Patient clustering by serum inflammatory mediators stratifies early disease-modifying anti-rheumatic drug-naive rheumatoid arthritis and reveals distinct pathobiological signatures: a prospective observational cohort

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Abstract

Objective

Rheumatoid arthritis (RA) is a heterogeneous disease; therefore, a one-size-fits-all treatment approach is suboptimal. This study aimed to explore biological heterogeneity in treatment-naive patients with early RA using serum inflammatory mediator profiles to identify distinct subgroups and investigate their longitudinal dynamics.

Methods

We conducted an exploratory, hypothesis-generating post-hoc analysis of 204 disease-modifying anti-rheumatic drug (DMARD)-naive patients with early RA from a prospective cohort. Thirteen baseline serum inflammatory mediators, including interleukin (IL)-1β, IL-4, IL-6, IL-10, IL-12p40, IL-12p70, IL-23, IL-1RA, tumour necrosis factor-alpha, interferon-gamma, C-X-C motif chemokine ligand 10, C-C motif chemokine ligand 17, and arginase, were quantified. Patients were stratified using unsupervised hierarchical clustering, followed by correlation and regression analyses to compare clusters and examine response pathways. The predictive value of routine clinical markers for cluster membership was assessed using the receiver operating characteristic curve analysis.

Results

Two distinct patient clusters were identified based on differing serological and inflammatory mediator profiles. Despite these biological differences, the baseline disease activity and 52-week outcomes were largely similar. However, the pathways of clinical improvement varied by cluster; IL-6 dynamics were uniquely associated with improvement in cluster 1, whereas no dominant mediator was identified in cluster 2. In contrast to C-reactive protein, a low rheumatoid factor titre effectively identified the IL-6-driven subgroup (area under the curve=0.785).

Conclusion

Baseline serum inflammatory mediator profiles can stratify DMARD-naive patients with early RA into subgroups with distinct pathobiological mechanisms. These subgroup-specific signatures, including those of the IL-6-driven pathway, offer a basis for developing biomarker-guided therapeutic strategies.

Key words

rheumatoid arthritis, prospective studies, cluster analysis, pharmacological biormarkers, inflammation mediators, interleukin-6, tumour necrosis factor-alpha, precision medicine

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Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterised by substantial heterogeneity in clinical course and response to therapy (1). This variability complicates effective disease management and underscores the limitations of uniform treatment strategies. To address this challenge, patient stratification – the classification of patients into distinct subgroups based on their underlying pathogenic mechanisms – is crucial (2, 3). This approach is fundamental for the development of targeted treatments and advancementof personalised medicine for patients with RA (4).

Previous studies have used data-driven clustering approaches for various data types, such as synovial molecular data and clinical outcomes, to identify RA subtypes (5-7). Analysis of serum inflammatory mediator profiles represents a promising avenue for stratifying patients with RA in a minimally invasive manner (8-12). Although previous studies have explored the association between mediator levels and clinical outcomes, identifying robust biomarkers in peripheral blood remains an unresolved challenge (13). A significant limitation of existing research is that most of the studies included patients already receiving therapies known to influence mediator expression, thereby obscuring the intrinsic biological state of the disease. Therefore, investigation of treatment-naive populations is essential. Reliance on static, single-timepoint analyses has prevailed, neglecting the valuable insights gained from longitudinal assessments of mediator dynamics, which are critical for validating biomarkers and elucidating treatment response mechanisms.

To address these gaps, the primary objective of this exploratory, hypothesis-generating study was to identify distinct patient subgroups by applying unsupervised clustering to baseline serum inflammatory mediator profiles within a cohort of disease-modifying anti-rheumatic drug (DMARD)-naive patients with early RA. Second, we explored the pathobiological mechanisms within these subgroups by analysing the association between the longitudinal dynamics of these mediators and clinical improvement under a standard treat-to-target (T2T) strategy. This approach allows the investigation of both fundamental biological heterogeneity at disease onset and dynamic responses to intervention.

Materials and methods

Study design and population

This investigation was an exploratory, hypothesis-generating *post-hoc* analysis of data from the 'Three Arrow Study', a prospective, multicentre inception cohort study that enrolled 204 patients with early RA from 11 institutions in Hiroshima Prefecture between January 2018 and March 2021 (registered in the Clinical Innovation Network no. 644). The inclusion criteria were:
1) an RA diagnosis confirmed by a rheumatologist based on the 2010 American College of Rheumatology/European League Against Rheumatism classification criteria, 2) age of 20 years or older, 3) no prior exposure to DMARDs at enrolment (14).

Subsequently, all patients received treatment based on a T2T strategy at the discretion of their attending physicians, which was based on the latest Japan College of Rheumatology guidelines at the time. The specific therapeutic agents administered to the cohort during the 52-week follow-up are shown in Supplementary Table S1 and Supplementary Figure S1. This analysis was based on the complete dataset of 204 eligible participants (Suppl. Fig. S2).

Data collection and measurements
- Clinical, serological, and radiographic assessments

Data were collected at baseline and weeks 24 and 52. The baseline patient profile included demographic and personal history (age, sex, medical/family history, and smoking status) and serological markers (rheumatoid factor [RF] and anti-citrullinated protein antibody [ACPA] positivity and titres). Disease activity was comprehensively evaluated using several indices at each of three time points. These included the Clinical Disease Activity Index (CDAI) and Simplified Disease Activity Index. Their components, namely, tender ioint count 28, swollen joint

count 28, patient's global assessment of disease activity, and physician's global assessment, were recorded. Additional measures included the pain visual analogue scale and serum C-reactive protein (CRP) levels. Information on the type of therapeutic drug administered to each patient was documented during each visit. Radiographic joint damage was quantified at all three visits using the modified total sharp score (mTSS), which includes erosion and joint space narrowing scores (15). The final mTSS for analysis was reported as the average of scores from two independent specialists, with a third reader adjudicating in cases of significant (≥10 points) or qualitative (progression *vs*. repair) discrepancies.

