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Type 1 inflammatory endotype relates to low compliance, lung fibrosis, and severe complications in COVID-19

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ABSTRACT

Background: Coronavirus disease 2019 (COVID-19) is an acute respiratory disease; approximately 5% of patients developing severe COVID-19. It is known that cytokine release is associated with disease severity, but the relationship between the different clinical phenotypes and inflammatory endotypes is not well understood.
Objective: This study investigated the association between inflammatory biomarker-based endotypes and severe COVID-19 phenotypes.
Methods: Interleukin (IL) -6, C-reactive protein (CRP), C–X–C motif chemokine (CXCL) 9, IL-18, C–C motif chemokine (CCL) 3, CCL17, IL-10, and vascular endothelial growth factor (VEGF) were measured in 57 COVID-19 patients, and their association with clinical characteristics was examined using a cluster analysis.
Results: Significantly higher blood levels of the eight inflammatory markers were noted in patients who developed acute respiratory distress syndrome (ARDS) than in those who did not develop ARDS (non-ARDS). Using a cluster analysis, the patient groups were classified into four clusters, of which two had patients with high IL-6 and CRP levels. In the cluster with high levels of Type 1 (T1) inflammatory markers such as CXCL9 and IL-18, 85% of the patients had ARDS, 65% of the patients developed acute kidney injury (AKI), and 78% of the patients developed pulmonary fibrosis.
Conclusions: In the cluster with high levels of T1 inflammatory markers, the patients frequently suffered from tissue damage, manifested as ARDS and AKI. Our findings identified distinct T1 inflammatory endotypes of COVID-19 and suggest the importance of controlling inflammation by monitoring T1 biomarkers and treating accordingly to limit the severity of the disease.

1. Introduction

The coronavirus disease 2019 (COVID-19) pandemic, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is still a serious public health problem, with second and third waves of infection causing difficulties in many countries. The World Health Organization announced that the total number of cases had reached 82 million worldwide by 2 January 2021. COVID-19 poses a serious threat to public health and the socioeconomics of civilised society. Currently, treatment options for this disease are few, and infection control is still the main control measure until mass vaccinations can be established.

The asymptomatic infection period of COVID-19 is approximately 3–5 days. After the onset of the initial symptoms, patients can develop pneumonia, fever, and catarrhal symptoms for about a week. About 80% of these patients recover [1]. However, approximately 20% of patients develop further symptoms, with about 5% developing several critical conditions such as acute respiratory distress syndrome (ARDS), septic shock, intravascular thrombosis, and multiple organ failure. These

Abbreviations: ARDS, patients who already suffered from acute respiratory distress syndrome (ARDS) at the time of blood collection; pre-ARDS, patients who developed ARDS after blood collection; non-ARDS, patients who did not develop ARDS during the observation period; AKI, patients developed acute kidney injury (AKI); pre-AKI, patients who developed AKI after blood collection; non-AKI, patients who did not develop AKI during the observation period.

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conditions are caused by a severe inflammatory immune reaction with the release of a large amounts of pro-inflammatory cytokines, causing a "cytokine storm" or cytokine release syndrome (CRS) [2]. Several studies have attempted to understand the inflammatory pathophysiology by analysing the biomarkers in the peripheral blood. Thus far, elevated levels of IL-6, CRP, lactate dehydrogenase (LD), and neutrophils have been observed in patients with severe disease outcomes [3,4]. These findings collectively indicate that systemic inflammation occurs during severe COVID-19. Blood levels of IL-6 were increased before severe symptoms appeared in the patients who eventually died. This evidence indicates that peripheral blood biomarkers can predict disease prognosis before symptom onset [5]. Serum levels of various types of inflammatory biomarkers such as CXCL10 and IL-4 have also been reported to be increased in severe patients [6]. These biomarker levels were not equally elevated in all severe patients, suggesting that patients can be classified into distinct endotypes.

Therefore, in this study, in addition to IL-6, serum biomarkers of type 1 (T1) and type 2 (T2) inflammation were analysed retrospectively, and the relationships between inflammatory endotypes and the clinical characteristics of patients with COVID-19 were examined.

2. Methods

2.1. Patient selection

A total of 80 patients with COVID-19 who were admitted to Kobe City Medical Centre General Hospital, Kobe, Japan, between 10 March 2020 and 28 April 2020 were recruited for this study. One patient was excluded owing to cryptogenic organising pneumonia (COP) before the SARS-CoV-2 infection, and 22 patients were excluded because samples could not be obtained within three days of hospital admittance. After exclusion, 57 patients were included in this study. COVID-19 was confirmed using a SARS-CoV-2 specific polymerase chain reaction (PCR) test [7].

The study complied with the requirements of the Declaration of Helsinki and was approved by the Medical Research Ethics Committee of Kobe City Medical Centre General Hospital (20041/200636) and Sysmex Corporation (2020–11). Informed consent was obtained from all the patients using the following method: In compliance with the Ethical Guidelines for Medical and Health Research Involving Human Subjects, the study information was published on the website, and patients had the opportunity to withdraw their consent at any stage of the study. Therefore, the requirement for written informed consent from the enrolled patients was waived by the ethics committee.

2.2. Study design

This was a retrospective cohort study. All serum samples were collected from the patients after hospital admission. Clinical information was obtained from the medical records.

2.3. Data collection

Serum levels of IL-6, IL-10, IL-18, CXCL9, CCL3, CCL17, and VEGF were measured using a fully automatic immune analyser (HISCL-5000; Sysmex Corp., Hyogo, Japan). Serum levels of surfactant protein A (SP-A), Krebs von den Lungen-6 (KL-6), amino-terminal pro-brain brain sodium peptide (NT-proBNP), and presepsin were also measured on the HISCL-5000. The analytical performance is shown in Supplemental Figs. 4 and 5. Data on CRP, haematology, and biochemical tests were obtained from the medical records.

