants and by an early down-regulation of  
72 FoxP3-positive T regulatory cells predominantly in patients with a faster disease progression  
73 [5, 6]. In SOD1G93A transgenic mouse models of ALS, symptomatic disease can develop  
74 with a slow and fast progression in C57 and 129Sv genetic backgrounds respectively (here  
75 defined as Sym-SOD1C57 and Sym-SOD1129S). In these SOD1G93A transgenic mice,  
76 impairment of the protein quality control in the spinal cord and the activation of the major  
77 histocompatibility complex I (MHCI) expression in axons and neuromuscular junctions are  
78 considered indices of fast disease progression [7, 8].

79

80 The inflammatory response in ALS co-exists with a state of deranged lipid metabolism and  
81 altered total daily energy expenditure [6, 9, 10], associated also with the effects of genetic  
82 mutations of the TDP-43 and C9orf72 genes linked to familial ALS [11-14]. Importantly, it is  
83 also acknowledged that functionally competent and high energy-demanding motor neurons  
84 are vulnerable to chronic inflammation and changes in metabolism [15-17] while aging, one  
85 of the main risk factors for ALS, may increase neuronal vulnerability with a decline in cell  
86 glucose uptake and mitochondrial energy production [18].

87

88 Over 50 randomized controlled clinical trials in ALS have been unsuccessful, despite many  
89 compounds having shown a disease-modifying effect in animal models [19-21]. There is  
90 therefore a critical need to improve translation of pre-clinical results into clinical trial  
91 outcomes in ALS, through the identification of molecular factors driving disease progression  
92 in humans and rodents which can be used for phenotypic stratification across species. To this  
93 end, a viable strategy is the molecular profiling of accessible biofluids and of Peripheral  
94 Blood Mononuclear Cells (PBMC), central to many aspects of the systemic immunological  
95 reaction to neurodegeneration [22, 23]. TMTcalibrator™, an unbiased proteomics method, is  
96 ideally placed to combine tissue and fluid proteomics in a single experiment, providing  
97 significant gains in sensitivity and a direct link between protein expression in diseased tissue  
98 and in matched fluids [24].

99

100 In this study, we have used TMTcalibrator™ to investigate biological features which are  
101 regulated in plasma and are also coherently expressed in matched PBMC samples from  
102 pre-symptomatic and symptomatic SOD1G93A transgenic mouse models of ALS with a fast  
103 (129Sv strain) and a slow (C57 strain) progression of the disease. We have used the same  
104 approach to test the plasma/PBMC proteome in fast and slow progressing ALS patients  
105 (defined as ALS-Fast and ALS-Slow), at an early and late stage of the disease. We have  
106 detected an early inflammatory and acute phase response in both human and animal model,  
107 while perturbed metabolism and an overall switch to cellular senescence is seen in fast  
108 progressing human pathology. We found only a partial overlap of the plasma/PBMC  
109 proteome between rodents and humans, suggesting a variability which is disease-stage  
110 dependent.

111

112 **METHODS**

## 113 **Animal models**

114 Female superoxide dismutase 1 (SOD1) transgenic mice with a G93A mutation in a  
115 C57BL/6J01aHsd (C57SOD1G93A) and in a 129SvHsd (129SvSOD1G93A) genetic  
116 background and wild-type female littermates (defined as WTC57 and WT129Sv) were used in  
117 this study (Jackson Laboratories, B6SJL-TgNSOD-1-SOD1G93A-1Gur). The development of  
118 the symptomatic stage of the disease differed significantly in the two SOD1G93A transgenic  
119 mouse strains, with regards to disease onset and duration [8, 25]. In this study, fast developing  
120 symptomatic SOD1G93A transgenic mice are defined as Sym-SOD1129Sv (129Sv pre-  
121 symptomatic mice as Pre-SOD1129Sv), while slow symptomatic progressing mice as Sym-  
122 SOD1C57 (C57 pre-symptomatic mice as Pre-SOD1C57). SOD1G93A transgenic animals  
123 expressed 20 copies of the human Gly93Ala SOD1 gene substitution in the C5701aHsd and  
124 129SvHsd backgrounds for more than 30 and 10 generations respectively. Mice were  
125 maintained in a specific pathogen-free environment (21°C temperature, 10% relative humidity  
126 in a 12 hour of light/dark cycle) with food/water supplied ad libitum and adaptations for  
127 animals with a substantial motor impairment. Mice of both strains were considered in a  
128 symptomatic stage when they exhibited a 50% decrease in latency of grip strength and a 45%  
129 body weight decline from the peak values in the pre-symptomatic stage [26]. Plasma samples  
130 from Wild type (WT) animals used in the re-test experiments were obtained at the same time  
131 of blood collection from both pre-symptomatic and symptomatic SOD1G93A transgenic  
132 animal models.

133

## 134 **Patient selection**

135 ALS patients and biological samples were selected from the ALS biomarkers study  
136 biorepository (Ethical approval: London and the City Research Ethics Committee 1  
137 09/H0703/27). Exclusion criteria were neuroinflammatory and neurodegenerative

138 comorbidities, recent mechanical injuries and/or infections, systemic autoimmune disorders,  
139 cancer, pro-thrombotic states, family history of ALS or frontotemporal dementia (FTD) as well  
140 as known genetic mutation linked to ALS or FTD. The Functional Rating Scale-Revised  
141 (ALSFRS-R; range 1 to 48, with increasing levels of neurological impairment with lower  
142 scores) was used to define the level of neurological impairment, with early ALS corresponding  
143 to a score  $> 40$  and late ALS  $< 35$ . Disease progression to last visit (PRL) was calculated as  
144 “48 - ALSFRS-R at the last visit, divided for disease duration from onset to the last visit in  
145 months” (fast ALS progression:  $PRL > 0.7$ ; slow ALS progression:  $PRL < 0.5$ )[6] .

146

#### 147 **Sample collection, plasma and mononuclear cell extraction and processing**

148 18 ml blood were collected in EDTA tubes and centrifuged at 800g for 10 minutes at room  
149 temperature. Plasma was recovered and centrifuged at 3500rpm for 10 minutes and stored at -  
150 80°C. Plasma samples were albumin and IgG depleted (ProteoPrep® Immunoaffinity kit).  
151 PBMC were isolated by density-gradient 2,000 rpm centrifugation for 40 minutes at 20°C using  
152 Lymphoprep™ (Alere) and subsequently washed in Dulbecco’s phosphate-buffered saline  
153 (Gibco). PBMC pellets were stored at -80°C in a freezing solution (10% DMSO in foetal  
154 bovine serum). After 24 hours, PBMC aliquots were transferred into liquid nitrogen. PBMC  
155 samples were thawed at 37°C and re-suspended in 10 ml warm (37°C) Dulbecco phosphate  
156 buffer. Cell suspension was centrifuged at room temperature and the pellet was re-suspended  
157 in 100 µl of lysis buffer (8 M urea, 75 mM NaCl, 50 mM Tris, pH 8.2, protease inhibitors  
158 cocktail, cOmplete™, Mini Protease Inhibitor Co, Sigma). Blood was collected from mice  
159 cheek and diluted in 3 ml medium (2.5 mM EDTA, 2% FBS in PBS) for PBMC and plasma  
160 fractions isolation as reported above. PBMC samples were lysed, sonicated and stored at -80°  
161 C.

