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Abbreviations

AE, acute exacerbation; GAP, gender, age, and physiology; IPF, idiopathic pulmonary

fibrosis; KL-6, Krebs von den Lungen-6; SP-D, surfactant protein D; WBC, white blood cell; 6MWT, 6-min walk test

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ABSTRACT

Background: Acute exacerbation of idiopathic pulmonary fibrosis (AE-IPF) is a devastating and life-threatening condition during its clinical course. Biomarkers for precisely anticipating the prognosis of AE-IPF remain to be fully established. The objective of this study was to clarify whether S100A8 and S100A9, which are calcium-binding proteins mainly produced by activated neutrophils, are significant prognostic biomarkers in AE-IPF.

Methods: Thirty-seven patients with AE-IPF who were diagnosed and treated at our hospital were retrospectively evaluated. The serum levels of S100A8 and S100A9 were measured using enzyme-linked immunosorbent assay, and the relationships between these levels and clinical parameters or prognosis were evaluated.

Results: The serum levels of S100A8 (median 386.5 ng/mL) and S100A9 (median 60.2 ng/mL) in patients with AE-IPF were significantly higher than those in age-matched healthy controls and in patients at IPF diagnosis ($p < 0.001$ for all combinations). The serum levels of S100A8 negatively correlated with percent forced vital capacity ($r = -0.356$, $p = 0.049$) and positively correlated with peripheral white blood cell number ($r = 0.509$, $p = 0.002$). Immunohistochemical staining of autopsy lung specimens showed that neutrophils, present mainly in the alveolar septum, were positive for S100A8 and S100A9. Patients with AE-IPF with higher levels of S100A8 or S100A9 showed significantly worse 3-month survival than those with lower levels (log-rank test, both $p = 0.028$). Finally, in multivariate analysis, the serum levels of both S100A8 and S100A9 were significant prognostic factors (hazard ratio 4.032, $p = 0.023$ and hazard ratio 4.327, $p = 0.012$).

Conclusion: The serum levels of S100A8 and S100A9 at AE were significant prognostic biomarkers in patients with AE-IPF.

Keywords: S100A8; S100A9; Calgranulin; Acute exacerbation; Idiopathic pulmonary fibrosis

1. Introduction

Idiopathic pulmonary fibrosis (IPF) has the poorest prognosis among the idiopathic interstitial pneumonias [1, 2]. Acute exacerbation of IPF (AE-IPF) [3] more frequently occurs in the advanced stage of IPF than in the early stages [4] and accounts for 30%–40% of all deaths [5, 6]. Although several serum biomarkers, such as Krebs von den Lungen-6 (KL-6), surfactant protein D (SP-D), and chemokine ligand 18 [1], which have elevated levels in patients with interstitial pneumonias, have been reported, no standard biomarker can precisely predict the prognosis of AE-IPF. We previously reported that serum ferritin [7] or the serum adiponectin/leptin ratio [8] were significant prognostic factors in patients with AE-IPF. However, additional novel biological molecules should be examined in clinical practice for precisely predicting clinical outcomes.

In the pathogenesis of AE-IPF, neutrophils play a role in the progression of diffuse alveolar damage, especially in the very early phase of AE-IPF [9, 10]. In addition, S100A8 (MRP8) and S100A9 (MRP14), which are calcium-binding proteins produced and released mainly by activated neutrophils, promote inflammation [11] and decrease vascular endothelial cell integrity [12], consequently inducing further neutrophil extravasation [13]. S100A8 and S100A9 are reportedly induced in patients with cystic fibrosis [14-16]. In diffuse lung diseases, S100A8 and S100A9 are more often produced in patients with IPF with chronic-phase disease than in patients with other diseases such as sarcoidosis or connective tissue disease-related interstitial pneumonia [17-19]. However, the blood kinetics and functions of S100A8 and S100A9 are unknown in patients with AE-IPF.

In the present study, we retrospectively investigated patients with AE-IPF and evaluated the serum levels of S100A8 or S100A9 and other data. To the best of our knowledge, this is the first study to show the relationship between S100A8/A9 levels and clinical parameters and how these relate to prognosis in patients with AE-IPF.

2. Patients and methods

2.1. Study design and patients

Thirty-seven patients who were diagnosed with AE-IPF at our hospital between 1995 and 2018 and had available serum samples were retrospectively reviewed. Seventeen patients were thoroughly examined through surgical lung biopsy before developing AE-IPF and met the 2018 international consensus criteria for IPF [1] (Table 1). The remaining 20 patients showed clinical and high-resolution computed tomography (HRCT) features compatible with IPF and were diagnosed with IPF without surgical lung biopsy [1]. Patients diagnosed with any connective tissue disorders were excluded from the study. The patients enrolled in this study also met the 2016 AE-IPF criteria [10]. Briefly, the criteria for the diagnosis of AE-IPF were as follows: 1) previous or concurrent diagnosis of IPF, 2) acute worsening or development of dyspnea typically within 1 month, 3) HRCT findings of new bilateral ground-glass opacity and/or consolidation superimposed on a background reticular or honeycomb pattern, and 4) deterioration not fully explained by cardiac failure or fluid overload.

The study protocol was approved by the Ethical Committee of Hamamatsu University School of Medicine (approved on September 1, 2018, approval no. 18-085), and this study was performed in accordance with the approved protocol. The need for patient approval and informed consent was waived owing to the retrospective nature of the study.

2.2. Data collection

Clinical data were obtained from the medical records. The disease severity of IPF within 12 months before the AE event was assessed using the GAP (gender, age, and physiology) staging system [20] and the Japanese Respiratory Society (JRS) severity scale of interstitial

pneumonia [4]. The GAP staging system considers sex, age, and two lung physiology variables: percent predicted forced vital capacity (%FVC) and diffusion lung capacity for carbon monoxide (%DLCO) [20]. The JRS severity scale is based on the partial pressure of arterial oxygen (PaO_2) at rest and minimum partial oxygen saturation during the 6-min walk test (6MWT) [4]. The extent of lung opacities was measured on three HRCT slices, as previously described [21, 22]. The sum of the scores from five lobes (0–25) was used to express the extent of lung opacities in each patient. The HRCT images were reviewed by two observers.

2.3. Measurement of serum S100A8 and S100A9 levels

All blood samples were collected on the first or second day of admission before starting treatments for AE-IPF. Blood samples were also collected at the time of IPF diagnosis in 23 patients and in 15 age-matched healthy controls. The serum levels of S100A8 and S100A9 were measured using enzyme-linked immunosorbent assay (ELISA) with the cooperation of Medical & Biological Laboratories (MBL, Nagoya, Japan; CircuLex™ S100A8/MRP8 and CircuLex™ S100A9/MRP14 ELISA kits, respectively).

2.4. Immunohistochemical staining of S100A8 or S100A9

Autopsy lung specimens were immunohistochemically stained for S100A8 or S100A9. Briefly, heat-mediated antigen retrieval was performed before staining. Deparaffinized sections were steeped in 0.3% hydrogen peroxide to inactivate endogenous peroxidase activity and were blocked with 10% normal goat serum. The sections were incubated with rabbit polyclonal antibody against S100A8 (rabbit anti-MRP8 antibody; Abcam, Boston, MA, USA) or S100A9 (rabbit anti-S100A9 antibody, Abcam). After rinsing with phosphate-buffered saline, the sections were incubated with biotin-conjugated goat anti-rabbit

IgG polyclonal antibody (Nichirei, Tokyo, Japan). Thereafter, the sections were incubated with streptavidin–peroxidase complex (Nichirei). The antigen–antibody complex was visualized with 3,3′-diaminobenzidine (Nichirei) and counterstained with hematoxylin.

2.5. Statistical analysis

Statistical analyses were performed using JMP-14.0.0 (SAS Institute Inc., Cary, NC, USA). Continuous data were compared using the Wilcoxon rank-sum test. Continuous data at different time points in the same patient were compared using the Wilcoxon signed-rank test. Categorical data were compared using the chi-square test or Fisher's exact probability test for independence. Multiple comparisons were performed using the Steel–Dwass test. The relationships between continuous variables were analyzed using Pearson's correlation coefficients, and those between continuous variables and discrete ordinal variables were analyzed using Spearman's rank correlation coefficients. The survival rates of patient groups were evaluated using Kaplan–Meier curves and compared between groups using the log-rank test. The relationships between variables and mortality were assessed through Cox proportional hazard regression analysis. All tests were two-sided, and statistical significance was set at $p < 0.05$.

