1 **Diagnostic and prognostic significance of plasma and CSF**

2 **NfL, TDP-43, and tau in ALS**

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29 **Key words**: 1) Amyotrophic lateral sclerosis, 2) biomarker, 3) TDP-43, 4)

30 neurofilament light chain, 5) Simoa.

31 **List of Disclosure**

34 **Abstract**

35 Objective

36 To determine the diagnostic and prognostic significance of neurofilament light chain

37 (NfL), TAR DNA-binding protein 43 (TDP-43), and total tau (t-tau) in cerebrospinal

38 fluid (CSF) and plasma of patients with amyotrophic lateral sclerosis (ALS).

39 Methods

40 This was a single-center, prospective, longitudinal study. CSF and plasma samples were

41 collected at the time of enrollment from a discovery cohort of 29 patients with ALS and

42 29 age-matched controls without neurodegenerative disease. In a validation cohort,

43 there were 46 patients with ALS, and 46 control (not age-matched) patients with motor

44 weakness resulting from neuromuscular diseases. NfL, TDP-43, and t-tau levels in CSF

45 and plasma were measured using ultrasensitive single molecule assay (Simoa)

46 technology.

47 Results

48 The following findings were reproducibly observed among the discovery and validation

49 cohorts: increased levels of CSF NfL, plasma NfL, and CSF TDP-43 in ALS compared

50 with control groups; shorter survival associated with higher levels of CSF and plasma

51 NfL. When the CSF NfL and CSF TDP-43 levels were combined, the areas under the

52 ROC curves (AUC) were slightly improved relative to AUCs for each biomarker alone.

53 Conclusion

54 CSF and plasma NfL may not only serve as diagnostic biomarkers but also provide a

55 measure of disease progression. CSF TDP-43 is also useful as a diagnostic biomarker of

56 ALS, but has no prognostic value. The combined use of CSF NfL and CSF TDP-43 may

57   be a useful biomarker for the diagnosis of ALS.

58   Key words: Amyotrophic lateral sclerosis, biomarker, TDP-43, neurofilament light

59   chain, Simoa.

60 **Introduction**

61   There is an urgent need for molecular biomarkers in biofluids for the diagnosis of

62 amyotrophic lateral sclerosis (ALS) [1]. At present, the most promising biomarker for

63 ALS is neurofilament light chain (NfL). Elevated levels of NfL in CSF and blood

64 plasma/serum have been reported in patients with ALS compared with controls;

65 moreover, they were associated with poor outcomes [2-3]. TAR DNA-binding protein 43

66 (TDP-43) positive inclusions are found in approximately 97% of patients with ALS.

67 This has led to the investigation of TDP-43 as a potential molecular biomarker for ALS.

68 Overall, these studies have identified increased levels of TDP-43 in CSF from ALS

69 patients compared with controls [4]. An elevated level of TDP-43 has also been reported

70 in plasma from ALS patients in one case-control study [5]. However, the absolute

71 concentrations of TDP-43 in CSF and plasma have varied across studies, suggesting that

72 TDP-43 immunoassays are inconsistent for measuring this protein within biofluids [4].

73 The other candidate is Tau. Recent studies reporting elevated levels of CSF total-Tau (t-

74 tau) in ALS patients compared with controls have generated novel interest in the

75 diagnostic potential of t-tau for ALS [6, 7]. However, there are conflicting results [8, 9] and

76 the prognostic significance of plasma t-tau in ALS has so far received little attention.

77   Considering the lack of comprehensive analysis of these three biomarkers for ALS, we

78 conducted the present study to determine the diagnostic and prognostic potential of

79 TDP-43 and t-tau as molecular biomarkers, compared with NfL not only in CSF but

80 also in blood plasma.

