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Identifying two pathways to poor prognosis in patients with anti-MDA5 antibodies: insights from prognostic factor and cytokines analysis

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Abstract

Objective To identify pathways linking cytokine abnormalities to mortality via prognostic factors in patients with anti-melanoma differentiation-associated protein 5 antibodies (anti-MDA5 Ab).

Methods This study included patients with anti-MDA5 Ab whose serum was available. Serum cytokine levels were measured using a multiplex bead assay. Prognostic factors were identified using Cox regression and log-rank test. Prognostic factor groups were identified using principal component analysis (PCA) and factor and cluster analyses. The association between cytokine levels and prognostic factors (groups) was examined using PCA and correlation and path analyses. A prognosis-prediction model was developed using prognostic factors from the different groups.

Results Thirty-five patients were included in this study, of whom 31 had rapidly progressive interstitial lung disease (RP-ILD), and 14 died. We identified white blood cell (WBC), gamma-glutamyl transpeptidase (γ-GTP), lactate dehydrogenase (LDH), C-reactive protein (CRP), ferritin, and ILD-related factors (Krebs von den Lungen-6 [KL-6], surfactant protein D [SP-D], and CT score) as prognostic factors, in addition to von Willebrand factor and thrombomodulin. Two prognostic factor groups were found: Group 1 included WBC, CRP, and ILD-related factors, and Group 2 included ferritin, LDH, and γ-GTP. Both groups contributed to mortality. Group 1 was associated with IL-6, and Group 2 was related to IL-6, IL-10, and IP-10, and indirectly with TNF-α. A model using CRP (Group1) and γ-GTP (Group2) achieved an area under the curve of 0.84, which was not inferior to previously reported models.

Conclusions Two pathways leading to poor prognosis were identified in anti-MDA5-Ab-positive patients, each marked by specific cytokine abnormalities.

Keywords Anti-MDA5 antibody, Dermatomyositis, Interstitial lung disease, Prognostic factors, Cytokines, Grouping, Prediction model

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Introduction

Anti-melanoma differentiation-associated protein 5 antibody-positive dermatomyositis (MDA5 + DM) is a subtype of dermatomyositis characterized by the presence of anti-MDA5 antibodies, skin rashes with ulceration, tender palmar papules, minimal or absent muscle involvement, and rapidly progressive interstitial lung disease (RP-ILD) [1, 2]. ILD, a severe manifestation of MDA5 + DM, significantly influences patient prognosis [3]. Additionally, macrophage activation syndrome, hemorrhagic myositis, and pneumomediastinum have been reported as potentially lethal complications of MDA5 + DM [4].

The pathogenesis and pathophysiology of MDA5 + DM remain largely unknown. However, recent studies have shown the important role of cytokine abnormalities, particularly those of type I interferon (IFN) [1, 2, 5]. Macrophage activation also plays a critical role in organ damage, and serum levels of ferritin, secreted by activated macrophages, are linked to disease activity and prognosis [1, 6]. Additionally, vasculopathy may contribute to organ damage, including skin ulcers and potentially ILD [2].

Several studies have reported on various prognostic factors in MDA5+DM [7, 8], with RP-ILD emerging as the most significant factor of poor prognosis [7, 9, 10]. The prognostic factors include demographic variables such as old age and male sex [7, 10, 11]; hematological factors such as increased white blood cell (WBC), neutrophilia, lymphopenia, and increased neutrophil-to-lymphocyte (N/L) ratio [12, 13]; laboratory parameters related to tissue damage such as elevated levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), and gamma-glutamyl transpeptidase (γ-GTP) [10, 12, 14, 15]; inflammatory markers such as increased levels of C- reactive protein (CRP) and ferritin [9, 10, 15–17]; ILD-related markers such as decreased pulmonary function, extensive pulmonary abnormalities, increased Krebs von den Lungen-6 (KL-6) and surfactant protein-D (SP-D) levels [9, 14, 16–19]; and increased cytokine levels of IL-6, IL-15, and IFN-λ [17, 20, 21]. However, the relationships between these prognostic factors remain unclear, and it is yet to be determined whether they form several prognostic groups. Furthermore, the association between immunological and cytokine abnormalities and the prognostic factor groups is still uncertain.

This study aims to identify pathways linking cytokine abnormalities to prognosis using prognostic factor groups, assessing their impact on mortality, and analyzing the relationship between cytokine levels and the prognostic factor groups. Furthermore, we sought to develop a prognostic model using selected factors from each pathway as variables.

Methods

Study design

We conducted a retrospective cross-sectional study involving patients with anti-MDA5 antibody whose serum samples were available before treatment initiation. This study adhered to the Declaration of Helsinki and was approved by the Bioethics Committee of Dokkyo Medical University (#22081). Written informed consent was obtained from each participant for the use of their serum samples and their clinical and laboratory data.

We gathered the following data by reviewing medical records: demographics, medical history, clinical manifestations, organ involvement, and laboratory and radiological findings.

Patient selection

Participants included consecutive patients with anti-MDA5 antibody who were admitted to Dokkyo Medical University Hospital between 2010 and 2022 for initial induction therapy and whose serum was available. Idiopathic inflammatory myopathies (IIMs) and amyopathic dermatomyositis (ADM) were diagnosed according to the Bohan and Peter criteria [22] and the definition given by Kang et al. [23], respectively. Patients with reticular opacities, ground-glass opacity (GGO), or a honeycomb appearance on high-resolution computed tomography (HR-CT) were considered to have ILD. RP-ILD was diagnosed when the following conditions were met: worsening dyspnea on exertion, a decrease in partial pressure of oxygen (PaO2) by >10 mmHg within 4 weeks, newly emerging or expanding GGO on radiographic or CT imaging within 4 weeks, and exclusion of other causes such as infection and drugs [24]. Anti-MDA5 antibodies were detected using enzyme-linked immunosorbent assay (ELISA) kits (MBL, Tokyo, Japan). Anti-Ro 52 antibodies were detected using the EUROLINE Myositis Antigen Profile 3 (EUROIMMUNE, Lübeck, Germany). Serum samples for evaluation were collected prior to treatment on the same day immunosuppressive therapy was initiated. HR-CT scans for evaluation were conducted within 0–3 days before the start of immunosuppressive therapy. Control serum samples were obtained from healthy volunteers.