162

163 **In-Solution Tryptic Digest, Tandem mass tag (TMT®) labelling and TMT**  
164 **calibrator™**

165 Protein quantification was carried out using a Bradford Protein Assay (Bio Rad). 100 µg (30  
166 µg for mice) protein from depleted plasma and 1 mg (750 µg for mice) from the PBMC pool  
167 were dried down in a vacuum centrifuge (SpeedVac, Thermo Scientific). Following  
168 solubilisation and denaturation in 100 mM Triethylammonium bicarbonate (TEAB)/0.1%  
169 (w/v) SDS), samples were reduced with 1 mM tris (2-carboxyethyl) phosphine 10 (TCEP) at  
170 55 °C for 60 minutes and alkylated with 7.5mM iodoacetamide at room temperature (RT) for  
171 60 minutes. Trypsin (MS grade, Promega) was added at a 1:25 (w/w) ratio to total protein and  
172 incubated at 37°C overnight. Digestion products were labelled with TMT®10plex reagents  
173 (Thermo Scientific) and incubated at RT for 60 minutes. Six of the 10 isobaric TMT® reagents  
174 were used to label individual plasma samples and the total amount of PBMC protein pool was  
175 divided among the remaining 4 TMT channels (the four-point calibrator) comprising 1/21,  
176 4/21, 6/21 and 10/21 total protein respectively. To quench the TMT® reaction, 0.25%  
177 hydroxylamine was added. The samples were then combined to form the analytical 10plex sets,  
178 desalted in RP18 columns and dried under vacuum. **The human plasma samples were divided**  
179 **into four TMT®10plex sets, two for the early and two for the late time point plasma samples.**  
180 **Each set contained three ALS-Fast and three ALS-Slow plasma samples in combination with**  
181 **the four-point PBMC pool lysate, used as calibrator. The same experimental design was applied**  
182 **to the animal model study, where four different TMT®10plex sets were used to compare 24**  
183 **samples in total. Two TMT®10plex sets included samples from transgenic mice, one for Pre-**  
184 **SOD1129Sv and Pre-SOD1C57, and the other one for Sym-SOD1129Sv and Sym-SOD1C57**  
185 **(n=3 for each genetic background plus 4 channels in each set for the PBMC calibrator). The**



186 remaining two TMT<sup>®</sup>10plex sets included plasma samples from the equivalent Wild type (WT)  
187 animals for both 129Sv and C57genetic backgrounds (n:3 per group), collected at the same  
188 time blood was taken from pre-symptomatic and symptomatic animal (additional file 2,  
189 supplementary fig 1 A, shows sample distribution across TMT<sup>®</sup>10plexes).

190

## 191 **Strong Cation exchange (SCX) Fractionation and liquid chromatography tandem** 192 **mass spectrometry (LC-MS/MS)**

193 Each analytical 10plex sample was reconstituted and loaded onto a Polysulfoethyl-A column  
194 (4.6 × 100mm, 5 μm, 200 Å, PolyLC) attached to a Waters 2695 HPLC. Quantitative analysis  
195 was performed using an Orbitrap Fusion<sup>™</sup> Tribrid<sup>™</sup> mass spectrometer in positive ion mode  
196 with an EASY nLC1000 system and 50cm EASY-Spray column (all Thermo Scientific). More  
197 details on the SCX and LC-MS/MS methodologies are reported in the additional file 1.

198

## 199 **Mass spectrometry analysis and computational proteomics**

200 All plasma samples and pooled PBMC lysates used as tissue calibrator passed sample quality  
201 control protocols (data not shown) and were divided into four TMT<sup>®</sup>10plex sets. All mass  
202 spectrometry files were inspected independently and passed internal quality control metrics  
203 (data not shown). Outputs of computational proteomics from the 40 raw data files from each  
204 TMT<sup>®</sup>10plex study (human and animal model study) were assembled into a single dataset and  
205 processed by Proteome Sciences' proprietary workflows for TMTcalibrator<sup>™</sup> including data  
206 integration (CalDIT), pre-processing and feature selection (FeaST). Normalised quantitative  
207 data were provided for all plasma peptides channels as a ratio against the calculated mid-point

208 value of the PBMC calibrator channels (see additional file 2, supplementary figure 1 describing  
209 the experimental workflow).

## 210 **Peptide identification and quantification**

211 For each experiment, raw mass spectrometry data files were submitted to Proteome Discoverer  
212 (PD) v1.4 (Thermo Scientific), using the Spectrum Files node. The spectrum selector was set  
213 to its default values while the SEQUEST-HT node was set to search data against the human or  
214 mouse (supplemented with the human SOD1 sequence) FASTA UniProtKB and Swiss-Prot  
215 database respectively. A more detailed description of the peptide identification and  
216 quantification process is reported in additional file 1.

217

## 218 **Data assembly, pre-processing and normalisation**

219 In the first step of the CalDIT workflow, reporter ion intensities were corrected to remove the  
220 contribution of signals from adjacent reporter ion channels. Subsequently, within each mass  
221 spectrometry run, intensity values across the four calibrant channels (129C, 130N, 130C, 131)  
222 were normalised (median-scaling) and, for each peptide spectrum match (PSM), a reference  
223 intensity value was calculated based on the calibrant intensity distribution. This reference value  
224 was then used to calculate  $\text{Log}_2$ -transformed PSM ratio values for each of the experimental  
225 channels, resulting in lower variance between PSM-level quantifications of the same peptide  
226 sequence at different points of the elution profile, or MS runs. To generate peptide expression  
227 (ratio) values, median values were then computed across quantified PSMs of the same peptide  
228 sequence.

229 In the first step of the FeaST workflow, peptides with more than ~ 35% missing quantitative  
230 values within an experimental group (*i.e.* ALS-Fast-Early; ALS-Fast-Late; ALS-Slow-Early;  
231 ALS-Slow-Late) were removed from subsequent analysis. Remaining peptides, with values

232 below the percentage threshold, were replaced by values imputed using the k-nearest  
233 neighbours (n=2) imputation method, applied to samples within each experimental group. In  
234 order to reduce the batch effect created by the use of multiple TMT<sup>®</sup>10 plexes, a LIMMA-  
235 based batch effect correction procedure was applied (additional file 2, supplementary figure 2),  
236 using a linear model constructed on the TMT<sup>®</sup> 10plex batch number and TMT<sup>®</sup> channel and  
237 specifying the experimental groups (PCA before batch effect correction additional file 2,  
238 supplementary figure 2). For protein-level analysis, the same procedure was applied in a  
239 parallel analysis and, subsequently, expression values were computed by averaging (trimmed  
240 mean, trim factor: 0.2) ratios of all non-phosphorylated peptides which matched uniquely to  
241 the gene identifier.

242

243 Two quality control metrics per sample were calculated: the median (measure of central  
244 tendency) and the inter-quartile range (IQR), measure of scale, using peptide and protein  
245 distributions. A sample was considered as a strong outlier if either QC metric value was more  
246 than three standard deviations from the overall mean.

247

## 248 **Immunoassays**

249 Expression analysis of 5 protein candidates in mouse and human plasma samples was  
250 undertaken by enzyme-linked immunosorbent assay (ELISA) using commercial kits, a  
251 electrochemiluminescence (ECL)-based Meso Scale Discovery (MSD) platform and by  
252 Western blot. Plasma samples were processed, aliquoted and frozen at -80°C within 1 hour  
253 from blood collection, according to standard consensus procedures. ECL-MSD was used to  
254 quantify Apolipoprotein E (APOE; R-PLEX Antibody Set F212I), Galectine-3 (R-PLEX  
255 Antibody Set F214F) and transforming growth factor beta-1 (TGFB1; U-PLEX kit

256 K151XWK), in human and mouse plasma, and Apolipoprotein A1 (ApoA1; R-PLEX Antibody  
257 Set F21PR) in human. A commercial ELISA was used to analyse ITGB3 and Apo A1 in mouse  
258 plasma (Biorbyt orb408222 and Abbexa abx254777 respectively) as well ITGB3 in human  
259 plasma (Biorbyt orb407522). Standards, primary and secondary antibodies, detection range  
260 including lower and upper limits of detection were specified in the manufacturer's conditions.  
261 Plasma samples from Wild-type (WT) and SOD1G93A transgenic mice (both pre-symptomatic  
262 and symptomatic), from ALS-Fast, ALS-Slow and healthy controls were equally distributed  
263 on each plate and measured in duplicate. Each plate contained a target-specific calibrator:  
264 APOA1 (0-1,000,000pg/ml for human and 7.81-500ng/ml for mouse); APOE (0-  
265 200,000pg/ml); Galectin-3 (03,000pg/ml), TGF $\beta$ 1 (0-56,600pg/ml) and ITGB3 (125-8000  
266 pg/ml for human 62.5-4000pg/ml for mice).