3. Results

3.1. Characteristics; laboratory, physiologic, and radiologic findings; and treatments in patients with AE-IPF

The clinical characteristics of 37 patients with first AE-IPF are shown in Table 1. The median age was 69 years, and 33 of the 37 patients were men. The median period from diagnosis to AE was 72 months. Pulmonary physiologic tests and severity scores were evaluated within 12

months before AE-IPF. The median %FVC and %DL_{CO} before AE were 53.7% and 50.1%, respectively. More than half of the patients had advanced-stage IPF based on the JRS or GAP severity grading system before AE (24 were in stage III or IV in the JRS system and 22 were in stage II or III in the GAP system). Nineteen patients (51.4%) had preceding treatments. Of them, 11 received immunosuppressive therapy, 7 received antifibrotic therapy, and 1 received both immunosuppressive and antifibrotic therapy. In addition, nine (24.3%) patients received preceding oxygen therapy. With respect to the data at AE, the median PaO_2/fraction of inspired oxygen (P/F) ratio was as low as 180. The median white blood cell (WBC) number (9900/μL) and neutrophil number (7300/μL) were slightly increased. The serum levels of KL-6 and SP-D were high at AE (median 1520 U/mL and 368 ng/mL, respectively). The median extent score on HRCT (full score 25) was as high as 20 at AE. All patients were treated with steroid pulse therapy at AE.

3.2. Serum S100A8 and S100A9 levels evaluated with ELISA

The serum levels of S100A8 and S100A9 were measured in all 37 patients at AE. Furthermore, S100A8 and S100A9 levels were also evaluated in 23 patients at IPF diagnosis and were compared with those in 15 age-matched healthy controls (Figure 1A and Supplementary Figure 1A). The serum S100A8 levels in patients with AE-IPF were significantly higher than those in healthy controls or in patients at IPF diagnosis (in the chronic phase; Figure 1A; median 386.5 vs. 73.9 ng/mL, $p < 0.001$ and vs. 120.0 ng/mL, $p < 0.001$). The results of the serum S100A9 levels were almost the same as those of S100A8 (Supplementary Figure 1A; median 60.2 vs. 11.0 ng/mL, $p < 0.001$ and vs. 15.5 ng/mL, $p < 0.001$). In 18 patients with IPF in whom the serum S100A8 levels were sequentially measured at both IPF diagnosis and AE-IPF, the levels at AE were significantly higher than those at IPF diagnosis (Figure 1B; median 338.3 vs. 114.1 ng/mL, $p < 0.001$). Similarly, the results of the

serum S100A9 levels were almost the same as those of S100A8 (Supplementary Figure 1B; median 56.4 vs. 15.6 ng/mL, $p < 0.001$).

3.3. Relationships between serum S100A8 or S100A9 levels and several clinical parameters

The correlations between serum S100A8 levels at AE-IPF and several clinical parameters are shown in Figure 2. A significantly negative correlation was found between S100A8 levels and %FVC (Figure 2A; $p = 0.049$, $r = –0.356$), although the WBC number was not significantly correlated with %FVC ($p = 0.336$, $r = –0.182$). The S100A8 levels were not correlated with the P/F ratio and extent scores at AE (Figure 2B and 2C; $p = 0.808$ and $p = 0.462$, respectively). A moderately positive correlation was found between S100A8 levels and peripheral WBC number (Figure 2D; $p = 0.002$, $r = 0.569$). Furthermore, the serum S100A8 levels were strongly and positively correlated with the serum S100A9 levels (Figure 2E; $p < 0.001$, $r = 0.838$). In the same way, the correlations between the serum S100A9 levels at AE-IPF and several clinical parameters are shown in Supplementary Figure 2. The S100A9 levels were not significantly correlated with %FVC (Supplementary Figure 2A, $p = 0.103$), P/F ratio (Supplementary Figure 2B, $p = 0.935$), or extent scores at AE (Supplementary Figure 2C, $p = 0.337$), but were positively and weakly correlated with peripheral WBC number (Supplementary Figure 2D; $p = 0.030$, $r = 0.363$). Furthermore, the serum S100A8 levels at the diagnosis of IPF in the chronic phase were not significantly correlated with the %FVC values, which were measured at the same time ($p = 0.476$, $r = 0.179$).

3.4. Immunohistochemical staining of S100A8 or S100A9 in autopsy lung specimens

To elucidate the source of S100A8 and S100A9, autopsy lung specimens were subjected to S100A8 and S100A9 immunohistochemical staining (Figure 3 and Supplementary Figure 3).

The specimens were from a 52-year-old male patient with high serum S100A8 and S100A9 levels at AE (823.6 and 63.9 ng/mL, respectively), who died on day 8 from the onset of AE-IPF. Immunohistochemical staining of the autopsy lung specimens, having diffuse alveolar damage with hyaline membrane (Figure 3A and Supplementary Figure 3A), showed that neutrophils were positive for both S100A8 and S100A9 (Figure 3B and 3C, Supplementary Figure 3B and 3C). More neutrophils with S100A8 or S100A9 existed in the alveolar septa than in the alveolar spaces (for S100A8: 79.8% vs. 20.2% and for S100A9: 80.6% vs. 19.4%).

3.5. Comparisons of data between patients with higher and lower S100A8 or S100A9

On the basis of the median values of S100A8 (median 386.5 ng/mL) and S100A9 (60.2 ng/mL) at AE-IPF in 37 patients, the patients were divided into two groups. Comparisons of clinical data between patients with higher and lower S100A8 are shown in Table 2. Although the distance in 6MWT was significantly shorter in the S100A8 higher group ($p = 0.034$), the %FVC ($p = 0.138$) and severity grades (JRS, $p = 0.433$ and GAP, $p = 0.083$) before AE were not different between groups. At AE, the peripheral blood WBC number was significantly higher in the S100A8 higher group than in the lower group (11.0 vs. 9.2 × $10^3/\mu L$, $p = 0.031$). The serum C-reactive protein level tended to be higher in the S100A8 higher group than in the lower group (9.1 vs. 4.9 mg/dL, $p = 0.125$); however, the P/F ratio ($p = 0.879$) or extent scores on HRCT ($p = 0.367$) at AE were not different between groups. Similarly, comparisons of clinical data between patients with higher and lower S100A9 are shown in Supplementary Table 1. Although %FVC was significantly lower in the S100A9 higher group ($p = 0.023$), the severity grades (JRS, $p = 0.471$ and GAP, $p = 0.248$) before AE and the P/F ratio ($p = 0.715$) at AE were not different between groups. All patients received steroid pulse therapy. Other treatments did not differ between groups (Table 2 and

Supplementary Table 1).

3.6. Impact of serum S100A8 or S100A9 on survival

Among 37 patients with AE-IPF, 8 died within 1 month (1-month mortality rate 21.6%), 12 died within 3 months (3-month mortality rate 32.4%), and 28 died within 12 months (12-month mortality rate 75.7%) from the onset of AE-IPF. Of the 12 patients who died within 3 months from the onset of AE-IPF, 8 died of respiratory failure and 4 died of infectious pneumonia. Of the 28 patients who died within 12 months, 23 died of respiratory failure and 5 died of infectious pneumonia. The Kaplan–Meier survival curves from the AE-IPF onset are shown in Figure 4. When patients were divided into two groups based on the median value of S100A8 levels, the 3-month survival rate was significantly lower in patients with higher S100A8 levels than in those with lower S100A8 levels (Figure 4; log-rank test, $p = 0.028$). Similarly, the 3-month survival rate was significantly lower in patients with higher S100A9 levels than in those with lower S100A9 levels (Supplementary Figure 4; log-rank test, $p = 0.028$). Lastly, prognostic factors for 3-month survival were evaluated using Cox proportional hazard models (Table 3). In univariate analysis, the JRS severity grade before AE and the P/F ratio at AE tended to be prognostic factors (hazard ratio [HR] 1.980, $p = 0.072$ and HR 0.994, $p = 0.081$, respectively). Preceding oxygen therapy (HR 4.649, $p = 0.012$), extent scores on HRCT at AE (HR 1.282, $p = 0.007$), and peripheral blood WBC number at AE (HR 3.621, $p = 0.037$) were significant prognostic factors. In addition, higher serum levels of S100A8 and S100A9 at AE were also significant factors in patients with AE-IPF (HR 3.887, $p = 0.027$ and HR 3.887, $p = 0.027$, respectively). In multivariate models adjusted for the P/F ratio at AE, preceding oxygen therapy (HR 3.971, $p = 0.025$) and extent scores on HRCT at AE (HR 1.265, $p = 0.017$) remained significant. Furthermore, higher serum levels of S100A8 and S100A9 at AE were also significant prognostic factors in

patients with AE-IPF (HR 4.032, p = 0.023 and HR 4.327, p = 0.012, respectively). When higher levels of S100A8 were adjusted for age, sex, or %FVC before AE-IPF, the HRs were 3.886 (p = 0.027), 3.848 (p = 0.030), and 3.427 (p = 0.055), respectively. In addition, when higher levels of S100A9 were adjusted for in the same way, the HRs were 3.899 (p = 0.027), 3.848 (p = 0.030), and 3.446 (p = 0.060), respectively. With respect to continuous variables, S100A8 and WBC number were both significant prognostic factors (univariable: HR 1.001, p = 0.016 and HR 1.000, p = 0.007; multivariable: HR 1.001, p = 0.011 and HR 1.000, p = 0.025, respectively). Conversely, S100A9 was not a significant factor (univariable: HR 1.013, p = 0.052 and multivariable: HR 1.012, p = 0.089).

