Clinical significance of cold-inducible RNA-binding protein in idiopathic pulmonary fibrosis

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43 **Abbreviations:** AE, acute exacerbation; CI, confidence interval; CIRBP, cold-inducible

44 RNA-binding protein; %DL_{CO}, percent predicted diffusing capacity of the lung carbon monoxide;

45 ER, endoplasmic reticulum; %FVC, percent predicted forced vital capacity; GAP, Gender–Age–

46 Physiology; HC, healthy control subject; HE, haematoxylin–Eosin stain; HR, hazard ratio; IPF,

47 idiopathic pulmonary fibrosis; OR, odds ratio; PaO_2, arterial oxygen pressure; ROC,

48 receiver-operating characteristic

49

Abstract

BACKGROUND: Idiopathic pulmonary fibrosis (IPF) is associated with a poor prognosis with variable clinical course. Early identification of patients at high risk for disease progression and death would lead to early therapeutic intervention and thereby improvement of outcomes. Cold-inducible RNA-binding protein (CIRBP) is produced in response to cellular stresses, which is implicated in multiple biological processes, including cell survival and proliferation.

RESEARCH QUESTION: Is CIRBP a useful biomarker for predicting the outcomes of patients with IPF?

STUDY DESIGN AND METHODS: This study included 95 and 93 patients with IPF from two independent hospitals (derivation and validation cohorts, respectively). The associations of serum CIRBP level upon IPF diagnosis with disease progression within 1 year after diagnosis (i.e. ≥10% relative decline in percent predicted forced vital capacity or death) and all-cause mortality were retrospectively analysed. Discrimination performances for predicting these outcomes were evaluated using the c-index.

RESULTS: Serum and lung tissue CIRBP levels were higher in patients with IPF than in control subjects. In the derivation cohort, the CIRBP [high] sub-group had significantly higher 1-year disease progression rates and lower cumulative survival rates than the CIRBP [low] sub-group, and the results were replicated in the validation cohort. In multivariate analyses, high serum CIRBP level was independently associated with higher 1-year disease progression and all-cause mortality rates in both cohorts. Combining the Gender–Age–Physiology and serum CIRBP models improved the c-indices for predicting 1-year disease progression and all-cause mortality compared with that of each model alone. The c-indices of serum CIRBP were particularly high in patients with Gender–Age–Physiology stage I.

INTERPRETATION: This study successfully validated that serum CIRBP level was an independent predictor of 1-year disease progression and all-cause mortality in IPF. CIRBP is a promising

75   biomarker that can help identify high-risk patients with IPF, especially in the early stage.

76   Idiopathic pulmonary fibrosis (IPF) is a progressive fibrotic interstitial lung disease that has a high

77   mortality, with a median survival time of 3–5 years.[1-5] Although the pathogenesis of IPF is not fully

78   understood, factors, including oxidative and endoplasmic reticulum (ER) stress, are considered to be

79   associated with lung fibrosis.[6,7] IPF has a natural history that is characterised by insidious decline in

80   lung function, resulting in progression to respiratory failure and death; however, its disease course is

81   highly variable and unpredictable.[8] Some patients have a rapid decline in lung function, whereas

82   others have a stable clinical course with slow disease progression even without treatment. The

83   current guideline suggested some therapeutic options, including antifibrotic agents and lung

84   transplantation,[4] but these treatments may be associated with the risk for adverse events, reduced

85   quality of life and increased medical costs. Noninvasive biomarkers that can predict disease

86   progression may be beneficial in guiding decisions regarding the appropriate treatment and optimal

87   timing. However, such a clinically applicable serum biomarker has not yet been identified.

88   Cold-inducible RNA-binding protein (CIRBP, also termed CIRP) is a stress response protein

89   that is ubiquitously expressed in various tissues, including the brain, lungs, heart and others, and is

90   induced by cellular stresses, such as cold shock, hypoxemia, oxidative stress and ER stress.[9-12]

91   Studies have shown that CIRBP is implicated in multiple biological processes, including cell survival,

92   proliferation, inflammation, apoptosis and telomere maintenance.[9-12] CIRBP is predominantly

93   located in the nucleus but can migrate to the cytoplasm or extracellular space in response to the

94   stresses, against which it plays a protective role.[10-12] Therefore, we hypothesised that in patients with

95   IPF, CIRBP may reflect the cellular stresses in the lungs and serve as a novel biomarker of disease

96   progression. The primary objective of this study is to investigate associations between serum levels

97   of CIRBP and disease progression in patients with IPF and validate the clinical significance of this

98   biomarker using two independent IPF cohorts.

99

100 **Methods**

5

101 ***Study population and diagnosis***

102 This retrospective study included consecutive patients with IPF and who had available stored serum

103 samples collected at the time of IPF diagnosis from two institutions, including the Hamamatsu

104 University Hospital (Hamamatsu, Shizuoka, Japan) between 2004 and 2016 (cohort 1, for derivation)

105 and the Tosei General Hospital (Seto, Aichi, Japan) between 2008 and 2016 (cohort 2, for validation).

106 The diagnosis of IPF was made via retrospective multidisciplinary discussion based on the current

107 international guideline.[5] Patients with severe comorbidities, including advanced malignancy, liver

108 cirrhosis, renal failure requiring dialysis or acute exacerbation of IPF (AE-IPF), at the time of IPF

109 diagnosis were excluded. AE-IPF was diagnosed retrospectively based on the 2016 International

110 Working Group report.[13] Consequently, a total of 188 IPF patients (n = 95 from the cohort 1 and n =

111 93 from the cohort 2) were enrolled. The observation period was calculated from the date of IPF

112 diagnosis until the last visit (the date of censoring or the date of death). Patients were censored if

113 they remained alive until 31 March 2020. All patients were regularly followed up for at least >1

114 year/until death. We included and collected serum samples from 50 healthy control subjects (HC)

115 without comorbidities, such as interstitial lung disease, advanced malignancy, liver cirrhosis, or renal

116 failure requiring dialysis. This study was conducted in accordance with the Declaration of Helsinki.

117 Signed consent forms were obtained from all study participants, except those who died before 2018.

118 The institutional review board of the Hamamatsu University School of Medicine waived the

119 informed consent requirement for deceased patients and approved this study (approval number

120 17-164).

121   Data pertaining to the following variables were collected from the medical records: age, sex,

122 smoking history, arterial oxygen pressure (PaO_2), percent predicted forced vital capacity (%FVC),

123 percent predicted diffusing capacity of the lung for carbon monoxide (%DL_{CO}), Gender–Age–

124 Physiology (GAP) stage,[14] high-resolution computed tomography, lung biopsy, treatment details, AE

125 and survival outcomes.

126

Serum and tissue CIRBP

128 Baseline serum samples were collected at the time of diagnosis and stored at −80°C until further

129 analysis. Serum CIRBP levels were retrospectively measured using commercially available

130 enzyme-linked immunosorbent assay (CircuLex Human CIRP ELISA Kit; MBL, Japan), according

131 to the manufacturer's instructions. The measurement range for this kit is from 0.4–25.6 ng/mL, with

132 a lower limit of detection of 0.201 ng/mL; concentrations higher than 25.6 ng/mL can be measured

133 by dilution. The CIRBP level in the serum CIRBP undetectable cases was assumed to be 0 ng/mL.

134 Formalin-fixed, paraffin-embedded sections (5 µm thickness) of surgically resected lung

135 biopsy specimens from patients with IPF were analysed. Normal lung tissues obtained from sites

136 distant from the lesion in patients who had lung cancer without IPF were used as control subjects.