81

82

83 # Methods

84 ***Study design, ethical approvals, and subject recruitment***

85   All study subjects provided written informed consent before participation and the

86 study protocols were approved by the University Ethics Committee (ERB-G-12, Kyoto

87 Prefectural University of Medicine, Kyoto, Japan). Informed consent from patients was

88 obtained when possible and also from the nearest relative. Study procedures were

89 designed and performed in accordance with the Declaration of Helsinki. The discovery

90 cohort consisted of 29 individuals with possible, probable, or definite ALS diagnosed

91 according to the revised El Escorial criteria (the ALS group of the discovery cohort) [10]

92 and 29 age-matched controls (the control group of the discovery cohort). All patients

93 with possible ALS when their CSF and plasma were measured, were confirmed to show

94 conversion to probable or definite ALS within the follow-up period. The control group

95 participants had non-neurodegenerative diseases and presented with no neurological

96 symptoms. They were enrolled from the registration for neurodegenerative and

97 dementia disorders in Kyoto Prefectural University of Medicine (KPUM) from

98 September 2009 to March 2014. All participants of the discovery cohort underwent CSF

99 and plasma collection. The sample size of the discovery cohort was set according to the

100 effect size of previous biomarker studies [11] [12]. The validation cohort comprised 46

101 individuals with suspected, possible, probable, or definite ALS diagnosed with the same

102 criteria as for the discovery cohort (the ALS group of the discovery cohort) and 46

103 patients with motor weakness resulting from neuromuscular diseases (the control group

104 of the validation cohort), comprising: chronic inflammatory demyelinating

105 polyneuropathy (CIDP: N=17), Gullain-Barre syndrome (GBS: N=18), multifocal

106 motor neuropathy (MMN: N=6), and inclusion body myositis (IBM: N=5). As described

107 above, suspected and possible ALS patients were confirmed to show conversion to

108 probable or definite ALS within the follow-up period. They were enrolled from KPUM

109 from April 2014 to May 2018. The sample size of the discovery cohort was set based on

110 the data from discovery cohort. Of note, not all participants in the validation cohort

111 provided both blood and CSF samples. Because relatively young individuals were

112 included in the control group, the ALS and control groups were not age-matched in the

113 validation. All measurements of the biomarkers were done on a Simoa HD-1 Analyzer

114 (Quanterix, Lexington, MA, USA) by commercially available kits. TDP43 kit used in

115 the study was developed with antibodies against the amino acid residues between 203 –

116 209 and the C-terminal region and therefore mainly target the C-terminal part of the

117 protein. For detailed information about plasma and CSF sampling, measurements of the

118 biomarkers as well as statistical analyses see supplementary methods.

119

120 *Bias*

121 Our data are from patients who agreed to participate in this study and agreed to receive

122 plasma collection or lumbar puncture for the diagnosis of ALS or other disorders.

123

124 *Data availability statement*

125 Any anonymized data not published in the article will be shared upon request from any

126 qualified investigator.

127

Results

Patient characteristics.

The demographic characteristics of the discovery and validation cohorts are summarized in Table 1 (for clinical information and raw data on biomarker concentrations, see Supplementary Tables 1 and 2). There was no significant difference in age (P=1.000) or sex (P=0.7840) between the ALS and control groups in the discovery cohort. In the validation cohort, the median age of the control group was significantly younger than that of the ALS group (P<0.0001), while there was no significant difference in sex between the two groups (P=0.3696).

137 **Table 1**

Category	Specific diagnosis	N	Sex(M:F)	Age
The discovery cohort				
ALS		29	18:11	65.41±12.34
Control (non-neurodegenerative control)	See Supplementary Table 1B	29	19:10	66.40±9.2
	Difference between the groups:		P=1.000	P=0.7840
The validation cohort				
ALS		46	29:17	71.36±9.27
Control (patients with motor weakness from neuromuscular diseases)		46	34:12	69.83 ±20.18
	Difference between the groups:		P=0.3696	P<0.0001
	CIDP	17	14:3	60.06±14.45
	GBS	18	11:7	50.67±23.80
	MMN	6	5:1	48.50±21.03
	IBM	5	4:1	76.00±2.45

138

139 GBS: Gullain-Barre syndrome, MFS: Millar-Fisher syndrome, CIDP: chronic

140 inflammatory demyelinating polyneuropathy, MMN: multifocal motor neuropathy

141

142 **Concentrations of biomarkers in the discovery cohort.**

143   The concentrations of TDP-43, NfL, and t-tau in the samples from the discovery

144 cohort are summarized in Figure 1. In the case of TDP-43, both plasma (P=0.0035,