4. Discussion

In the present study, serum S100A8 and S100A9 levels and their prognostic significance were retrospectively evaluated in patients with AE-IPF. The serum levels of S100A8/A9 at AE were significantly higher than those in age-matched healthy controls and in patients at IPF diagnosis in the chronic phase. The serum levels of S100A8 negatively correlated with %FVC and positively correlated with the peripheral WBC number. Immunohistochemical staining showed that infiltrating alveolar neutrophils were positive for S100A8 and S100A9. Patients with AE-IPF with higher levels of S100A8/A9 showed significantly worse survival than those with lower levels. Lastly, the serum levels of S100A8/A9 were significant prognostic factors in patients with AE-IPF. From these results, it seems that serum S100A8/A9 levels in an advanced stage of IPF are diagnostic markers for detecting AE-IPF and are also prognostic markers in patients with AE-IPF.

S100A8 and S100A9 are calcium-binding proteins that are mainly released by activated neutrophils and monocytes [11]. S100A8/A9 act through an autocrine/paracrine mechanism,

and extracellular S100A8/A9 function as endogenous damage-associated molecular patterns, Toll-like receptor 4 (TLR-4) ligands, and receptor for advanced glycation end-product ligands [11]. Consequently, these proteins facilitate inflammation; therefore, S100A8/A9 are known as endogenous danger signals called “alarmins” [11, 23]. In addition, S100A8/A9 have bipolar functions. Extracellular S100A8/A9, mainly produced by neutrophils, have pro-inflammatory functions by enhancing TLR-4 signaling and activating macrophages [24] and type-1 helper T-lymphocytes [23, 25]. Moreover, S100A8/A9 decrease vascular endothelial cell integrity [12] and facilitate further neutrophil extravasation [13]. In contrast, S100A8/A9 also have anti-inflammatory functions in different micromilieu. S100A8/A9 suppress the differentiation and functions of dendritic cells [25], induce myeloid-derived suppressor cells [11], and increase the functions of regulatory T-lymphocytes [26]. Furthermore, S100A8/A9 promote tissue proliferation and repair at low levels, whereas they have deleterious effects on inflammatory tissues at high levels [27]. As mentioned above, the biological functions of S100A8/A9 differ depending on the types of cells [25] and on their levels in involved tissues [27]. In our study, the peripheral blood WBC number at AE was significantly higher and the serum C-reactive protein level also tended to be higher in the S100A8 higher group. In addition, immunohistochemistry revealed that infiltrating alveolar neutrophils were a source of S100A8/A9. This positive correlation of S100A8 and peripheral WBC number, and the S100A8 positivity of infiltrating alveolar neutrophils seem to be consistent with the above-mentioned pathogenesis. In addition, the negative correlation of S100A8 levels and %FVC may be related to inappropriate tissue proliferation and repair induced by S100A8 even just before AE-IPF. Furthermore, higher levels of S100A8 were associated with poor survival in patients with AE-IPF. Thus, the pro-inflammatory effects followed by anti-inflammatory effects of S100A8/A9 may be related to inappropriate tissue repair and progression of lung fibrosis like M1 and M2 macrophages [28].

In lung diseases, increased S100A8 and S100A9 were found in patients with cystic fibrosis [14-16]. Additionally, in diffuse lung diseases, higher levels of S100A8 and S100A9 were detected in patients with IPF in the chronic phase than in patients with sarcoidosis [17, 18] or nonspecific interstitial pneumonia/cryptogenic organizing pneumonia/connective tissue disease-related interstitial pneumonia [19]. Moreover, in patients with systemic sclerosis and interstitial pneumonia, the S100A8/A9 levels in bronchoalveolar lavage fluid were higher in patients with extensive fibrosis on HRCT than in those with limited fibrosis and were positively correlated with the neutrophil number in bronchoalveolar lavage fluid [29]. In terms of fibroblasts, which play a central role in fibrosis, S100A8/A9 activated cardiac fibroblasts [30] and dermal fibroblasts [31]. These studies indicated that S100A8/A9 are related to the progression of fibrosis. In the present study, the serum levels of S100A8 negatively correlated with %FVC, and patients with AE-IPF with higher levels of S100A8/A9 showed significantly worse survival than those with lower levels. Therefore, although little is known about S100A8/A9 in patients with AE-IPF, these proteins may play important roles in the progression of lung fibrosis even in AE-IPF. Furthermore, as the results for S100A8 and S100A9 were highly similar, it seems that simultaneous evaluation of S100A8 and S100A9 is not beneficial. However, S100A8 was significantly and negatively correlated with %FVC before AE but S100A9 was not. In addition, S100A8 as a continuous variable was also a significant prognostic factor, whereas S100A9 was not. Therefore, a single evaluation of S100A8 may be sufficient in clinical practice.

This study had several limitations. First, only a small number of patients with AE-IPF were included. Second, the data were retrospectively collected. Third, immunohistochemical analysis was conducted in only one patient with AE-IPF owing to the limited number of patients. Fourth, the treatment for AE-IPF was not uniform. Therefore, a larger and prospective study is needed to precisely evaluate the pathogenic role of S100A8/A9 in

AE-IPF.

5. Conclusions

We retrospectively evaluated the serum S100A8 and S100A9 levels and their prognostic significance in patients with AE-IPF. The serum levels of S100A8/A9 at AE were significantly higher than those in age-matched healthy controls and in patients at IPF diagnosis. Immunohistochemical staining showed that infiltrating alveolar neutrophils were positive for S100A8/A9. Finally, patients with AE-IPF with higher levels of S100A8/A9 showed significantly worse survival, and the serum levels of S100A8/A9 were significant prognostic factors in patients with AE-IPF. S100A8/A9 produced by activated neutrophils are potential novel therapeutic targets in this devastating disease, which has no effective treatment at the time of this reporting. Therefore, further studies are needed to improve the poor survival of patients with AE-IPF.

Conflict of interest

All authors do not have any potential conflicts of interest. This research was not funded.

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FIGURE LEGENDS

Figure 1. Serum levels of S100A8 in 37 patients with AE-IPF. S100A8 levels were also evaluated in 23 patients at IPF diagnosis before AE and were compared with those in 15 age-matched healthy controls (HC). The serum S100A8 levels in patients with AE-IPF were significantly higher than those in HC or in patients at IPF diagnosis (A; median 386.5 vs. 73.9 ng/mL, $p < 0.001$ and vs. 120.0 ng/mL, $p < 0.001$). In 18 patients with IPF in whom serum S100A8 levels were sequentially measured at both IPF diagnosis and AE-IPF, the levels at AE were significantly higher than those at IPF diagnosis (B; median 338.3 vs. 114.1 ng/mL, $p < 0.001$). Abbreviations: HC, healthy controls; AE, acute exacerbation; IPF, idiopathic pulmonary fibrosis.

Figure 2. Correlations between serum S100A8 levels at AE of idiopathic pulmonary fibrosis and several clinical parameters. A negative correlation was found between S100A8 levels and %FVC (A; $p = 0.049$, $r = -0.356$). The S100A8 levels were not correlated with the P/F ratio or extent scores on HRCT at AE (B and C; $p = 0.808$ and $p = 0.462$, respectively). A moderately positive correlation was found between S100A8 levels and peripheral WBC number (D; $p = 0.002$, $r = 0.569$). Furthermore, serum S100A8 levels were strongly and positively correlated with serum S100A9 levels (E; $p < 0.001$, $r = 0.838$). Abbreviations: AE, acute exacerbation; %FVC, percent predicted forced vital capacity; P/F, partial pressure of arterial oxygen/fraction of inspired oxygen ratio; HRCT, high-resolution computed tomography ; WBC, white blood cell.