Treatment

Patients were treated with 'triple therapy' (high-dose glucocorticoid (GC), intravenous cyclophosphamide (CY), and calcineurin inhibitors (CI) as soon as possible after admission. Patients with less severe disease [no respiratory symptoms with normal oxygen saturation, normal ferritin or Kl-6 levels, and small extents of pulmonary abnormalities on HRCT (<5% of the total lung)] were started treatment with GC and CI and added CY if necessary. Janus kinase (JAK) inhibitors were added to the

above triple therapy in patients with poor response to treatment and poor prognostic factors, as previously reported [25] within 7 days of starting treatment.

HR-CT scoring

HR-CT imaging was reviewed by investigators blinded to the patients' clinical information and scored according to the modified methods of Kazerooni et al. [26] The extent of ILD in each lung field was semi-quantitatively scored as follows: 0, absent; 1, < 10%, 2, 10–25%; 3, 25–50%; 4, 50–75%; and 5, > 75% of the lung field. The CT score was calculated by summing the ILD scores from the six lung fields for each patient, ranging 0–30.

Identification of prognostic factors

We first conducted a comparative analysis of demographics and clinical characteristics between survivors and non-survivors and a univariate Cox regression analysis. Variables with P-values < 0.05 in either analysis were selected as candidate variables. If a log-rank test yielded a P-value < 0.05 for a candidate variable, it was identified as a prognostic factor. Cut-off points were determined using receiver operating characteristic (ROC) curves.

Measurement of serum cytokine levels

Serum samples were obtained with written permission from the patients and stored at − 80 °C. We measured the serum levels of IL-1β, IL-6, Il-10, IL-15, Il-17, GM-CSF, IFN-α, IFN-γ, IP-10, MCP-1, MIG, and TNF-α using a multiplex bead assay. All reagents were purchased from Bio-Rad Laboratories (Hercules, CA, USA), and the samples were prepared according to the manufacturer's instructions. The plate was measured on a Bioplex 200 system, and the data were analyzed using the Bio-Plex Manager (Bio-Rad Laboratories). Measurements and analyses were performed by Bio-Rad Laboratories (Tokyo, Japan). When the value was low or out of range, it was set to zero.

Statistical analysis

All statistical analyses, except path analysis, were performed using the JMP14 software (SAS Institute Japan, Tokyo, Japan). Path analysis was performed using Jamovi Desktop ver2.3.28 (https://www.jamovi.org/).

Normally distributed continuous data were analyzed using a two-sample t-test or one-way analysis of variance and were presented as median and interquartile range (IQR). Categorical data were examined using a chi-squared test or a Fisher's exact test. Statistical significance was defined as $p < 0.05$.

To examine patterns in prognostic factors and cytokine levels, we conducted principal component analysis (PCA) and factor and cluster analyses using the identified prognostic factors and elevated cytokine levels as variables. Factor and cluster analyses were performed using Promax rotation and Ward's method, respectively. Survival analysis was performed using log-rank tests and Cox regression analysis. In survival analysis, treatment initiation was defined as time zero for the analyses. The criterion for censoring was loss to follow-up within 365 days of observation.

Pearson's correlation test was used for correlation analyses. The area under the curve (AUC) obtained by an ROC analysis was used to compare the models.

When appropriate, the values of the prognostic factors and cytokine levels were logarithmized and standardized (standardized score). If a variable included zero, logarithmization was performed by adding the variable with the smallest value greater than zero to the analyzed variables.

The 'total weighted standardized score' was used to evaluate the effect of each prognostic factor group on mortality including Cox regression analysis. Since the impact on prognosis differs among factors, a weighted adjustment was made to account for the contribution of each factor to mortality using odd ratio. The 'total weighted standardized score' was calculated as the average of the 'weighted standardized scores' for the factors within the group. Each 'weighted standardized score' was obtained by multiplying the standardized score of the factor by its corresponding standardized odds ratio for death. The standardized odds ratios were calculated using standardized variables in logistic regression analysis. 'Total weighted standardized scores 'were further applied in correlation and path analyses to explore relationships among prognostic factors and their impact on mortality.

Path analysis was conducted using standardized cytokine scores as exogenous variables and total weighted standardized prognostic scores for the groups as endogenous variables. All the cytokines were initially included as variables in the analysis. Variables whose association with prognostic factors did not meet the P-value<0.10 were excluded. The analysis was continued until the final path diagram was obtained.

Results

Demographics and clinical features of patients with MDA5 DM

Thirty-five participants were enrolled in this study, including 19 females, with a mean age of 55. The patient demographics and clinical characteristics are shown in Table 1. All patients had ILD, and 31 developed RP-ILD. Fourteen patients died, with 13 deaths resulting from respiratory failure due to ILD. Survival curves are shown in Fig. 1A.