2.4. Diagnostic definitions

Fever was defined as an axillary temperature of at least 37.3 °C. Sepsis and septic shock were defined according to the 2016 Third International Consensus Definition for Sepsis and Septic Shock [8]. AKI was diagnosed according to the KDIGO clinical practice guidelines [9], and ARDS was diagnosed according to the Berlin definition [10]. The diagnostic values of the CT score were based on a scoring system described in a previous study [11]. Oxygen therapy was defined as supplemental oxygen or ventilatory management with an increased oxygen fraction. "At the time of admission" was defined as a specimen collected within three days of admission. The estimated glomerular filtration rate (eGFR) was calculated based on age, gender, and serum creatinine levels [12].

Table 1
Demographics and clinical characteristics of COVID-19 patients.

Demographics and clinical characteristics	Median (IQR)
Age (Years)	59 (45–72)
Sex (male)	33/57 (57.9%)
SpO_2 (%)	95 (94–98)
Nasal cannula oxygen therapy	12/57 (21.16%)
Invasive mechanical ventilation	13/57 (22.8%)
RR (times/min)	20 (16–24)
Fever (°C)	38.0 (36.9–38.4)
Dyspnea	29/57 (50.9%)
ARDS	28/57 (49.1%)
AKI	16/57 (28.1%)
SOFA	2 (0.75–4.25)
CT score	15 (6.3–21)
Pneumonia	47/57 (82.5%)
ICU	18/57 (31.6%)
Medication	
Favipiravir	23/57 (40.4%)
Steroid	5/57 (8.8%)
Ciclesonide	7/57 (12.3%)
Heparin	15/57 (26.3%)
Macrolide	25/57 (44.0%)
Other antibiotics	22/57 (38.6%)
Antipyretic	25/57 (43.9%)
Laboratory findings	Median (IQR)
Neutrophil (%)	75.1 (68.8–87)
Lymphocyte (%)	15.5 (10–23)
CRP ($\times 10^4$ μg/L)	6.3 (1.2–12.6)
LD (IU/L)	291 (202–411)
AST (IU/L)	32 (26–52)
ALT (IU/L)	23 (15–41)
BUN (mmol/L)	5.4(4.0–7.0)
Creatine (μmol/L)	70.7 (53.0–79.6)
eGFR (mL/min/1.73 m^2)	83.5 (68.5–260)
Comorbidities and smoking status	
COPD	1/57 (1.8%)
Asthma	2/57 (3.5%)
ILD	1/57 (1.8%)
Hypertension	16/57 (28.1%)
Diabetes mellitus	13/57 (22.3%)
Smoker	6/49† (12.4%)
Ex-Smoker	13/49 (26.5%)

Data are presented as median (IQR), n (%), or n/N (%), where N is the total number of patients with available data. †:8 patients lack smoking condition.

2.5. Statistical analysis

Owing to the non-normal distribution of data, data were described in terms of medians and interquartile ranges (IQR). Two-tailed P values < 0.05 were considered significant. Fisher's exact test, the Steel–Dwass test, and Mann–Whitney U test were applied using R (r-project) [13]. The Wilcoxon test was used to analyse the biomarker changes accompanying disease progression. Unsupervised hierarchical cluster analysis was performed using Cluster 3.0 (University of Tokyo Human Genome Centre). The cluster analysis was performed using a complete linkage based on Euclidean distance.

Table 2
Biomarkers associated with acute respiratory distress syndrome.

	Non-ARDS	Pre ARDS	ARDS	*P*-value		
	(n = 29)	(n = 8)	(n = 20)	PreARDS vs Non ARDS	ARDS vs Non ARDS	ARDS vs pre ARDS
IL-6, pg/mL	44.9 (15.8–97.9)	127 (95.8–140.6)	199.2 (91.2–310)	0.069	<0.001	0.194
IL-10, pg/mL	7.1 (4.4–16.4)	18.2 (14.3–21.7)	19.1 (12.6–26.4)	0.069	0.014	0.987
IL-18, pg/mL	464.6 (410.5–590.4)	656.6 (594.7–855.8)	686.9 (502.2–890.7)	0.013	0.010	0.933
VEGF, pg/mL	538.1 (296.2–1028.3)	360.5 (107–1252.4)	1063.3 (674.3–1534.5)	0.650	0.095	0.176
CCL3, pg/mL	40.9 (24.7–50.8)	63.4 (37.3–69.6)	71.4 (50.5–121.1)	0.303	0.002	0.534
CCL17, pg/mL	174 (85.6–290.6)	82.8 (59.3–169.6)	77.1 (56.2–129.7)	0.390	0.037	0.891
CXCL9, pg/mL	71.7 (52.3–126.4)	141.4 (112.9–193.6)	219.2 (147.5–417.1)	0.027	<0.001	0.502
WBC count, × 10^6 /L	5350 (3675–6075)[a]	5550 (4250–6600)	7050 (6150–9075)	0.950	0.001	0.083
Eosinophil, %	0.3 (0–1) [b]	0 (0–1.2)[c]	0 (0–0)[d]	0.898	0.054	0.528
Neutrophil, %	69 (63–81.5)[b]	76.6 (72.8–82)	86 (76–89)[e]	0.217	0.002	0.243
Basophil, %	0.2 (0–0.4)[b]	0.1 (0–0.4)	0 (0–0.3)[f]	0.992	0.733	0.901
Lymphocyte, %	22 (13.3–30)[b]	16.8 (13.3–19.5)	10.1 (7.5–13)[e]	0.354	0.003	0.140
Red blood cell, × 10^{10}/L	452 (419.5–486.5)[a]	448 (372.3–469.8)	460 (408.8–495)	0.891	0.899	0.771
Haemoglobin, g/L	135.5 (122–148.3)[a]	135 (118.8–138)	138.5 (128.8–152.3)	0.874	0.673	0.486
Platelet count, × 10^9 /L	200.5 (171.8–240)[a]	170 (136.5–206.8)	173.5 (146.3–219.3)	0.377	0.362	0.942
CRP, × 10^4μg/L	1.6 (0.4–5.2)	6.3 (5.0–8.0)	13.3 (9.1–17.9)	0.108	<0.001	0.083
LD, IU/L	215 (184–259)	310 (214.8–359.8)	450.5 (402.8–560.3)	0.144	<0.001	0.009
Presepsin, pg/mL	503 (282–966)	751.5 (572.5–2265)	1181 (715.5–1788.5)	0.340	0.007	0.867
NT-proBNP, pg/mL	39 (15–63)	119 (101.3–129)	214 (107.5–795)	0.046	<0.001	0.471
KL-6, U/mL	165 (137–245)	344.5 (212.3–470.8)	379 (215.3–597.3)	0.017	0.001	0.902
SP-A, ng/mL	26.4 (19.9–38.2)	48.2 (39.6–51.9)	51.6 (26–80.5)	0.123	0.039	0.815
Total bilirubin, μmol/L	8.6 (6.8–10.3)[a]	9.4 (8.2–11.6)	8.6 (6.8–10.8)	0.728	0.955	0.888
AST, IU/L	27 (22–32)	40 (26.8–83.5)	50 (36.5–79)	0.235	0.000	0.726
ALT, IU/L	19 (13–30)	28 (15.8–47.5)	32.5 (20.5–52.5)	0.661	0.111	0.977
BUN, mmol/L	4.3 (3.1–5.4)	6.1 (5.1–7.5)	6.9 (6–9)	0.149	0.001	0.614
Creatine, μmol/L	61 (53.9–71.6)	76.9 (56.6–117.6)	77.8 (59.2–96.4)	0.379	0.150	0.842
eGFR, mL/min/1.73 m^2	234.6 (81.3–283.7)	72 (44.3–94.8)	76.9 (62.8–132)	0.114	0.099	0.695