Figure 3. Immunohistochemical staining of S100A8 in autopsy lung specimens. Autopsy lung specimens were subjected to S100A8 immunohistochemical staining. The specimens were

from a 52-year-old male patient with a high serum S100A8 level at acute exacerbation of idiopathic pulmonary fibrosis (AE-IPF) (823.6 ng/mL), who died on day 8 from the onset of AE-IPF. Immunohistochemical staining of autopsy lung specimens, having diffuse alveolar damage with hyaline membrane (A), showed that infiltrating alveolar neutrophils were positive for S100A8 (B and C). More neutrophils with S100A8 existed in the alveolar septa than in the alveolar space. Abbreviation: HE, hematoxylin and eosin.

Figure 4. Serum S100A8 level and Kaplan–Meier survival curves from the onset of acute exacerbation of idiopathic pulmonary fibrosis. When patients were divided into two groups based on the median value of S100A8 (386.5 ng/mL), the 3-month survival rate was significantly lower in patients with higher S100A8 levels than in those with lower S100A8 levels (log-rank test, $p = 0.028$).

Supplementary Figure 1. Serum levels of S100A9 were measured in all 37 patients at AE-IPF. Furthermore, S100A9 levels were also evaluated in 23 patients at IPF diagnosis, and were compared with those in 15 age-matched healthy controls (HC). The serum S100A9 levels in patients with AE-IPF were significantly higher than those in HC or in patients at IPF diagnosis (A; median 60.2 vs. 11.0 ng/mL, $p < 0.001$ and vs. 15.5 ng/mL, $p < 0.001$). In 18 patients with IPF in whom serum S100A9 levels were sequentially measured at both IPF diagnosis and AE-IPF, the levels at AE were significantly higher than those at IPF diagnosis (B; median 56.4 vs. 15.6 ng/mL, $p < 0.001$). Abbreviations: HC, healthy controls; AE, acute exacerbation; IPF, idiopathic pulmonary fibrosis.

Supplementary Figure 2. Correlations between serum S100A9 levels at AE-IPF and several clinical parameters. S100A9 levels were not significantly correlated with %FVC (A; $p = 0.103$), P/F ratio (B; $p = 0.935$), or extent scores on HRCT at AE (C; $p = 0.337$), but were positively and weakly correlated with peripheral WBC number (D; $p = 0.030$, $r = 0.363$). Abbreviations: AE, acute exacerbation; IPF, idiopathic pulmonary fibrosis; %FVC, percent predicted forced vital capacity; P/F, partial pressure of arterial oxygen/fraction of inspired oxygen ratio; WBC, white blood cell.

Supplementary Figure 3. Immunohistochemical staining of S100A9 in autopsy lung specimens. Autopsy lung specimens were subjected to S100A9 immunostaining. The specimens were from a 52-year-old male patient with a high serum S100A9 level at acute exacerbation of idiopathic pulmonary fibrosis (AE-IPF) (63.9 ng/mL), who died on day 8 from the onset of AE-IPF. Immunohistochemical staining of autopsy lung specimens, having diffuse alveolar damage with hyaline membrane (A), showed that infiltrating alveolar neutrophils were positive for S100A9 (B and C). More neutrophils with S100A9 existed in the alveolar septa than in the alveolar space. Abbreviations: HE, hematoxylin and eosin.

Supplementary Figure 4. Serum S100A9 level and Kaplan–Meier survival curves from the AE-IPF onset. When patients were divided into two groups based on the median value of S100A9 (60.2 ng/mL), the 3-month survival rate was significantly lower in patients with higher S100A9 levels than in those with lower S100A9 levels (log-rank test, $p = 0.028$). Abbreviations: AE, acute exacerbation; IPF, idiopathic pulmonary fibrosis.

Table 1. Characteristics, physiologic and laboratory data, and treatments in patients with AE-IPF

	n = 37, Median (range)
Age, y	69 (50–92)
Sex, male/female	33/4
Smoking, never/ex/current	6/27/4
Smoking, pack-years	35 (0–81)
Diagnosis, surgical lung biopsy/clinical	17/20
Period from IPF diagnosis to AE, mo	72 (0–203)
Observation period, mo	5 (0–45)
Data before AE*	
FVC before AE, % pred	53.7 (37.5–89.3)
DL_{CO} before AE, % pred	50.1 (34.6–88.2)
PaO_2 at rest, Torr	71.4 (40.3–86.6)
Distance in 6MWT, m	427 (160–507)
Minimum SpO_2 in 6MWT, %	83 (60–95)
JRS severity grade of IPF I/II/III/IV/unknown	6/0/11/13/7
GAP staging system I/II/III/unknown	8/11/11/7
Preceding treatments for IPF Immunosuppressive/antifibrotic/immunosuppressive+antifibrotic/none	11/7/1/18
Preceding oxygen therapy, +/−	9/28
Data at AE	
Peripheral blood WBC, × 10^3/μL	9.9 (1.9–20.0)
Peripheral blood neutrophils, × 10^3/μL (n = 32)	7.3 (1.5–18.2)
Peripheral blood monocytes, × 10^3/μL (n = 23)	0.6 (0.1–1.2)
Serum CRP, mg/dL	7.4 (0.1–20.3)
Serum LDH, IU/L	338 (183–602)
Serum KL-6 at AE, U/mL	1520 (634–6070)
Serum SP-D at AE, ng/mL	368 (23–1330)
P/F ratio at AE	180 (38–386)
HRCT extent scores at AE (full score 25)	20 (13–25)
Period from admission to the beginning of AE treatment, d	1 (0–17)
Administration of steroid pulse therapy, +/−	37/0
Administration of immunosuppressants, +/−	25/12
Treatment with PMX-DHP, +/−	17/20

*Physiologic tests and severity scores were evaluated within 12 months before AE-IPF.
Abbreviations: AE, acute exacerbation; IPF, idiopathic pulmonary fibrosis; FVC, forced vital capacity; % pred, percent predicted; DL_{CO}, diffusion lung capacity for carbon monoxide; PaO_2, partial pressure of arterial oxygen; 6MWT, 6-min walk test; SpO_2, partial oxygen saturation; JRS, Japanese Respiratory Society; GAP, gender, age, and physiology; WBC, white blood cell; CRP, C-reactive protein; LDH, lactate dehydrogenase; KL-6, Krebs von den Lungen-6; SP-D, surfactant protein D; P/F: partial pressure of arterial oxygen/fraction of inspired oxygen ratio; HRCT, high-resolution computed tomography; PMX-DHP, polymyxin B-immobilized fiber column-direct hemoperfusion.

Table 2. Comparison of data between patients with higher and lower serum S100A8 levels

	S100A8 high*, n = 18, median (range)	**S100A8 low,** n = 19, median (range)	**p Value**
Age, y	69.5 (50–92)	69.0 (55–82)	0.964
Sex, male/female	17/1	16/3	0.604
Smoking, never/ex/current	4/14/0	2/13/4	0.096
Pack-years of smoking	38.5 (0–80)	34.0 (0–81)	0.976
Diagnosis, surgical lung biopsy/clinical/unknown	8/9/1	9/10/0	0.581
Observation period from AE onset, mo	2 (0–45)	7 (0–43)	0.173
Data before AE†			
FVC, % pred	50.3 (37.5–84.1)	58.4 (40.4–89.3)	0.138
DL_{CO}, % pred (n = 20)	47.4 (38.0–79.8)	67.7 (34.4–88.2)	0.425
PaO_2 at rest, Torr	74 (49–87)	67 (40–80)	0.254
Distance in 6MWT, m	375 (160–450)	472 (175–507)	0.034
Minimum SpO_2 in 6MWT, %	83 (62–95)	83 (60–89)	0.915
JRS severity grade I/II/III/IV/unknown	4/0/4/5/5	2/0/7/8/2	0.433
GAP staging system I/II/III/unknown	2/8/4/4	6/3/7/3	0.083
Preceding treatment for IPF, +/−	11/7	8/11	0.330
Preceding oxygen therapy, +/−	6/12	3/16	0.269
Data at AE			
Peripheral blood WBC, × 10^3/μL	11.0 (1.9–20)	9.2 (5.8–16.1)	0.031
Peripheral blood neutrophils, × 10^3/μL	9.5 (1.5–18.2)	7.0 (4.1–14.4)	0.212
Peripheral blood monocytes, × 10^3/μL	0.5 (0.3–1.2)	0.6 (0.1–1.1)	0.620
Serum CRP, mg/dL	9.1 (1.0–17.0)	4.9 (0.1–20.3)	0.125
Serum LDH, IU/L	338 (187–602)	338 (183–477)	0.899
Serum KL-6, U/mL	2053 (807–6070)	1464 (634–3540)	0.515
Serum SP-D, ng/mL	351 (102–966)	368 (23–1330)	0.986
P/F ratio	182 (44–386)	176 (38–368)	0.879
Extent scores on HRCT (full score 25)	21.5 (13–25)	19.5 (14–25)	0.367
Period from admission to the beginning of AE treatment, d	0 (0–5)	2 (0–17)	0.054
Administration of steroid pulse therapy‡, +/−	18/0	19/0	1.000
Administration of immunosuppressants, +/−	10/8	15/4	0.170
Treatment with PMX-DHP, +/−	8/10	9/10	0.873
Intubation, +/−	7/11	5/14	0.414