137 The detailed methods are presented in e-Appendix 1.

138

Statistical analysis

140 Continuous and categorical variables were expressed as mean ± standard deviation or median

141 (interquartile range) and number (%), respectively. Welch's unequal variances t-test and Fisher's

142 exact test or Chi-square test were used for between-group comparisons. List-wise deletion was

143 performed when handling missing data. Correlation among the different variables was analysed

144 using the Pearson correlation coefficient. One-year disease progression was defined as ≥10% relative

145 decline in %FVC or death within 1 year after IPF diagnosis. Logistic regression analysis was used to

146 identify variables that were associated with 1-year disease progression. Cox proportional hazards

147 regression analysis with time-dependent covariates was used to identify the prognostic factors. For

148 assessing the association between serum CIRBP and these outcomes, GAP stage and antifibrotic

149 treatment exposure were included in multivariate analyses. Sub-distribution hazard analysis was

150 performed, according to the method of Fine and Gray, to identify the predictive factors of AE,

151   treating death as a competing event. Receiver-operating characteristic (ROC) curve analysis was

152   performed to identify the optimal cut-off value, which was decided as the point that had the highest

153   value of sensitivity + specificity – 1. The discrimination performance (c-index) for predicting 1-year

154   disease progression and mortality was evaluated using ROC and time-dependent ROC analyses,

155   respectively.[15] The cumulative survival rates were calculated using the Kaplan–Meier method; the

156   log-rank test was used to assess between-group differences. In all analyses, $P < 0.05$ was considered

157   statistically significant. All data were analysed using JMP version 13.2.1 (SAS Institute Inc., NC,

158   USA); R software version 4.0.2 (The R Foundation for Statistical Computing, Austria) and Prism

159   version 7.04 (GraphPad Software Inc., CA, USA).

160

161   **RESULTS**

162   *Characteristics*

163   The baseline characteristics of cohorts 1 and 2 are summarised in Table 1. Compared with cohort 2,

164   cohort 1 had more impaired lung function and more advanced GAP stages. In cohorts 1 and 2, 34

165   (35.8%) and 39 (41.9%) patients, respectively, met the definition of 1-year disease progression and

166   51 (53.7%) and 65 (70.7%), respectively, died during the observation period. The characteristics of

167   the HC are shown in e-Table 1.

168   The details of antifibrotic treatment, including pirfenidone and nintedanib, are presented in

169   e-Table 2. In cohorts 1 and 2, antifibrotic treatment was introduced to 29 (30.5%) and 42 (45.2%)

170   patients, respectively, within 1 year after IPF diagnosis and to 50 (52.6%) and 66 (71.0%) patients,

171   respectively, anytime during the observation period.

172

173   *Serum and tissue CIRBP*

174   Serum CIRBP level was detectable in 36 (37.9%) of the 95 patients in cohort 1 and 59 (63.4%) of the

175   93 patients in cohort 2, but in only 2 (4.0%) of the 50 HCs. The proportion of patients with

8

176   detectable serum CIRBP levels was higher in both cohorts 1 and 2 compared to the HCs ($P < 0.0001$

177   and $P < 0.0001$, respectively). The baseline serum CIRBP levels in cohorts 1 and 2 and the HC are

178   presented in Figure 1. The mean ± standard deviation of serum CIRBP levels in cohort 1, cohort 2,

179   and the HCs were 7.72 ± 12.7, 8.49 ± 10.9, and 0.32 ± 1.91 ng/mL, respectively. Serum CIRBP level

180   was higher in cohorts 1 ($P < 0.0001$) and 2 than in the HCs ($P < 0.0001$); however, the difference

181   between cohorts 1 and cohort 2 was not significant ($P = 0.6150$).

182   The results of immunohistochemical experiments are presented in Figure 2. CIRBP was

183   faintly expressed in the control lungs (n = 4) (Figure 2a) but was diffusely expressed mainly in the

184   fibrotic area of the lung tissues of patients with IPF (n = 9) (Figure 2b). Particularly, CIRBP was

185   strongly expressed in the nuclei of cells among and around the fibroblastic foci (Figure 2c).

186

187   ***CIRBP ^{high} and CIRBP ^{low} sub-groups***

188   ROC curve analysis was performed to identify the optimal cut-off value of serum CIRBP for

189   predicting 1-year disease progression in cohort 1 (e-Figure 1). Using 11.439 ng/mL as the cut-off

190   value of serum CIRBP, the sensitivity, specificity, positive predictive value, and negative predictive

191   value were found to be 44.1%, 85.2%, 62.5%, and 73.2%, respectively. Based on this cut-off value,

192   patients were categorised into the CIRBP ^{low} (serum CIRBP level < 11.439 ng/mL) and CIRBP ^{high}

193   (serum CIRBP level ≥ 11.439 ng/mL) sub-groups. Sensitivity analyses also supported this cut-off

194   value (e-Figure 2).

195   The baseline characteristics of cohorts 1 and 2 as per CIRBP category are presented in

196   e-Table 3 and e-Table 4, respectively. The 1-year disease progression rates were significantly higher

197   in the CIRBP ^{high} sub-group than in the CIRBP ^{low} sub-group in cohort 1 (62.5% vs. 26.8%, $P =$

198   0.0027). Using the same cut-off value, similar results were observed in cohort 2 (66.7% vs. 33.3%, in

199   the CIRBP ^{high} and CIRBP ^{low} sub-groups, respectively; $P = 0.0075$). Figure 3 shows the Kaplan–

200   Meier survival curves. In both cohorts 1 and 2 and in their pooled groups, the cumulative survival

201 rate was significantly lower in the CIRBP [high] sub-group than in the CIRBP [low] sub-group.

202

Serum CIRBP and outcomes

204 The correlations of CIRBP with the clinical parameters are shown in e-Table 5. There was no

205 reproducible correlation between serum CIRBP levels and clinical parameters, such as age,

206 PaO_2, %FVC and %DL_{CO} in cohorts 1 and 2.

207   The results of the logistic regression analysis of 1-year disease progression are presented in

208 Table 2. In cohort 1, the multivariate analysis, adjusted for GAP stage and antifibrotic treatment

209 exposure within 1 year of IPF diagnosis, demonstrated that high serum CIRBP level was

210 independently associated with high 1-year disease progression rate in both model 1, which evaluated

211 serum CIRBP as a continuous variable, and model 2, which compared the CIRBP [high] and CIRBP [low]

212 sub-groups. Similar results were observed in cohort 2. In the sub-analyses of patients for whom

213 antifibrotic treatment was introduced within 1 year after IPF diagnosis (i.e., treated patients) and

214 those for whom it was not introduced within 1 year after IPF diagnosis (i.e., untreated patients)

215 (e-Figure 3 and e-Table 6), multivariate analysis, adjusted for GAP stage, demonstrated that high

216 serum CIRBP level was not associated with 1-year disease progression in the treated patient cohort 1

217 but was an independent predictor of 1-year disease progression in the treated patient cohort 2 and

218 untreated patient cohorts 1 and 2.

219   The results of Cox proportional hazard analysis of mortality are presented in Table 3. In

220 cohort 1, multivariate analysis, adjusted for GAP stage and antifibrotic treatment exposure

221 (time-dependent covariate), in both models 1 and 2 demonstrated an independent association of high

222 serum CIRBP level with increased mortality. Similar results were demonstrated in cohort 2.

223   The results of the sub-distribution hazards analysis of AE development are presented in

224 e-Table 7. Serum CIRBP level was not significantly associated with the development of AE, even

225 after adjustment for GAP stage.

226

227 ***Serum CIRBP and GAP models***

228 Pooled analyses were performed to examine the potential clinical application of a GAP and CIRBP

229 combination model (e-Figure 4). In the pooled group of patients from cohorts 1 and 2 for whom GAP

230 stage at IPF diagnosis was available, the 1-year disease progression rate was higher in the following

231 order: patients with GAP stage III (n = 19), patients with GAP stage II (n = 41), and patients with

232 GAP stage I (n = 100) (68.4% vs. 43.9% vs. 31.0%, *P* = 0.0071). It was also higher in the CIRBP [high]

233 sub-group (n = 37) than in the CIRBP [low] sub-group (n = 123) (67.6% vs. 30.1%, *P* < 0.0001). The

234 c-index for predicting 1-year disease progression was 0.618 using the GAP model and 0.647 using

235 the serum CIRBP model, but it improved to 0.745 when the GAP and serum CIRBP models were

236 combined. Therefore, the performance of serum CIRBP model was evaluated at each GAP stage. In

237 patients with GAP stage I, the 1-year disease progression rate was higher in the CIRBP [high] sub-group

238 (n = 25) than in the CIRBP [low] sub-group (n = 75) (64% vs. 20%, *P* < 0.0001); the c-index of the

239 serum CIRBP model was 0.730. Further, patients with GAP stages II and III were evaluated together

240 owing to their small sample size. In patients with GAP stage II/III, the 1-year disease progression

241 rate tended to be higher in the CIRBP [high] sub-group (n = 12) than in the CIRBP [low] sub-group (n =

242 48) (75.0% vs. 45.8%, *P* = 0.1070); the c-index of serum CIRBP model was 0.593.