Data are presented as median (interquartile range) or n (%). *P* values were calculated using Steel-Dwass, pre-ARDS: Patients before onset of ARDS at blood collection; [a]n = 28, [b]n = 27, [c]n = 7, [d]n = 15, [e]n = 17, [f]n = 16.

3. Results

Fifty-seven adult patients (18 admitted to the intensive care unit: ICU, 39 non-ICU) were included in the study (Table 1). The median age was 59 years, and the median oxygen saturation (SpO_2) was 95% (IQR; 94–98%); 12 patients received oxygen inhalation therapy, and 13 patients received invasive mechanical ventilation. Twenty-eight patients had ARDS during the observation period, and 20 of them already had ARDS upon admission. Furthermore, 16 patients had AKI during hospitalisation, but only seven patients had AKI upon admission.

The relationship between the severity of COVID-19 and each blood biomarker was examined (Table 2). The patients were divided into three groups: patients who did not develop ARDS during the observation period (non-ARDS), patients who developed ARDS after blood collection (pre-ARDS), and patients who already suffered from ARDS at the time of blood collection (ARDS). Pre-ARDS developed into ARDS in a median of 4 days (IQR: 2–5.5) after blood collection. As previously reported, the blood levels of IL-6, CRP, blood neutrophil percentage, presepsin, NT-proBNP, KL-6, and LD were significantly higher in ARDS than in non-ARDS. In contrast, the levels of lymphocytes and CCL17 were significantly lower in ARDS. Furthermore, the blood levels of the T1-inflammatory markers CXCL9, CCL3 and IL-18 were higher in the patients with ARDS. In comparing pre-ARDS with non-ARDS, the blood levels of IL-18, CXCL9, NT-proBNP, and KL-6 were significantly higher in pre-ARDS. eGFR levels were also lower in ARDS and pre-ARDS than in non-ARDS.

A similar trend was also observed when the blood biomarker levels were compared based on the presence or absence of oxygen therapy, ICU management and AKI. (Table S1-3). Blood levels of T2-inflammatory markers, such as CCL17 and eosinophils, were low in the patients with severe disease, while the blood levels of T1-inflammatory markers such as CXCL9, IL-18, and CCL3 were high in these patients. The levels of systemic inflammatory markers such as IL-6, CRP, and IL-10 were also high in the patients with severe disease.

To examine the relationship between blood levels of inflammatory markers and various clinical pathological parameters, correlations between each parameter were analysed (Fig. 1, Supplemental Fig. 1). The levels of CXCL9 and CCL3 showed a strong positive correlation (r_s = 0.70), and the CXCL9 levels were positively correlated with those of IL-6, CRP, neutrophil percentage, IL-10, and IL-18 (r_s = 0.49, 0.56, 0.51, 0.47, and 0.46 respectively). The CRP levels were also positively correlated with the levels of T1-inflammatory markers such as CCL3 and IL-18 (r_s = 0.46, r_s = 0.43). CXCL9 levels showed a weak negative correlation (r_s = −0.32) with the levels of CCL17, a T2 inflammatory marker but did not show a significant correlation with the eosinophil percentage. The CXCL9 levels were positively correlated with the LD levels (r_s = 0.58) and negatively correlated with the lymphocyte ratio (r_s = −0.49). Positive correlations were seen between the CXCL9 levels and the levels of renal function markers, such as blood urea nitrogen (BUN) and creatine (Cr) (r_s = 0.53, 0.45), while a negative correlation was seen with eGFR levels (r_s = −0.43). Positive correlations were also obtained between the CXCL9 levels and the levels of aspartate aminotransferase (AST) and NT-proBNP (r_s = 0.52, r_s = 0.46) (Fig. 1, Supplemental Fig. 1)

The correlation coefficients were analysed using Spearman's correlation analysis. Grey squares indicate a lack of statistical significance; positive correlations are in red and negative correlations in blue. *P-SEP* presepsin, *WBC* white blood cells, *RR* respiratory rate per minute, *Neu* neutrophil %, *LD* lactate dehydrogenase, *Cr* creatinine, *BT* body temperature, *Eo* eosinophil %, *Lym* lymphocyte %, *Baso* basophil %, *Plt* platelet count, *dBP* diastolic blood pressure, *sBP* systolic blood pressure, *MAP* mean arterial pressure, *RBC* red blood cell count, *Hb* haemoglobin, *Tbil* total bilirubin.