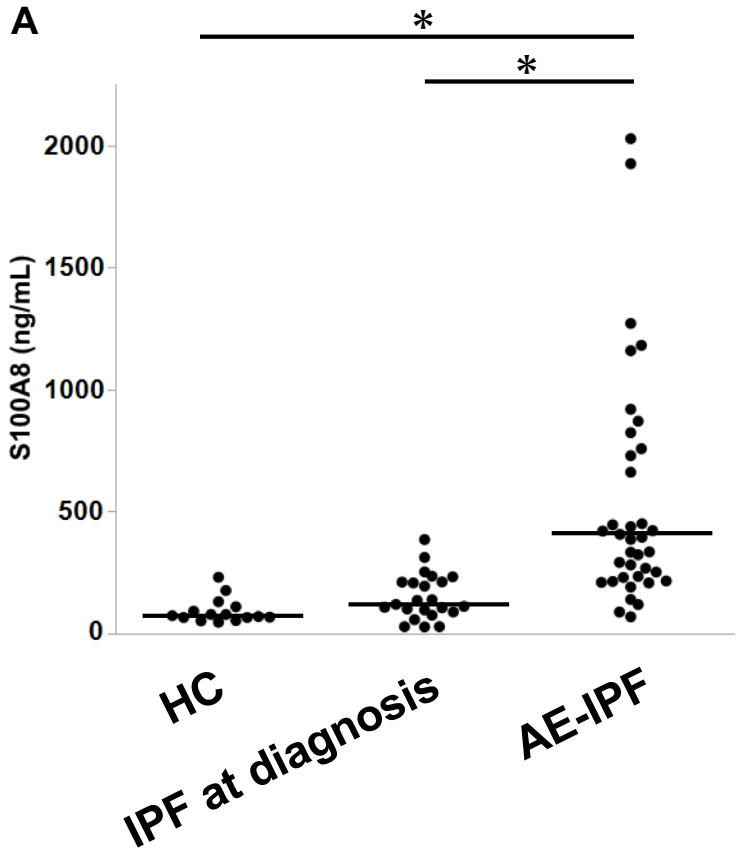
*S100A8 levels were divided into two groups based on the median value of 386.5 ng/mL.
†Physiologic tests and severity scores were evaluated within 12 months before AE-IPF.
‡Methylprednisolone 1000 mg/day for 3 days.
Abbreviations: AE, acute exacerbation; IPF, idiopathic pulmonary fibrosis; FVC, forced vital capacity; % pred, percent predicted; DL_{CO}, diffusion lung capacity for carbon monoxide; 6MWT, 6-min walk test; JRS, Japanese Respiratory Society; GAP, gender, age, and physiology; WBC, white blood cell; CRP, C-reactive protein; LDH, lactate dehydrogenase; KL-6, Krebs von den Lungen-6; SP-D, surfactant protein D; P/F, partial pressure of arterial oxygen/fraction of inspired oxygen ratio; HRCT, high-resolution computed tomography; PMX-DHP, polymyxin B-immobilized fiber column-direct hemoperfusion.

Table 3. Cox proportional hazard models of mortality from AE-IPF onset

Variable	Hazard ratio	95% CI		p Value
		Lower	Upper	
Univariate models				
Age, y	1.009	0.943	1.077	0.801
Sex, male	1.490	0.290	27.236	0.687
Smoking, never vs. former/current	1.007	0.155	3.827	0.993
Pack-years of smoking	1.011	0.991	1.032	0.278
FVC before AE*, % pred	0.983	0.937	1.024	0.428
DL_{CO} before AE, % pred	0.978	0.926	1.022	0.330
PaO_2 at rest before AE, Torr	1.014	0.948	1.100	0.697
Distance in 6MWT before AE, m	0.997	0.990	1.005	0.456
Minimum SpO_2 in 6MWT before AE, %	0.946	0.874	1.029	0.184
JRS severity grade before AE	1.980	0.950	6.082	0.072
GAP staging system before AE	0.997	0.460	2.228	0.995
Preceding treatments for IPF, +	2.130	0.670	7.989	0.203
Preceding treatments with antifibrotic agents, +	1.384	0.307	4.648	0.636
Preceding immunosuppressive treatments, +	2.367	0.740	7.574	0.142
Preceding oxygen therapy, +	4.649	1.439	15.01	0.012
P/F ratio at AE	0.994	0.987	1.001	0.081
Extent scores on HRCT at AE (full score 25)	1.282	1.067	1.601	0.007
Peripheral blood WBC at AE, high (vs. low)	3.621	1.079	16.335	0.037
Serum KL-6 at AE, U/mL	1.000	1.000	1.000	0.737
Serum SP-D at AE, ng/mL	0.999	0.997	1.001	0.475
Serum S100A8 at AE, high† (vs. low)	3.887	1.158	17.538	0.027
Serum S100A9 at AE, high‡ (vs. low)	3.887	1.158	17.538	0.027
Multivariate models adjusted for P/F ratio§				
JRS severity grade before AE	1.729	0.782	5.495	0.197
Preceding oxygen therapy, +	3.971	1.205	13.11	0.025
Extent scores on HRCT at AE (full score 25)	1.265	1.040	1.594	0.017
Peripheral blood WBC at AE, high (vs. low)	3.110	0.915	14.17	0.070
Serum S100A8 at AE, high† (vs. low)	4.032	1.200	18.21	0.023
Serum S100A9 at AE, high‡ (vs. low)	4.327	1.286	19.55	0.012

*Physiologic tests and severity scores were evaluated within 12 months before AE-IPF.
†S100A8 levels were divided into two groups based on the median value of 386.5 ng/mL.
‡S100A9 levels were divided into two groups based on the median value of 60.2 ng/mL.
§Multivariate models were adjusted for P/F ratio.
Abbreviations: CI, confidence interval; AE, acute exacerbation; IPF, idiopathic pulmonary fibrosis; FVC, forced vital capacity; % pred, percent predicted; DL_{CO}, diffusion lung capacity for carbon monoxide; 6MWT, 6-min walk test; JRS, Japanese Respiratory Society; GAP, gender, age, and physiology; P/F, partial pressure of arterial oxygen/fraction of inspired oxygen ratio; HRCT, high-resolution computed tomography; WBC, white blood cell; KL-6, Krebs von den Lungen-6; SP-D, surfactant protein D.