267 For Western blot analysis of ITGB3, 40  $\mu$ g of albumin-depleted proteins were diluted in  
268 Laemmli buffer and loaded onto 10% acrylamide gels. After electrophoresis, proteins were  
269 transferred onto nitrocellulose membranes and blocked with Tris buffered saline 0.1% Tween  
270 (TBS-T) containing 5% non-fat dry milk powder and -20 for 1h at room temperature.  
271 Membranes were then incubated overnight with rabbit anti-Integrin  $\beta$ 3 antibody 1:1000  
272 (Integrin  $\beta$ 3, D7X3P XP<sup>®</sup> Rabbit mAb #13166. Cell Signaling Technology, Inc.) and with  
273 anti-Galectin-3 antibody (Mouse monoclonal Galectin 3 ab2785, Abcam Ltd.) in TBS-T  
274 (0.1%) containing 5% bovine serum albumin (BSA) and further incubated with horseradish  
275 peroxidase (HRP)-conjugated swine anti-rabbit 1:2500 (Dako) as secondary antibody in TBS-  
276 T (0.1%) containing 5% BSA. Enhanced chemiluminescence (ECL kit; GE Healthcare), the  
277 ChemiDoc XRS+ imaging system and the image lab 5.2.1 (Bio-Rad) software were used for  
278 signal detection acquisition and analysis.

279

## 280 **Statistical analysis and data mining**

281 All statistical methods applied for the proteomic data analysis were performed using an in-  
282 house workflow called FeaST, developed in R statistical programming environment. Principal  
283 component analysis (PCA) score and loading plots were generated to study the variance  
284 structure of the data sets, indicating technical and biological factors and the influence of each  
285 step in the data pre-processing and normalisation workflow. Multifactorial linear modelling  
286 (LIMMA) was applied to determine significantly regulated features (peptides or proteins),  
287 using the following linear model:  $\log\text{Ratio} \sim \text{Class} + \text{Age} + \text{Stage} + \text{Group (ALS/Control)}$ .  
288  $\log_2$  fold changes (logFC) and p-values were calculated for all peptides and proteins that  
289 passed the filtering criteria described (above). The significance criterion  $\alpha$  was standardly set  
290 to 0.05 to consider a feature as “regulated”. Multiple testing was performed using a Benjamini-  
291 Hochberg correction.

292

293 Proteome Sciences’ proprietary workflows for functional analysis (FAT) were used to identify  
294 differences among phenotypic variants in biological processes extracted from plasma  
295 proteomes. Significance of *enrichment* was evaluated based on the results of Fisher’s Exact  
296 Test and multiple test corrections were applied (Benjamini-Hochberg). Functional terms used  
297 in this analysis included Gene Ontology Biological Processes and Reactome Pathways. Human  
298 and mouse-specific annotations were extracted from publicly-available data repositories. A  
299 minimum of two matched gene names was required and terms were considered significant. for  
300 a 3-group comparison.

301

302 For the immunoassay data, statistical analysis was performed using GraphPad Prism 6.  
303 Continuous variables were presented in median (interquartile range) and nonparametric  
304 analysis for group comparisons (with Dunn’s multiple comparisons test) as well as correlation

305 analysis were applied. We used log rank analysis (Mantel-Cox test) to compare survival (fixed  
306 date was used to censor data for survival analysis). Receiver operating characteristic curve  
307 analysis was used to assess assay sensitivity/specificity and diagnostic performance. A *p* value  
308 of less than 0.05 was considered statistically significant.

309

310 We have used a RNA-Seq transcriptome and splicing database of glia, neurons and vascular  
311 cells of the cerebral cortex to look at cell type-specific expression in the central nervous system  
312 (CNS) of Galectin-3, TGFb1 and ITGB3  
313 ([https://web.stanford.edu/group/barres\\_lab/brain\\_rnaseq.html](https://web.stanford.edu/group/barres_lab/brain_rnaseq.html)).

314

## 315 **RESULTS**

### 316 **Patients and controls**

317 17 patients with a diagnosis of possible, probable, laboratory-supported and definite ALS  
318 according to the El Escorial criteria [27] were enrolled in the discovery study (demographic  
319 and clinical features reported in Table 1A, B). Plasma samples from 12 of these, including six  
320 ALS-Fast (progression rate to last visit (PRL) >0.7; male/female ratio 3/3, average age at  
321 disease onset 61.7, 48-67; average disease duration to death or last visit 20.5 months (11-32))  
322 and six with a slow rate of progression (PRL<0.5; 5 male/1 female, average age at disease onset  
323 58.1, 35-71; average disease duration to death or last visit 107 months (97, 254)) were included  
324 in the exploratory proteomics as analytical samples, while PBMC samples isolated from blood  
325 donated by the remaining five patients were used in the tissue calibrant channels (PRL range:  
326 0.023 – 2.5; three male / two female, average age at disease onset 65.8, 57-68), Table 1).

327

328 Blood samples from an additional cohort of 47 ALS patients, including 24 ALS-Fast and 23  
329 ALS-Slow, and 29 healthy age and gender-matched controls were used to re-test plasma

330 expression of selected protein candidates using immunodetection. The average age of disease  
331 onset among the ALS sub-groups was comparable to the average age of sampling in the healthy  
332 control group (Table 1). The most common genetic mutations linked to familial ALS were  
333 excluded and all ALS patients had plasma CRP and ferritin levels within normal limits at the  
334 time of sampling (normal values: CRP < 5 mg/L; ferritin 10 - 160 µg/L) [6].

335

336

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338

<b>A. DISCOVERY EXPERIMENT: ALS PLASMA SAMPLES</b>									
ALS type	ALSFRS-R early	ALSFRS-R late	Gender M/F	Age at onset (years)	PRL	Time from onset to death or last visit (months)	Time from onset to first sample (months)	Time between first and last sample (months)	Site of disease onset limb/ bulbar
<i>Slow</i> (n:6)	43 (41-45)	31.5 (27-35)	5M / 1F	58.1 (35-71)	0.25 (0.1 – 0.4)	107 (97, 254)	76 (30-192)	58 (48-69)	4 limb /2 bulbar
<i>Fast</i> (n:6)	43.8 (41-46)	28.6 (22-35)	3M / 3F	61.3 (48-67)	1.5 (1-1.9)	20.5 (11-32)	10.2 (5-19)	13.8 (7-22)	3 limb /3 bulbar
<b>B. DISCOVERY EXPERIMENT: ALS PBMC SAMPLE TO FORM THE REFERENCE POOL</b>									
ALS Pool	ALSRS-R	Gender M/F		Age at onset (years)	PRL	Time from onset to death or last visit (months)	Time from onset to sampling (months)	Site of disease onset limb/ bulbar	
<i>Slow</i> (3) <i>Fast</i> (2) n:5	31 (22-42)	3M /2 F		65.8 (57-68)	0.8 (0.02–2.5)	64.6 (8-120)	19 (7 – 44)	3 limb /2 bulbar	
<b>C. RE-TEST EXPERIMENT: ALS PLASMA SAMPLES</b>									
ALS type	ALSRS-R	Gender M/F		Age at onset (years)	PRL	Time from onset to death or last visit (months)	Time from onset to sampling (months)	Site of disease onset limb/ bulbar	
<i>Slow</i> (n: 23)	39 (18-47)	18M / 5F		65.25 (37-86.9)	0.216 (0.03 – 0.47)	120.4 (28-335.2)	62.8 (7 -248.8)	15 limb /8 bulbar	
<i>Fast</i> (n: 24)	33 (13-43)	8M / 16F		62.8 (34.8-82.4)	1.48 (0.74 – 3.6)	28.1 (5 – 50)	14.8 (2.8 – 29.8)	13 limb /11 bulbar	
<b>D. RE-TEST EXPERIMENT: HEALTHY CONTROLS PLASMA SAMPLES</b>									
n: 29	Gender M/F				Age at sampling (years)				
	8M / 17F				60.9 (50.8 – 73)				