243   In the pooled group, the 2-year cumulative survival rate was lower in the following order:

244 patients with GAP stage III, patients with GAP stage II, and patients with GAP stage I (17.0% vs.

245 64.9% vs. 79.7%, *P* < 0.0001, e-Figure 5a). It was also found to be lower in the CIRBP [high] sub-group

246 than in the CIRBP [low] sub-group (41.9% vs. 76.7%, *P* = 0.0032, e-Figure 5b). In patients with GAP

247 stage I, the 2-year cumulative survival rate was lower in the CIRBP [high] sub-group than in the CIRBP

248 [low] sub-group (52.0% vs. 89.1%, *P* = 0.0061, e-Figure 5c). In patients with GAP stage II/III, the

249 2-year cumulative survival rate was lower in the CIRBP [high] sub-group than in the CIRBP [low]

250 sub-group (19.1% vs. 57.3%, *P* = 0.0273, e-Figure 5d). The discrimination performance for

251 predicting mortality is shown in Figure 4. The c-index was found to be higher using the GAP model

252 than the serum CIRBP model, and it improved when the GAP and serum CIRBP models were

253 combined (e.g., 0.662, 0.645, and 0.747 for predicting 2-year mortality; Figure 4a). The c-index

254 using the serum CIRBP model was particularly high when predicting mortality at 1.5–2 years in

255 patients with GAP stage I (Figure 4b).

256

257 **DISCUSSION**

258 To our best knowledge, this was the first study to determine the clinical significance of CIRBP as an

259 IPF biomarker to predict disease progression and mortality. In this study, serum and tissue CIRBP

260 levels were higher in patients with IPF than in the control group. Moreover, high serum CIRBP level

261 was an independent risk factor for 1-year disease progression and an independent prognostic factor

262 for death. These novel findings were successfully validated in the two independent IPF cohorts.

263 More interestingly, it was suggested that CIRBP measurements could help identify patients who had

264 rapid disease progression or high mortality, especially among those who were classified as GAP

265 stage I. Therefore, the combination of CIRBP measurements and the GAP model showed the

266 potential for further risk classification of patients with IPF.

267   In this study, immunohistochemistry demonstrated that CIRBP expression was strongly

268 expressed in the nuclei of cells among the fibrotic lesions, including the fibroblastic foci and cells

269 around the fibrotic lesions. These suggested that in patients with IPF, CIRBP may play a role in

270 fibrogenesis and may potentially come from fibrotic lung tissues. Moreover, the independent

271 association of high serum CIRBP with high 1-year disease progression rate may account for the high

272 mortality rate in patients with high serum CIRBP. Therefore, validation of these significant results on

273 two independent cohorts that had different baseline characteristics was the strength of this study. We

274 believed that CIRBP may be a clinically applicable and useful serum biomarker for predicting

275 disease progression and mortality in patients with IPF.

276   This study suggested that the combination of the GAP and serum CIRBP models could

277   improve the prognostic classification of patients with IPF; patients with high serum CIRBP had a

278   relatively high 1-year disease progression rate and a relatively low cumulative survival rate, and the

279   c-indices of serum CIRBP for predicting the outcomes were particularly high in those with GAP

280   stage I. The GAP model has been proven to be the most easy and applicable tool for predicting IPF

281   outcome. However, even patients with GAP stage I have been shown to have 15% to 20% 3-year

282   mortality rates.[14,16-19] Therefore, among patients with GAP stage I, identification of those with

283   high-risk for rapid disease progression and death would be clinically meaningful for facilitating early

284   therapeutic decisions (e.g., treatment induction). Moreover, the results showed that in patients with

285   GAP stage II/III, the CIRBP[high] group tended to have a higher 1-year disease progression rate than

286   the CIRBP[low] group (although there was no significant difference). In addition, the CIRBP[high] group

287   had a significantly lower cumulative survival rate than the CIRBP[low] group. However, owing to the

288   small sample size, we could not analyse patients with GAP stage II or III each. Therefore, further

289   investigation of the utility of CIRBP in patients with GAP stage II or III may be necessary.

290   Collectively, these results suggested that assessment of serum CIRBP can contribute to further

291   classification of patients with IPF, especially in GAP stage I, in terms of disease progression and

292   mortality. Therefore, earlier therapeutic intervention may be considered for patients with IPF and

293   high serum CIRBP, even those in the early stage of the disease. CIRBP may provide valuable

294   information for attending physicians who would need to weigh the benefits against the potential

295   burdens of treatment, including antifibrotic agents and lung transplantation.

296   The precise mechanism of enhanced CIRBP expression in the lungs with IPF is unknown.

297   Studies have revealed that CIRBP was up-regulated in response to cellular stresses, including

298   hypoxia, oxidative stress and ER stress;[9-12] such stresses were suggested to be involved in the

299   pathogenesis of IPF.[6,7] Therefore, it is speculated that serum CIRBP level may reflect the degree of

300   cellular stress in the IPF lungs and may play a regulatory role in the proliferation of fibroblasts/

301   myofibroblasts. In some reports, CIRBP was shown to accelerate cell-cycle progression in mouse

302   embryonic fibroblasts,[20] and its overexpression enhanced cell proliferation in mammalian kidney

303   cells.[21] Interestingly, in a mouse model of sepsis, deficiency of CIRBP attenuated lung injury.[22]

304   Conversely, the intravenous administration of recombinant CIRBP induced lung injury.[23] Future

305   basic researches on CIRBP are needed to elucidate its association with IPF pathogenesis and to

306   examine its potential as a therapeutic target in IPF.

307   The present study had several limitations. First, the retrospective design rendered it

308   vulnerable to several biases. The two IPF cohorts were from regional interstitial lung disease referral

309   centres, and this may have introduced an element of selection bias. Second, the changes in serum

310   CIRBP levels during the clinical course were difficult to evaluate. Therefore, future studies are

311   needed to analyse the relationships of changes in serum CIRBP levels to treatment and outcome.

312   Third, the cut-off CIRBP value for disease progression was applicable exclusively to this study

313   population. Further validation is needed to determine the optimal cut-off value for the general

314   population. Fourth, the results of the sub-analysis of treated patients should be interpreted with

315   caution due to differences in the timings of treatment induction, agents, and response to treatment

316   among each patient. Finally, in cohort 1, some $\%DL_{CO}$ data were missing; this may have influenced

317   the results of multivariate models.

318

319   Interpretation

320   In conclusion, the present study successfully validated that serum CIRBP level was independently

321   associated with 1-year disease progression and mortality in two independent IPF cohorts. This

322   promising biomarker may help identify patients who have a potential for rapid disease progression or

323   mortality, especially among those at an early stage of the disease, thereby facilitating therapeutic

324   interventions at an optimal timing. A prospective multicentre study is required in the future.

325

Acknowledgments

Guarantor statement

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411

412 **Take-Home Point**

413 **Question:** Can Cold-inducible RNA-binding protein (CIRBP), a stress response protein, be a useful

414 biomarker for predicting the outcome of patients with idiopathic pulmonary fibrosis (IPF)?

415 **Results:** The prognostic significance of patient classification based on serum CIRBP cut-off value

416 and the independent association of serum CIRBP levels with 1-year disease progression and

417 mortality were successfully validated by using two independent cohorts of patients with IPF.

418 Combining the Gender–Age–Physiology and serum CIRBP models improved the discrimination

419 performances to predict these outcomes, compared with each model alone, with the performances of

420 serum CIRBP being particularly high in patients with Gender–Age–Physiology stage I.