- Measurement of serum inflammatory mediators

Serum samples collected at baseline (week 0) and week 24 were used for analysis. The concentrations of 13 inflammatory mediators were quantified simultaneously using the LEGENDplex Human Macrophage/Microglia Panel (13-plex) (BioLegend, San Diego, CA, USA). This panel was selected because it provides a comprehensive measurement of the key molecules involved in the complex pathophysiology of RA, including major pro-inflammatory cytokines, anti-inflammatory cytokines, and chemokines (16-18). The bead-based multiplex assay was analysed via flow cytometry according to the manufacturer's protocol, and it included the following mediators: tumour necrosis factor-alpha (TNF-α), interleukin (IL)-6, IL-1β, IL-4, IL-10, IL-12p70, IL-12p40, IL-23, IL-1 receptor antagonist (IL-1RA), interferon-gamma (IFN-γ), C-X-C motif chemokine ligand 10, C-C motif chemokine ligand 17, and arginase-1.

Patient clustering

Baseline concentrations of the 13 inflammatory mediators were prepared for clustering using a multi-step consensus clustering approach. First, to handle values below the limit of detection (LOD), we treated them as left-censored data. All mediator values were log-transformed before imputation. We then performed multiple imputation (m=20) using a regression-based approach (fully conditional specifications). For each mediator, the baseline and 24-week values were used to predict each other, and the values for the censored data points were drawn from a truncated normal distribution bounded by the log-transformed LOD to generate 20 complete datasets (19). Second, for each of the 20 imputed datasets, the log-transformed values were standardised using Z-scores. We then performed consensus clustering on the standardised datasets. Hierarchical clustering (using Euclidean distance and Ward.D2 linkage) was performed for each dataset, and the results were aggregated into a consensus matrix, that represents the stability of patient pairings (20). This matrix was then re-clustered to derive the final, stable patient assignments (Suppl. Fig. S3). Third, to determine the optimal number of clusters, we quantitatively compared the performance of k=2, 3, and 4 solutions using multiple validation metrics, including the average silhouette width and the proportion of ambiguous clustering(21). The solution with k=2 was identified as the most stable and appropriate solution (Suppl. Table S2). Therefore, we adopted the two-cluster solution for all primary analyses. Fourth, the robustness of the chosen clustering algorithm (Euclidean distance and Ward.D2 linkage) was validated against alternative specifications by quantifying the concordance between partitions using the Adjusted Rand Index (ARI) (Suppl. Table S3).

Statistical analysis

- Comparison of group characteristics

Baseline characteristics were compared across the two patient clusters. The non-parametric Kruskal-Wallis test was used to test for differences in all continuous variables (age and baseline CDAI score). The distribution of categorical variables such as sex and seropositivity was compared among clusters using the chi-square test.

- Analysis of associations and predictive modelling

A multifaceted analytical approach was used to investigate the relationships between variables. The initial exploratory analysis involved calculating Spearman's rank correlation between the 24-week changes in mediators and various clinical/radiographic parameters, which were visualised as heatmaps for each cluster. For exploratory variable selection in the presence of multicollinearity, elastic net regression was used to identify the inflammatory mediator dynamics associated with CDAI(22). A linear mixed-effects model was used to model disease progression over 52 weeks. The model included fixed effects for time (treated as a categorical factor: 0, 24, and 52 weeks), cluster assignment, and their interaction, while adjusting for age, sex, and baseline CDAI. A random intercept for each patient was included to account for repeated measures (23). In a targeted subgroup analysis of patients treated with conventional synthetic (cs)DMARDs, the Wilcoxon rank-sum test was used to compare the baseline predictors between responders and non-responders. Finally, we developed a series of multivariate logistic regression models to determine whether routine clinical markers could predict membership in cluster 1. These models were built sequentially to assess the incremental predictive value of key clinical markers, starting with the RF titre, which was the most discriminant variable in the baseline comparisons. Next, we then evaluated the contribution of CRP given its conventional association with IL-6, age, and ACPA titre, which are clinically important variables in RA. The discriminative abilities of these nested models were compared using receiver operating characteristic curve analysis and the area under the curve (AUC).

- Sensitivity analysis

Several sensitivity analyses were performed. First, to evaluate potential selection bias from patients with missing 24-week serum samples, we compared baseline characteristics between those with and without complete data using absolute standardised mean differences (SMDs), with an $|SMD| > 0.2$ considered a meaningful difference. Second, we assessed the stability of the primary k=2 solution by comparing it to a k=3

Table I. Baseline patient characteristics stratified by clusters.