Table 1 Demographics and clinical features of patients with anti-melanoma differentiation-associated protein 5 antibody

	Total (*n*=35)	Survivor (*n*=21)	Non-survivor (*n*=14)	*P*-value*
Age, year	55.1±11.2**	50.9±10.9	61.3±8.9	**0.004**
Sex(M/F)	16/19‡	7/14	9/5	0.07
Smoking (current-/ex-/non-)	5/15/15	3/9/9	2/6/6	1.0
Disease duration§. weak	8 (4 – 2)***	5 (4–12)	8 (6.7–12.5)	0.19
Anti-Ro52 antibody	24	11	13	0.01
Symptoms				
Fever	22	12	10	0.38
Cough	31	18	13	0.56
Dyspnea	27	14	13	0.10
Organ involvement				
Lung	35	21	14	1.0
ILD	35	21	14	1.0
RP-ILD	31	18	13	1.0
Skin	35	21	14	1.0
Gottron's sign	34	20	14	1.0
Heliotrope rash	14	8	6	0.77
Skin ulcer	6	3	3	0.66
Raynaud's symptoms	0	0	0	1.0
Muscle	21	13	8	0.77
Muscle pain	10	7	3	0.70
Muscle weakness	15	8	7	0.48
Joint pain	18	13	5	0.17
Malignancy	2	2	0	0.23
Treatment				
Glucocorticoid	35	21	14	1.0
CsA/TAC	35	21	14	0.50
IVCY	30	17	13	0.62
JAK inhibitor	11	7	4	0.76
Triple therapy	27	15	12	0.43

*P-value; survivor vs. non-survivor, ** mean ± standard deviation, *** median (interquartile range)† number of patients, § Disease duration: From initial symptoms (rash, muscle symptom) to diagnosis, ‡ bold indicates statistically significant,

CsA, cyclosporine A; ILD, interstitial lung disease; JAK, Janus kinase; MDA5, melanoma differentiation-associated protein 5; RP-ILD, rapidly progressive interstitial disease; TAC, tacrolimus; triple therapy, high-dose glucocorticoid, cyclophosphamide, and calcineurin inhibitors

Poor prognostic risk factors in patients with MDA5 DM

As shown in Supplementary Table S1 and Fig. 1, older age, elevated WBC, elevated serum levels of LDH, γ-GTP, CRP, Kl-6, and SP-D, along with a large extent of inflammatory abnormalities in HRCT (high CT Score) were identified as poor prognostic factors. Elevated serum thrombomodulin (TM) and plasma von Willebrand factor (vWF) activity have also been identified as poor prognostic factors. However, male sex, neutrophil and lymphocyte numbers, and serum AST, ALT, and creatine kinase (CK) levels were not prognostic factors.

Grouping of poor prognostic factors

To summarize the numerous prognostic factors, we performed PCA using the laboratory and imaging prognostic factors measured in all cases. As shown in Fig. 2A, two components were identified. Component 1 was interpreted as a high-risk factor for death because non-survivors were frequently found on the right side of the score plot (Fig. 2B). Component 2 was interpreted as a different characteristic of prognostic factors, as indicated by the two groups of factors identified in the loading plot (Fig. 2A). To find shared underlying mechanisms, factor analysis was performed and confirmed two groups of prognostic factors (Fig. 2C). Cluster analysis, which was conducted for grouping of patients and cytokines, revealed three patient clusters and two prognostic factors clusters that were the same as the results of PCA and factor analysis (Fig. 2D).

Taken together, two groups of prognostic factors were identified: Group 1 consisted of WBC, CRP, KL-6, SP-D, and CT scores; the last three factors were markers for the severity of ILD. Group 2 consisted of ferritin, LDH, and γ-GTP. TM and vWF, which were not measured in all cases, were categorized into Group 2 and Group 1 respectively. However, correlations between prognostic factors and their groups were found (Supplementary Fig. S1).

Contribution of two prognostic factor groups to mortality

We used a Cox regression model to assess the contribution of two groups of prognostic factors to mortality, using 'total weighted standardized scores' for prognostic factor Groups 1 and 2. The analysis revealed that both groups were independent prognostic factors for mortality (Fig. 3A).

Each group was further divided into high and low according to the median value of 'the total weighted standardized scores (cut off values: Group 1: 0.15, Group 2: -0.12). Patients were classified into four subgroups: low/low, high/low, low/high, and high/high for Groups 1 and 2, and their survival rates were examined. Clinical features of patients in each group were shown in Supplementary Table S2. As shown in Fig. 3B, the survival rates of high/low and low/high patients were similar. The survival of patients with high/high was poorer than that of patients with high/low and low/high.

Additionally, we examined the impact of components of PCA on mortality. Cox regression analysis using individual component scores as variables identified Component 1 as a significant risk factor, with a hazard ratio of 1.71 (95% CI: 1.31–2.35, P=0.002). In contrast, Component 2 was not identified as a risk factor, with a hazard ratio of 0.98 (95% CI: 0.67–1.31, P=0.98). These results revealed that Component 1 was associated with mortality but not Component 2. The result that Component 2 was