In order to analyse the relationship between inflammatory markers and pathological conditions in more detail, the patients were classified into four clusters based on the inflammatory markers defined in the

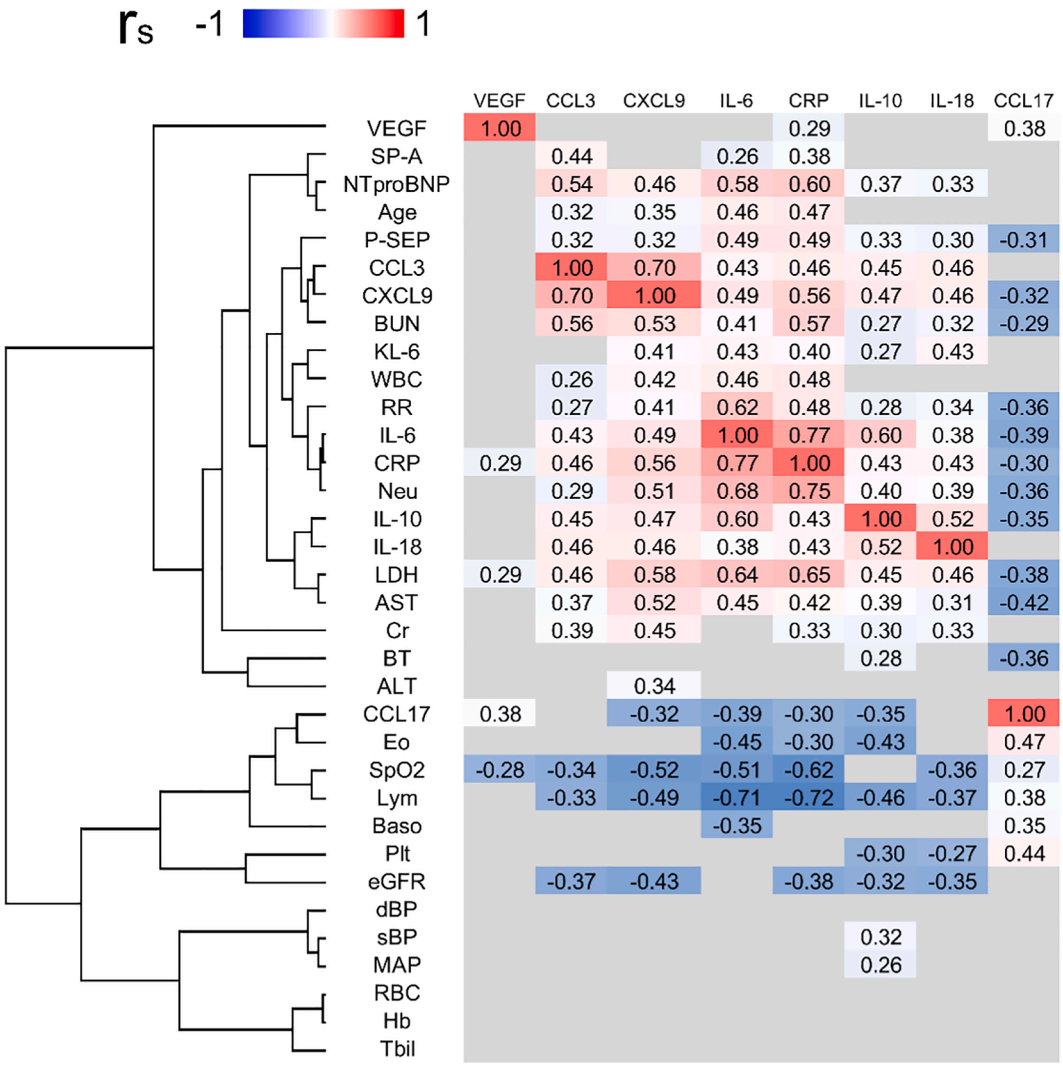

Fig. 1. Correlation coefficients of the clinical and biological parameters.

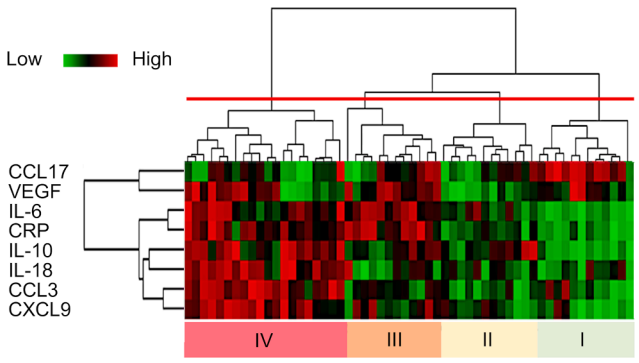

Fig. 2. Unsupervised hierarchical clustering analysis of serum inflammatory marker levels. The cluster analysis was performed by a complete linkage based on Euclidean distance.

unsupervised hierarchical cluster analysis: Cluster I, where only CCL17 was high; Cluster II, where all the inflammatory factors were relatively low; Cluster III, where VEGF, IL-6, and CRP were high; and Cluster IV which showed high levels of IL-10 and T1-inflammatory markers in addition to the Cluster III inflammation markers (Figs. 2 and 3).

Significant differences were not observed between Clusters III and IV for IL-6, CRP, lymphocyte %, neutrophil %, and LD (Fig. 3C, D, I, J, and K). The CT score, oxygen therapy frequency, SpO_2, ICU management ratio, invasive mechanical ventilation management ratio, and ARDS ratio worsened from Cluster I to IV depending on the strength of inflammation. (Fig. 4A, B, C, D, E, and F). Virus load was not significantly different among the clusters (Supplementary Fig. 4). And there was also no significant difference in the ratio of hypertension, diabetes, or smoker among the cytokine clusters (Supplementary Figure 6).

In Cluster IV, 70% of the patients received oxygen therapy, 65% were admitted to the ICU, and 85% suffered from ARDS or pre-ARDS. This cluster also included all type H patients, i.e. patients with severe disease, decreased pulmonary compliance, extensive CT consolidation, higher lung weight, high response to PEEP (lung recruitability), and a poor prognosis. 78% of patients in this cluster developed lung fibrosis (Fig. 4G and H). In addition, 20% of patients in this cluster already suffered from AKI, while 45% developed AKI after few days; Cr, eGFR, BUN, and AST values were significantly different from other clusters (Fig. 4I, J, K, L, and M).