	Cluster 1 (n=117)	Cluster 2 (n=87)	Overall (n=204)	*p*-value
Age at diagnosis, median [IQR]	69.00 [57.00, 77.00]	66.00 [55.25, 74.75]	68.00 [56.00, 76.00]	0.234
Female sex, n (%)	72 (61.5)	60 (69.0)	132 (64.7)	0.342
RF positivity, n (%)	71 (60.7)	78 (89.7)	149 (73.0)	<0.001*
RF titre (IU/mL), median [IQR]	26.80 [9.00, 83.50]	114.80 [58.40, 225.00]	61.40 [18.53, 143.60]	<0.001*
ACPA positivity, n (%)	63 (54.3)	75 (86.2)	138 (68.0)	<0.001*
ACPA titre (U/mL), median [IQR]	43.70 [1.30, 282.50]	96.90 [33.10, 430.00]	74.90 [6.15, 340.35]	0.002*
Any comorbidity, n (%)	75 (64.1)	63 (72.4)	138 (67.6)	0.270
Respiratory disease, n (%)	11 (9.4)	14 (16.1)	25 (12.3)	0.220
Diabetes mellitus, n (%)	21 (17.9)	14 (16.1)	35 (17.2)	0.873
Cardiovascular disease, n (%)	21 (17.9)	15 (17.2)	36 (17.6)	1.000
Renal disease, n (%)	11 (9.4)	2 (2.3)	13 (6.4)	0.078
Family history of RA, n (%)	25 (21.4)	18 (20.7)	43 (21.1)	1.000
Smoking status, n (%)		0.712		
current	16 (13.7)	15 (17.4)	31 (15.3)	
former	34 (29.1)	22 (25.6)	56 (27.6)	
never	67 (57.3)	49 (57.0)	116 (57.1)	
Disease Activity Measures				
PhVAS, median [IQR]	40.00 [30.00, 55.00]	40.00 [30.00, 59.50]	40.00 [30.00, 58.25]	0.707
PainVAS, median [IQR]	50.00 [30.00, 72.00]	51.00 [30.00, 70.00]	50.00 [30.00, 70.00]	0.995
PtGA, median [IQR]	50.00 [35.00, 70.00]	50.00 [27.00, 66.50]	50.00 [30.00, 70.00]	0.239
TJC28, median [IQR]	3.00 [1.00, 6.00]	3.00 [1.00, 4.50]	3.00 [1.00, 5.00]	0.974
SJC28, median [IQR]	4.00 [2.00, 8.00]	5.00 [2.00, 8.00]	5.00 [2.00, 8.00]	0.542
ESR, median [IQR]	45.00 [19.00, 70.00]	48.50 [27.00, 73.75]	46.00 [22.00, 71.50]	0.255
CRP, median [IQR]	1.19 [0.25, 2.92]	1.18 [0.26, 2.65]	1.19 [0.26, 2.86]	0.982
SDAI, median [IQR]	19.20 [13.80, 28.70]	17.80 [11.85, 26.10]	18.85 [12.52, 27.38]	0.310
CDAI, median [IQR]	18.60 [12.80, 26.00]	16.90 [11.20, 23.05]	17.00 [11.50, 24.27]	0.259
Radiographic damage				
mTSS (units), median [IQR]	1.00 [0.00, 3.50]	0.15 [0.00, 2.38]	1.00 [0.00, 3.00]	0.210
erosion (units), median [IQR]	0.00 [0.00, 0.50]	0.00 [0.00, 0.50]	0.00 [0.00, 0.50]	0.821
JSN (units), median [IQR]	1.00 [0.00, 3.00]	0.00 [0.00, 2.00]	0.00 [0.00, 2.50]	0.117
Initial treatments (baseline to 24 weeks)				
MTX, n (%)	100 (85.5)	74 (85.1)	174 (85.3)	1.000
bDMARDs, n (%)	19 (16.2)	16 (18.4)	35 (17.2)	0.829
TNFi, n (%)	14 (12.0)	9 (10.3)	23 (11.3)	N/A
IL-6Ri, n (%)	4 (21.1)	2 (12.5)	6 (17.1)	N/A
CTLA4-Ig, n (%)	1 (5.3)	4 (25.0)	5 (14.3)	N/A
JAK inhibitor, n (%)	0 (0.0)	1 (6.2)	1 (2.9)	N/A
Glucocorticoid, n (%)	35 (29.9)	22 (25.3)	57 (27.9)	0.956
Glucocorticoid dose for users (prednisolone equivalent, mg/day)	3.00 [1.50, 4.00]	2.25 [1.00, 4.00]	3.00 [1.00, 4.00]	0.432

Data are presented as median [interquartile range (IQR)] for continuous variables or n (%) for categorical variables, unless otherwise stated. Differences between the three clusters were assessed using the Kruskal-Wallis test for continuous variables and the Chi-squared test or Fisher's exact test for categorical variables. *p*-values are presented for comparisons across the three clusters. An asterisk (*) indicates $p<0.05$. Comorbidities were defined based on physician diagnosis documented in the medical records. Respiratory disease included a broad range of conditions such as interstitial lung disease, obstructive lung diseases (*e.g.* chronic obstructive pulmonary disease, asthma), and bronchiectasis. Renal disease included chronic kidney disease, renal cancer, or kidney stones. ACPA: anti-citrullinated protein antibody; CDAI: Clinical Disease Activity Index; csDMARDs: conventional synthetic disease-modifying anti-rheumatic drugs; CTLA4-Ig: cytotoxic T-lymphocyte antigen 4-immunoglobulin; CRP: C-reactive protein; DMARDs: disease-modifying anti-rheumatic drugs; ESR: erythrocyte sedimentation rate; IL-6Ri: interleukin-6 receptor inhibitor; IQR: interquartile range; JAKi: Janus kinase inhibitor; JSN: joint space narrowing; MTX: methotrexate; mTSS: modified Total Sharp Score; N/A: not applicable; PhVAS: Physician Global Assessment of disease activity (Visual Analogue Scale); PtGA: Patient Global Assessment of disease activity; RA: rheumatoid arthritis; RF: rheumatoid factor; SDAI: Simplified Disease Activity Index; SJC28: swollen joint count in 28 joints; TJC28: tender joint count in 28 joints; TNFi: tumour necrosis factor inhibitor.

partition, evaluating cluster correspondence and the resulting patterns of mediator–clinical correlations. Finally, to test the stability of our longitudinal model findings, we fitted two additional models with expanded covariate adjustment. The first model included additional baseline covariates for RF positivity, ACPA positivity, and a history of respiratory or renal disease. The second model was built upon the first model by adding time-varying covariates for glucocorticoid and biological/targeted synthetic (b/ts) DMARD use to adjust for the effects of treatment.

All statistical tests were two-sided and performed using R software (v. 4.4.2). Given the exploratory, hypothesis-generating nature of this study, *p*-values were not adjusted for multiple comparisons and should be interpreted as descriptive metrics alongside their corresponding confidence intervals. For longitudinal analyses involving 24-week serum mediators, a complete-case approach was used, thereby excluding patients with missing data at this time point. A detailed sensitivity analysis was performed to assess potential selection bias resulting from this exclusion, as described in the *Sensitivity Analyses* subsection.