340

341

342



343

344

345 **Table 1. Demographics and clinical characteristics of ALS (A,B,C) and healthy control**  
346 **(D) individuals included in the discovery proteomics (A, B) and in the re-test**  
347 **immunoassays (C, D).**

348 The ALS functional rating scale revised (ALSFRS-R; 1 to 48, higher level of neurological  
349 disability with lower score) was used to define early (ALSFRS-R  $\geq$  40) and late (ALSFRS-R  
350  $\leq$  35) time points in the ALS cohort used for the discovery experiment. ALS patients were  
351 stratified according to disease progression rate to last visit (PRL: 48 minus the ALSFRS-R  
352 score at the last visit, divided per disease duration from onset in months) in slow (PRL $<$ 0.5)  
353 and fast (PRL $>$ 0.7). PBMC samples were extracted from blood taken from 5 ALS patients with  
354 a variable rate of disease progression (B). Age at onset and gender for both patients and healthy  
355 matched controls are also reported. All individuals belonged to the same ethnic group  
356 (Caucasian; except for one patients who was of Asian ethnicity). Duration of the disease from  
357 onset of first symptoms (e.g. weakness or speech impairment) to both sampling time and  
358 death/last visit is reported. For each sub-group, values are reported as mean (average).

359

### 360 **Exploratory analysis using the Feature Selection Tool (FeaST)**

361 Differentially regulated plasma protein signatures across the four phenotypic variants were  
362 analysed using principal component analysis (PCA), as illustrated in Fig. 1A. The strongest  
363 driver of variance in the data matrix was stage of disease (early vs. late), which was captured  
364 along the first principal component (Fig. 1A: x-axis) and accounted for approximately 36% of  
365 total variance. The rate of disease progression was defined by the second principal component  
366 (Fig. 1A: y-axis) and accounted for 15% of total variance (Fig. 1 B, C and D).

367

## 368 **Functional analysis**

369 Proteome Sciences' proprietary tool for functional analysis (FAT) was used to identify  
370 significantly enriched pathways and biological processes in the human and mouse proteomics  
371 data. A cross-sectional analysis was undertaken at the early (Fig. 2) and late (additional file 2;  
372 supplementary Table 1,1) time points individually using fast vs slow progressing ALS patients  
373 as terms of comparison. Fast and slow ALS phenotypic variants were also analysed separately  
374 in a longitudinal study, comparing early and late **time points (additional file 2; supplementary**  
375 **Table 1, (2-3)). The same analytical approach was used to process data from pre-symptomatic**  
376 **and symptomatic fast and slow progressing SOD1G93A transgenic animal models (additional**  
377 **file 2, supplementary Table 1(2-7): cross-sectional and longitudinal analysis; additional file 2**  
378 **supplementary Table 4: cross-sectional analysis between SOD1G93A transgenic animals and**  
379 **Wild type littermates )**

380

## 381 Pathway enrichment

382 To avoid redundancy, only those pathways with the highest enrichment values were shown  
383 when multiple closely related pathways were identified by FAT. The list of selected pathways  
384 is presented in descending order of statistical significance (Fig. 2). In the early stage (Fig. 2A),  
385 the strongest differential expression between fast and slow progressing ALS patients was  
386 related to proteins involved in ER to Golgi anterograde and retrograde transport, programmed  
387 cell death, the immune response linked to MHCII antigen presentation and to pathogenic  
388 Escherichia Coli infection. Pathways found to be regulated in both late stage and earlier time  
389 point plasma samples included cell cycle (mitosis), apoptosis, protein degradation, post-  
390 translational modifications and folding, parkin-ubiquitin proteasomal system, organelle  
391 maintenance and membrane transport linked pathways (organelle biogenesis and maintenance,

392 phagosome, and translocation of glucose transporter 4 (GLUT4) to the plasma membrane; Fig.  
393 2B). Pathways showing significant enrichment only at the later time point included cellular  
394 responses to stress, senescence, vesicular transport, RHO GTPases, RNA regulatory and  
395 metabolic processes (Fig. 2C).

396

397 When the slow and fast progressing ALS patients were analysed separately and longitudinally  
398 (Fig. 3), the most enriched pathways in slow progressors (Fig. 3A) included DNA damage and  
399 telomere stress induced senescence, inflammation and metabolism. In fast progressing ALS  
400 patients (Fig. 3C), changes in signal transduction and parkin-ubiquitin proteasomal system  
401 pathways were among the most enriched, followed by a cluster of five different pathways  
402 sharing a role in the immune response, RNA regulation and protein transport. Notably, in  
403 common with the cross-sectional study, the longitudinal analysis identified regulation of  
404 proteins involved in cell cycle and signalling, Rho GTPase, membrane trafficking, organelle  
405 mediated transport and translocation of GLUT4 to the plasma membrane (Fig. 3 B).

406

#### 407 Gene Ontology (GO) biological processes:

408 In the cross-sectional study, cytoskeleton organization was found enriched in both early and  
409 late time points while phagocytosis, epidermis development, membrane organization, innate  
410 immune response, ageing and cell division were among the most enriched biological processes  
411 only in the early time point. In the late time point, regulation of endopeptidase activity was the  
412 top enriched term along with fibrinolysis and proteolysis (additional file 2, supplementary Fig.  
413 3).

414

415 In the longitudinal study and in the fast progressing ALS patients, proteins involved in  
416 cytoskeletal organization, folding and stabilization, cell adhesion, negative regulation of

417 endopeptidase activity, innate immune response and substantia nigra development were within  
418 the most significantly enriched biological features (additional file 2, supplementary Fig. 4B).  
419 The analysis of the early versus late time points in the slow progressing patients did not show  
420 any significantly enriched biological process.

421

#### 422 Regulated protein candidates:

423 The early disease time point was chosen as the source of most informative differentially  
424 expressed plasma protein candidates when comparing ALS-Fast *versus* ALS-Slow (Table 2;  
425 proteins containing two or more peptides shown in a top-down order of fold-changes).

426 Additional file 2, table 1 shows the most regulated proteins comparing fast *versus* slow ALS  
427 patients in late disease (1), early versus late time points in both fast (2) and Slow (3)  
428 progressing ALS patients, as well as those identified in the same cross-sectional (4-5) and  
429 longitudinal (6-7) experiment in animal models. The most regulated proteins emerging from  
430 the comparison of pre-symptomatic and symptomatic SOD1G93A transgenic mice with their  
431 related genetic back-ground WT littermates are reported in additional file 2, table 3.

432 (*For reasons of space and length of the table, Table 3 has been included further down in the*  
433 *paper*)

434

#### 435 **Plasma proteomic profile in fast vs slow SOD1 G93A mouse models of ALS and** 436 **comparability with human pathology**

437 Functional analysis for enriched pathways and biological processes (GO terms) in the plasma  
438 proteome from the ALS animal models was performed in the same way as for human cases  
439 (additional file 2. Supplementary Table 2). Enriched features were compared with those seen  
440 in the human arm of the study. There was only a partial overlap in enriched pathways between  
441 the human and mouse proteome changes comparing data from the different time points in the

442 two species. Chaperonin mediated protein folding and mitotic processes (pre-symptomatic  
443 mouse model, early and late human ALS; Fig. 4A); RHO GTPase activators of IQGAPs  
444 (symptomatic mouse model, late human ALS) and apoptosis, anchoring of the basal body to  
445 the plasma membrane and AURKA Activation by TPX2 (symptomatic mouse model, early  
446 human ALS) (Fig. 4C) were among the pathways shared by human and mouse ALS.