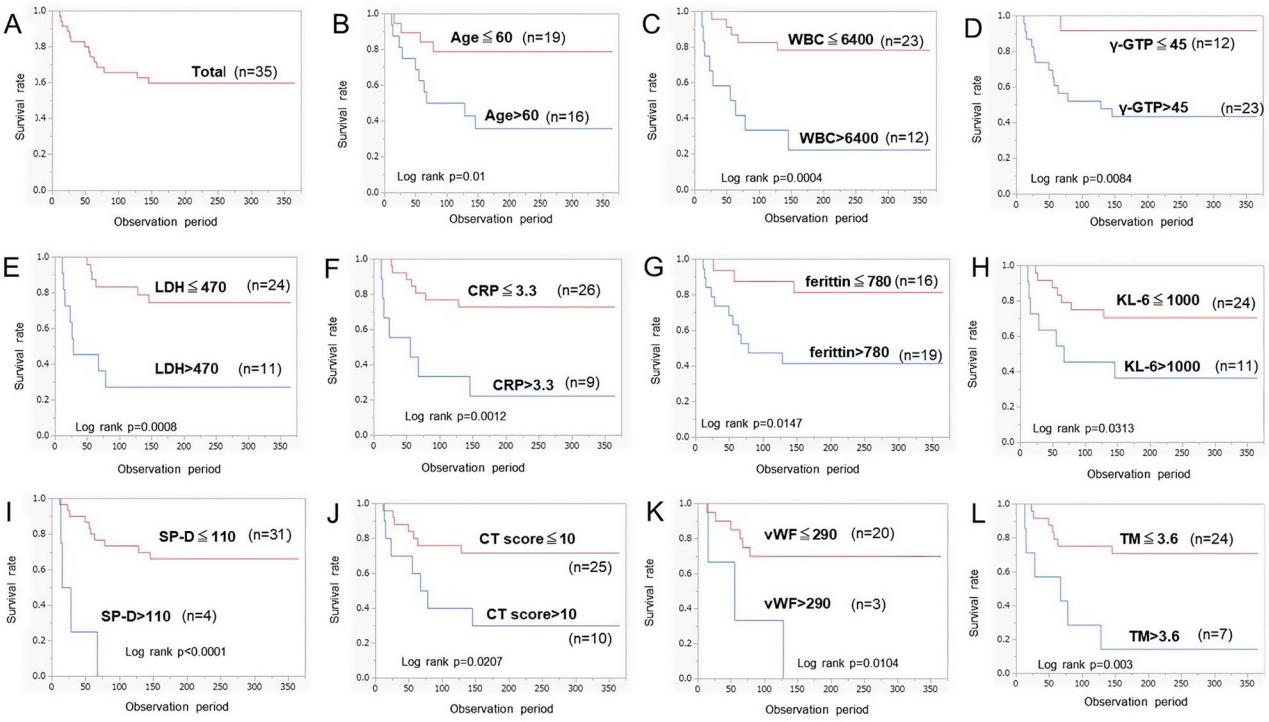


Fig. 1 Poor prognostic factors identified in patients with anti-melanoma differentiation-associated protein 5 antibody. Each figure shows the survival curve by Kaplan–Meier analysis. Survival curves for all patients: (**A**) survival curves of patients divided by the cut-off levels of the indicated factors: age (**B**-**L**). (**B**) age; (**C**) white blood cell count (WBC); (**D**) γ-glutamyl transpeptidase (γ-GTP); (**E**) lactate dehydrogenase (LDH); (**F**) C-reactive protein (CRP); (**G**) ferritin; (**H**) Krebs von den Lungen-6 (KL-6); (**I**) surfactant Protein-D (SP-D); (**J**) CT-Score; (**K**) von Willebrand factor (vWF); (**J**) thrombomodulin (TM)

not a poor prognostic factor may suggest that the two groups independently contribute to mortality because Component 2 reflects the different nature of prognostic factors (groups).

Together, these two poor prognostic factor groups contributed to mortality in patients with anti-MDA5 antibody.

Cytokine profiles in patients with MDA5 DM

Serum cytokine levels were measured before initiating immunosuppressive therapy. As shown in Supplementary Table S3, serum levels of IL-6, IL-10, IFN-γ, IP-10, MCP-1, MIG, and TNF-α were elevated compared to those in healthy controls. IL-6 and IFN-γ levels were higher in non-survivors than survivors (Supplementary Table S3) and were identified as poor prognostic factors (Supplementary Fig.S2).

Cytokine abnormalities underlying two groups of poor prognostic factors

We assessed the relationship between the prognostic factors and elevated cytokine levels. First, we added cytokines as supplemental variables to the PCA prognostic factor. As shown in Fig. 4A, IL-6, IFN-γ, and MCP-1 were grouped with WBC, CRP, and ILD markers (Group 1), while IL-10, IP-10, MIG, and TNF-α were grouped with ferritin, LDH, and γ-GTP (Group 2). We also conducted PCA, including elevated cytokine and laboratory tests compared to healthy subjects as covariates (Supplementary Fig.S3). Similarly, IL-6, IFN-γ, and MCP-1 were associated with Group 1, while IL-10, IP-10, MIG, and TNF-α, as well as AST, ALT and CK, were associated with Group 2.

Correlation analysis revealed that Group 1 and its related prognostic factors were correlated with IL-6, while Group 2 and its prognostic factors were correlated with IL-10, IP-10, TNFα, and IL-6 (Fig. 4B).

Path analysis confirmed that IL-6 was associated with both groups, whereas IL-10 and IP-10 were associated with Group 2 although there was an interaction between Groups 1 and 2 (Fig. 4C).

When compared with the contributions of cytokines to Group 2 (coefficient of determination, R2: 0.40, $p<0.001$), the contribution of IL-6 to Group 1 (R2=0.13, $p=0.04$) was small, suggesting that IL-6 partially explained the development of Group 1.

Notably, there was a bidirectional interaction between Groups 1 and 2. We tested models that explored the influence of Group 1 on Group 2 and vice versa. Both analyses (Supplementary Fig. S4) showed comparable fit indices to the original model in Fig. 4C (Goodness of fit index (GFI): 0.97 in Fig. 4C, 0.99 in S-Fig. 3AA, 0.95