Ethics approval and consent to participate

This study was conducted in accord-

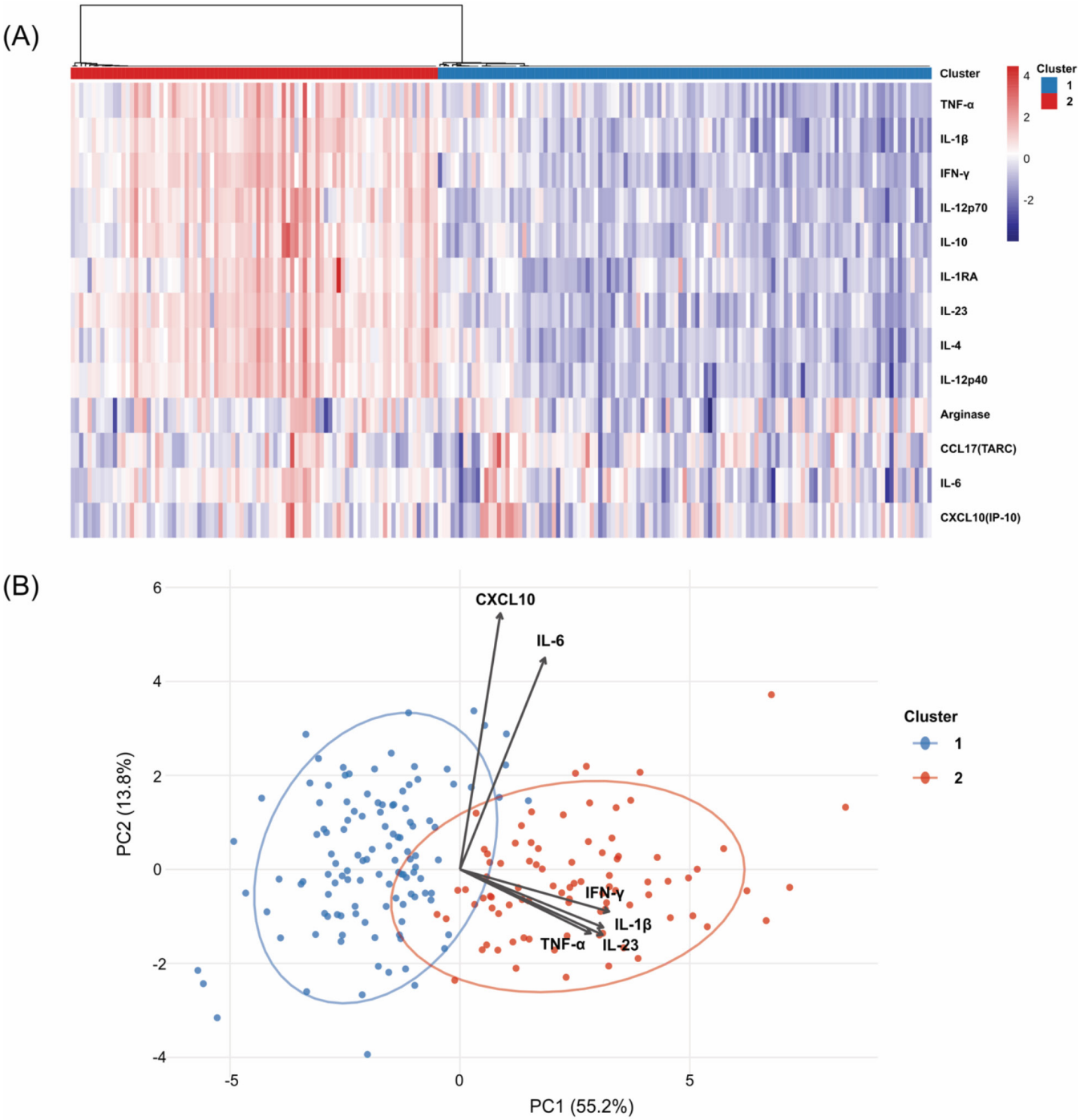

Fig. 1. Patient clustering based on inflammatory mediator profiles
A: Heatmap of Z-scored, log-transformed baseline levels of 13 inflammatory mediators, with patients (columns) grouped and ordered by cluster assignment (top annotation bar). Mediators (rows) are hierarchically clustered.
B: Principal Component Analysis (PCA) plot based on the same data. Patients are plotted by PC1 (55.2% variance) and PC2 (13.8% variance) scores and coloured by cluster. Ellipses represent 95% confidence intervals. Arrows indicate loading vectors for six selected inflammatory mediators (TNF-α, IL-6, IL-1β, IFN-γ, CXCL10 (IP-10), and IL-23).
PCA: principal component analysis; PC1: principal component 1; PC2: principal component 2; CI, confidence interval

ance with the Declaration of Helsinki and approved by the Institutional Review Board of Hiroshima University (approval no: 2024-39). Written informed consent was obtained from all the participants.

Results

Patient clustering and baseline characteristics

Two patient clusters were identified through hierarchical clustering of baseline inflammatory mediators (cluster 1: n=117; cluster 2: n=87; Fig. 1A). The principal component analysis plot illustrates two distinct, albeit overlapping, clusters in a low-dimensional space (Fig. 1B).At baseline, the clusters were well differentiated based on their biological