447

448 However, four pathways; assembly of the primary cilium, organelle biogenesis and  
449 maintenance, regulation of PLK1 Activity at G2/M transition and translocation of GLUT4 to  
450 the plasma membrane were found enriched in both animal models and human disease at all  
451 stages considered (Fig. 4A/ 4C).

452

453 With regards to biological processes, cytoskeleton organization was the only feature enriched  
454 when comparing fast *versus* slow plasma proteomes from animal and human at the early and  
455 late time points (Fig. 4B/ 4D). The acute-phase response GO term was consistently enriched in  
456 the pre-symptomatic and symptomatic mouse proteome, but only enriched in the late stage of  
457 human disease. The dysregulation of proteolysis pathway was observed only at the late stage  
458 in both species. Phagocytosis engulfment was shared between the human early and the  
459 symptomatic mouse time points (Fig. 4D).

460

#### 461 **Immunodetection studies**

462 Using immunodetection, we have re-tested the plasma expression of protein candidates  
463 belonging to the regulated pathways identified by plasma TMT proteomic analysis in  
464 SOD1G93A transgenic mice and in ALS patients, including metabolic processes (APOE,  
465 APOA1), acute response, inflammation and cell senescence (ITGB3, Galectin-3, TGFB1).  
466 These proteins appear also as regulated in the proteomic analysis comparing WT and

467 SOD1G93A transgenic mice in both genetic back-grounds (additional file 2, **Supplementary**  
468 **Table 5**). The re-test experiment was performed using plasma samples from WT129Sv, Pre-  
469 SOD1129Sv, Sym-SOD1129Sv, WTC57, Pre-SOD1C57 and Sym-SOD1C57 mice (n:36, 6  
470 per sub-group) as well as from ALS patients of the discovery cohort (n:12), re-test cohort (n:  
471 47) and from healthy controls (n: 29; Table 1).

472

473 *SOD1G93A transgenic mice*: ITGB3 was significantly up-regulated in plasma from pre-  
474 symptomatic SOD1G93A transgenic mice of both fast (129S) and slow (C57) genetic  
475 backgrounds compared to both WT and symptomatic mice (Figure 5 A2; Pre-SOD1129Sv p=  
476 0.003, Pre-SOD157 p: 0.020). Within the same genotypes, ITGB3 expression decreased  
477 significantly in symptomatic SOD1G93A transgenic mice compared to pre-symptomatic  
478 (Sym-SOD1C57 p: 0.013; Sym-SOD1129S p: 0.039; Figure 5A). Galectin-3 was down-  
479 regulated in plasma from pre-symptomatic and symptomatic SOD1G93A transgenic mice with  
480 fast genetic background compared to their WT littermates (Pre-SOD1129Sv p: 0.009; Sym-  
481 SOD1129Sv p: 0.004; Figure 5A1), but not in the slow progressing genetic background (Figure  
482 5A1). TGFB1 plasma expression increased significantly in pre-symptomatic and symptomatic  
483 slow SOD1G93A transgenic mice compared to their WT littermates (Figure 5 A2: Pre-SOD157  
484 p: 0.013; Sym-SOD1C57 p: 0.001). Conversely, TGFB1 was down-regulated in pre-  
485 symptomatic and symptomatic fast SOD1G93A transgenic mice compared to their WT  
486 littermates (Pre-SOD1129Sv p= 0.039; Sym-SOD1129S p: 0.039). There was no statistically  
487 significant change of APOE and APOA1 plasma expression between WT and SOD1G93A  
488 transgenic mice of both genetic backgrounds (data not shown). For all 5 protein candidates,  
489 immunodetection supported the same pattern of expression found in proteomics.

490

491 Analysis of RNA expression of Galectin-3, TGFB1 and ITGB3 in a range of cortical cells [28]  
492 showed predominant microglia expression for all three proteins (Figure 5; B, B1, B2 -  
493 [https://web.stanford.edu/group/barres\\_lab/brain\\_rnaseq.html](https://web.stanford.edu/group/barres_lab/brain_rnaseq.html)).

494

495 *ALS patients:* APOE was up-regulated in plasma from ALS-Fast compared to ALS-Slow and  
496 healthy controls (p: 0.035 and p: 0.041 respectively; Figure 5C). Regression analysis showed  
497 a correlation between APOE plasma levels and PRL (p= 0.041; Figure 5C1). Kaplan Mayer  
498 analysis identified reduced survival in the higher APOE tertile compared to middle and lower  
499 tertiles (p: 0.0156; Figure 4A2) while diagnostic performance by ROC analysis showed APOE  
500 levels separate ALS-Fast from ALS-Slow (Area: 0.6759; Std. Error: 0.07162; p: 0.0204).

501 APOE analysis in plasma from the discovery cohort (n:12) showed a trend of over-expression  
502 in ALS-fast compared to ALS slow which did not reach significance (data not shown; p: 0.069).

503 There was no statistically significant regulation of Galectine-3, TGFb1, ApoA1 and ITGB3  
504 plasma levels at group analysis comparing healthy controls to ALS-FAST and ALS-Slow in  
505 both discovery and re-test cohorts, although a trend of up-regulation in ALS-Fast compared to  
506 ALS-Slow was seen in both cohorts for these protein candidates (data not shown).

507

508 ITGB3 and Galectin-3 WB analysis was performed on plasma samples from the discovery and  
509 re-test cohort, including ALS-Fast (n: 12), ALS-Slow (n:12) and healthy controls (n:10). WB  
510 showed a trend of up-regulation for both Galectin-3 and ITGB3 in ALS-Fast compared to ALS-  
511 slow which was not significant (data not shown). These results are in line but not in complete  
512 agreement with the level of differential regulation seen in the proteomic experiment of the early  
513 and late time points (ITGB3 adjusted p value: 0.001111; Galectin adjusted p value: 0.002369;  
514 Table 2, additional file 2 supplementary Table 1).

515

## 516 **DISCUSSION**

517 This study has three unique aspects: 1) a novel quantitative proteomics approach which  
518 combines tissue and fluid in a single experiment using TMT<sup>®</sup> labelling and liquid  
519 chromatography combined to mass spectrometry, 2) the use of PBMC, peripheral reporters of  
520 central neuro-inflammation [29] as source tissue for plasma biomarker identification and 3) the  
521 study of two species (human, mouse) with a comparable phenotypic heterogeneity of the  
522 disease.

523

524 We have observed that the rate of ALS progression, and above all the disease stage, are linked  
525 to substantial changes in the abundance of a wide range of plasma proteins. In ALS patients  
526 and animal models, regulated plasma proteins support a range of inflammatory events and  
527 metabolic modifications, while senescence-related alterations are mostly seen with the disease  
528 progression in the human ALS proteome. Studying similar phenotypic variants in mouse  
529 models and patients with ALS, we have identified only a partial overlap in the overall plasma  
530 proteomes acquired in time points which may not necessarily be comparable in the natural  
531 history of the disease in humans and rodents. Furthermore, the monogenetic driver of disease  
532 in an inbred animal model may only partially reflect the pathological processes of adaptation  
533 seen in sporadic human disease, explaining this discrepancy. It is nevertheless encouraging that  
534 we could dissect common features that could be developed as translational biomarkers to bridge  
535 pre-clinical and human studies.