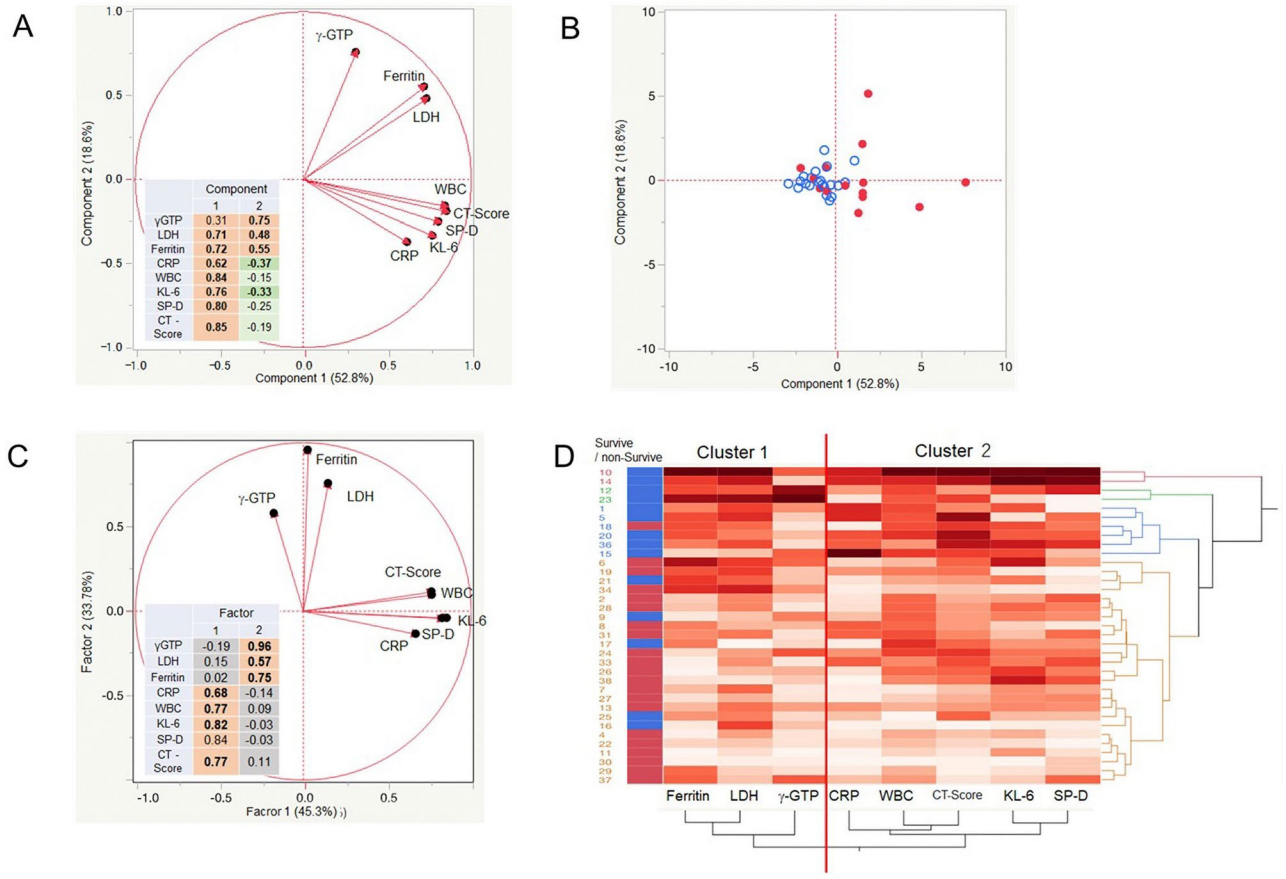


Fig. 2 Two groups of prognostic factors. (**A**) Loading plot by principle component analysis (PCA) of prognostic factors. A table indicates the loading values of the variables for each component. (**B**) Scatter plot of prognostic factors by PCA; red circles indicate non-survivors, and blue open circles indicate survivors. (**C**) Prognostic factor analysis. This table indicates the factor loading values of the variables for each factor. (**D**) Cluster analysis of patients according to the prognostic factors. Blue and red cells indicate non-survivors and survivors, respectively

in S-Fig. 3B), suggesting bidirectional interactions. In these path models, IL-6 is linked to one risk factor group, through which IL-6 is indirectly connected to another group.

In summary, Group 1 was linked to IL-6, while Group 2 was associated with IL-6, IL-10, and IP-10 with a possible indirect association with TNF-α. There was significant bidirectional interaction between Group 1 and Group 2, where IL-6 was involved.

Prognostic-prediction model using variables from two groups of prognostic factors

We developed a simple prognostic model using a pair of factors from the two groups of prognostic factors. To compare the effectiveness of the models using different group pairs versus same-group pairs, we calculated the AUC of the models using a pair of variables (Fig. 5A). The difference between the AUC with the paired variables and the average AUCs with the original variables was high when using a different group variable pair compared to using the same group pairs (different group: 0.083±0.015 vs. same group: 0.062±0.020, p=0.01) (Fig. 5B). These results suggest that models with different group pairs may be more effective than those with the same group pairs.

We created a prognostic prediction model using WBC from Group 1 and γ-GTP from Group 2, which had the highest AUC of 0.84. Using a scoring system that assigns one point for each g-GTP>45 or WBC>6,400, the one-year survival rates of patients with zero, one, and two points were 93%, 73%, and 10%, respectively (Fig. 5C). When comparing the usefulness of our models with previously reported models using our participants, our model was not inferior to previously reported models such as FLAIR [9] with AUC 0.72 (Fig. 5D), MSK [16] with AUC 0.70 (Fig. 5E), and CROSS [11] with AUC 0.66 (Fig. 5F).

Discussion

The present study revealed that the prognostic factors can be categorized into two groups. Group 1 included WBC, CRP, and ILD markers, while Group 2 included

A

	Uni-variate			Multi-variate		
	RR	CI	p	RR	CI	p
Group 1 (WBC CRP LUNG)*	1.72	1.27-2.43	0.0002	1.65	1.22-2.34	0.008
Group 2 (Fer LDH g GTP)*	1.40	1.11-1.75	0.004	1.38	1.04-1.78	0.02

B

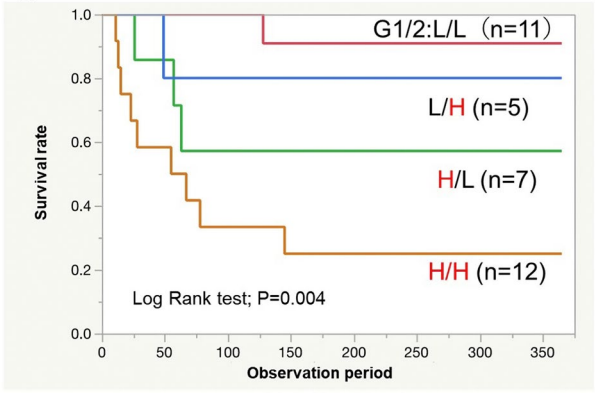


Fig. 3 Contribution of two groups of prognostic factors to mortality. (**A**) Cox regression analysis of two groups of poor prognostic factors. (**B**) Survival curves of patients divided into four groups by prognostic scores of Groups 1 and 2 (Group 1/2: low/low, low/high, high/ low, and high/high). Cut-off values for classification were 0.15 for Group 1 and −0.12 for Group 2, based on the total weighted standardized score. CI, confidence interval; HR, hazard ratio

ferritin, LDH, and γ-GTP. These groups were linked to specific cytokine abnormalities: Group 1 was associated with IL-6, and Group 2 was associated with IL-6, IL-10, IP-10, and, indirectly, with TNF-α, although there were bidirectional interactions between the two groups. These findings suggest that these two pathways lead to a poor prognosis with characteristic cytokine abnormalities in patients with MDA5+DM.