Fig. 1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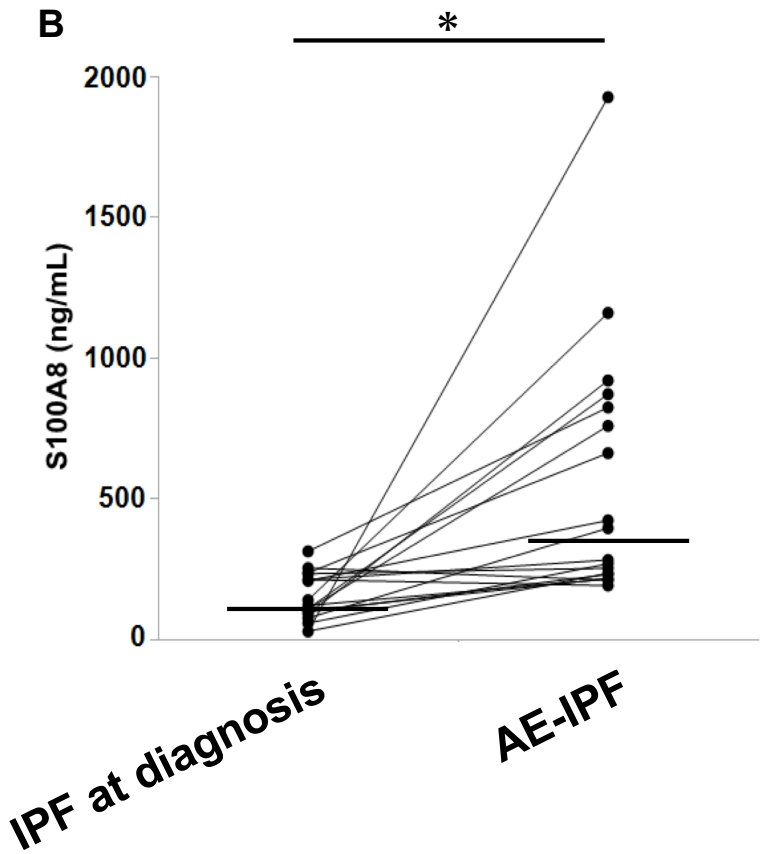
S100A8 (ng/mL)
HC
IPF at diagnosis
AE-IPF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*
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1500
1000
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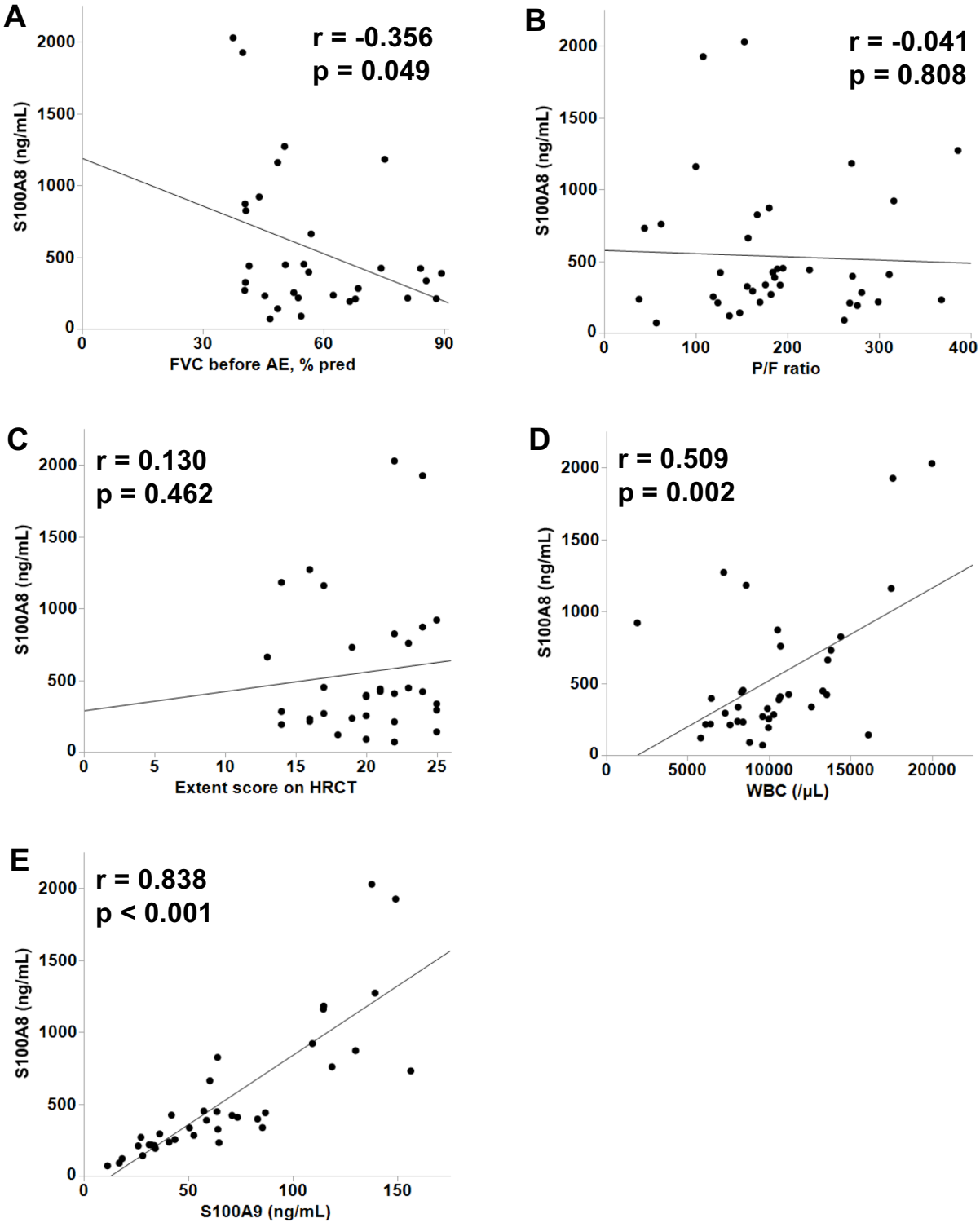
S100A8 (ng/mL)
IPF at diagnosis
AE-IPF

Fig. 2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FVC before AE, % pred
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S100A8 (ng/mL)
P/F ratio
C
r = 0.130
p = 0.462
S100A8 (ng/mL)
Extent score on HRCT
D
r = 0.509
p = 0.002
S100A8 (ng/mL)
WBC (/μL)
E
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S100A8 (ng/mL)
S100A9 (ng/mL)