536 The cross-sectional proteomic studies in human and animals were based on a relatively small  
537 sample size. The risk of obtaining non-specific biological signals was mitigated by the  
538 inclusion of phenotypic variants and disease time points to test the longitudinal profile of the  
539 disease, while a re-test experiment in larger cohorts of WT and transgenic animals as well as  
540 of ALS patients and healthy controls, helped to confirm the regulation of relevant protein



541 candidates observed using proteomics, particularly in mouse model. Further investigation of  
542 the regulated immuno-metabolic and senescence pathways showed how plasma expression of  
543 APOE differentiates ALS patients based on rate of disease progression, while the study of other  
544 biomarkers like ITGB3, Galectin-3 and TGFB1 confirmed the pattern of regulation across WT  
545 and SOD1G93A transgenic animal models seen in the proteomic experiment. The re-test study  
546 by immunodetection did not show the same degree of regulation for ITG3, Galectin-3 and  
547 TGFB1 shown in ALS-Fast compared to ALS-Slow using deep proteomics. This can be  
548 explained by the small sample size of the discovery cohort used in the proteomic experiment  
549 and by the concomitant high phenotypic (and genetic) variability of the disease in human. MS  
550 and antibody-based detection techniques have also different analytical sensitivity and  
551 specificity, which could also depend on the pre-analytical albumin depletion utilized in  
552 proteomics but not in ELISA, as albumin's chaperoning and protein-binding activity may affect  
553 the abundance of specific proteins [30]. In the more homogeneous animal models of ALS,  
554 proteomic and immunodetection provided comparable results.

555

### 556 **The plasma/PBMC proteome as a reporter of disease progression in human ALS**

557 PCA indicates that differences in the plasma proteome are more significant between early and  
558 late stages of disease, independent of speed of progression (Fig. 1). The selection of ALS  
559 patients in an "early" disease stage is complicated by unavoidable diagnostic delays and by  
560 poor detection of early signs of "phenoconversion" in asymptomatic individuals. Therefore, in  
561 our study, participants were enrolled and sampled at or shortly after diagnosis, and re-sampled  
562 after an interval of 6 to 24 and of 48 to 56 months for fast and slow progressing ALS patients  
563 respectively (fast and slow ALS patients had comparable ALSFRS-R at the late stage). The  
564 change of the plasma proteomic profile in this time-frame includes the early activation of the  
565 innate immune response (MCH II signalling), initiation of apoptosis and dysfunction of the

566 proteasome systems which only partially overlap with the pre-symptomatic mouse plasma  
567 proteome, while later alterations related to metabolism and gluconeogenesis as well as RHO  
568 GTPase activation are in common with the animal model proteomic (Fig. 2). In our study, RHO  
569 GTPase activity is closely linked to a marked regulation of the cytoskeletal proteins  
570 organization in both species. RHO GTPase binding to plasma membrane-associated actin  
571 cytoskeleton is fundamental to maintain cell-cell and/or cell extracellular matrix (ECM)  
572 adhesion [31]. The early immune response activation in our plasma proteome endorses  
573 previous reports of a systemic inflammatory response in ALS, involving  
574 monocyte/macrophages and a decrease of peripheral T-regulatory cells (Treg) particularly in  
575 fast progressing ALS patients, but may also relate to auto-immune disorders which are co-  
576 morbid or precede ALS as previously reported [5, 17, 32-34]. However, none of the cases in  
577 study had such a history and their blood levels of acute phase reactants, including CRP and  
578 ferritin, were within normal limits.

579

580 The prominent expression of proteins involved in the translocation of GLUT4 to the plasma  
581 membrane observed in all disease stages under investigation in human and mice, suggests an  
582 altered glucose uptake from the blood-stream and a dysmetabolic state (Fig. 2). Lactate, a by-  
583 product of cell metabolism which has been reported as raised in blood and CSF from ALS  
584 patients, is critically involved in glucose utilization for energy production [35-37]. Recently, it  
585 has been proposed that the adenosine triphosphate (ATP)-dependent lactate flow between  
586 muscle and neurons at the neuromuscular junction (NMJ) may become disrupted in ALS [38].  
587 Extracellular lactate has been shown to cause T cell entrapment at sites of inflammation by  
588 inhibition of CD4+ and CD8+ T cells motility, inducing chronic inflammation and a switch of  
589 CD4+ T helper cells to a Th17 phenotype with the release of pro-inflammatory cytokines [39].  
590 Hence lactate may facilitate chronic inflammation, a key feature of neurodegeneration, while

591 causing NMJ disruption and motor neuron death. Lactate dyscrasia and translocation of  
592 GLUT4 to plasma membrane have also been reported to interfere with muscle contraction by  
593 loss of GTPase activity [40-44]. The altered GLUT4 translocation and lactate dyscrasia are  
594 among those features potentially bridging human to animal pathology, representing a  
595 biologically plausible biomarker and treatment target in ALS.

596

### 597 **Inflammation, cell senescence and microglial biomarkers in ALS across species**

598 When the differential plasma proteomic profiles of pre-symptomatic and symptomatic  
599 transgenic animals were compared to early and late ALS cases, we found inter-species  
600 commonalities which included, among others, changes in the acute phase and innate immune  
601 responses, protein folding and GTPase Rho signaling (Figure 5). Further analysis of  
602 differentially regulated features in our study pointed to microglia activation: 1) the early up-  
603 regulation of proteins involved in apoptosis, phagocytosis and of the proteasome system  
604 reflecting microglia/macrophage activation which leads to removal of cellular debris and  
605 protein aggregates by phagocytosis [45] and 2) ITGB3, Galctin-3 and TGFB1 are selective  
606 brain microglia markers, according to a large RNA-Seq transcriptome and splicing database  
607 of glia, neurons, and vascular cells of the cerebral cortex  
608 ([https://web.stanford.edu/group/barres\\_lab/brain\\_error\\_3.html](https://web.stanford.edu/group/barres_lab/brain_error_3.html); Figure 5, B,B1,B2).

609

610 Integrins, Galectin-3 and TGFB1 have been implicated in different biological processes  
611 including angiogenesis, fibrosis and wound healing [46, 47]. These proteins are also central to  
612 the development of the acute phase response leading to chronic tissue injury [48], a molecular  
613 feature shared by animal and human ALS plasma proteomes. Critically, ITGB3 has been  
614 shown to be a key factor in cell senescence and in the interplay between membrane and ECM  
615 through a molecular cascade that includes activation of TGFB1 [49]. Our study also show

616 activation of telomere stress induced senescence with disease progression, implicating ITGB3  
617 and TGB1 further in replicative senescence, where telomere length becomes fundamental in  
618 loss of cellular homeostasis observed with aging [50]. Astrocyte over-expression of  
619 TGFB1 accelerates disease progression in ALS mice [51]. ALS skeletal muscle and plasma also  
620 show enhanced TGFB1 signaling, which can lead to muscle fibrosis [52, 53]. The up-regulation  
621 of ITGB3 and TGFB1 adds to our previous finding of increased levels of pro-inflammatory  
622 cytokines like IL-6 in plasma from ALS patients, a concerted response described as  
623 senescence-associated secretory phenotype [6, 54-56].

624

625

#### 626 **ALS: choosing the right therapeutic target and timing for intervention**

627 Any novel treatment strategy for ALS will have to be tested in a more personalized approach,  
628 using biomarkers and genetics for clinical stratification [57]. In humans, the lack of any mean  
629 of accurate prognostication makes ALS clinical heterogeneity the main obstacle to the  
630 development of effective treatments [32]. Conversely, in the relatively homogeneous *SOD1*  
631 animal models of ALS, phenotypic variations depend only on expression levels of mutant  
632 SOD1, gender, genetic background and possibly on breeding conditions, all features that can  
633 be manipulated in a controlled experimental setting [58].