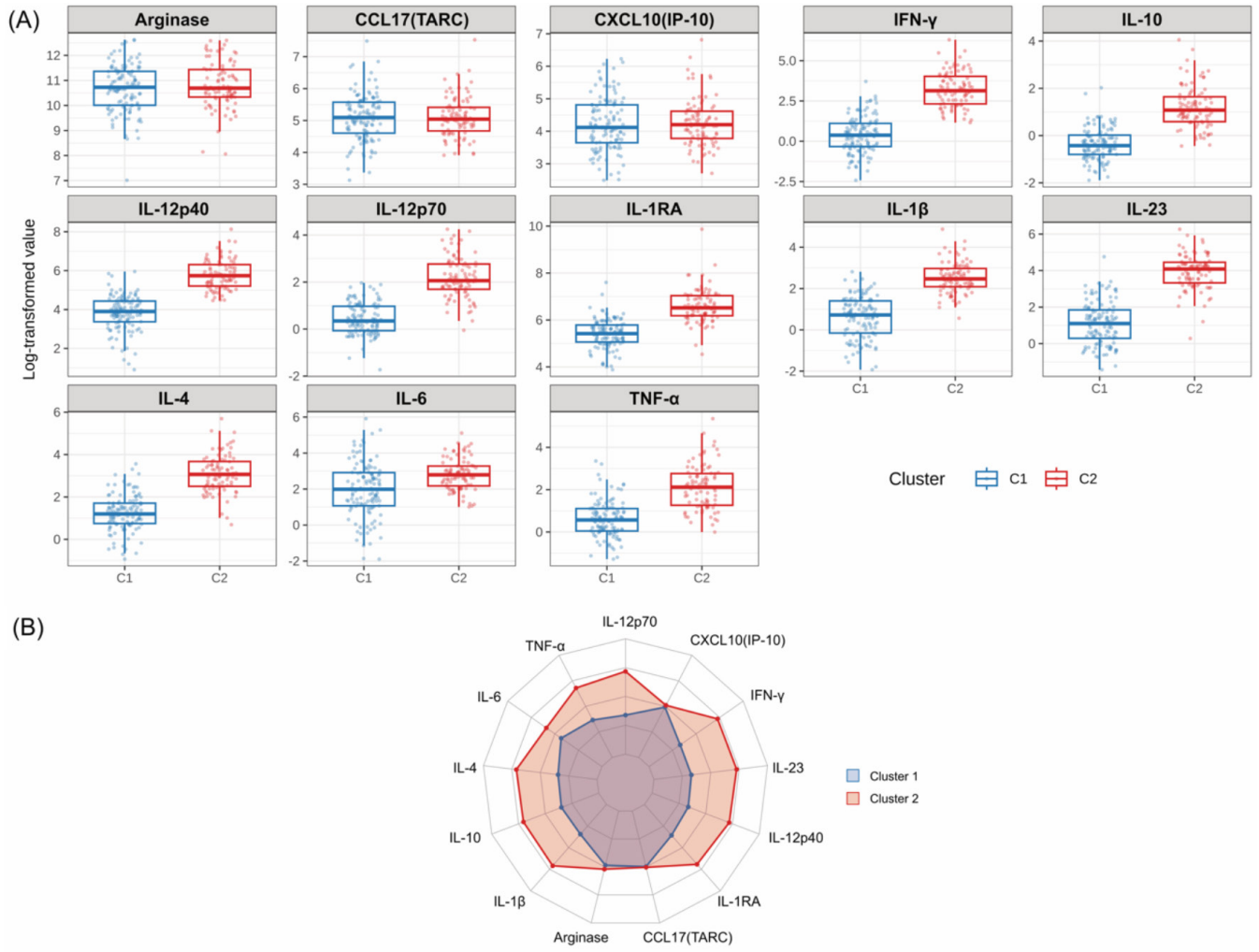

Fig. 2. Cluster comparison of baseline inflammatory mediator levels.
A: Distribution of log-transformed baseline levels for each inflammatory mediator shown via boxplots and individual data points, coloured by cluster.
B: Radar chart comparing the mean baseline Z-score profile for each cluster across the 13 inflammatory mediators.

characteristics (Table I; Fig. 2). RF and ACPA positivity and titres were lower in cluster 1 compared with cluster 2.In contrast, clinical disease activity scores were comparable across groups, with no significant difference in baseline CDAI (p=0.259). No significant differences were observed in the demographic characteristics or comorbidity rates.

Cluster-specific associations between mediator dynamics and clinical parameters

Analysis of the relationship between the 24-week changes in inflammatory mediators and clinical parameters revealed distinct pathobiological signatures in each cluster. Initial exploratory analysis using Spearman correlation heatmaps showed that while cluster 2 exhibited widespread positive correlations across many mediators, cluster 1 was uniquely characterised by strong positive correlations primarily involving the change in IL-6 (ΔIL-6) (Fig. 3). An elastic net regression model, used to further identify key predictors of clinical improvement (ΔCDAI), confirmed these distinct signatures. In cluster 1, a reduction in IL-6 was the sole significant predictor associated with a reduction in CDAI. In contrast, no single mediator was robustly selected in cluster 2, although exploratory analysis suggested that changes in arginase and IL-1β were the most frequently selected variables (Fig. 4; Suppl. Fig. S4).

Prediction of cluster membership using clinical markers

Given the distinct biological signatures, we tested whether they could be identified using routine clinical markers. A series of logistic regression models demonstrated that the baseline RF titre alone could effectively discriminate patients in cluster 1 (AUC = 0.785), with no meaningful improvement gained by adding other demographic or serological markers such as CRP (Suppl. Table S4; Fig. 5).