In patients with pre-ARDS and pre-AKI, we examined whether the serum levels of inflammatory markers increased with ARDS or AKI onset. In Pre-ARDS to ARDS onset, serum levels of both CCL3 and CXCL9 tended to increase with the onset of ARDS except for patient 54, who already had high serum levels and had already developed AKI at the pre-ARDS period (Fig. 5A and B). This patient subsequently died. VEGF was the only inflammatory marker whose serum level significantly increased with ARDS progression. SP-A and KL-6, which are interstitial lung

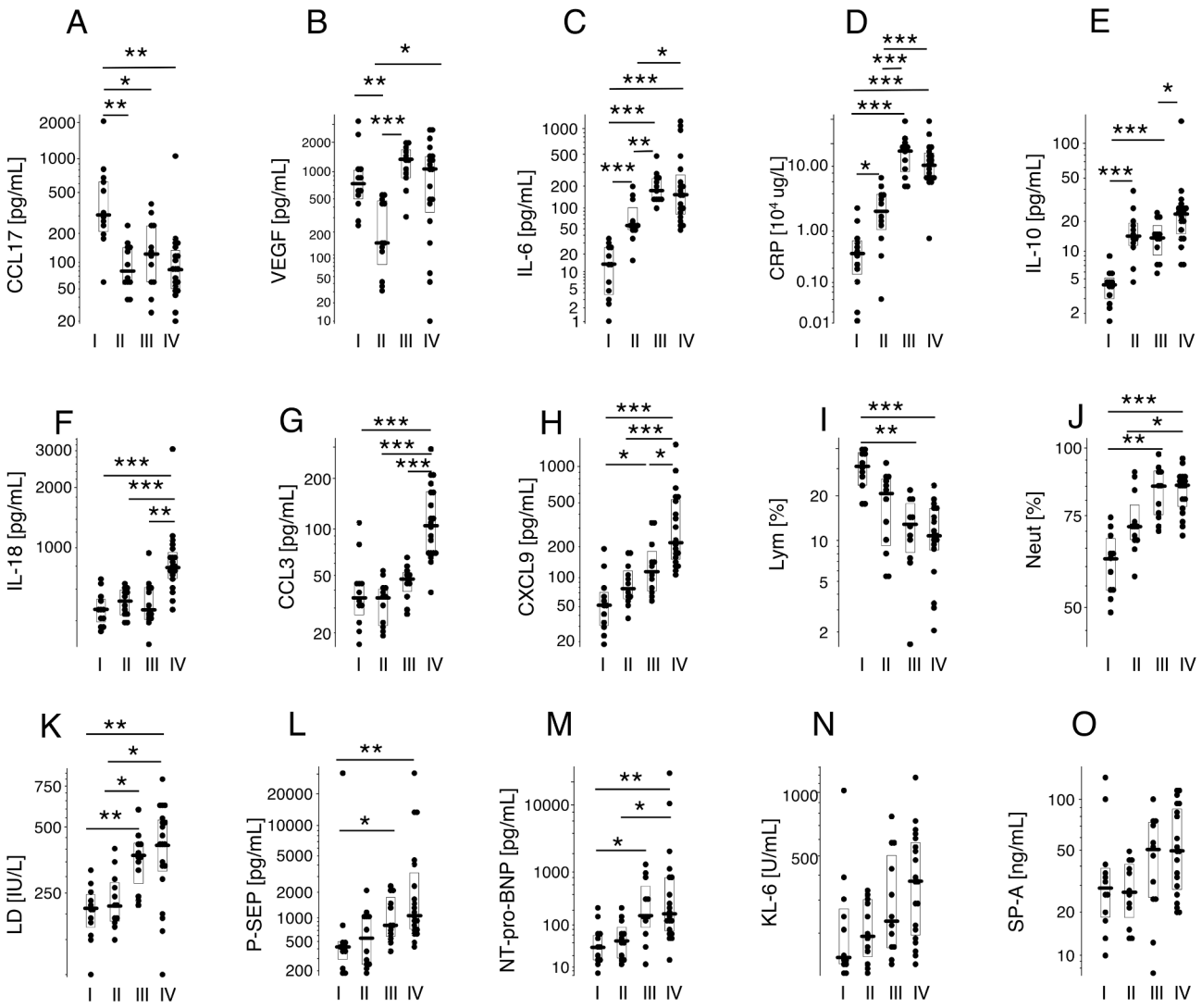

Fig. 3. Blood levels of inflammatory markers in the four identified clusters. CCL17 (A), VEGF-A (B), IL-6 (C), CRP (D), IL-10 (E), IL-18 (F), CCL3 (G), CXCL9 (H), lymphocyte % (I), neutrophil % (J), lactate dehydrogenase (K), presepsin (l), NT-pro-BNP (m), KL-6 (n), and SP-A (O). The results are shown as individual data points with a median (bar) and an interquartile range (box). The clusters are indicated at the bottom of each graph. *P*-values were calculated using the Steel–Dwass test. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

disease (ILD) markers, also tended to increase with the onset of ARDS; however, only SP-A was statistically significant (Supplemental Fig. 2).

Serum levels of IL-18 also increased significantly during the progression from pre-AKI to AKI. In patient 73, serum CXCL9 levels decreased at the onset of AKI, while the serum levels in other samples increased at the onset of AKI (Fig. 5G). Patient 73 received corticosteroid treatment during the sampling period and recovered from AKI, six days after the final blood collection. The CXCL9 levels were likely affected by treatment. The serum levels of CCL17, a T2-inflammatory marker, and VEGF were significantly elevated at the onset of AKI, and the IL-10 serum levels were significantly decreased. Furthermore, serum levels of SP-A and KL-6 were significantly elevated at the onset of AKI (Supplemental Fig. 2).

4. Discussion

This study showed that the blood levels of non-T2 inflammatory markers tended to be high in patients with severe disease. Furthermore, the correlation analysis indicated that the biomarkers reflected a variety of pathophysiological conditions in the patients based on their characteristics. The cluster analysis revealed the existence of a T1 endotype that is related to the type H phenotype, indicating progressive multiple organ failure such as AKI, and pulmonary fibrosis.

COVID-19 patients develop symptoms about 3–5 days after infection, after which many patients develop pneumonia [1]. It is known that the virus concentration is highest at the time of onset of pneumonia. The process by which COVID-19 progresses to ARDS and sepsis is due to CRS, triggered by an antiviral response [2].