Although many researchers have identified various biomarkers related to prognosis [7–21], few have explored the relationship between these prognostic factors. Correlations among ILD-related markers, including the extent of ILD, pulmonary function test results, and KL-6 levels, have been reported in MDA5+ILD [27], consistent with findings in ILD more broadly [28].

Additionally, associations between KL-6, LDH, and ferritin levels have been observed. These correlations may suggest a true association due to shared mechanisms, although pseudo-correlations cannot be ruled out. Given that all factors, as shown in PC1 in the PCA, indicate a high risk of mortality, correlations between factors from different groups in this study are not unexpected. However, the true nature of the relationship between these prognostic factors remains uncertain. This study is the first to demonstrate distinct groups of prognostic factors.

Group 1 included ILD markers, CRP, and WBC, but not ferritin or LDH, which was an unexpected finding as both ferritin and ILD markers like KL-6 are widely recognized biomarkers for disease activity and prognosis (9–10, 15, 16, 17). Our findings indicate that ferritin and ILD markers belong to different groups. The correlation between ferritin and ILD markers may be due to pseudo-correlation associated with high mortality risk, although several studies have reported macrophage activation and ferritin production in the lungs [29]. LDH, a general marker of ILD severity, reflects lung injury causing LDH release [30]. In MDA5 + DM, LDH has been reported as a risk factor for RP-ILD and poor prognosis [9–11]. The separation of LDH from ILD markers into different groups may be due to the greater significance of LDH release in systemic cell and tissue damage, driven by macrophage activation and accompanied by ferritin production, rather than in lung injury.

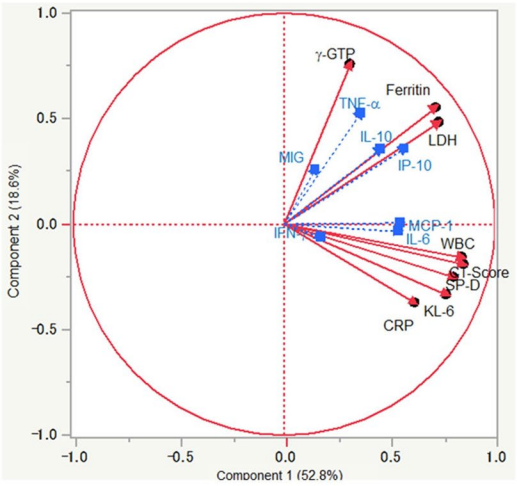
The mechanism linking ILD with CRP levels and WBC count remains unclear. Inflammation characterized by elevated CRP and WBC count may play a more significant role in ILD development compared to inflammation driven by macrophage activation. IL-6, an inducer of CRP production, may be involved in ILD development.

However, it is noteworthy that additional unknown factors may be important in developing ILD as the contribution of IL-6 alone was insufficient to fully explain its pathogenesis (Fig. 4C). fully (Fig. 4C). IFN-γ and MCP-1 could be potential contributing factors, as they were associated with Group 1 in PCA analysis (Fig. 4A). Type 1 IFN may also play a role in ILD development, although IFN-α was not sufficiently detected in this study. The Type 1 IFN signature is upregulated in the lungs of patients with MDA5-ILD [31]. We recently reported that IFN-α and IL-6 are elevated in the BALF of patients with MDA5-ILD, and that PCA analysis of BALF showed IL-6 and IFN-α belong to the same component [32]. Identifying these unknown factors may be a key to developing novel treatments for refractory MDA5+ILD.

Group 2 consisted of ferritin, LDH, and γ-GTP with IL-6, IL-10, IP-10, and TNF-α. Since ferritin, IP-10, and TNF-α are produced by activated macrophages [6], Group 2 likely reflects macrophage activation and associated cell and tissue damage.

Together, these two pathways—the development of ILD and macrophage activation—appear to contribute to mortality in anti-MDA5+patients. Importantly, these pathways may interact indirectly, as evidenced by correlations between the two biomarker groups and the involvement of IL-6 in both pathways. These interactions suggest the involvement of macrophage activation and its related cytokines in the development of ILD. Alternatively, the two groups might represent different stages of disease progression, given that the temporal and causal relationships between prognostic factors remain unclear. Further studies are needed to elucidate the pathways

A



B

	IL-6	MCP1	IFN γ	MIG	IL-10	IP10	TNFα
Group1*	0.43						
CRP*	0.43						
WBC*	0.42						
KL-6*	0.37						
SP-D*							
CT-Score*	0.35						
Group2*	0.39				0.54	0.56	0.53
γ-GTP*					0.56	0.35	0.37
LDH*	0.44				0.45	0.56	0.52
Ferritin*	0.37					0.48	0.43

