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Paediatric rheumatology

Identification and validation of interferon-driven gene signature as a predictor of response to methotrexate in juvenile idiopathic arthritis

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ARTICLE INFO

ABSTRACT

Objectives: To identify and validate gene expression biomarkers of response to methotrexate (MTX) treatment in peripheral blood of children with juvenile idiopathic arthritis (JIA) measured before starting MTX treatment.

Methods: RNA sequencing was performed on sorted CD4+, CD8+, CD14+, and CD19+ cells, as well as peripheral blood mononuclear cells (PBMC) taken pre-treatment in a discovery cohort (n = 97) and 2 validation cohorts (n = 26 and n = 47, respectively) of patients with non-systemic JIA. Clinical data were recorded at baseline (timepoint 1) prior to treatment and 6 months (timepoint 2) of MTX treatment. Analysis tested the association of gene expression in specific cell types with treatment response using limma-voom, gene set enrichment analysis, and a novel 51-gene score against response to treatment. Parallel analysis, also using pre-treatment gene expression data, was performed in adult rheumatoid arthritis (RA) data (n = 240).

Results: In patients with JIA, the baseline expression of genes driven by interferon (IFN) alpha (type-I) or gamma (type-II) was associated with response to treatment at 6 months in 3 independent JIA cohorts. The direction of the association indicated that children with higher baseline expression of IFN-stimulated genes prior to MTX were more likely to be good responders. Comparison with adult RA indicated differences between PBMC and whole blood gene expression associations with response.

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Conclusions: In children with JIA, a high IFN-driven gene signature is associated with a better response to MTX than those with a low IFN-driven gene signature. These data could pave the way to clinically validated tools to identify those most likely to require medications in addition to MTX to control inflammation.

WHAT IS ALREADY KNOWN ON THIS TOPIC

- Methotrexate (MTX) is the first line of treatment for most forms of non-systemic juvenile idiopathic arthritis (JIA) before biologic agents are used; however, adequate response to treatment is achieved in only 50% of patients, yet most patients suffer side effects from the use of MTX.
- Achievement of early clinical remission is correlated with better long-term outcomes in JIA.
- There are no validated biomarker tests yet that are used in clinical practice to predict children who need early escalation to biologic agents.

WHAT THIS STUDY ADDS

- This study shows that genes in the interferon (IFN) alpha (IFNα, type-I) and gamma (IFNγ, type-II) response pathways are associated with response to treatment with MTX in JIA.
- This finding was replicated in 2 validation cohorts of JIA, and the association was confirmed using a score of only 5 IFN-driven genes.
- The association between IFN-driven pathways and response differs between children with JIA and adults with rheumatoid arthritis (RA).

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- An IFN-gene score detected in a small blood test could be built into models to develop a tool to define the risk of nonresponse to MTX and inform treatment decisions early in JIA.
- Accurate prediction of non-response to medication would enable 'patient-precise' medicine and prevent disability.
- This study emphasises the need for age-specific research in inflammatory arthritis, and that results in RA cannot be extrapolated to JIA.

INTRODUCTION

Juvenile idiopathic arthritis (JIA) is a group of conditions presenting with inflammation of synovial joints in children and young people starting before the 16th birthday. JIA continues to be a major cause of childhood pain, morbidity, and disability [1–3]. When active inflammation remains uncontrolled or children suffer repeated flares of disease, JIA significantly lowers children's quality of life, impacting education, development, and mental health during vital formative years [4]. Despite an increasing number of treatments available, the choice of medication remains largely by historical precedent rather than through an evidence base since there are no validated robust biomarkers that reliably predict response to specific therapeutics. This 'trial-and-error' method of prescribing can result in years of ineffective drug treatment and unpleasant side effects, during which time uncontrolled arthritis causes irreversible joint damage.