421 **Interpretation:** CIRBP is a promising biomarker that can help predict rapid disease progression and

422 high mortality in IPF and may serve to identify patients with high-risk IPF, especially in the early

423 stage.

424   18

425 **Table 1. Baseline characteristics and outcome**

	Cohort 1, n = 95	Cohort 2, n = 93
Age, years	70.3 ± 8.0	65.7 ± 8.3
Male/female	83 (87.4)/12 (12.6)	73 (78.5)/20 (21.5)
Smokers, former or current	76 (80.0)	62 (66.7)
Diagnosis, clinically/pathologically proven	72 (75.8)/ 23 (24.2)	45 (48.4)/ 48 (51.6)
PaO$_2$, Torr	76.6 ± 12.7	81.5 ± 13.4
%FVC, %	73.9 ± 20.0	80.9 ± 19.5
%DL$_{CO}$, % [a]	65.2 ± 24.8	59.8 ± 19.8
GAP stage, I/II/III [b]	33 (49.3)/19 (28.4)/15 (22.4)	67 (72.0)/22 (23.7)/4 (4.3)
One-year disease progression	34 (35.8)	39 (41.9)
10% relative decline in %FVC	19 (20)	31 (33.3)
Death	15 (15.8)	8 (8.6)
Observation period, months	33.5 (15.1–55.1)	45.6 (16.4–67.3)
AE [c, d]	26 (27.4)	26 (28.0)
Death [c]	51 (53.7)	65 (70.7)
from respiratory failure [c]	45 (47.3)	47 (51.1)

426 Data are presented as mean ± standard deviation, median (interquartile range) or number (%)

427 [a] cohort 1, n = 58; cohort 2, n = 93

428 [b] cohort 1, n = 67; cohort 2, n = 93

429 [c] During the observation period

430 [d] The 1-year cumulative AE incidence in cohorts 1 and 2 was 10.8% and 8.7%, respectively.

431 PaO$_2$, arterial oxygen pressure; %FVC, percent predicted forced vital capacity; %DL$_{CO}$, percent

432 predicted diffusing capacity of the lung for carbon monoxide; GAP, Gender–Age–Physiology Index;

433 AE, acute exacerbation

434 **Table 2. Logistic regression analysis of CIRBP for 1-year disease progression**

	Cohort 1, n = 95			Cohort 2, n = 93		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Model 1 [a]						
Univariate analysis	1.07	1.03–1.11	0.0014*	1.07	1.02–1.12	0.0063*
Multivariate analysis [c]	1.06	1.02–1.11	0.0053*	1.11	1.04–1.18	0.0011*
Model 2 [b]						
Univariate analysis	4.56	1.71–12.1	0.0024*	4.00	1.49–10.7	0.0058*
Multivariate analysis [c]	3.39	1.16–9.94	0.0259*	6.54	2.12–20.2	0.0011*

435 *$P < 0.05$

436 [a] CIRBP, per 1 ng/mL increase

437 [b] CIRBP [high] (serum CIRBP level ≥11.439 ng/mL) sub-group, vs. CIRBP [low] (serum CIRBP level <

438 11.439 ng/mL) sub-group

439 [c] Adjusted for Gender–Age–Physiology stage and antifibrotic treatment exposure within 1 year of

440 diagnosis

441 OR, odds ratio; 95% CI, 95% confidence interval; CIRBP, cold-inducible RNA-binding protein

442 **Table 3. Cox proportional hazards regression analysis of CIRBP for mortality**

	Cohort 1, n = 95			Cohort 2, n = 93		
	HR	**95% CI**	*P*-value	**HR**	**95% CI**	*P*-value
Model 1 [a]						
Univariate analysis	1.03	1.01–1.05	0.0063*	1.03	1.002–1.05	0.0299*
Multivariate analysis [c]	1.03	1.003–1.05	0.0258*	1.04	1.02–1.07	0.0005*
Model 2 [b]						
Univariate analysis	2.22	1.27–3.87	0.0049*	1.76	1.05–2.95	0.0321*
Multivariate analysis [c]	1.96	1.07–3.56	0.0275*	2.10	1.24–3.55	0.0057*

443 *$P < 0.05$

444 [a] CIRBP, per 1 ng/mL increase

445 [b] CIRBP [high] (serum CIRBP level ≥11.439 ng/mL) sub-group, vs. CIRBP [low] (serum CIRBP level <

446 11.439 ng/mL) sub-group

447 [c] Adjusted for GAP stage and antifibrotic treatment exposure (time-dependent covariate)

448 HR, hazard ratio; 95% CI, 95% confidence interval; CIRBP, cold-inducible RNA-binding protein
449

450 **FIGURE LEGENDS**

451 **Figure 1. Baseline serum CIRBP levels**

452 Serum CIRBP was detected in 36 (37.9%) of the 95 patients in cohort 1, 59 (63.4%) of the 93

453 patients in cohort 2, and 2 (4.0%) of the 50 HCs. The proportion of patients with detectable serum

454 CIRBP levels was higher in both cohorts 1 and 2 than in the HCs ($P < 0.0001$ and $P < 0.0001$,

455 respectively). When the CIRBP level in the patients with undetectable serum CIRBP levels was

456 assumed to be 0 ng/mL, the mean ± standard deviation of serum CIRBP levels in cohort 1, cohort 2,

457 and the HCs were 7.72 ± 12.7, 8.49 ± 10.9, and 0.32 ± 1.91 ng/mL, respectively. Serum CIRBP

458 levels were higher in cohorts 1 ($P < 0.0001$) and 2 than in the HCs ($P < 0.0001$), but the difference

459 between cohorts 1 and cohort 2 was not significant ($P = 0.6150$).

460 CIRBP, cold-inducible RNA-binding protein; HC, healthy control subject

461

462 **Figure 2. Representative images of CIRBP immunostaining of lung specimens**

463 a) A control lung showing a faint CIRBP expression (arrow)

464 (×100 magnification, scale bar: 50 µm)

465 b) A lung tissue from a patient with IPF showing a diffusely positive CIRBP expression in the

466 fibrotic area (×40 magnification, scale bar: 200 µm)

467 c) A lung tissue from a patient with IPF showing CIRBP expression in the nuclei of the cells among

468 (arrowhead) and around (arrow) a fibroblastic focus (original magnification ×100, scale bar: 50

469 µm)

470 CIRBP, cold-inducible RNA-binding protein; HE, Haematoxylin–Eosin stain; IPF, idiopathic

471 pulmonary fibrosis

472

473 **Figure 3. Kaplan–Meier survival curve**

474 The 2-year cumulative survival rate was significantly lower in the CIRBP [high] sub-group than in the

475   CIRBP [low] sub-group in a) cohort 1 (39.4% vs. 73.5%, P = 0.0183 by the log-rank test), b) cohort 2

476   (45.8% vs. 74.8%, P = 0.0299 by the log-rank test), and c) their pooled groups (44.8% vs. 74.1%, P

477   = 0.0030 by the log-rank test).