Longitudinal clinical and radiographic outcomes

Longitudinal analyses of clinical and mediator changes were conducted on 184 patients (90.2%) who had complete serum data at baseline and 24 weeks. A comparison of baseline characteristics revealed several meaningful imbalances (|SMD| >0.2) between these patients and the 20 patients with missing 24-week data (Suppl. Table S5). Lon-

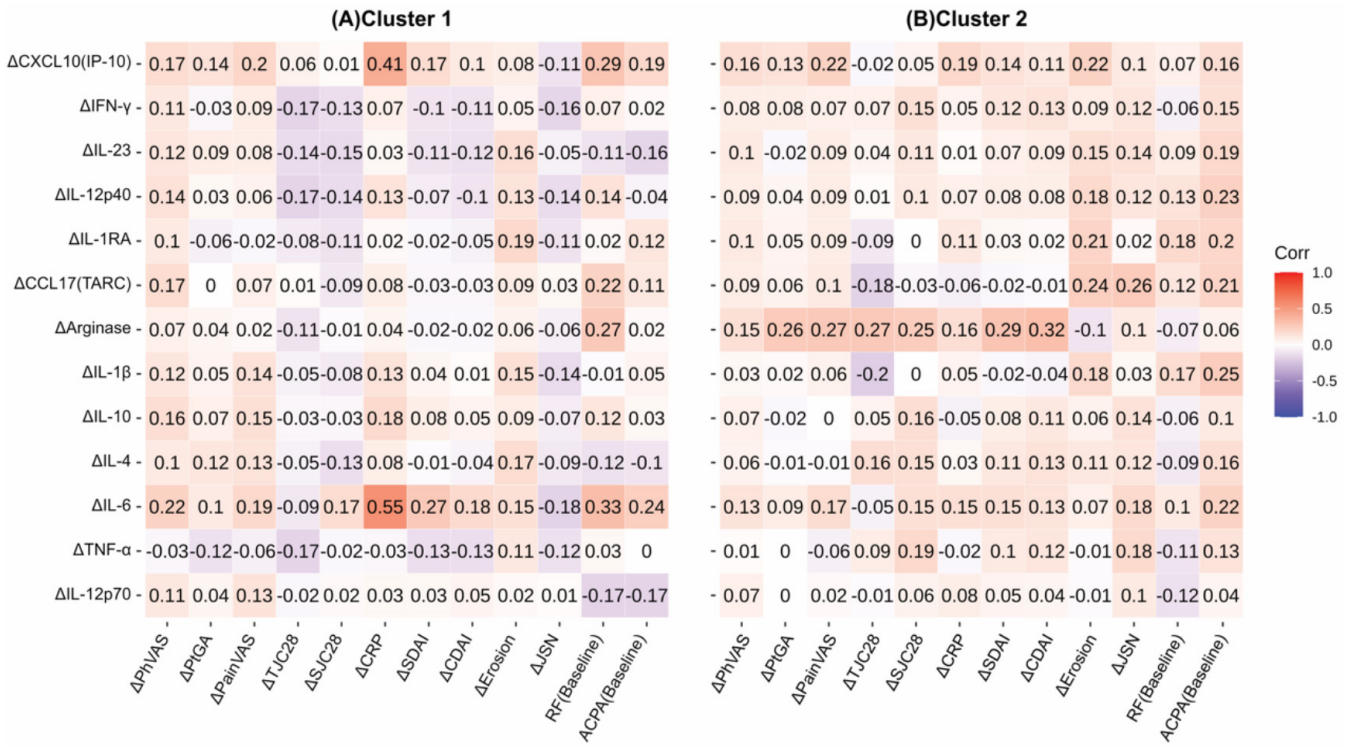

Fig. 3. Cluster-specific correlations between 0–24 week changes in serum inflammatory mediators and clinical parameters.
Spearman correlation coefficients (ϱ) are visualised in heatmaps for (**A**) Cluster 1 (n=117), and (**B**) Cluster 2 (n=87). The heatmaps depict correlations between 0–24 week changes (Δ) in 13 log-transformed serum inflammatory mediator level (rows) and 0–24 week changes (Δ) in key clinical parameters (columns). Colour intensity and hue represent the strength and direction of correlations.
CDAI: Clinical Disease Activity Index; SDAI, Simplified Disease Activity Index; DAS28, Disease Activity Score using 28 joints; TJC28, tender joint count in 28 joints; SJC28, swollen joint count in 28 joints; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; PtGA, Patient Global Assessment; VAS, Visual Analogue Scale; Erosion, erosion score (component of mTSS); JSN, Joint Space Narrowing score (component of mTSS); RF, rheumatoid factor; ACPA, anti-citrullinated protein antibody.

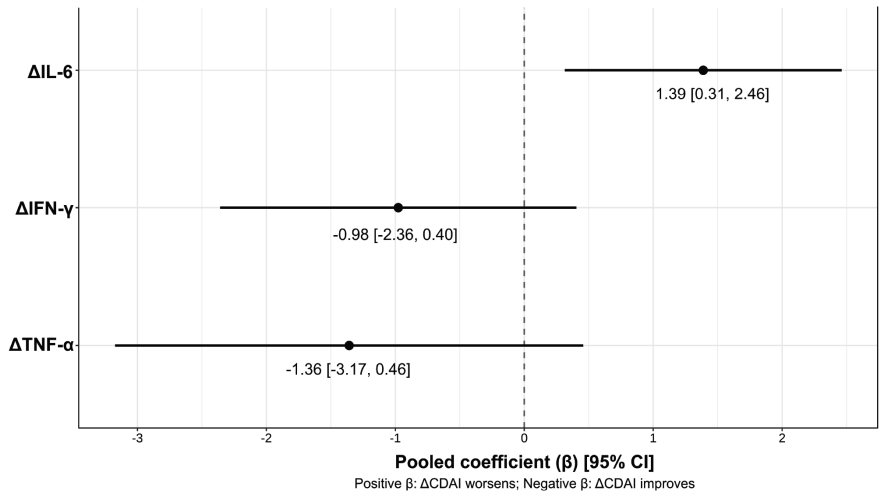

Fig. 4. Associations between 0–24 week changes in inflammatory mediators and ΔCDAI in cluster 1.
Standardised coefficients (β) and 95% confidence intervals (CIs) from a lambda.1se regularised elastic net model. The model predicts the 24-week change in CDAI (ΔCDAI) based on the Z-standardised log-change of 13 serum mediators for patients in Cluster 1. Positive β coefficients represent an association with a less favourable clinical response.
CDAI: Clinical Disease Activity Index; CI: confidence interval.

gitudinal analysis of this complete-case cohort under a standard T2T strategy showed a significant improvement in the CDAI in both clusters over 52 weeks (Suppl. Fig. S5) (24). Although a trend towards a slower rate of improvement was observed in cluster 2 compared to cluster 1, the adjusted mean difference between the clusters at 52 weeks was not statistically significant (1.46; 95% CI, -0.22 to 3.14). Consistent with the primary analysis, no significant inter-cluster differences were detected in radiographic measures, including the mTSS and its components (Suppl. Fig. S6). The detailed results of these linear mixed-effects models are presented in Supplementary Table S6.