Methotrexate (MTX) remains the first-line disease-modifying agent of choice for many types of JIA [5], where a simple intra-articular steroid joint injection does not control the disease. However, typically, 50% of patients have a poor response to MTX at 6 months of treatment [6]; these children may benefit from earlier intervention with biologic agents if they could be reliably identified early. We have shown that the JIA subtype cannot predict a good response to MTX [7]. The CLUSTER (Childhood arthritis and its associated uveitis: stratification through endotypes and mechanism to deliver benefit) Consortium, a UK-wide multidisciplinary collaborative network, was established to address this urgent need for stratifiers to enable a precision approach to treatment for children and young people with JIA [8].

In this study, we aimed to define blood biomarkers that are associated with response to MTX in JIA, with the ultimate goal of generating predictive biomarkers to help guide treatment choice. We took a hypothesis-free approach and interrogated transcriptome data from sorted peripheral blood immune cells and total peripheral blood mononuclear cells (PBMC), taken just prior to MTX start, against treatment response after 6 months in patients with JIA. We identified pathways that are associated with response, with interferon (IFN)-driven pathways being the most significant pathways identified. We generated a univariate gene score of 51 transcripts that were robustly associated with response in JIA and showed that this score was also associated with response. We validated these findings in 2 further JIA replication cohorts. We looked at the relationship between IFN-driven pathways and MTX response at 6 months in adult rheumatoid arthritis patients and found an association in the opposite direction to the results seen in JIA.

This large, comprehensive study which set out to define specific immune cell biomarkers measured by gene expression to predict outcomes in response to MTX treatment, provides a significant step towards the possibility of precision medicine in JIA.

METHODS

Detailed methods are provided in the Supplementary Methods. In brief, we recruited a discovery cohort of 97 non-systemic JIA patients, naive to disease-modifying antirheumatic drugs (DMARDs), and recorded core outcome variables at baseline (timepoint 1 [t1]) and again after MTX treatment (timepoint 2 [t2], median follow-up 6.6 months). We quantified gene expression in sorted immune cell populations from blood using RNA sequencing (RNAseq). We examined the association between gene expression and change in outcome variables using limma-voom [9–11] and gene set enrichment analysis [12]. We used false discovery rates (FDR) to adjust for multiple hypothesis testing. We attempted to replicate findings in 2 external validation cohorts (1 UK and 1 USA) of JIA patients [13]. We used 2 measures of replication: the correlation between effect estimates in discovery and validation cohorts (ρ) and an overall measure of association (λ, which captures the deviation of a set of P values from their expected distribution if the null hypothesis is true for all tests, defined fully in the Supplementary Methods). We also compared the results with those of the RA-MAP (Rheumatoid Arthritis MRC–ABPI consortium) study of adult rheumatoid arthritis (RA) patients [14]; all 3 cohorts included patients naive to DMARDs and treated with MTX for approximately 6 months.

RESULTS

Study overview

An overview of the study is shown in Figure 1A. Patients with all categories of JIA other than systemic JIA (sJIA) were