478   CIRBP, cold-inducible RNA-binding protein

479

480   **Figure 4. Discrimination performance of each model for predicting mortality based on**
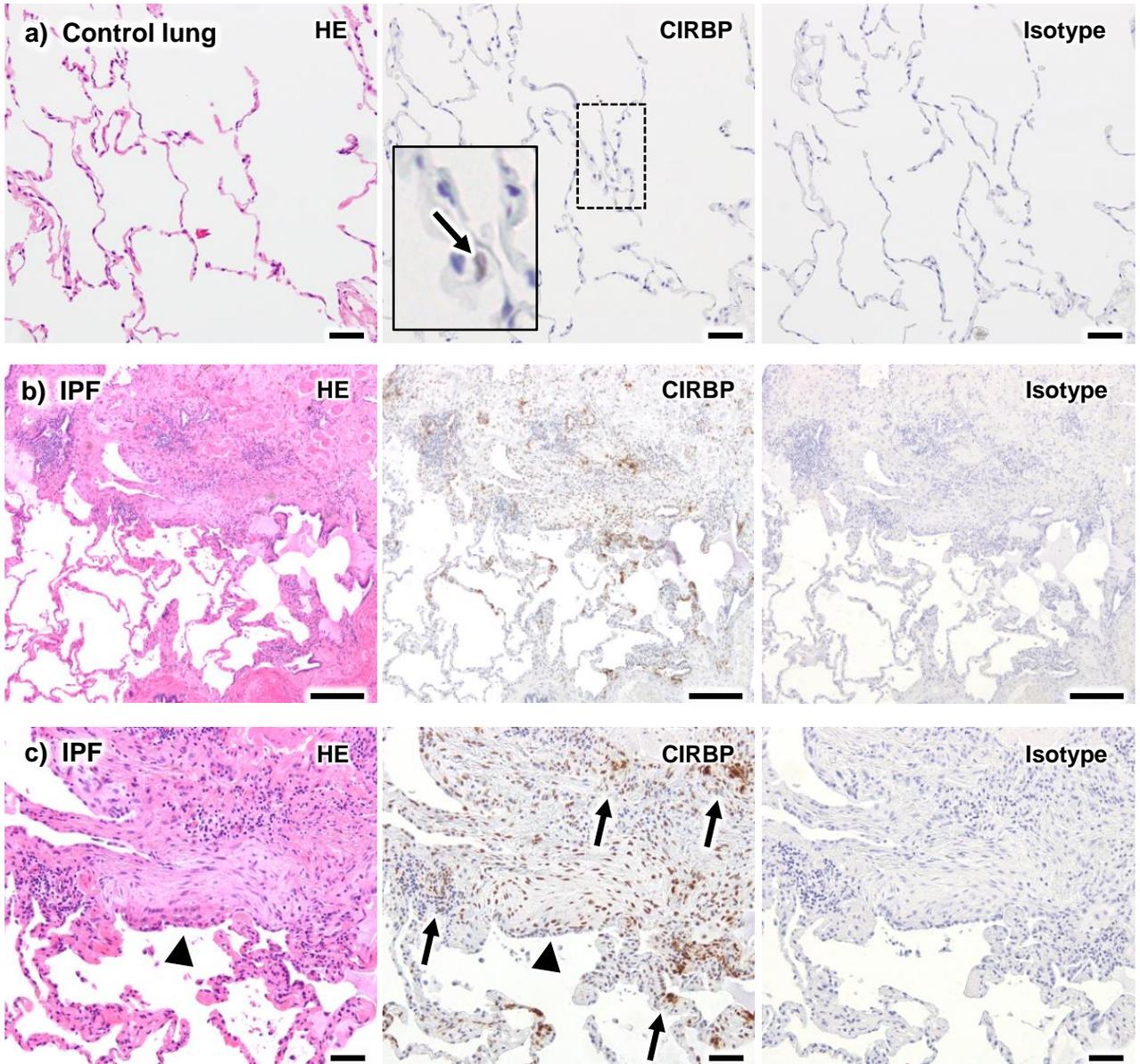
481   **time-dependent ROC analysis**

482   a)   The c-indices of the serum CIRBP model, GAP model, and combination of CIRBP and GAP

483   model for predicting 2-year mortality were 0.645, 0.662, and 0.747, respectively.

484   b)   The c-indices of the serum CIRBP model for predicting 2-year mortality in patients with GAP

485   stage I and those with GAP stage II/III were 0.712 and 0.663, respectively.
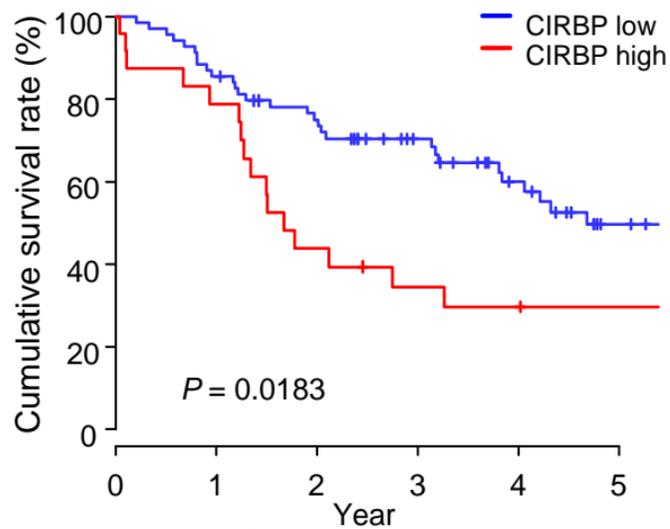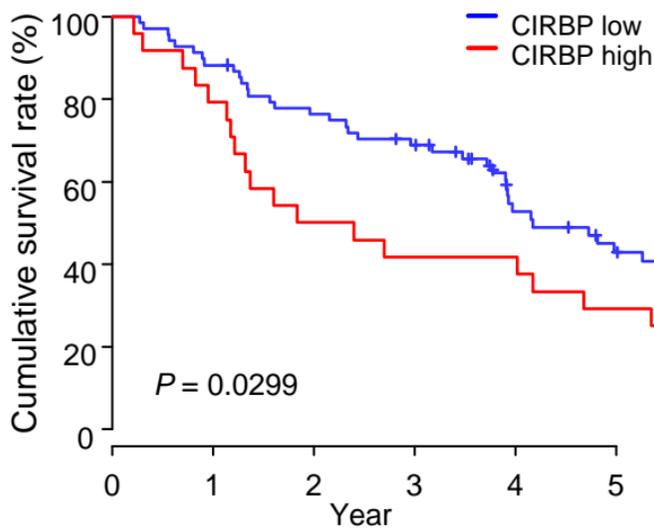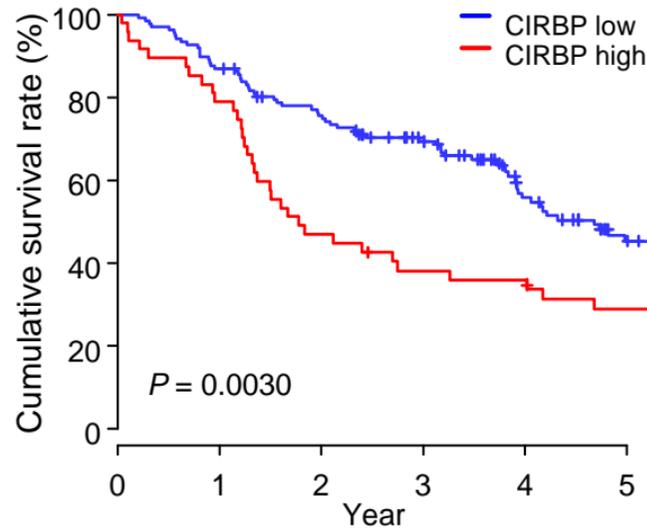
486

Figure 1

Figure 2

Figure 3

a) Cohort 1

b) Cohort 2

c) Pooled

a) Cohort 1 — Cumulative survival rate (%) vs Year; CIRBP low, CIRBP high; $P = 0.0183$

Number at risk

	0	1	2	3	4	5
CIRBP low	71	59	49	36	25	12
CIRBP high	24	18	10	7	6	5

b) Cohort 2 — Cumulative survival rate (%) vs Year; CIRBP low, CIRBP high; $P = 0.0299$

Number at risk

	0	1	2	3	4	5
CIRBP low	69	60	51	45	28	21
CIRBP high	24	19	12	10	10	7

c) Pooled — Cumulative survival rate (%) vs Year; CIRBP low, CIRBP high; $P = 0.0030$

Number at risk

	0	1	2	3	4	5
CIRBP low	140	119	100	81	53	33
CIRBP high	48	37	22	17	16	12

Figure 4

Supplemental materials

Title: Clinical significance of cold-inducible RNA-binding protein in idiopathic pulmonary fibrosis