145 Figure 1A) and CSF levels (P<0.0001, Figure 1B) of this marker were elevated in the

146 ALS group compared with the control group. This was also the case for NfL with

147 increased levels found in both plasma (P=0.0299, Figure 1C) and CSF (P<0.0001,

148 Figure 1D) from the ALS group compared with the control group. Finally, t-tau levels

149 were significantly lower in the ALS group only in plasma (P=0.0178, Figure 1E), and

150 not in CSF (P=0.1062, Figure 1F).

151 **ROC analysis of biomarkers in the discovery cohort.** (for data, see Supplementary

152 Figure 1)

153   According to ROC analysis of the discovery cohort, CSF NfL generated the highest

154 area under the curve (AUC) value (AUC=0.8347, Supplementary Figure 1D). The

155 second highest AUC value was observed with CSF TDP-43 (AUC=0.8205,

156 Supplementary Figure 1B).

157 **Correlation between levels of biomarkers in CSF and plasma in the discovery**

158 **cohort.** (for data, see Supplementary Figure 2)

159   There was a significant positive correlation between NfL levels of plasma and CSF

160 taken from each patient with ALS in the discovery cohort (solid line, P<0.0001,). Such

161 a significant CSF-plasma correlation was also identified in the control group (dashed

162 line, P=0.0013) (Supplementary Figure 2B). Neither TDP-43 nor t-tau levels showed

163 any plasma-CSF correlation in either of the groups (TDP-43 in the ALS group:

164 P=0.2279, TDP-43 in the control group: P=0.9252, t-tau in the ALS group: P=0.1024, t-

165 tau in the control group: P=0.3463) (Supplementary Figure 2A and C).

Biomarkers and survival times in the discovery cohort.

All members of the ALS group in the discovery cohort were included in log-rank

analysis (Figure 2). Nineteen patients reached the endpoint of death, tracheostomy, or

invasive ventilation during the follow-up period. Survival times ranged from 17 to

2,793 days (median: 575 days) (Supplementary Table 1B). Patients with ALS were

subdivided into two groups according to the levels for each of the biomarkers: a low-

level group (< median value), and a high-level group ($\geq$ median value). When

comparing the high and low level groups, significant differences were noted in plasma

NfL (P=0.0248, Figure 2C), CSF NfL (P=0.0207, Figure 2D), and CSF t-tau (P=0.0124,

Figure 2F), while there is no significant difference in plasma TDP-43, CSF TDP-43, or

plasma t-tau (Figure 2A, B, E). The high-level groups were associated with shorter

survival compared with the low-level groups, for plasma NFL, CSF NfL, and CSF t-tau.

After age-adjustment in multivariate analysis, the high levels of plasma and CSF NfL

still retained significant prognostic value (plasma NfL, Hazard ratio (HR) = 6.800,

P=0.003; CSF NfL, HR=7.727, P=0.002), while the association between CSF t-tau and

survival did not reach significance (CSF t-tau, HR=2.875, P=0.065).

Concentrations of biomarkers in the validation cohort.

The concentrations of TDP-43, NfL, and t-tau in the validation cohort are

summarized in Figure 3. On comparing ALS and control groups, significant elevations

of biomarker concentrations in the ALS group were reproduced for CSF TDP-43

(P=0.087, Figure 3B), plasma NfL (P=0.0031, Figure 3C), and CSF NfL (P<0.0001,

Figure 3D), while neither plasma TDP-43 nor plasma t-tau levels were different

between the groups, in contrast to those in the discovery cohort. CSF t-tau levels in the

ALS group were significantly higher than those in the control group in the validation

190 cohort, although such a difference was not observed in the discovery cohort. Those

191 significant differences were reproducibly confirmed by multiple comparison with the

192 Kruskal-Wallis test among the ALS group and subgroups of the controls (CIDP, GBS,

193 MMN, and IBM). Post-hoc analysis of Dunn's multiple comparison tests revealed

194 significantly higher levels of CSF TDP-43 in the ALS group compared with those in the

195 CIDP subgroup, CSF NfL in the ALS group compared with those in the CIDP and GBS

196 subgroups, and CSF t-tau in the ALS group compared with those in the CIDP subgroup.