634

635 Our study has shown a convergence between species in molecular mechanisms centered around  
636 regulation of the immune response and of cell metabolism. These shared regulated pathways  
637 support the potential for early immune-modulatory strategies, including the use of anti-  
638 inflammatory compounds, stem-cell based immune-effective treatment or protein kinase  
639 inhibitors, used to stem microglia-mediated neuroinflammation [21, 59, 60]. While fighting  
640 this innate immune response as early as possible may be plausible, it is worth considering that

641 macrophages at this stage and potentially throughout the disease course clear debris from cells  
642 undergoing degeneration. This “beneficial” inflammatory response may later shift towards a  
643 chronic and harmful process [6, 45, 61-65]. Therefore, timing of immunomodulation in ALS  
644 is crucial and interventions must aim at the window of therapeutic opportunity to arrest any  
645 development to chronic inflammation, which is an integral part of cell senescence and of the  
646 SASP response. Inducers of heat shock response have been shown to efficiently inhibit and  
647 reduce chronic inflammation in obesity and the same therapeutic paradigm may be relevant in  
648 neuroinflammation [66].

649

650 Our findings support also treatment strategies which target the enzymatic chain of glucose  
651 metabolism leading to the production of lactate, based on the combination of drugs that inhibit  
652 lactate accumulation at the NMJ and its effect in reducing T-cell migratory capabilities,  
653 enhancing respiratory chain function, and/or promoting re-innervation [38]. Interestingly,  
654 nutritional supplements acting at the interface between inflammation and metabolism have  
655 been shown to dampen the inflammatory environment. For example, omega-3 essential fatty  
656 acids decrease the levels of IL-1, IL-6, TNF $\alpha$  and CRP [67] and improve cognitive function in  
657 aged mice [68].

658

## 659 **CONCLUSIONS**

660

661 This study provides one of the most in-depth qualitative and quantitative proteomics studies  
662 performed in ALS, using the plasma/PBMC interface to investigate crucial aspects of  
663 phenotypic heterogeneity of this condition and across species. Researchers working on ALS  
664 and on other neurodegenerative disorders will be able to draw on the data we have generated  
665 to steer the development of novel biomarkers and therapeutic strategies in ALS.

666

667 **LIST OF ABBREVIATIONS**

ALS (amyotrophic lateral sclerosis)

ALSFRS-R (amyotrophic lateral sclerosis Functional Rating Scale-  
Revised)

CRP (C-reactive protein)

ECM (extracellular matrix)

EDTA (Ethylenediaminetetraacetic acid)

ER (Endoplasmatic Reticulum)

FBS (Fetal bovine serum)

FTD (frontotemporal dementia)

HRP (horseradish peroxidase)

IL (interleukin)

ITGB3 (integrin Beta 3)

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

LIMMA (linear models for microarray data )

logFC (Log2 fold changes)

MHC (major histocompatibility complex)

NMJ (neuromuscular junction)

PBMC (peripheral Blood Mononuclear Cells)

PBS (phosphate buffered saline)

PCA (principal component analysis)

PRL (disease progression to last visit )

PSM (peptide spectrum matches),

RT (room temperature)

SASP (senescence-associated secretory phenotype)

SCX (Strong Cation exchange)

SOD1 (superoxide dismutase 1)

TBST (tris buffered saline- tween)

TCEP (tris (2-carboxyethyl) phosphine 10)

TDP-43 (TAR DNA-binding protein 43)

TEAB (triethylammonium bicarbonate)

TGF- $\beta$ 1 (transforming growth factor beta 1)

TMT (tandem mass tag)

TNF $\alpha$  (tumor necrosis factor alpha)

## 668 **DECLARATIONS**

### 669 **Ethics approval and consent to participate**

670 Ethical approval was obtained from the East London and the City Research Ethics Committee  
671 1 (09/H0703/27). All participants provided written consent to the study (or gave verbal  
672 permission for a carer to sign on their behalf).

673 Procedures involving animals and their care were conducted in conformity with institutional  
674 guidelines that comply with national (Legislative Degree 26.03.2014) and international (EEC  
675 Council Directive 2010/63, August, 2013) laws and policies. Animals studies were approved  
676 by the Mario Negri Institute Animal Care and Use Committee and by the Italian Ministerial  
677 decree no. 84-2013.

### 678 **Consent for publication**

679 Not applicable

### 680 **Availability of data and material**

681 All data generated or analysed during this study are included in this published article and its  
682 supplementary information files as additional files.

683 Additional file 1 (pdf). Detailed protocols for Sample fractionation, LC-MS/MS analysis and  
684 peptide identification and quantification: methods are explained in more detail in this additional  
685 file.

686 Additional file 2 (pdf.) Supplementary figures and tables: four supplementary figures and 2  
687 supplementary tables are presented in this file. Supplementary figure 1 represents and scheme  
688 of the workflow followed to perform this study. Supplementary figure 2 shows the PCA before  
689 batch effect correction, Supplementary figure 3, illustrates the uncut and un edited western blot  
690 results together with the Ponceau staining for the membranes, supplementary figure 4 shows  
691 enriched biological processes for the cross-sectional and longitudinal study. Additional table 1  
692 shows the top regulated proteins in plasma from 1) fast versus slow progressing ALS patients  
693 at the late stage disease, early versus late time points for slow and fast progressing ALS patients  
694 and mouse model: cross sectional and longitudinal studies. Supplementary table 2 where the  
695 functional analysis of the animal model proteomic data are presented.

696 Additional file 3 (csv.) contains all the raw data generated in the human analysis

697 Additional file 4 (csv.) contains all the raw data generated in the animal model analysis.

698

### 699 **Competing interests**

700 The authors declare that they have no competing interests.

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#### 704 **Author contribution**

705 IZ is the lead author who has worked on the design, conduction of experiments and analysis of  
706 the data and contributed to manuscript writing. VL has performed all the Mesoscale assays and  
707 validation processes. MB and VM were responsible for data processing, bioinformatics method  
708 development and execution. MB has also developed the functional analysis tool employed in  
709 this study. GN and CB have been in charge of breeding and sampling of the animal model used  
710 in this study. RA, VL, OY and CHL have worked on the human samples collection, processing  
711 and storage, while contributing to sample selection, RA has also performed some of the Integrin  
712 beta 3 western blot experiments. EL, MW, PY contributed to the experimental design. AM and  
713 IP have conceived the study design and led on all the aspects including bio banking,  
714 experimental procedures, data interpretation and manuscript writing. AM was awarded he  
715 grant that has made this study possible. LG has contributed to the project design and  
716 supervision. AM was the group leader of this project leading on patient recruitment, guidance,  
717 paper writing and study design.

718

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724 the mass spectrometry data acquisition.

725

#### 726 **FIGURESLEGENDS AND TABLE**

727

728 **Figure 1. Distribution of differentially regulated features and ALS patient phenotypic**  
729 **variants:** (A) Principal component analysis (PCA) using **scores** plots before feature selection  
730 **(colour codes: fast-early: yellow; fast-late: green; slow-early: red; slow-late: brown).** The  
731 disease stage dimension is the main contributor to the separation in the first component  
732 (35.86%) and the rate of disease progression is the main contributor to the separation on the  
733 second component (15%). (B) PCA **loadings plot** generated with data derived from regulated  
734 features shows a more significant separation in the first compared to second component (40.6%  
735 and 17.5% respectively). (C) Volcano plot showing significantly regulated features comparing  
736 fast and slow progressing ALS patients in the early time point (cross-sectional study). (D)  
737 Volcano plot showing significantly regulated features comparing early and late time points for  
738 the slow progressing ALS patients (longitudinal study). Volcano plots agree with PCA showing  
739 a more significant difference between early and late time points when compared to fast versus  
740 slow disease progression.