Fig. 3

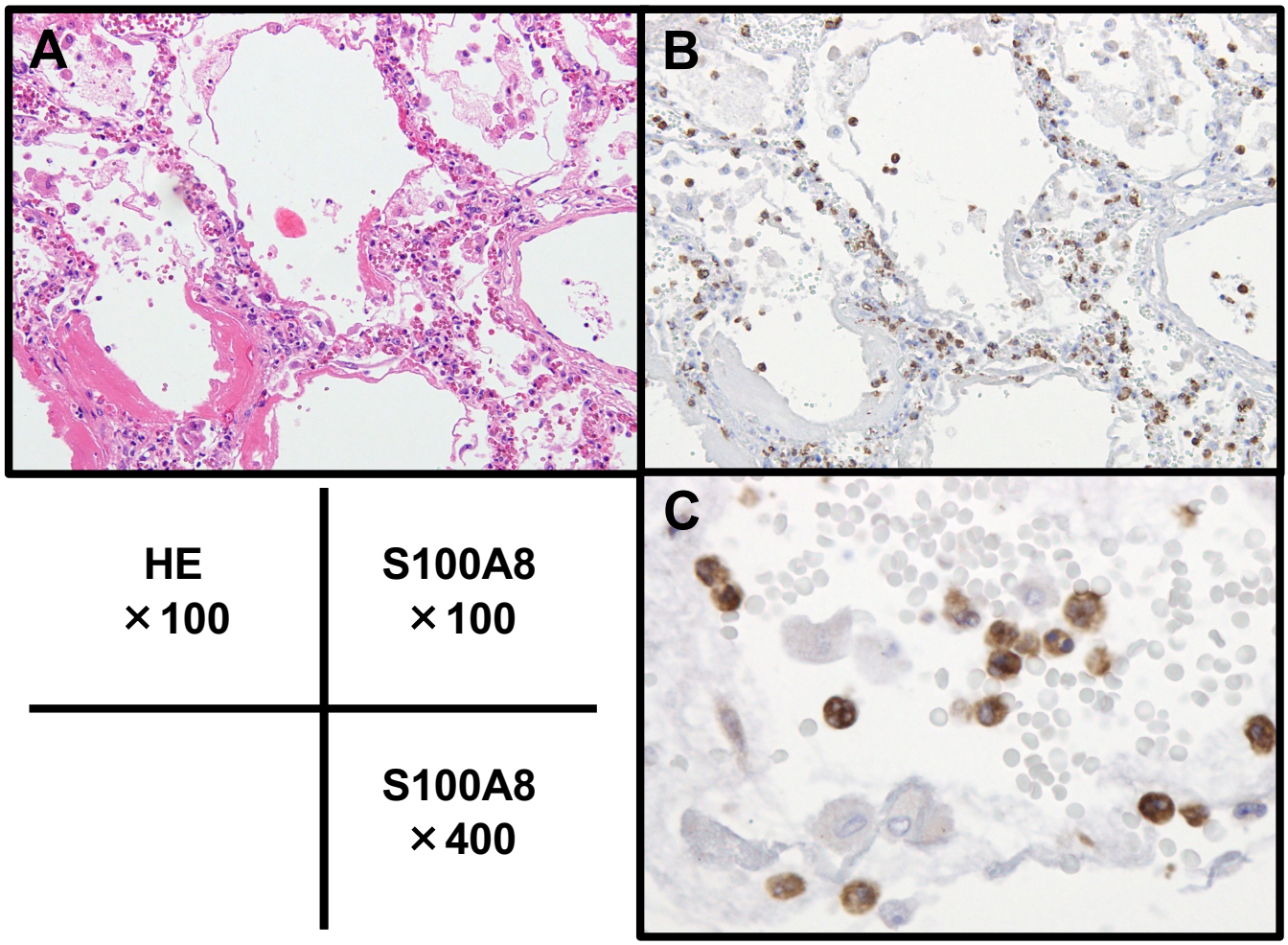
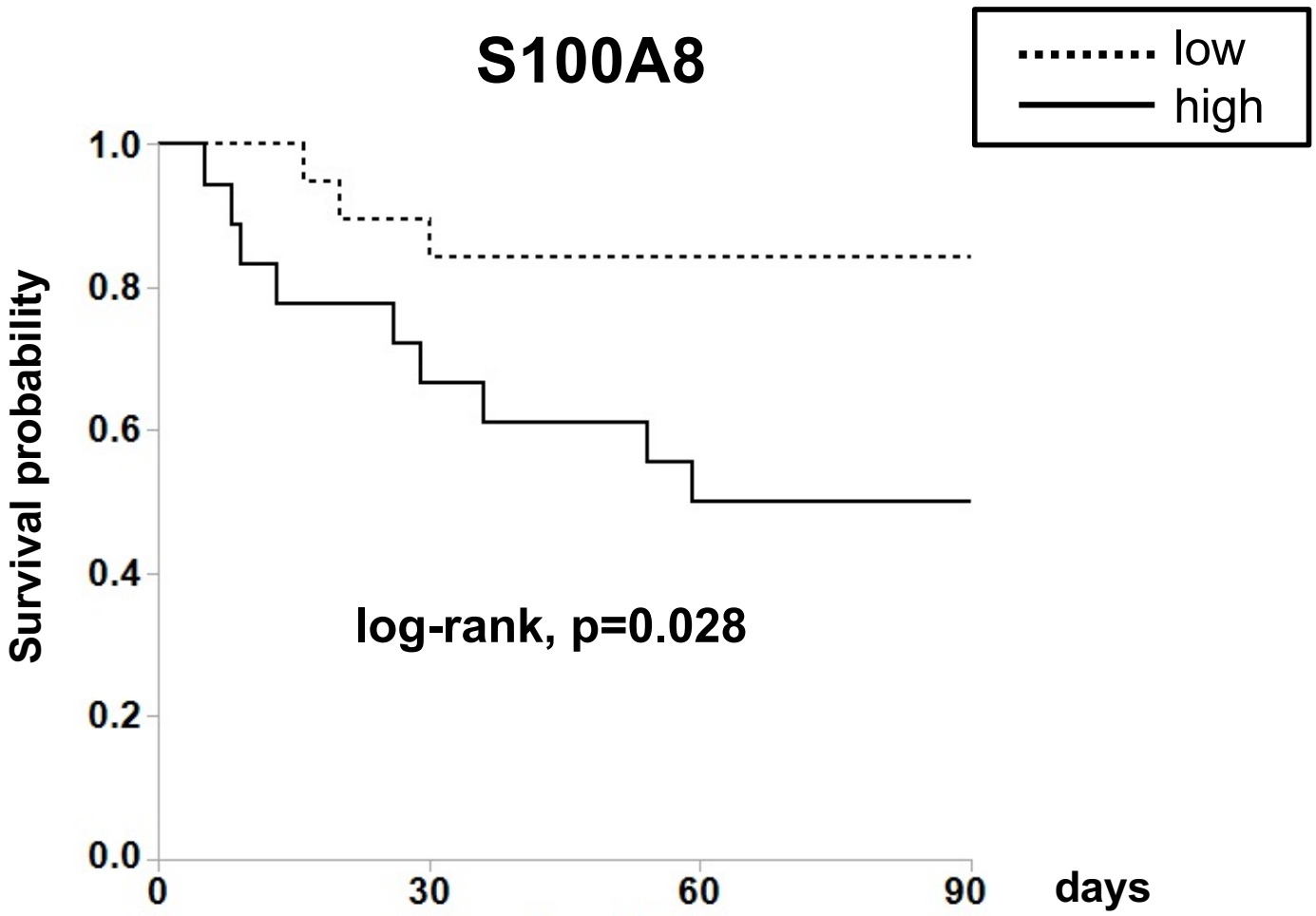