Treatment response and baseline predictors

An analysis of the treatment response at 24 weeks stratified by the therapeutic mode of action is shown in Supplementary Figure S7. The response rates to csDMARDs were comparable among the three clusters. For b/tsDMARDs, small sample sizes precluded formal inference; descriptive heatmaps suggest heterogeneity by mechanism of action without a consistent between-cluster divergence. Finally, we explored the baseline predictors of the response to csDMARD therapy at 24

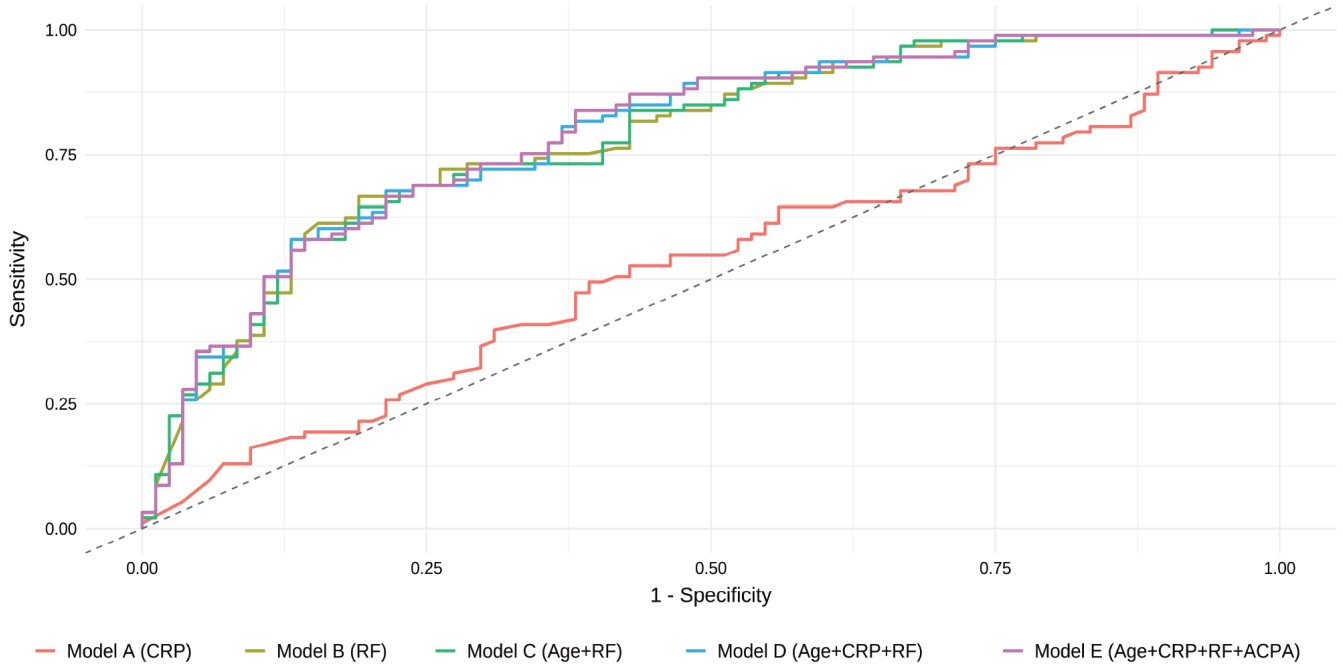

Fig. 5. Receiver operating characteristic (ROC) curves for models predicting Cluster 1 membership.
Receiver operating characteristic (ROC) curves compare the discriminative ability of five logistic regression models for predicting membership in Cluster 1: Model A (CRP), Model B (RF), Model C (Age+RF), Model D (Age+CRP+RF), and Model E (Age+CRP+RF+ACPA).Detailed performance metrics and comparisons between models are presented in Supplementary Table S4.
ACPA: anti-citrullinated protein antibody; CRP: C-reactive protein; RF: rheumatoid factor.

weeks by comparing responders and non-responders (Suppl. Table S7). I In the overall cohort, non-responders generally exhibited higher baseline disease activity and a greater inflammatory burden. Although this analysis was also stratified by cluster, these subgroup findings were exploratory and should be interpreted with caution due to the small number of non-responders.

Sensitivity analysis

The primary two-cluster solution was found to be robust. It demonstrated high concordance with an alternative specification using Manhattan distance and average linkage (median ARI = 0.813), while showing modest agreement with other settings (Suppl. Table S3). Furthermore, the main findings from the longitudinal CDAI analysis remained consistent across the two pre-specified sensitivity models with expanded covariate adjustment (Suppl. Table S8). The k=3 sensitivity analysis supported the primary solution, showing that cluster 1 remained highly stable while cluster 2 separated into two subgroups with distinct correlation profiles (Suppl. Table S9). Specifically, correlation heatmaps confirmed that cluster 1 retained its ΔIL-6 signature, while the split of cluster 2 revealed a highly pro-inflammatory subgroup (C2b) and an intermediate subgroup (C2a) (Suppl. Fig. S8).

Discussion

This study offers new insights into the heterogeneity of treatment-naive populations with early RA by stratifying patientsinto two distinct clusters based on their baseline serum inflammatory mediator profiles. Furthermore, we demonstrated that these clusters differed at baseline and exhibited unique pathobiological signatures, as the specific mediator dynamics associated with clinical improvement were different for each subgroup. These results address the challenge of the "one-size-fits-all" approach by providing a framework for identifying patient subgroups with distinct underlying disease mechanisms, which is a foundational step in the development of targeted therapies.