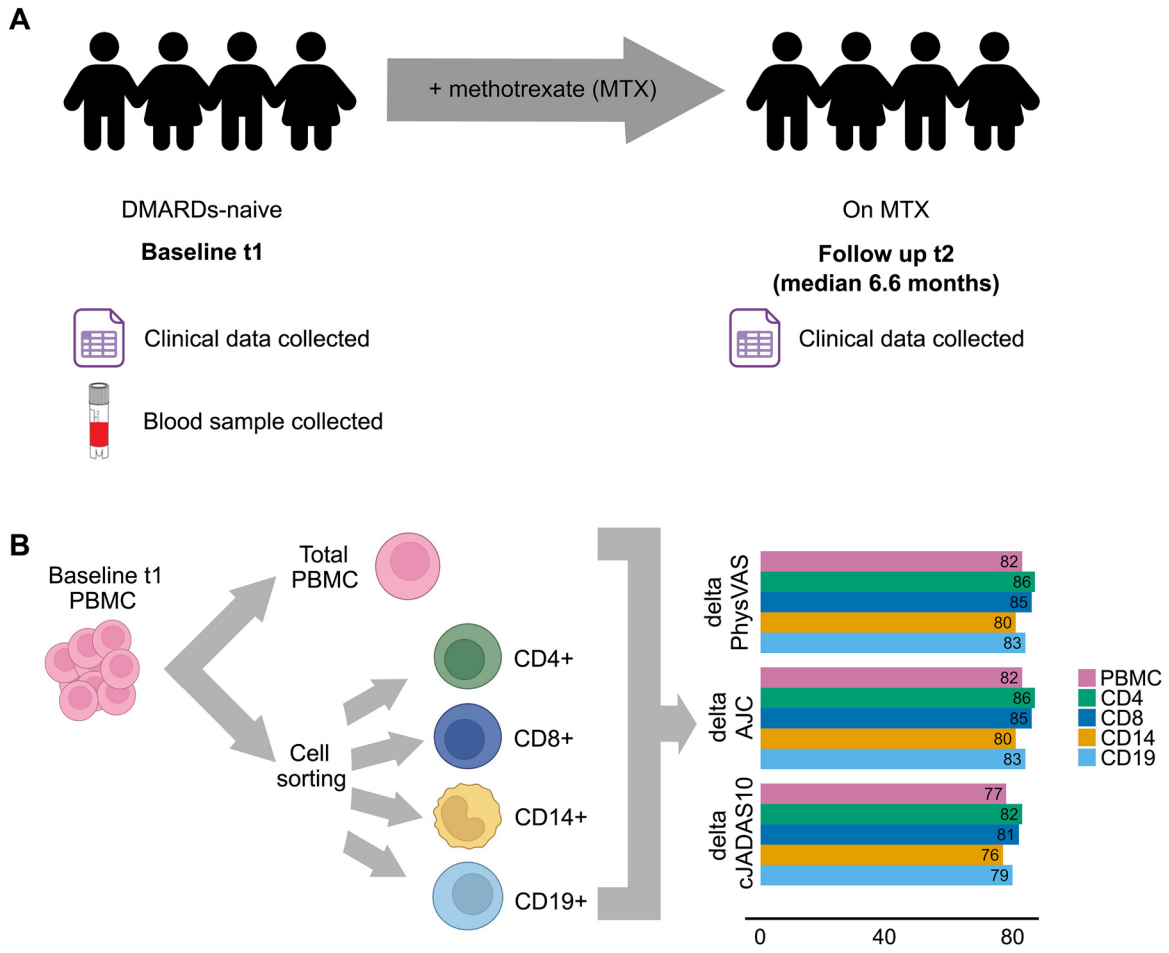

Figure 1. Study design and RNA sequencing (RNAseq) data generation. (A) A total of 97 juvenile idiopathic arthritis (JIA) patients with active JIA (excluding systemic JIA) who were about to start methotrexate (MTX) were included. Blood samples were collected at the time of diagnosis (baseline timepoint 1 [t1]), prior to receiving MTX (pre-MTX). Clinical data were collected at baseline t1 and follow-up timepoint 2 (t2) to calculate the response. (B) Peripheral blood mononuclear cells (PBMC) were isolated from whole blood and sorted into 4 different immune cell types. Sorted immune cells and total PBMC were sequenced. The final number of available RNAseq data for each cell type and each outcome variable is shown in the bar plot. delta-AJC, change in active joint count; delta-cJADAS10, change in clinical Juvenile Arthritis Disease Activity Score 10; delta-PhysVAS, change in Physician Visual Analogue Score (VAS); DMARDs, disease-modifying antirheumatic drugs.

recruited when they were about to start MTX for active arthritis. sJIA was excluded since MTX alone is no longer the standard of care for sJIA. A total of 416 RNAseq data files from 97 patients were included in the discovery analysis, as illustrated in Figure 1B. Patients were predominantly in the polyarticular rheumatoid factor-negative and oligoarticular categories of JIA, with smaller numbers in other categories (Table) [15]. PBMC isolated from blood samples collected prior to starting MTX treatment (naive) were sorted into 4 immune cell types: CD4+ T cells, CD8+ T cells, CD14+ monocytes, and CD19+ B cells (Fig 1B). To define predictors of response to MTX in JIA, we performed RNAseq on these sorted immune cell populations and total PBMC.