197 Considering the age difference between the ALS and control groups, we reanalyzed

198 those comparisons after the exclusion of individuals younger than 60 years old

199 (Supplementary Figure 3). There was no significant difference in age between the ALS

200 (n=42) and control (n=24) groups, consisting of individuals aged no younger than 60. In

201 these advanced age groups, comparisons between groups regarding biomarkers showing

202 significant differences between the groups based on raw data (CSF TDP-43, CSF NfL,

203 plasma NfL, and CSF t-tau) were conducted. Significant elevation of CSF TDP-43 and

204 CSF NfL and plasma NfL levels in the ALS group compared with those in controls was

205 preserved (P=0.004 in Supplementary Figure 3A, P=0002 in Supplementary Figure 3B,

206 and P=0.0156 in Supplementary Figure 3C, respectively), while the difference between

207 the groups regarding CSF t-tau did not reach significance (Supplementary Fig. 3D).

208 **Biomarkers and survival times in the validation cohort.**

209   Not all patients with ALS in the validation cohort were included in the log-rank

210 analysis due to missing samples. We performed survival analysis involving 20 ALS

211 patients with plasma biomarker data and 41 ALS patients with CSF biomarker data

212 (Figure 4). In those patients, 10 patients in plasma biomarker analysis and 18 patients in

213 CSF biomarker analysis reached the endpoint. Survival times ranged from 28 to 1,592

214   days (median: 305 days) (Supplementary Table 2B). The high-level group showed

215   significantly shorter survival compared with the low-level group for plasma NfL

216   (P=0.0178, Figure 4C) and CSF NfL (P=0.0284, Figure 4D), corresponding with the

217   results in the discovery cohort. However, the significant difference in CSF t-tau was not

218   reproduced (Figure 4F). After age-adjustment, the high levels of plasma and CSF NfL

219   still exhibited significant prognostic values (HR=12.262, p=0.041 and HR=4.83,

220   P=0.01, respectively).

221   **ROC analysis of composite biomarkers in discovery and validation cohorts**

222   **regarding CSF TDP-43, CSF NfL and plasma NfL**

223   Regarding the CSF TDP-43, CSF NfL, and plasma NfL that showed significant

224   elevation in the ALS compared with control groups for both discovery and validation

225   cohorts, we calculated composite parameters of the products of CSF NfL x CSF TDP-

226   43, of CSF NfL x plasma NfL, and of plasma NfL x CSF TDP-43 (Figure 5). In both

227   cohorts, the composition of CSF NfL and CSF TDP-43 provided better performance in

228   terms of the AUC value compared to those in each biomarker alone (AUC=0.8430 and

229   0.9493 in the discovery and validation cohorts, respectively, whereas the

230   discriminability in the product of CSF NfL x plasma NfL was inferior to that in the CSF

231   NfL alone in the discovery cohort. The AUC value for composition of plasma NfL and

232   CSF TDP-43 (0.6813) could not exceed that in CSF TDP-43 alone. The combined

233   analyses for the CSF and plasma biomarkers in the validation were not performed

234   because more than half of participants of the validation cohort did not underwent both

235   plasma and CSF collection.

236   **Combined analysis of validation and discovery cohorts regarding plasma TDP-43,**

237   **CSF TDP-43, plasma t-tau, and CSF t-tau**

238   Regarding the levels of plasma TDP-43, plasma t-tau, and CSF t-tau, for which

239   inconsistent differences were found between ALS patients and controls when

240   comparing the two cohorts, we conducted a combined analysis based on data from

241   internal controls. Levels of plasma TDP-43 in the combined ALS group were higher

242   than those in the combined control group (P=0.0137). Levels of plasma t-tau were not

243   different between these groups (P=0.228), while CSF t-tau was significantly elevated in

244   the combined ALS group compared with the combined control group (P=0.0006)

245   (Figure 6). We also recalculated survival analyses in the combined ALS group for the

246   biomarkers. Both plasma and CSF NfL levels were associated with shorter survival

247   (P=0.0002 and P=0.0193, respectively). Those significances were still preserved after

248   age-adjustment (HR=7.611, P<0.001 and HR=4.567, P<0.001, respectively).