Authors: Hironao Hozumi, M.D., Ph.D., Kensuke Kataoka, M.D., Ph.D., Yasuhiro Kondoh, M.D., Ph.D., Takuya Isayama, Jun Okada, Katsunori Sugiura, Kazutaka Mori, M.D., Ph.D., Masato Kono, M.D., Ph.D., Yuzo Suzuki, M.D., Ph.D., Masato Karayama, M.D., Ph.D., Kazuki Furuhashi, M.D., Ph.D., Noriyuki Enomoto, M.D., Ph.D., Tomoyuki Fujisawa, M.D., Ph.D., Naoki Inui, M.D., Ph.D., Yutaro Nakamura, M.D., Ph.D., Takafumi Suda, M.D., Ph.D.

e-Appendix 1

The sections were deparaffinised and preheated for 30 minutes in Tris/EDTA buffer (pH 9.0). After blocking the endogenous peroxidase activity using 3% H_2O_2 for 15 minutes, the sections were incubated overnight at 4°C either with a primary rabbit anti-human CIRBP monoclonal antibody (1:1000; Anti-CIRP antibody; Abcam, Cambridge, UK) or with an isotype control IgG. Subsequently, the sections were incubated with a visualisation reagent (Histofine simple stain MAX-PO [M]; Nichirei, Tokyo, Japan) for 30 minutes. The immunoreaction was visualised using 3, 3-diaminobenzidine chromogen and the sections were counterstained with haematoxylin.

e-Table 1. Characteristics of healthy control subjects

	Healthy control subjects, n = 50	*P*-value (vs. cohort 1)	*P*-value (vs. cohort 2)
Age, years	65.6 ± 4.6	<0.0001	0.9558
Male/female	41 (82)/9 (18)	0.4579	0.6690

Data are presented as mean ± standard deviation or number (%)

e-Table 2. Antifibrotic treatment

	Cohort 1, n = 95	Cohort 2, n = 93
Introduced within 1 year after IPF diagnosis	29 (30.5)	42 (45.2)
Pirfenidone	26 (27.4)	37 (39.8)
Nintedanib	3 (3.2)	5 (5.4)
Anytime during the observation period	50 (52.6)	66 (71.0)
Pirfenidone	43 (45.3)	53 (57.0)
Nintedanib	12 (12.6)	22 (23.7)
Median time from IPF diagnosis to introduction of antifibrotic treatment, months	4.1 (0.1–31.4)	6.5 (1.8–18.2)

IPF, idiopathic pulmonary fibrosis

e-Table 3. Comparison of the CIRBP [high] and CIRBP [low] groups in cohort 1

	CIRBP [high], n = 24	CIRBP [low], n = 71	*P*-value
Age, years	71.4 ± 8.8	69.9 ± 7.7	0.4583
Male/female	19 (79.2)/5 (20.8)	64 (90.1)/7 (9.9)	0.1718
Smoker, former or current	16 (66.7)	60 (84.5)	0.0776
Diagnosis, clinical/pathologically proven	17 (70.8)/7 (29.2)	55 (77.5)/16 (22.5)	0.5839
PaO$_2$, Torr	76.9 ± 15.6	76.5 ± 11.6	0.9088
% FVC, %	65.8 ± 22.3	76.7 ± 18.6	0.0381*
% DL$_{CO}$, % [a]	54.4 ± 23.8	66.9 ± 24.7	0.1988
GAP stage, I/II/III [b]	4 (30.8)/2 (15.4)/7 (53.9)	29 (53.7)/17 (31.5)/8 (14.8)	0.0083*
One-year cumulative AE incidence, %	12.9	10.1	0.75 [c]
Observation period, months	19.3 (12.3–46.6)	38.1 (16.7–56.9)	0.5189
Antifibrotic treatment, yes [d]	12 (50.0)	38 (53.5)	0.8160
Pirfenidone	11 (45.8)	32 (45.1)	1.0000
Nintedanib	1 (4.2)	11 (15.5)	0.2842
AE [d]	8 (33.3)	18 (25.4)	0.4415
Death [d]	19 (79.2)	32 (45.1)	0.0045*
from respiratory failure [d]	18 (75.0)	26 (36.6)	

Data are presented as mean ± standard deviation, median (interquartile range), or number (%)

*P <0.05

[a]n = 58

[b]n = 67

[c]Gray's test

[d]During the observation period

CIRBP, cold-inducible RNA-binding protein; PaO$_2$, arterial oxygen pressure; %FVC, percent predicted forced vital capacity; %DL$_{CO}$, percent predicted diffusing capacity of the lung for carbon monoxide; GAP, Gender–Age–Physiology Index; AE, acute exacerbation

e-Table 4. Comparison of the CIRBP high and CIRBP low groups in cohort 2

	CIRBP high, n = 24	CIRBP low, n = 69	P-value
Age, years	64.1 ± 9.2	66.3 ± 8.0	0.3135
Male/female	19 (79.2)/5 (20.8)	54 (78.3)/15 (21.7)	1.0000
Smoker, former or current	14 (58.3)	48 (69.6)	0.3264
Diagnosis, clinical/pathologically proven	9 (37.5)/15 (62.5)	36 (52.2)/33 (47.8)	0.2435
PaO$_2$, Torr	82.6 ± 14.7	81.1 ± 13.0	0.6740
% FVC, %	78.8 ± 17.1	81.7 ± 20.3	0.5031
% DL$_{CO}$, %	61.6 ± 20.5	59.2 ± 19.7	0.6890
GAP stage, I/II/III	21 (87.5)/2 (8.3)/1 (4.2)	46 (66.7)/20 (29.0)/3 (4.4)	0.1174
One-year cumulative AE incidence, %	16.7	7.3	0.5960 [a]
Observation period, months	25.8 (14.0–67.4)	46.0 (21.8–67.3)	0.2043
Antifibrotic treatment, yes [b]	18 (75.0)	48 (69.6)	0.7949
Pirfenidone	14 (58.3)	39 (56.5)	1.0000
Nintedanib	5 (20.8)	17 (24.6)	0.7872
AE [b]	8 (33.3)	18 (26.1)	0.5686
Death [b]	22 (91.7)	43 (62.3)	0.0087*
from respiratory failure [b]	14 (58.3)	33 (47.8)	

Data are presented as mean ± standard deviation, median (interquartile range), or number (%)

*P <0.05

[a]Gray's test

[b]During the observation period

CIRBP, cold-inducible RNA-binding protein; PaO$_2$, arterial oxygen pressure; %FVC, percent predicted forced vital capacity; %DL$_{CO}$, percent predicted diffusing capacity of the lung for carbon monoxide; GAP, Gender–Age–Physiology Index; AE, acute exacerbation

e-Table 5. Correlation between the serum CIRBP level and clinical parameters

		Cohort 1, n = 95		Cohort 2, n = 93	
		r	*P*-value	*r*	*P*-value
CIRBP, ng/mL	Age, years	−0.02	0.8833	−0.19	0.0640
	PaO$_2$, Torr	0.07	0.5314	−0.02	0.8725
	% FVC, %	−0.24	0.0170	−0.003	0.9755
	% DL$_{CO}$, % [a]	−0.08	0.5677	0.005	0.9628
Age, years	PaO$_2$, Torr	−0.18	0.0812	−0.28	0.0073
	% FVC, %	−0.08	0.4453	−0.05	0.6049
	% DL$_{CO}$, % [a]	−0.26	0.0496	−0.07	0.5325
PaO$_2$, Torr	% FVC, %	0.25	0.0160	0.21	0.0421
	% DL$_{CO}$, % [a]	0.56	<0.0001	0.52	<0.0001
% FVC, %	% DL$_{CO}$, % [a]	0.51	<0.0001	0.59	<0.0001

[a]cohort 1, n = 58; cohort 2, n = 93

r, correlation coefficient; CIRBP, cold-inducible RNA-binding protein; PaO$_2$, arterial oxygen pressure; %FVC, percent predicted forced vital capacity; %DL$_{CO}$, percent predicted diffusing capacity of the lung for carbon monoxide

e-Table 6. Multivariate logistic analysis of 1-year disease progression in treated and untreated patients

	Cohort 1			Cohort 2		
	OR	**95% CI**	*P*-value	**OR**	**95% CI**	*P*-value
Treated patients [a]						
CIRBP, per 1 ng/mL increase [c]	1.06	0.96–1.17	0.2373	1.12	1.01–1.23	0.0342*
CIRBP [high] (vs. CIRBP [low]) [c]	4.10	0.47–36.2	0.2038	6.39	1.06–38.5	0.0430*
Untreated patients [b]						
CIRBP, per 1 ng/mL increase [c]	1.07	1.01–1.12	0.0048*	1.11	1.02–1.20	0.0124*
CIRBP [high] (vs. CIRBP [low]) [c]	4.98	1.27–19.6	0.0215*	8.05	1.83–35.3	0.0057*

*$P < 0.05$

[a] cohort 1, n = 29; cohort 2, n = 42

[b] cohort 1, n = 66; cohort 2, n = 51

[c] Adjusted for Gender–Age–Physiology stage

Treated patients: patients for whom antifibrotic treatment was introduced within 1 year of diagnosis.