Our recent study exploring the complexity of available tools for assessing response to treatment in 2 large JIA cohorts showed that an American College Rheumatology (ACR) definition of improvement (DOI) [16] or ACR paediatric DOI may be reached despite disparate changes in the core set variables of the tool [7]. Therefore, for initial analyses, we considered the core variables, the physician's Visual Analogue Score (PhysVAS), active joint count (AJC), and the disease activity score tool, clinical Juvenile Arthritis Disease Activity Score (cJADAS) 10 [15], as continuous variables over time. After thorough quality control, n = 76 to 86 cases had data per cell type across the different measures of response to treatment (Fig 1B). Subsequent analyses also used the ACR-DOI tool [16] in this study.

Analysis of gene expression across all cell types against outcome measures

Our first aim was to identify genes whose expression at baseline in specific cell types or total PBMC was significantly associated with MTX treatment response at 6 months. Gene expression analysis found a limited number of genes that were individually significantly associated with treatment response (FDR < 0.05) in any cell type (Fig 2A-C). In all, 14 genes across all cell types were significantly associated with a change in 1 or more outcome measures, 13 of which were significantly associated with monocytes (Fig 2D). Genes that reached significance with 1 measure of response (change in PhysVAS [delta-PhysVAS], AJC, or cJADAS10) generally had the same direction of association with other outcomes (Fig 2D). However, interestingly, some genes reaching significance in one cell type had a heterogeneous pattern of association across other cell types (Fig 2D).

Table
Demographic data of JIA patients in CLUSTER cohort

Characteristics	JIA patients (n = 97)
Age (y) at baseline, median (IQR)	8.5 (4.9-12.8)
Female, n (%)	61 (62.9)
Disease duration (y) at MTX start (t1), median (IQR)	0.9 (0.5-2.5)
Time to follow-up time point t2 (mo), median (IQR)	6.6 (5.8-7.4)
JIA subtype by ILAR classification, n (%)	
Oligoarthritis, persistent	18 (18.6)
Oligoarthritis, extended	19 (19.6)
Polyarthritis, RF negative	37 (38.1)
Polyarthritis, RF positive	7 (7.2)
Psoriatic arthritis	5 (5.2)
Enthesitis-related arthritis	11 (11.3)
Clinical variables	
Active joints t1, median (IQR)	5 (3-9)
Active joints t2, median (IQR)	0 (0-3)
Change in active joints, delta, median (IQR)	−4 (−7 to −1)
Physician global score t1, median (IQR)	4.4 (2.3-6)
Physician global score t2, median (IQR)	1.1 (0.5-2.7)
Change in physician global score delta, median (IQR)	−2.1 (−4.5 to −0.9)
cJADAS10 t1, median (IQR)	13.3 (8.2-19)[a]
cJADAS10 t2, median (IQR)	4.3 (1.5-8.9)[a]
Change in cJADAS10 score delta, median (IQR)	−7.7 (−13.6 to −3.1)[b]

cJADAS, clinical Juvenile Arthritis Disease Activity Score (range, 0-30) [15]; Delta, t2 − t1; ILAR, International League of Associations for Rheumatology; JIA, juvenile idiopathic arthritis; MTX, methotrexate; RF, rheumatoid factor; t1, baseline timepoint; t2, follow-up timepoint.
Summary of demographics and clinical characteristics of patients in the discovery analysis; IQR (25%, 75%).
[a] cJADAS10 available in n = 94 JIA patients.
[b] Delta-cJADAS10 available in n = 92 JIA patients.

Expression of IFN-driven genes at baseline is associated with response to MTX

Because the power to detect association with individual genes can be limited by the large multiple testing burden, we next used gene set enrichment analysis (GSEA) to ask whether particular pathways showed enrichment for association signals. First, we compared 2 popular methods: GSEA, as originally proposed [12], and the more recent fastGSEA (fGSEA) [17], using simulated traits in an independent set of JIA cases (US cohort). We found that fGSEA produced a large number of false positive results, whereas GSEA, as originally proposed, controlled the false positive rate well while maintaining similar power (Supplementary Fig S1A). Therefore, we used GSEA rather than fGSEA to explore biological drivers of response-associated gene expression in our data.