249   Meanwhile, there was no significant difference in survival between the high- and low-

250   level groups based on TDP-43 and t-tau levels in plasma and CSF (Figure 7).

251

Discussion

253   Biomarker profiles of TDP-43, NfL, and t-tau in ALS have been comprehensively

254   investigated [4]. However, most previous studies have focused on one or two of these

255   biomarkers. Moreover, the diagnostic or prognostic value of plasma TDP-43 or plasma

256   t-tau in ALS has remained uncertain because of the difficulty of stable measurement. To

257   the best of our knowledge, this study is the first to comprehensively measure levels of

258   all of these three candidate biomarkers, not only in CSF but also, simultaneously, in

259   plasma. The current study showed the following three major findings that were

260   consistent across the discovery and validation cohorts.

261   First, CSF NfL was significantly elevated in the ALS compared with control

262   groups. Furthermore, the potential prognostic value of elevated levels of CSF NfL, in

263   terms of shorter survival time, was observed after stratifying cohorts according to the

264   median CSF NfL levels. These confirm findings gathered in retrospective case-control

265   studies and prospective observations [2 3 13-18]. On the other hand, the AUC value used to

266   discriminate between ALS patients and controls in our study (0.8347) was slightly

267   lower than in a previous meta-analysis: 0.90; 95% confidence interval, 0.87–0.92 [18]. We

268   consider that this difference may be associated with the research design, control-group

269   choice, and ethnic differences.

270   Second, plasma NfL was significantly higher in the ALS group than in the controls,

271   and higher plasma NfL was associated with a shorter survival. Those results are in

272   agreement with observations in previous case-control studies using serum [17 19 20] and

273   plasma [14]. Overall, these findings support the possibility that NfL not only in CSF but

274   also plasma, can serve as a promising biomarker for the diagnosis and monitoring of

275   disease progression of ALS. The fact that CSF and plasma NfL shared the same

276   biomarker profile is reasonable when we consider the correlation between them in each

277   participant of the discovery cohort. Such plasma-CSF correlation in NfL has been

278   observed not only in patients with ALS [20] but also in patients with Alzheimer's disease,

279   multiple sclerosis, and control individuals [21 22]. The plasma-CSF correlation in our

280   controls was slightly irregular; actually, the association in the controls did not fit a linear

281   correlation, in contrast to that in the ALS group. This inconsistency may be due to

282   heterogeneity caused by the use of disease controls in this study.

283   Third, we noted significantly higher levels of TDP-43 in CSF of ALS patients than

284   those in controls. This result is consistent with previous observations, including two of

285 our studies and one meta-analysis [11] [12] [23-26]. TDP-43 is considered to be a disease-

286 specific biomarker reflecting TDP-43 pathology in the central nervous system As

287 expected, the AUC values, representing the ability to discriminate between ALS patients

288 and controls, were improved by combining CSF NfL with CSF TDP-43 relative to that

289 in each biomarker alone. This observation was consistently found across the both

290 cohort, suggesting that CSF TDP-43 could serve as a biomarker complementary to NfL

291 in the diagnosis of ALS. CSF NfL was recently reported to have a diagnostic potential

292 even for presymptomatic ALS [19]. However, at present, no one can predict which kind

293 of neurodegeneration will develop in individuals with elevated CSF NfL levels due to

294 its lack of disease specificity [27]. The combined use of CSF NfL and CSF TDP-43 may

295 be recommended for such people suspected to have neurodegeneration with

296 undetermined pathology. This biomarker-combination could also facilitate enrollments

297 of clinical trials toward preemptive therapy for ALS. Of note here, there is controversy

298 regarding the validity of the hypothesis that elevation of CSF TDP-43 is specifically