C

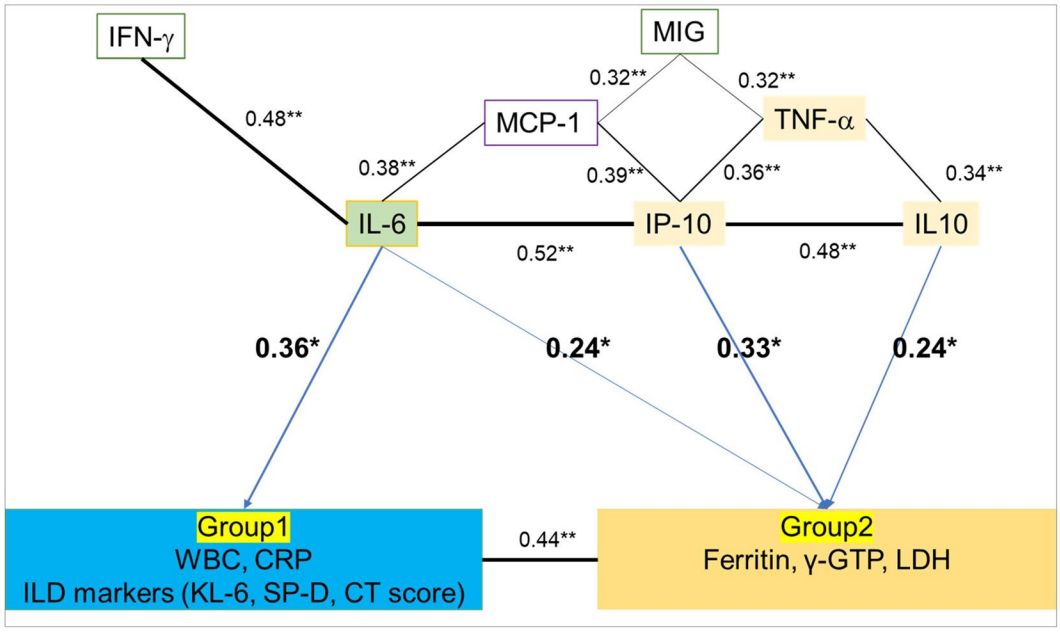


Fig. 4 Association between cytokines and prognostic factors (groups). (**A**) Loading plot by principal component analysis (PCA) of prognostic factors with cytokines added as supplemental variables. (**B**) Correlation analysis between cytokines and prognostic factors (groups). Prognostic factors (groups) were standardized. Brown-filled cells indicated significant correlations. (**C**) Correlations between cytokines and prognostic factors (groups) by path analysis. Variables were standardized. * Standardized partial regression coefficient (β). ** Correlation coefficient

linking cytokine abnormalities to prognostic factors and mortality.

The present study suggests that IL-6 is an important cytokine influencing the prognosis of anti-MDA5+patients, as IL-6 is involved in both Group 1 and Group 2 pathways and has been identified as a prognostic factor. The efficacy of tocilizumab and JAK inhibitors in treating RP-ILD in MDA5+patients has been reported [25, 33]. Therefore, IL-6 may be a potential therapeutic target for MDA5+DM.

MDA5+DM is a heterogeneous condition. Recent machine-learning studies have demonstrated the existence of several clusters with characteristic clinical phenotypes [13, 34, 35]. A cluster characterized by RP-ILD and a poor prognosis was identified in all studies. Other clusters, which varied between studies, were characterized by skin rash, myositis, arthralgia, and vasculopathy, with a favorable prognosis. However, the mechanisms underlying clinical subtypes of MDA5+DM remain unclear. The balance between the two identified pathways—related to ILD development and macrophage activation—may explain these clinical subtypes: concurrent activation of both pathways may contribute to