The blood levels of IL-6, CRP, and LD are increased in severe COVID-19 patients, and these indicators have been used in clinical COVID-19 management [3,4]. It has also been reported that blood levels of T1-inflammatory markers (IFN-γ, CXCL10, CCL2, and CCL3), T2-inflammatory markers (IL-4 and IL-13), and an angiogenic factor VEGF were increased in severe patients [6]. However, these studies have also shown that the blood levels of inflammatory markers did not increase equally in all patients with severe pathological conditions. Some of the severe patients showed relatively low blood levels of IL-6 or T1-markers. These results suggest that endotypes with distinct pathophysiological conditions exist in patients with severe disease. Hence, this study focused on examining the inflammatory endotype. The T1-inflammatory markers involved in the viral immune response (CXCL9, CCL3, IL-18), systemic inflammatory markers (IL-6 and CRP), T2

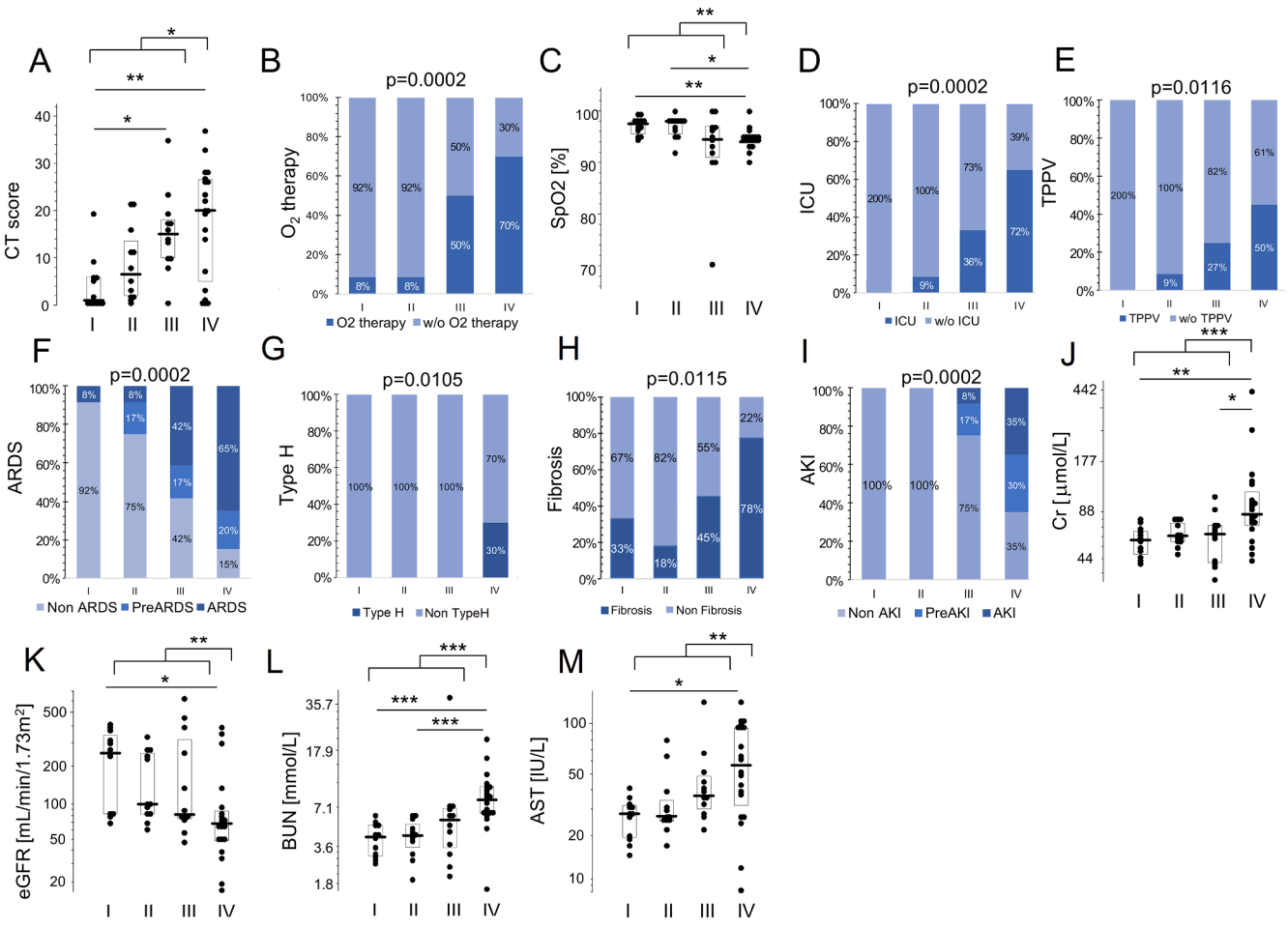

Fig. 4. A comparison of patient characteristics in the four identified clusters. CT score (A), oxygen therapy ratio (B), SpO_2 levels (C), ICU admission ratio (D), tracheostomy positive pressure ventilation: TPPV percentage (E), percentage of ARDS complication (F), percentage of CT confirmed fibrosis (H), percentage of AKI complication (I), serum creatinine levels (J), eGFR levels (K), BUN levels (L), serum ALT levels (M). The statistical significances between the clusters were calculated using Fisher's exact test (B, D, E-I) or the Steel–Dwass test (A, C, J-M). The statistical significance between Cluster IV and the other clusters was calculated using the Mann–Whitney *U* test (A, C, J-M). *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. *CT* Computed Tomography, *ICU* Intensive care unit, *Cr* Creatinine.

inflammatory marker CCL17, immunosuppression related cytokine IL-10 and VEGF were measured, and the inflammatory endotypes and clinical pathology of COVID-19 were examined. In addition, presepsin, NT-proBNP, KL-6, and SP-A were also analysed as related pathological markers [14–17].