299 caused by TDP-43 proteinopathy. Immunoblotting shows that the identification of TDP-

300 43 in biofluids by the commonly applied antibody combinations used for quantification

301 represent a 45-kDa full-length form of TDP-43, rather than disease-specific truncated

302 forms [23] [28]. Therefore, no evidence has been reported to date that the elevation of CSF

303 TDP-43 detected by our method results from TDP-43 pathology. Taking these facts into

304 consideration, it is possible that increased CSF TDP-43 in ALS might simply be a

305 consequence of neuronal cell damage, similar to NfL. To develop a more disease-

306 specific biomarker in the future, measurements of C-terminal truncated or

307 phosphorylated forms of TDP-43, if possible extracted from neuron-derived exosomes,

308 would be ideal candidates.

309   Levels of plasma TDP-43, plasma t-tau, and CSF t-tau were significantly different

310   between the ALS and control groups in both the discovery and validation cohorts,

311   although the results were not preserved across these cohorts. In the combined analysis,

312   the significant elevation of plasma TDP-43 and CSF t-tau in the ALS group was

313   repeatedly observed, whereas the significant difference in plasma t-tau between the

314   groups was not reproduced. The significant elevation of plasma TDP-43 in the ALS

315   group agrees with one case-control study [5]. The previous measurement of plasma TDP-

316   43 based on conventional immunoassay had the problem of low sensitivity, and actually

317   failed to accurately quantify more than 70% of samples due to signals being lower than

318   the detection limit [5]. In contrast, we could detect measurable signals from the whole

319   plasma samples. This advantage may be due to the SIMOA analyzer, with 100- to

320   1,000-fold higher sensitivity than conventional assays [29]. This result provides evidence

321   supporting the potential diagnostic value of plasma TDP-43 for ALS as well as

322   usefulness of such new digital analytical platforms for the development of blood-based

323   biomarkers of the disease.

324   No difference in CSF levels of t-tau were found in the discovery cohort, while levels

325   of this biomarker were significantly elevated in the ALS group compared with the

326   controls in the validation cohort, and on combined analysis. Previous studies have

327   yielded similar inconsistent results regarding CSF levels of t-tau in ALS patients, which

328   ranged between normal [16, 8, 9, 30, 31] and increased levels [6 7, 32]. This inconsistency might

329   be linked to the inherent variability of the disease; for example, variability in release of

330   tau from motor neurons during disease progression. Thus, differences in the disease

331   stage and disease progression rate of enrolled patients may have contributed to the

332   variable findings of CSF t-tau. On the other hand, levels of plasma t-tau were

333 significantly lower in the ALS than control group in the discovery cohort, but this result

334 was not reproduced in the validation cohort or in the combined study. There is one

335 published case control study on plasma t-tau in patients with FTD and controls, in

336 which levels of plasma t-tau were not different between patients with pathogenic

337 mutations causing TDP-43 proteinopathy (i.e., mutation of *GRN* or *C9orf72*) but they

338 were significantly elevated in patients with *MAPT* mutations compared with controls.

339 Our results on plasma t-tau agree with this report in that plasma t-tau levels were not

340 different between patients with TDP-43 proteinopathy and controls.

341   In survival analysis all of the biomarkers except for plasma and CSF NfL failed to

342 exhibit any prognostic value, consistently across the discovery and validation cohorts.

343 We previously reported that lower CSF TDP-43 levels were correlated with shorter

344 survival [12]. However, the current study did not reproduce the results in the discovery

345 and validation cohorts, or on combined analysis. ~~This discrepancy may be due to the~~

346 ~~confounder that levels of CSF TDP-43 vary depending on the stage of ALS [11].~~ A recent

347 study argued that higher levels of CSF t-tau are associated with shorter survival [6]. This

348 result was consistent with that in our discovery cohort, but was not reproduced in either

349 the validation cohort or on combined analysis. This inconsistency may have been

350 caused by the shortness of the follow-up period in the validation cohort, which was

351 around half of that in the previous study. Longer observation would be needed to

352 validate the usefulness of CSF t-tau as a prognostic biomarker.