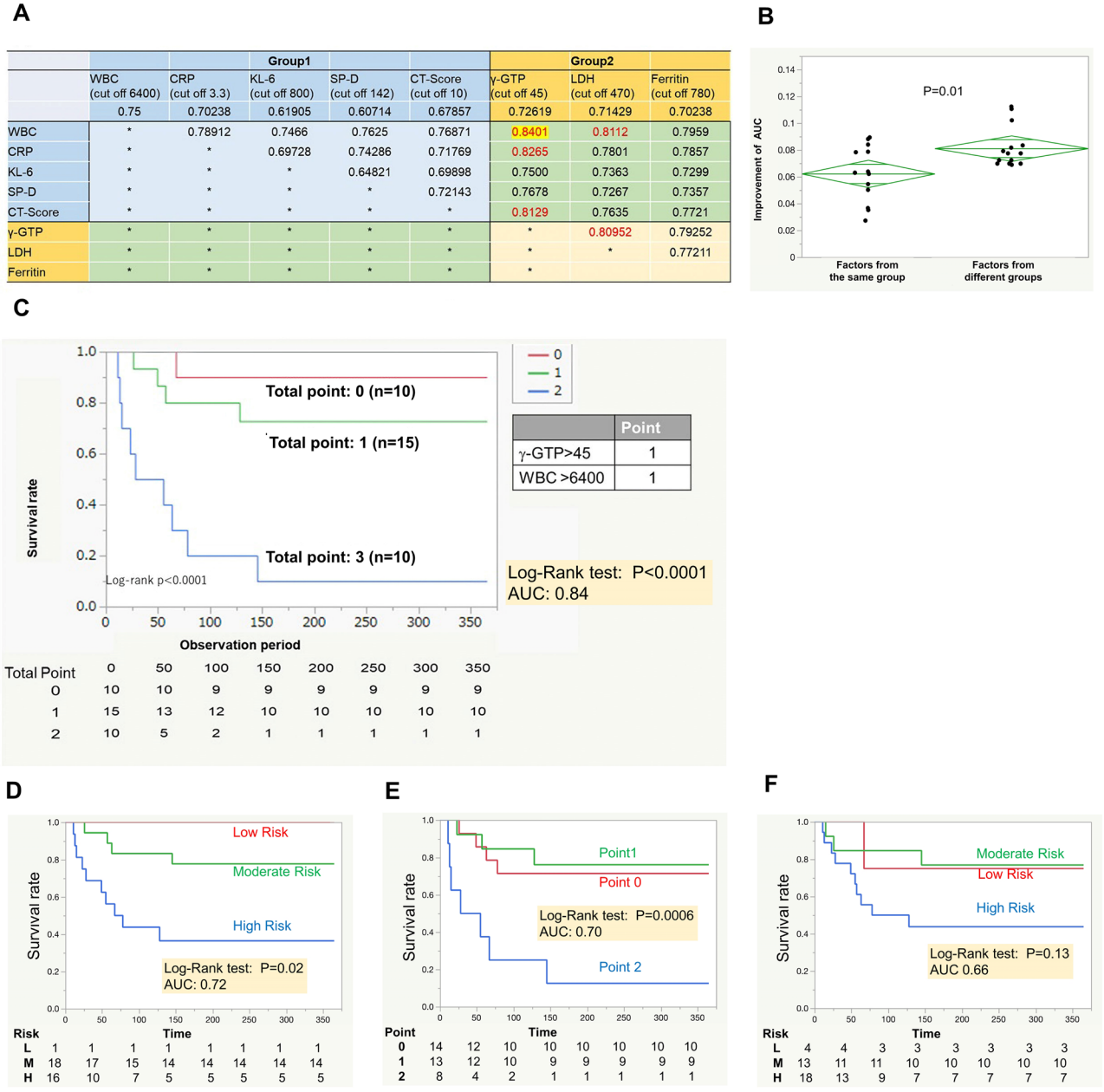


Fig. 5 Predictive model for prognosis with white blood cell (WBC) and γ-glutamyl transpeptidase (γ-GTP). (**A**) Area under the curve (AUC) by a pair of prognostic factors. (**B**) Improvement of AUC by pairing factors within the same and different groups of factors. Improvement of AUC is a difference in AUC between AUC by paring factors and the average of AUCs of the original two factors. (**C**) Survival curve by our predictive model using a scoring system that gives 1 point for each γ-GTP>45 or WBC>6,400. Patients were divided by total points. (**D**) Survival curve by FLAIR model [9]. (**E**) survival curve by MSK model [16]. (**F**) Survival curve by CROSS model [11]

RP-ILD, whereas activation of the macrophage pathway alone may result in non-ILD lesions.

Plasma TM levels and vWF activity were identified as novel prognostic factors. TM and vWF are markers of endothelial damage in various diseases [36, 37]. In MDA5+DM, vasculopathy, such as skin ulcers, was frequently observed [38]. Some researchers have proposed that pulmonary vasculopathy may be involved in ILD pathogenesis [2, 39]. Plasma levels of endothelial cell damage markers, including vWF, are higher in anti-MDA5+patients with ILD and skin ulcers than in other MDA5+DM cases [40]. However, vWF has not been previously reported as a prognostic factor. To the best of our knowledge, TM levels in patients with MDA5+DM have not been reported, although higher TM levels have been observed in patients with ILD in the context of myositis [41].

Several prognostic models have been proposed [9, 11, 12, 14, 16, 17]. These models were created by statistically selecting the prognostic factors. In contrast, we selected factors from the two identified pathways leading to mortality and combined them because a combination of factors from different pathways showed a higher AUC than those from the same pathway. Moreover, our model was not inferior to previously reported models. Our model does not require specialized laboratory tests such as ferritin, KL-6, or CT scores but uses the results of common laboratory tests like WBC and γ-GTP. This model allows for the prognostic prediction of patients with suspected MDA5+ILD before specific test results become available, thereby enabling personalized treatment.

The present study had some limitations. First, this was a single-center retrospective study with a small sample size, which hinders a clear understanding of the association between cytokines, prognostic factors, and mortality. Second, all participants had complicated ILD, most of which was RP-ILD, and thus may not accurately represent the heterogeneity of MDA5+DM. However, whether these pathways are present in patients without ILD remains unclear. Third, important prognostic factors, such as anti-MDA5 antibody titers [42], were not included because of unavailable data for approximately half of the patients. Fourth, we may have overlooked essential cytokines related to these two pathways, particularly the ILD pathway. Cytokines, such as IFN-α and IL-15, were not detected due to the limited sensitivity of the assays used in this study, and previously reported elevated cytokines, including IL-18 [43], and IFN-λ [21], were not measured. Finally, the prognostic significance of TM and vWF in MDA5+DM, as well as the validity of the proposed prognostic prediction model, requires confirmation in multicenter studies with larger patient cohorts.

In conclusion, our analysis identified two pathways that led to poor prognosis in MDA5+patients: one with ILD-related markers, CRP, and WBC, which was partially associated with IL-6, and another with ferritin, LDH, and γ-GTP, which was associated with IL-6, IL-10, IP-10, and TNF-α, although both pathways interact with each other. We also proposed a model combining selected variables from each group (WBC, γ-GTP). Our findings may not only provide clues to elucidate the pathogenesis and develop new therapies but also enable early patient stratification for better clinical management. However, further detailed investigations with a larger number of participants are needed to validate and extend our findings.

Supplementary Information

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Supplementary Material 1

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Author contributions
All authors contributed to the conception and design of this study. AS, KK, TH, AK, SK, YY, AH, TM, TA, SA, MA, and RM prepared the materials, collected, and analyzed the data. AS, KK, SA, and KI wrote the first draft of the manuscript, and all authors commented on previous versions of the manuscript. All the authors have read and approved the final version of the manuscript.

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Data availability
The data that support the findings of this study are not openly available due to reasons of sensitivity and are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
This study was conducted in accordance with the Declaration of Helsinki and the Ethical Guidelines for Clinical Studies 2011 of the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labour and Welfare, Japan. This study was approved by the Bioethics Committee of Dokkyo Medical University (#22081). Written informed consent was obtained from each patient for the use of serum samples, and clinical and laboratory data.

Competing interests
KK received research support from Asahi-Kasei, Boehringer-Ingelheim, Chugai, Eisai, the Japan Blood Products Organization, and Ono outside the submitted work. KI received research grant from Mitsubishi-Tanabe and Japan Blood Products Organization and speakers fees from Eli Lilly, Abbvie, Eisai, Gilead, Pfizer, AstraZeneca, Asahi-kasei, Novartis, Janssen, Taiho, UCB, Bristol-Myers Squibb, Ono, Chugai, Daiichi Sankyo, GSK, and Mitsubishi-Tanabe outside the submitted work. The other authors declare no conflicts of interest.

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