The median age of the patients in this study was 59 years; about 20% suffered from ARDS, and 23% received invasive mechanical ventilation. In a multicentre study of inpatients in China, the proportion of severe patients in their 60 s among those hospitalised was found to be 11.8% (IQR: 7.01–24.0); the patient group in this study was part of a population of patients visiting a community hospital and not a specific group of severe patients [18]. In the present study, the presence of pneumonia was confirmed in 82.5% of the PCR diagnosed COVID-19 patients; this result was comparable to previous findings [1,19]. In this study, along with the severe disease manifestations of COVID-19, such as ARDS, oxygen therapy requirement, ICU management, the blood levels of systemic-inflammatory markers, cardiac load, and pulmonary tissue injury markers, including CRP, IL-6, presepsin, NT-proBNP, and KL-6 were increased. This result was generally consistent with other studies [3–5,14,16,17].

The serum levels of T1-inflammatory markers CXCL9, CCL3, IL-18, and the suppressor cytokine IL-10 were increased in ARDS patients and severe patients requiring ICU admission. The serum levels of CXCL9 and IL-6 were higher than those in the healthy population, even in mild cases [20,21]. Furthermore, serum CCL17 levels in severe patients were lower than the median level of 274.4 pg/mL (IQR: 199.7– 338.9) in a healthy population [21]. The levels of T1-inflammatory markers were positively correlated with previously reported severity indicators and negatively correlated with those of the T2-inflammatory marker CCL17. The CXCL9 and CCL3 levels increased with onset of the ARDS. These results suggest that T1-inflammation predominates in patients with severe COVID-19 and highlights the importance of T1-inflammation in the development of severe pathophysiology. Furthermore, the levels of T1-inflammatory markers were positively correlated with those of NT-proBNP, BUN, creatinine, and AST, and negatively correlated with eGFP levels. Multiple regression analyses suggested that CXCL9 was significantly associated with AST and eGFR (Supplemental Table 4, 5). The IL-18 levels were significantly elevated with the onset of AKI. T1 inflammation was therefore increased in severe conditions and it may reflect tissue damage such as in renal failure. However, significant downregulation of IL-10 and significant upregulation of VEGF and CCL17 were also observed, indicating that not only T1 inflammation but also the overall immune response was activated with the onset of AKI.

The patients in this study were classified into four clusters based on the levels of serum inflammatory markers. In Cluster I, where the CCL17 levels were normal, only one case was accompanied by ARDS, and this patient subsequently recovered from ARDS. Meanwhile, in Cluster II, where the levels of CCL17 were lower than the levels seen in healthy subjects, two patients subsequently developed ARDS, and one had ARDS on admission. The CCL17 levels of Cluster II were lower than Cluster I,

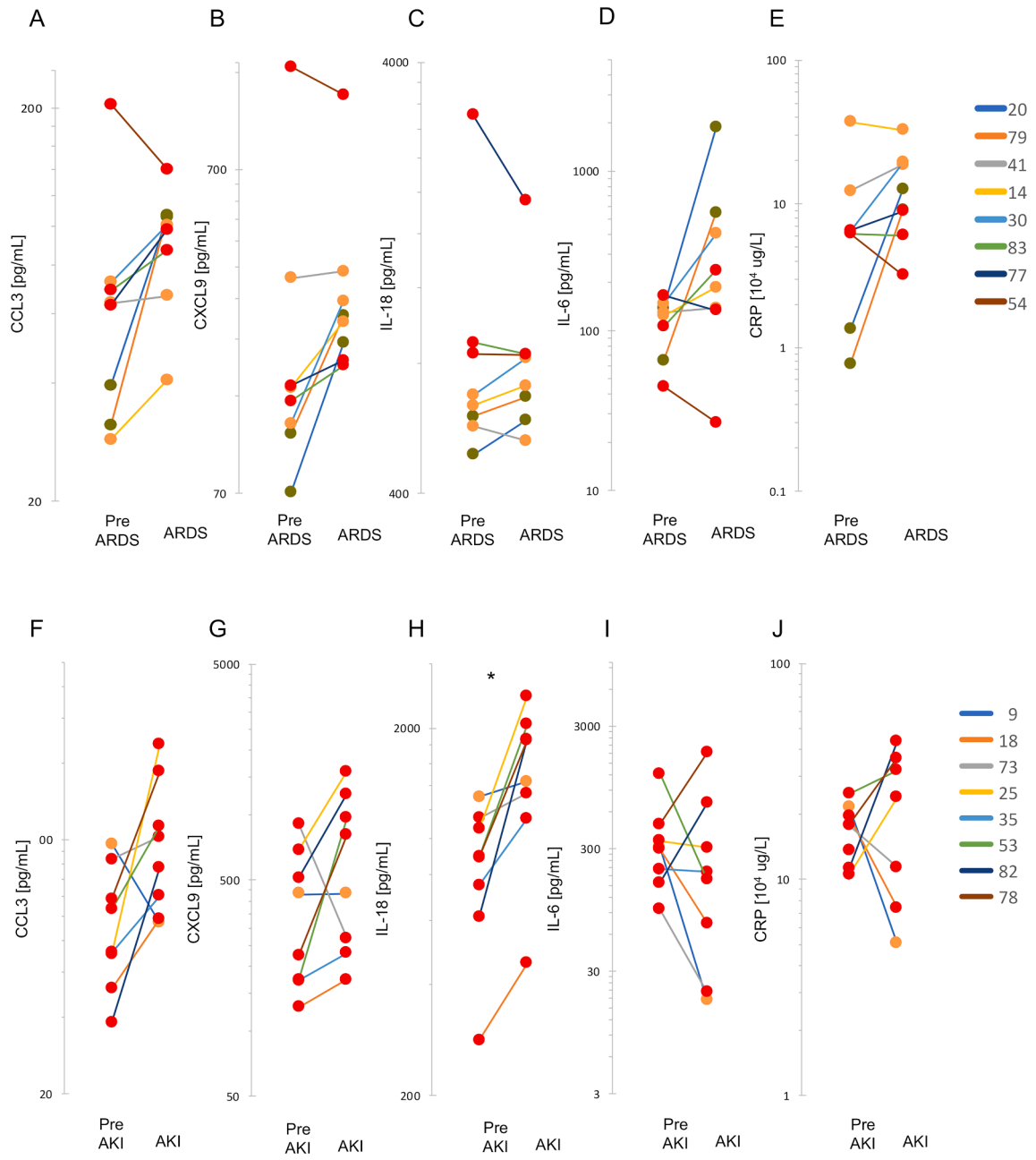

Fig. 5. . The serum levels of inflammatory markers before and after disease progression. Serum levels of CCL3 (A, F), CXCL9 (B, G), IL-18 (C, H), IL-6 (D, I), and CRP (E, J) were compared before and after ARDS (A-E) and AKI (E-J) using the Wilcoxon Test. Pre-ARDS: Patients before the onset of ARDS at blood collection; Pre-AKI: Patients before the onset of AKI at blood collection; *: $P < 0.05$.