Untreated patients: patients for whom antifibrotic treatment was not introduced within 1 year of diagnosis.

OR, odds ratio; CI, confidence interval; CIRBP, cold-inducible RNA-binding protein
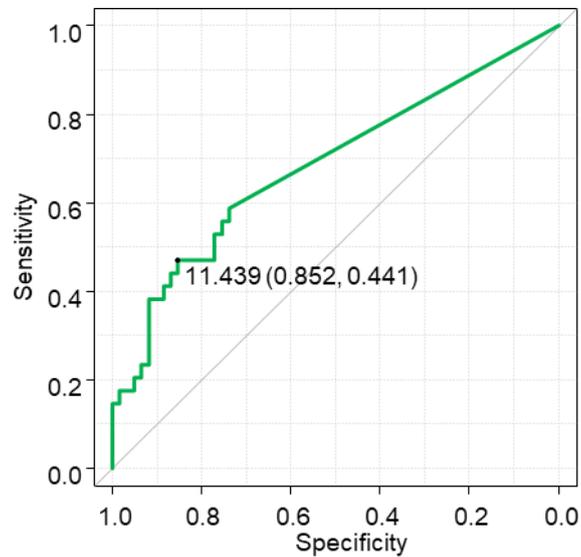
e-Table 7. Results of the fine-gray sub-distribution hazards analysis of CIRBP for AE development

	Cohort 1, n = 95			Cohort 2, n = 93		
	HR	**95% CI**	***P*-value**	**HR**	**95% CI**	***P*-value**
Model 1 [a]						
Univariate analysis	1.02	0.99–1.05	0.14	1.01	0.97–1.05	0.66
Multivariate analysis [c]	1.01	0.99–1.05	0.15	1.01	0.97–1.05	0.75
Model 2 [b]						
Univariate analysis	1.25	0.56–2.80	0.59	1.27	0.55–2.91	0.57
Multivariate analysis [c]	1.23	0.49–3.10	0.65	1.25	0.54–2.90	0.61

[*] $P < 0.05$

[a] CIRBP, per 1 ng/mL increase

[b] CIRBP [high] (vs. CIRBP [low])

[c] Adjusted for Gender–Age–Physiology stage

AE, acute exacerbation; HR, hazard ratio; 95% CI, 95% confidence interval; CIRBP, cold-inducible RNA-binding protein
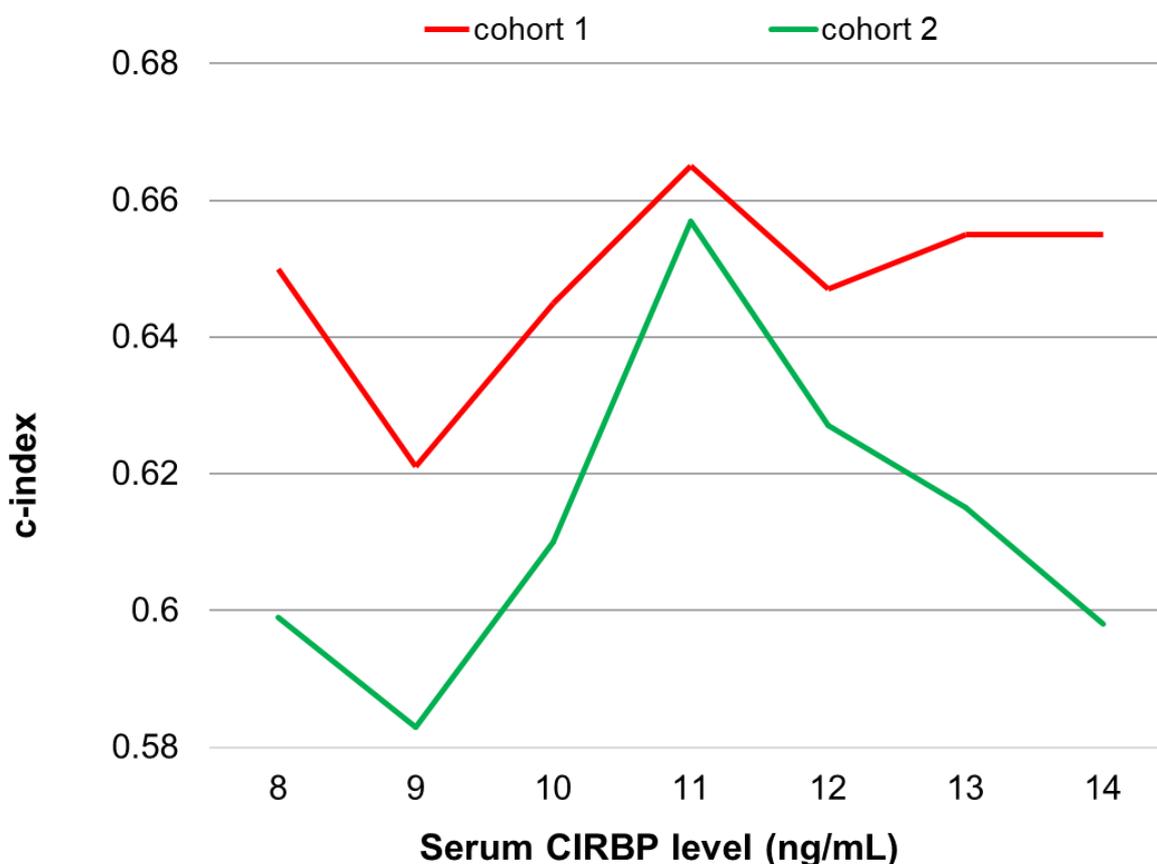
e-Figure 1. ROC curve analysis to identify the optimal cut-off value of serum CIRBP for predicting 1-year disease progression in cohort 1.

Using 11.439 ng/mL as the cut-off value of serum CIRBP, the sensitivity, specificity, positive predictive value, and negative predictive value were found to be 44.1%, 85.2%, 62.5%, and 73.2%, respectively. The area under the curve was 0.684 (95% confidence interval, 0.579–0.789).

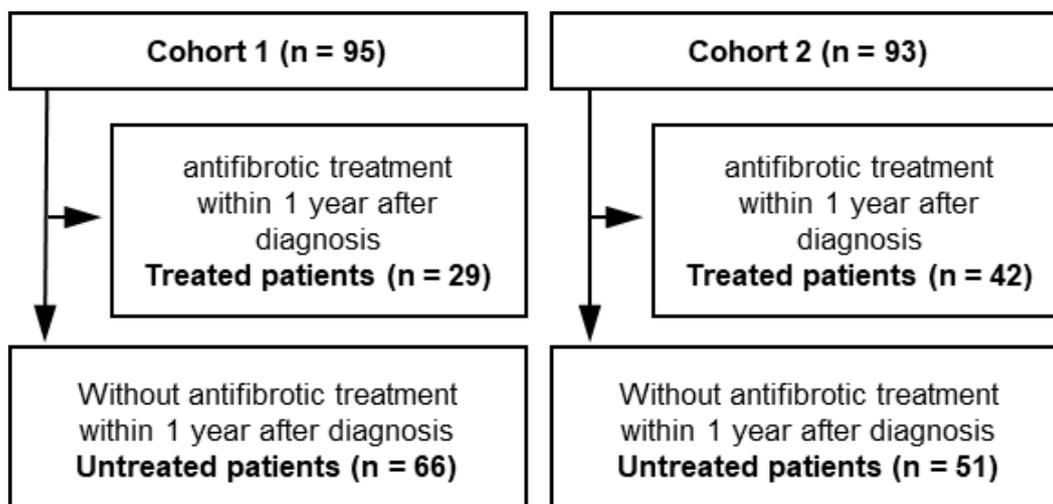CIRBP, cold-inducible RNA-binding protein