741 **Figure 2. Functional analysis of the proteomic data obtained comparing data from fast**  
742 **versus slow progressing ALS patients.**

743 The cross-sectional analysis was based on the list of regulated proteins (FC 1.3, p value <  
744 0.05). Pathways with a p value < 0.05 were considered significantly enriched and plotted  
745 with a -log<sub>10</sub> transformed p value. Functional analysis was performed for Reactome  
746 pathways, if not otherwise specified. Only the pathways with the highest enrichment were  
747 reported among the selected redundant pathways (mostly cell cycle and mitosis (early) and  
748 RHO GTPase (late)). Significantly enriched pathways in the early stage (A), late stage (C)  
749 and in both time points (B).

750

751 **Figure 3. Functional analysis of the proteomic data obtained comparing early versus late**  
752 **disease stage.**

753 The longitudinal analysis was undertaken using the list of total regulated proteins (FC 1.3, p  
754 value < 0.05) in slow and fast progressing ALS individuals independently. Pathways with a p  
755 value < 0.05 were considered significantly enriched and plotted with a - log 10 transformed p  
756 value in descending order of statistical significance. Functional analysis was performed for  
757 Reactome pathways, if not otherwise specified. Only pathways with the highest enrichment  
758 were reported among redundant pathways. Significantly enriched pathways in slow  
759 progressing patients (A), in fast progressing patients (C) and shared by slow and fast  
760 progressing patients (B).

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762 **Figure 5. Re-test of protein candidates using immunoassays**

763 ELISA and Meso Scale Discovery (MSD) analysis of selected protein candidates in plasma  
764 samples. **A.** APOE is up-regulated in plasma samples from ALS-Fast compared to ALS-Slow;  
765 **A1:** positive correlation between APOE plasma levels and PRL in ALS patients; **A2:** reduced  
766 survival for ALS patient with higher APOE levels (above 70776 pg/ml). **B.** Galectin-3 is  
767 significantly downregulated in pre-symptomatic (Pre-SOD1129Sv) and fast in progressing  
768 SOD1G93A transgenic mice (Sym-SOD1129Sv) compared to 129Sv WT animals (WT129Sv).  
769 **B1.** TGFB1 is significantly downregulated in plasma from Pre-SOD1C57 and from Sym-  
770 SOD1C57 compared to WTC57, while it upregulated up-regulated in Pre-SOD1129Sv and  
771 Sym-SOD1129Sv compared to WT129Sv. **B2.** ITGB3 is up-regulated in plasma from pre-  
772 symptomatic transgenic SOD1G93 animal models (Pre-SOD1129Sv, Pre-SOD1C57)  
773 compared to related WT animals, while ITGB3 plasma expression in the respective  
774 symptomatic animals are significantly reduces to WT levels. In each figure, reported upper p-  
775 values relate to Kruskal-Wallis while lower p-values to Dunn's multiple comparisons tests.

776 C. data mining using a RNA-Seq transcriptome and splicing database of glia, neurons, and  
777 vascular cells of the cerebral cortex. This interactive splicing browser shows predominant  
778 microglia expression of Galectin-3 (C), TGFB1(C1) and ITGB3 (C2)  
779 ([https://web.stanford.edu/group/barres\\_lab/brain\\_rnaseq.html](https://web.stanford.edu/group/barres_lab/brain_rnaseq.html)) in a range of cortical cells[28].

780 PRL: progression rate to last visit.

781 WT: wild type.

782 OPC: oligodendrocyte precursor cells.

783 FPKM: fragments per kilobase of transcript sequence per million mapped fragments.

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787 **Figure 6. Human-animal model comparison of enriched pathways (A and C) and**  
788 **biological processes (B and D) derived from regulated proteins in each specie.** (A) Shows  
789 the pathways and (B) the biological processes that were found enriched in both the mouse  
790 model pre-symptomatic stage and in the human ALS proteome at an early and a late disease  
791 stage respectively. (C) Shows the pathways and (D) the biological processes found enriched in  
792 both the mouse model symptomatic stage and the human ALS proteome at an early and late  
793 disease stage respectively. Pathways and biological processes, including translocation of  
794 GLUT4 to the plasma membrane or acute-phase response, that were found regulated in both  
795 mouse model and human plasma proteome at all time points are indicated with an asterisk (\*).

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	<b>Protein ID</b>	<b>Protein Descriptions</b>	<b>Gene Name</b>	<b>Peptide #</b>	<b>Early _Fast/Early Slow Log Fold Change</b>	<b>adjusted p-value</b>
Up regulated in FAST.  Early stage.	P05106	Integrin beta-3	ITGB3	2	2.224407	0.001111
	O95810	Serum deprivation-response protein	SDPR	3	2.102	0.001403
	H7BYX 6	Isoform of P13591, Neural cell adhesion molecule 1	NCAM1	2	2.027403	0.000418
	P04003	C4b-binding protein alpha chain	C4BPA	7	2.013732	8.04E-05
	P07437	Tubulin beta chain	TUBB	2	1.908567	0.00790
	P63173	60S ribosomal protein L38	RPL38	2	1.727873	0.00652
	Q14697	Neutral alpha-glucosidase AB	GANAB	2	1.638215	0.00134
	P02649	Apolipoprotein E	APOE	9	1.513701	8.53E-05
	P36957	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate	DLST	2	1.487187	0.00960

		dehydrogenase complex, mitochondrial				
	A0A0A0 MR02	Isoform of P45880, Voltage- dependent anion-selective channel protein 2	VDAC2	4	1.4397	0.00229
	P05090	Apolipoprotein D	APOD	6	1.293627	0.00240
	P80723	Brain acid soluble protein 1	BASP1	4	1.220442	0.00111
	P15169	Carboxypeptidase N catalytic chain	CPN1	3	1.17725	0.00616
	Q06033- 2	Isoform of Q06033, Isoform 2 of Inter-alpha-trypsin inhibitor heavy chain H3	ITIH3	4	1.176924	0.00172
	P48740- 2	Isoform of P48740, Isoform 2 of Mannan-binding lectin serine protease 1	MASP1	2	1.126878	0.00134
	P35527	Keratin, type I cytoskeletal 9	KRT9	6	1.061453	0.00827
	Q15582	Transforming growth factor- beta-induced protein ig-h3	TGFBI	3	1.026849	0.00652
	P07355	Annexin A2	ANXA2	6	1.021165	0.00788
Down regulated in FAST Early stage.	P69905	Hemoglobin subunit alpha	HBA1	6	-1.12687	0.00682
	E7ETH0	Isoform of P05156, Complement factor I	CFI	3	-1.14553	0.00190
	O75636- 2	Isoform of O75636, Isoform 2 of Ficolin-3	FCN3	4	-1.16238	0.00616
	Q96IY4	Carboxypeptidase B2	CPB2	2	-1.23801	0.00134

	P05452	Tetranectin	CLEC3B	2	-1.32297	0.00173
	P30050	60S ribosomal protein L12	RPL12	2	-1.50426	0.00172
	P20700	Lamin-B1	LMNB1	2	-1.86089	0.00728
	P00505	Aspartate aminotransferase, mitochondrial	GOT2	2	-2.47293	0.00172
	P02042	Hemoglobin subunit delta	HBD	3	-2.49876	0.00040
	Q5T123	Isoform of Q9H299, SH3 domain-binding glutamic acid- rich-like protein 3	SH3BGR L3	2	-2.75577	0.0011

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803 **Table 2. Regulated proteins in fast compared to slow progressing ALS patients in the early**  
804 **disease stage.** Only proteins identified with at least two unique peptides are shown. Up-  
805 regulated proteins are shown in the upper part of the table (grey), with Integrin beta-3 showing  
806 the highest fold change. Down-regulated proteins are shown in the bottom part of the table  
807 (light blue), with Isoform of Q9H299, SH3 domain-binding glutamic acid-rich-like protein 3,  
808 showing the highest fold change of all down-regulated proteins.

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