however the levels of CXCL9, IL-6, CRP, and IL-10 were higher in Cluster II than the levels of Cluster I or healthy populations [20,21]. These results indicated that inflammation was more significant in Cluster II than Cluster I with a shift to T1.

The CRP and IL-6 levels in Clusters III and IV were higher than in Cluster I and II, but those concentrations were not significantly different between Cluster III and IV. In addition, the levels of LD, neutrophil %, and lymphocyte %, which were previously reported as severity indicators, were similar in both Cluster III and IV. However, the levels of T1-inflammatory markers were significantly increased in Cluster IV with 85% of these patients having ARDS or pre-ARDS, which required ICU admission or mechanical ventilation. All cases of type H patients with severe disease were also included in this cluster [22]. In addition, in Cluster IV, CT scores were high, and 75% of the patients developed lung fibrosis, potentially due to incomplete recovery of severe lung damage in the acute phase. Furthermore, in Cluster IV, 75% of patients developed AKI with high blood levels of renal function markers. Serum levels of AST were also induced in this cluster. These results suggest that patients in Cluster IV may have a strong tendency to suffer from multiple organ failure.

In Clusters I and II, where serum levels of T1-inflammatory markers were not high upon admission, the T1 markers increased with the onset of ARDS or AKI. This result indicated that the inflammatory endotype of a patient was not fixed but changed according to pathological status.

In the recovery period from ARDS, the serum levels of T1-inflammatory markers such as IL-18, CCL3, and CXCL9 were higher with dyspnoea or renal failure, which suggests that T1-inflammatory markers reflect residual inflammation in these patients (Supplemental Fig. 3). It remains to be seen if there is a relationship between the residual inflammation from COVID-19 and induction or exacerbation of

chronic diseases such as ILD or COPD.

The relationship between tissue damage and T1- inflammatory markers has been reported in various studies. For example, high blood levels of CXCL9 were reported in diseases with pathophysiology that depends on tissue destruction by activation of cytotoxic T cells, such as chronic graft-versus-host disease, Stevens-Johnson syndrome, and toxic epidermal necrolysis [23,24]. It was also reported that the blood level of T1-inflammatory markers became higher in diffuse alveolar damage (DAD)-positive patients with CTD-ILD, autoimmune thyroid destruction, trauma-induced ARDS, and SARS [25–28].

IL-18 is a cytokine produced by tissue damage through the inflammation caused by the activation of damage-associated molecular patterns [28]. It is thought that COVID-19 is also involved in the mechanism of tissue damage [29]. In COVID-19 infections, the sustained production of IL-6, IL-18, TNF-a and IFN- I/III drives hyperinflammatory cascades. Activated macrophages or epithelial cells produce CXCL9/10/11 which recruit natural killer cells or CXCR3 positive T cells such as cytotoxic T lymphocytes or Th1 cells from the bloodstream. Activated macrophages also amplify dysregulated immune responses including production of CCL3, which induces neutrophilic NETosis and microthrombosis [30].

It was reported that the lymphocyte count in peripheral blood is reduced in patients with severe COVID-19. In this study, the lymphocyte percentage was low in patients with severe disease. However, HLA-DR - and Tim-3 positively activated CD8 cells, and natural killer cells were reported to be increased in the blood of severe COVID-19 patients [31,32,33]. In addition, in patients with influenza A H1N1pdm09, it was reported that there were many CD4 T cells, CD8 T cells, CD83 positive dendritic cells, and natural killer cells in the lung parenchyma of patients who died from DAD. This evidence suggests a link between the onset of DAD and cytotoxic inflammation [31,34]. Serum levels of T1 inflammatory markers might reflect the hyperinflammation that is triggered by SARS-CoV-2 infection.

The results of this study and the above findings suggest that persistent T1-inflammation reflects a state of active cytotoxic events which causes damage to the lungs, or other organ such as the kidneys, by direct or indirect mechanisms.

This study had some limitations. Being a single cohort retrospective study, it may not have included all the COVID-19 endotypes. Pathological or immune cell biological data were also missing. This study was limited to the inflammatory endotype, and abnormalities in haemostasis and coagulation, which are characteristics of pathological conditions of COVID-19, were not examined. The impact of other respiratory comorbidities on the results cannot be determined in this study due to the small number of cases. Further studies are also required to understand the impact of such as anti-inflammatory treatments.

5. Conclusions

This study identified a distinct inflammatory endotype of COVID-19 that is related to further severe symptoms such as poor lung compliance or multiple organ failure. In this endotype, T1-inflammation was strong and persistent, in addition to the previously reported severity markers such as IL-6 and CRP. Monitoring T1-inflammatory markers may provide an opportunity to reduce subsequent disorders such as tissue damage and reduced residual fibrosis by instigating appropriate anti-inflammatory therapy.

CRediT authorship contribution statement

Takehiro Hasegawa: Conceptualization, Supervision, Methodology, Software, Formal analysis, Visualization, Data curation, Writing - original draft, Writing - review & editing. **Atsushi Nakagawa:** Resources, Investigation, Writing - review & editing. **Kohjin Suzuki:** Methodology, Investigation, Writing - original draft. **Kazuto Yamashita:** Methodology, Investigation. **Saya Yamashita:** Methodology. **Niina Iwanaga:** Methodology, Investigation. **Eiya Tamada:** Methodology, Investigation. **Kenta Noda:** Supervision, Project administration, Funding acquisition, Writing - review & editing. **Keisuke Tomii:** Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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