353   We acknowledge that the relatively small sample size was a major limitation of the

354 study. Furthermore, as mentioned above, the short follow-up period may have weakened

355 the statistical power to detect an association between survival and the biomarkers. In the

356 future, case-control as well as longitudinal studies involving sufficient numbers of

Commented [K1]: I have erased the sentence because stage depending difference of CSF TDP43 was not found in my analysis, although this finding was observed in our first report).

357 participants with a longer follow-up period will be necessary to confirm our findings

358 and promote the clinical application of biomarker-supported diagnosis and progression

359 monitoring of ALS.

360

Conclusions

361

362   This is the first study comprehensively analyzed the three candidate biomarkers for

363 ALS in CSF and plasma. NfL levels in CSF and plasma were significantly elevated in

364 the ALS patients compared with controls. Moreover, higher levels of those markers

365 were associated with shorter survival. Both may serve as not only diagnostic biomarkers

366 but also measures of disease progression. TDP-43 levels in CSF, which were increased

367 in the ALS patients compared with controls but were not associated with survival

368 periods, may only be useful as a diagnostic biomarker. The discrimination ability

369 between ALS and control was improved by the combined use of CSF TDP-43 and CSF

370 NfL, therefore CSF TDP-43 could serve as a biomarker complementary to NfL in the

371 diagnosis of ALS. Plasma TDP-43 and CSF t-tau may be elevated in ALS patients and,

372 therefore, be of diagnostic value; however, the present results still need future validation

373 in a larger cohort.

374

Author Contributions

375

376 T. O. and Y.K assisted with patient enrollment, data analysis, and interpretation. H.T.,

377 F.K-M., and M.S. performed laboratory work and data analysis. Y.T. and Y.N.

378 contributed to data collection. D.A. and T.M. participated in review and revision of the

379 manuscript. T.K. and T.T were involved with conceptualization and design of the study,

380   patient enrollment, data collection, interpretation of the data, and review of the

381   manuscript. All authors reviewed the drafts and approved the final version of the

382   manuscript.

383

384   **Competing interests and funding**

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397 **Figure 1**

398 Scatter plots of biomarkers levels in the discovery cohort.

399 ALS (n=29) and control (n=29). Levels of plasma and CSF TDP-43 (A, B), NfL (B, C),

400 t-tau (D, E) are presented. Bars indicate median values. The P-value generated by

401 Mann-Whitney's U test is shown above each graph. n.s: not significant.

402

403 **Figure 2**

404 Kaplan-Meier survival curves in ALS patients of the discovery cohort according to

405 biomarkers levels.

406 (A): plasma TDP-43, (B): CSF TDP-43, (C): plasma NfL, (D): CSF NfL, (E): plasma t-

407 tau, (F): CSF t-tau. The squares and circles indicate an event (death, tracheostomy, or

408 invasive ventilation). Patients were subdivided into two groups according to the cut-off

409 biomarker levels. The cut-off value in each graph was set as the median value of the

410 corresponding biomarker within the ALS group. The red lines with red squares

411 represent patients with levels of biomarkers no lower than the cut-off (the high-level

412 group). The black lines with black circles represent those with levels lower than the cut-

413 off (the low-level group).

414

415 **Figure 3**

416 Scatter plots of biomarkers levels in the validation cohort.

417 Control (n=46) and ALS (n=46). Levels of plasma and CSF TDP-43 (A, B), NfL (B, C),

418 t-tau (D, E) are presented. Bars indicate median values. The P-value generated by

419 Mann-Whitney's U test between the ALS and whole control group is shown above each

420 graph. Significant differences were reproducibly confirmed by multiple comparison

421   tests with the Kruskal-Wallis test among the ALS group and subgroups of the controls

422   (CIDP, GBS, MMN, and IBM). Dashed bars and asterisks indicate significant

423   differences (P<0.05) between the groups by post-hoc analysis of Dunn's multiple

424   comparison procedure. n.s: not significant.

425

426   **Figure 4**

427   Kaplan-Meier survival curves in ALS patients of the validation cohort according to

428   biomarkers levels.