A key finding of this study was the identification of two distinct patient clusters in a treatment-naive early RA cohort based solely on baseline serum inflammatory mediator profiles. This stratification, derived from a noninvasive blood sample, shows some noteworthy similarities to classifications previously established through synovial tissue analysis (25, 26). Cluster 2, which is characterised by high seropositivity and broadly elevated levels of inflammatory mediators, may be analogous to the inflammatory lymphomyeloid subtype identified by Humby *et al*. (2) and the highly activated subtype described by Nakajima *et al*. (25). Conversely, cluster 1, with its low serological and inflammatory mediator activity, was broadly consistent with the 'pauci-immune' or 'fibroid' synovial pathotypes. This consistency between systemic (serum) and local (synovial) classifications supports the notion that circulating mediators can provide a window into the pathology at the site of inflammation.

The search for reliable theranostic biomarkers of RA is challenging. Our findings provide a potential explanation for these difficulties by demonstrating that the key molecular pathways associated with clinical improvement are not uniform across patients. We identified cluster-specific biological signatures. For example, an improvement in cluster

1 was strongly linked to the dynamics of IL-6, which is consistent with reports of an IL-6-driven phenotype in patients who have a low titre or are seronegative (16, 27). Cluster 2 reflected broader inflammatory activation, and no single mediator emerged as a stable predictor under the primary Elastic Net criterion (lambda.1se). A key clinical implication of our findings was the identification of patients with specific IL-6-driven disease signatures. Although a high CRP level is often used as a proxy for IL-6 activity, its utility may be limited by its lack of specificity. Our data suggest that a state of broad immune activation, such as that observed in cluster 2, can lead to high IL-6 levels, which may in turn contribute to CRP elevation. However, this may not truly represent the IL-6-*dominant* pathophysiology. Our analysis supports this notion, as the most effective marker for identifying IL-6-driven cluster 1 was not CRP, but rather a low baseline RF titre (AUC=0.785). A possible biological explanation relates to the immunopathology of high-titre RF disease, in which immune complexes are significant drivers. These complexes can trigger a multifaceted inflammatory cascade via complement activation and Fc receptors on various immune cells, inducing a broad spectrum of cytokines including both TNF-α and IL-6. In this widespread inflammatory state, a specific IL-6 signature may be obscured. In contrast, in low-titre RA with reduced immune complex activity, we hypothesised that IL-6 from other sources, particularly synovial fibroblasts, is a more prominent pathophysiological driver. This could allow the IL-6 pathway to be more readily identified as a dominant signature. Adding CRP to the RF-based model did not further improve its predictive power, which suggests that a low RF titre may be a more specific biomarker for identifying patients who are optimal candidates for IL-6-targeted therapies.

Based on our data, the direct clinical application of this clustering method for personalised treatment selection is not yet warranted. This study was designed as an exploratory analysis of pathophysiology and is not powered to detect differential responses to specific bDMARDs among the clusters. For instance, although favourable clinical responses to IL-6 inhibitors were noted in the few patients receiving this therapy across both clusters, such observations must be considered hypothesis-generating given the very small sample sizes. Therefore, the main implication of our findings is that this study is a foundational study that provides a strong biological basis and a practical, noninvasive framework for designing future clinical trials aimed at validating stratified medicine in RA.

This study has certain limitations. First, our analysis was limited to 13 mediators based on a pre-configured commercial panel, not a selection tailored to RA-specific biology; therefore other unmeasured mediators may have contributed to the underlying pathophysiology. For instance, incorporating additional cytokines such as interleukin IL-17A to identify a 'Th17-driven' phenotype, granulocyte-macrophage colony-stimulating factor a 'myeloid-high' pathotype, and B-cell activating factor a subgroup with high humoral immune activity, could provide a more granular classification. Therefore, although the dynamics of certain cytokines were key features in our analysis, we cannot exclude the possibility that other unmeasured pathways were involved. Nevertheless, the selected panel covered key pathways implicated in RA pathogenesis – including those related to Th1, Th2, and Th17 cells, as well as major pro-inflammatory cytokines such as TNF-α, IL-1β, and IFN-γ – making it suitable for our exploratory objectives. Second, the findings were not validated in an independent cohort. Third, our 24-week analyses relied on a complete-case cohort, and the sensitivity analysis revealed systematic baseline differences between the included and excluded patients (Suppl. Table S5). This indicates a potential selection bias, which could lead to an overestimation of treatment response and optimistic estimates of radiographic outcomes. Fourth, data regarding the concomitant use of non-steroidal anti-inflammatory drugs and analgesics were not systematically collected, which could be a source of unmeasured confounding. Fifth, the 52-week follow-up period was insufficient to definitively assess long-term radiographic outcomes. Sixth, our assessment of mediator 'dynamics' was based on two time points, capturing only linear change rather than more complex temporal patterns. Other limitations include the insufficient sample size to assess differential responses to specific bDMARDs and the potential lack of generalisability. Our findings are derived from a single-country (Japanese) cohort, and it is well established that both genetic risk factors and clinical disease expression in RA can differ significantly across ethnic populations (28). Given that ethnic and genetic factors can influence inflammatory mediator profiles, the specific subgroups identified here may not be directly applicable to other populations. Therefore, validation in independent, multi-ethnic cohorts is essential. Despite these limitations, this study provides a foundational, noninvasive framework for advancing stratified medicine in RA.

In conclusion, this study demonstrated that baseline serum inflammatory mediator profiles can be used to stratify DMARD-naive patients with early RA into two distinct clusters, each possessing a unique pathobiological signature. The identification of subgroup-specific mechanisms, such as the IL-6-driven pathway in one cluster, offers a deeper understanding of RA heterogeneity beyond a simple clinical presentation. These findings provide a strong biological rationale and noninvasive framework for the future development of biomarker-guided therapeutic strategies.

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