Fig. 4


S100A8
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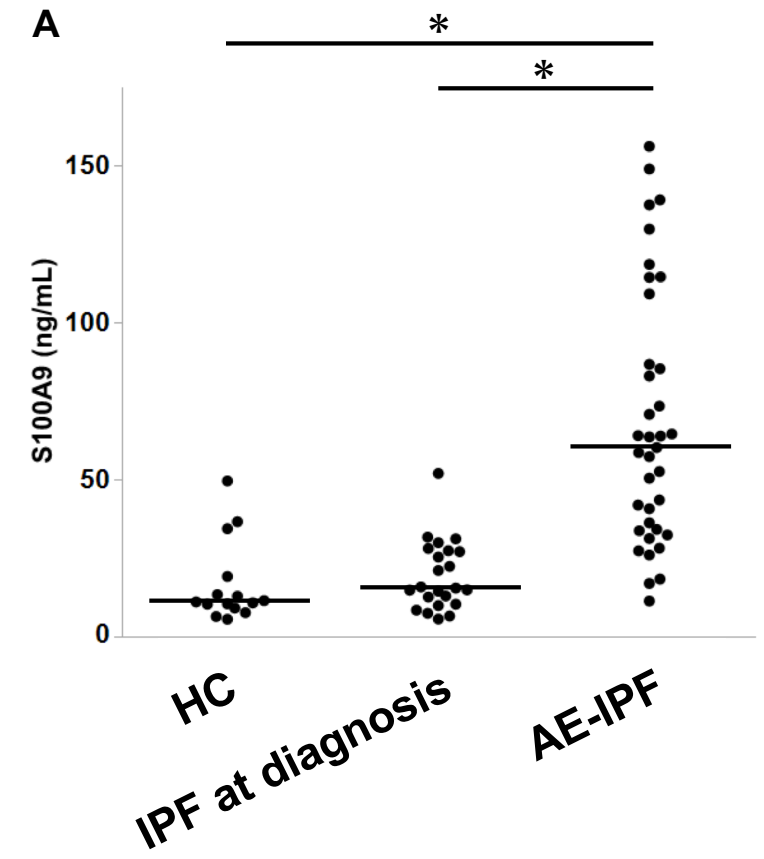
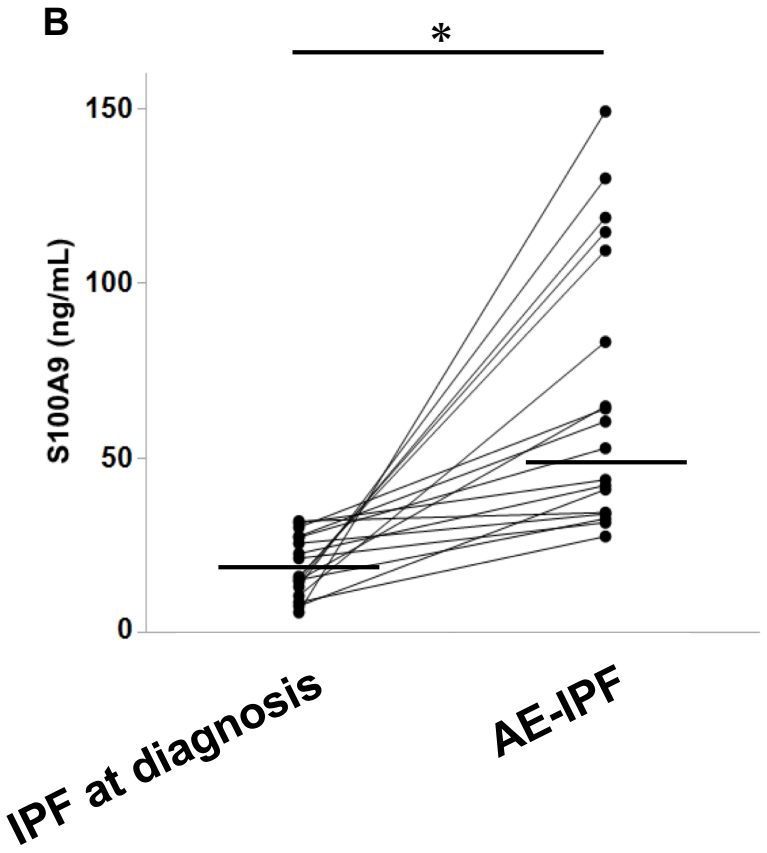
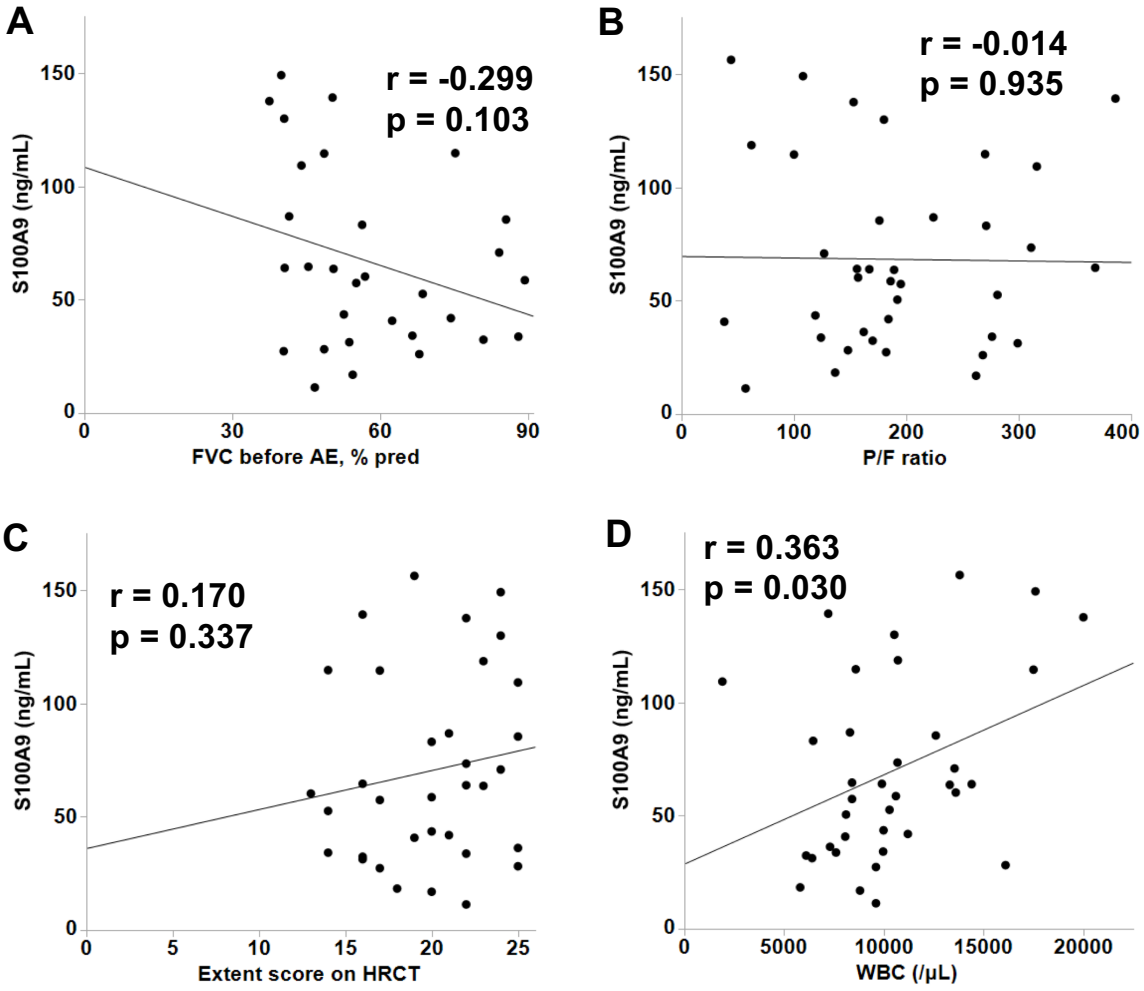
0
30
60
90
days

Supplementary Fig. 1

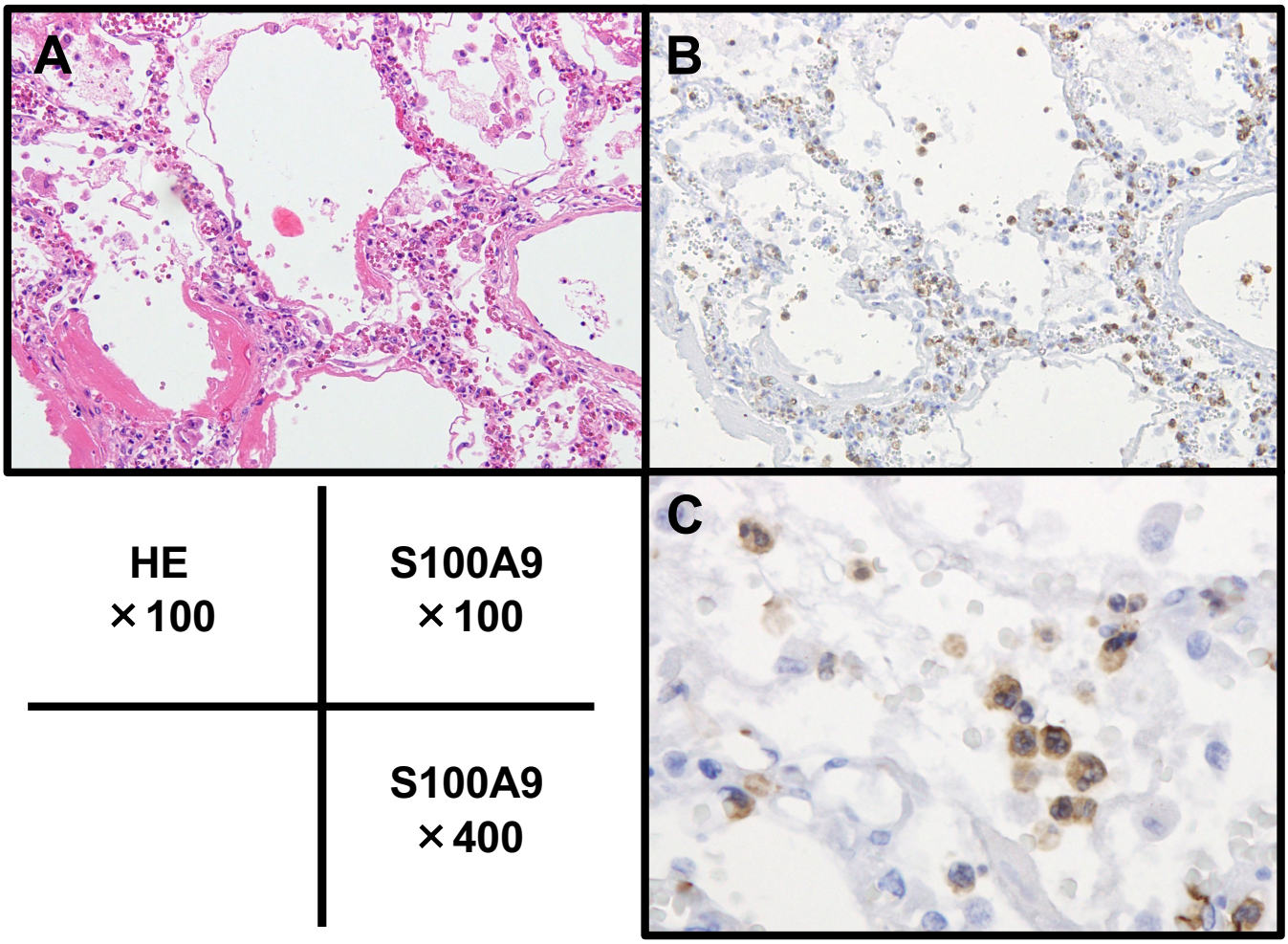




Supplementary Fig. 2



Supplementary Fig. 3



Supplementary Fig. 4