429   (A): plasma TDP-43, (B): CSF TDP-43, (C): plasma NfL, (D): CSF NfL, (E): plasma t-

430   tau, (F): CSF t-tau. Patients were subdivided into two groups according to the cut-off

431   biomarker levels. The cut-off value in each graph was set as the median value of the

432   corresponding biomarker within the ALS group. The squares and circles indicate an

433   event (death, tracheostomy, or invasive ventilation). The red lines with red squares

434   represent patients with levels of biomarkers no lower than the cut-off (the high-level

435   group). The black lines with black circles represent those with levels lower than the cut-

436   off (the low-level group).

437

438   **Figure 5**

439   ROC analyses for the composite parameters of the discovery and validation cohorts.

440   AUC values are indicated in the graphs. The title of each graph represents the biomarker

441   used as an independent variable on analysis: (A): the products of CSF NfL and CSF

442   TDP-43 in the discovery cohort; the red and blue dotted lines respectively indicate the

443   ROC curves of CSF NfL alone and CSF TDP 43 alone for reference (see Supplementary

444   Figure 1 and 3 regarding the ROC analyses of each biomarker for details). (B): the

445 products of plasma NfL and CSF NfL in the discovery cohort; the red and blue dotted

446 lines respectively indicate the ROC curves of CSF NfL alone and plasma NfL alone.

447 (C): the products of plasma NfL and CSF TDP-43 in the discovery cohorts; the red and

448 blue dotted lines respectively indicate the ROC curves of CSF TDP-43 alone and

449 plasma NfL alone. (D): the products of CSF NfL and CSF TDP-43 in the validation

450 cohort; the red and blue dotted lines respectively indicate the ROC curves of CSF NfL

451 alone and TDP-43 alone.

452

453 **Figure 6**

454 Scatter plots of biomarkers levels in combined analysis of the discovery and validation

455 cohorts.

456 Analyses of plasma biomarkers involved 49 ALS patients and 47 controls; CSF

457 biomarker analyses involved 71 ALS patients and 68 controls. Levels of plasma and

458 CSF TDP-43 (A, B), NfL (B, C), t-tau (D, E) are presented. Because of inter-assay

459 variation, we corrected the values of the validation cohort based on the correction

460 formula: raw values x correction factors. The correction factors were determined as the

461 mean value ratios between the discovery and validation assays based on four internal

462 controls for each biomarker. Bars indicate median values. The P-value generated by

463 Mann-Whitney's U test between the ALS and whole control groups is presented above

464 each graph. n.s: not significant.

465

466 **Figure 7**

467 Kaplan-Meier survival curves in ALS patients on combined analysis of the discovery

468 and validation cohorts.

469   Correction of interassay variation was conducted using the formula presented in Figure

470   5. (A): plasma TDP-43, (B): CSF TDP-43, (C): plasma NfL, (D): CSF NfL, (E): plasma

471   t-tau, (F): CSF t-tau. Patients were subdivided into two groups according to the cut-off

472   biomarker levels. The cut-off value in each graph was set as the median value of the

473   corresponding biomarker within the ALS group. The squares and circles indicate an

474   event (death, tracheostomy, or invasive ventilation). The red lines with red squares

475   represent patients with levels of biomarkers no lower than the cut-off (the high-level

476   group). The black lines with black circles represent those with levels lower than the cut-

477   off (the low-level group).

478

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594 **Supplementary Data**

595 **Supplementary Table 1**

596 Clinical information and concentrations of biomarkers in the control and ALS groups of

597 the discovery cohort.

598

599 **Supplementary Table 2**

600 Clinical information and concentrations of biomarkers in the control and ALS groups of

601 the validation cohort.

602

603 **Supplementary Figure 1**

604 ROC analyses of the discovery cohort.

605

606 **Supplementary Figure 2**

607 Scatter plots of levels of TDP-43, NfL, and t-tau in plasma and CSF.

608

609 **Supplementary Figure 3**

610 Scatter plots of biomarkers levels in individuals aged no younger than 60 in the

611 validation cohort.