e-Figure 2. Sensitivity analysis of cut-off values of serum CIRBP for predicting 1-year disease progression
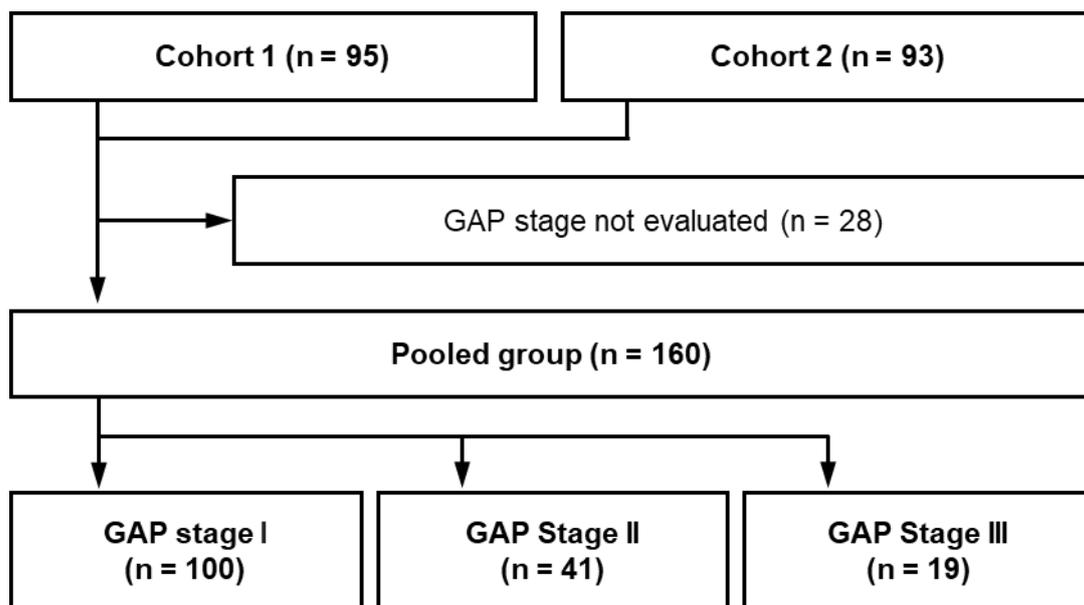

In sensitivity analyses of serum CIRBP levels between 8 and 14 ng/mL in 1-ng/mL increments, 11 ng/mL showed the highest discrimination performance for both cohorts 1 and 2 (c-index, 0.665 and 0.657, respectively).

CIRBP, cold-inducible RNA-binding protein; untreated, patients for whom antifibrotic treatment was not introduced within 1 year of IPF diagnosis
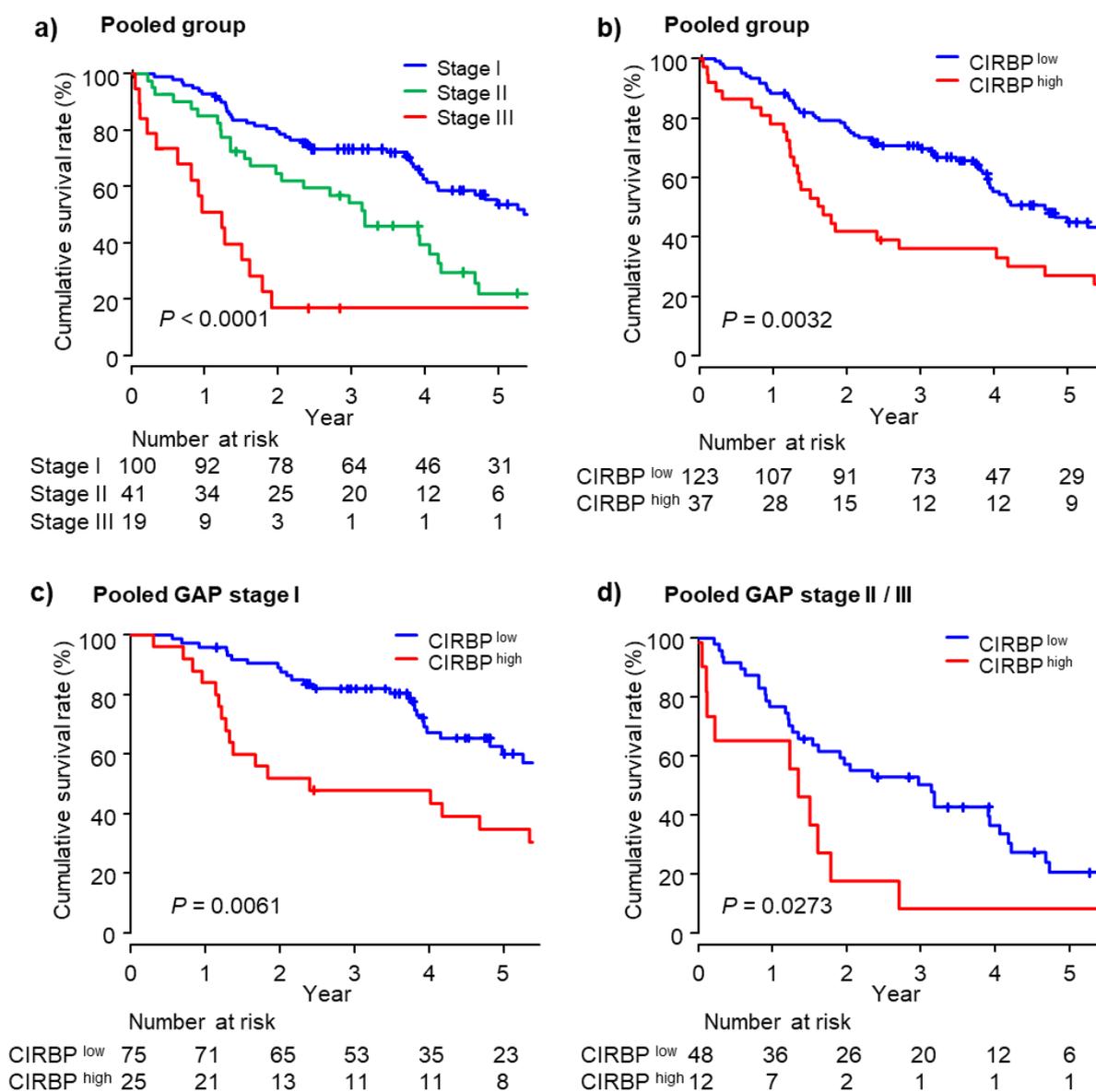
e-Figure 3. Flow diagram for extracting treated and untreated patients

e-Figure 4. Flow diagram representing the creation of the pooled group

e-Figure 5. Pooled analysis of cumulative survival rate

a) The 2-year cumulative survival rate was better in the following order: patients with GAP stage I, patients with GAP stage II, and patients with GAP stage III (79.7% vs. 64.9% vs. 17.0%, respectively, $P < 0.0001$).

b) In the pooled group, the 2-year cumulative survival rate was significantly lower in the CIRBP ^{high} sub-group than in the CIRBP ^{low} sub-group (41.9% vs. 76.7%, $P = 0.0032$).

c) In the pooled GAP stage I group, the 2-year cumulative survival rate was significantly lower in the CIRBP ^{high} sub-group than in the CIRBP ^{low} sub-group (52.0% vs. 89.1%, $P = 0.0061$).

d) In the pooled GAP stage II/III group, the 2-year cumulative survival rate was significantly lower in the CIRBP ^{high} sub-group than in the CIRBP ^{low} sub-group (19.1% vs. 57.3%, $P = 0.0273$).

GAP, Gender–Age–Physiology Index; CIRBP, cold-inducible RNA-binding protein