Using Molecular Signatures Database (MSigDB) Hallmark as a database reference [18], we found significant enrichment of genes in the IFN alpha (IFNα, type-I) and gamma (IFNγ, type-II) response pathways associated with delta-PhysVAS across PBMC, CD8, and CD19 cell types (FDR < 0.05 or FDR < 0.01; Fig 3A), with consistent direction of effect across the cell types and other outcome measures (Fig 3B). Specifically, higher expression of type-I and type-II IFN-driven genes prior to starting MTX was associated with a better response at 6 months. Of note, parallel analyses using PhysVAS at baseline and t2 showed higher expression of type-I IFN and type-II IFN-driven genes associated with higher PhysVAS at baseline and lower PhysVAS at t2, although enrichment was only significant in PBMC (IFNα and IFNγ) at t2 and in CD19 (IFNγ) at baseline (Supplementary Fig S1B). Interestingly, having defined IFN-stimulated genes as being associated with response to MTX in JIA, we then reanalysed the 14 significant genes initially identified by differential gene expression analysis (Fig 2D) using the Interferome database [19]; 12 of these 14 genes are associated with IFN-driven pathways (Supplementary Table S1).

To identify a set of IFN response genes for further analysis, we identified genes that drive enrichment (leading-edge genes) of these 2 IFN pathways (type-I and type-II IFN) from cell types (PBMC, CD8, and CD19) that were significantly associated with delta-PhysVAS and took these 127 genes for further analysis (Fig 4A, top table and Venn diagram). We also included *SIGLEC1* (CD169) since this gene has been reported in multiple autoimmune studies [20–23] to be associated with type-I IFN, but it is not annotated in MSigDB Hallmark IFN pathways. Of these 128 genes, 51 were nominally associated ($P < .05$) with PBMC from the CLUSTER cohort (Fig 4A, lower plot), which led to our definition of a 51-gene IFN-driven gene set (Supplementary Table S2) for further analysis in validation cohorts. We confirmed that the baseline expression of these 51 genes, expressed as the 51-IFN-gene score, correlated significantly with response at 6 months by delta-PhysVAS (Fig 4B) and change in cJADAS10 (delta-cJADAS10) (Supplementary Fig S2). Analysis of these 51 genes, annotated as type-I or type-II IFN-driven genes, showed that there was considerable overlap, with 30 of the 51 genes being annotated by the Hallmark database as both (Fig 4A, lower Venn diagram).

We next investigated whether we could replicate these findings in an independent cohort of JIA patients (Childhood Arthritis Response to Medication Study [CHARMS]) who were also naive to treatment and about to start MTX; demographics are shown in Supplementary Table S3. In this replication cohort (26 children), gene expression levels of the 51 genes were associated with delta-PhysVAS ($\lambda = 2.1$, $P = .043$; Fig 4C and Supplementary Fig S3A), with 15 reaching a nominal P value of $\leq .05$. The estimated log-fold changes for these 51 genes were significantly correlated between the replication CHARMS cohort and those from the primary CLUSTER dataset ($\rho = 0.765$, $P = .037$; Fig 4D and Supplementary Fig S3B).

Comparison with adult RA

Higher expression of genes in IFN response pathways at baseline was associated with an improved response to MTX in our JIA cohorts. This is in contrast to reports in adult RA where higher expression of IFN response genes in whole blood (WB) in treatment-naive patients has been observed to be associated with poor response to MTX treatment [24–26]. Previous analysis in RA WB samples was based on a 5-gene score (IGS5) comprised of the average expression of 5 canonical IFN response genes: *MX1, IFI44L, OAS1, ISG15*, and *IFI6*, only 2 of which were on our list of 51 genes. We, therefore, compared the expression of all these genes (the 51 genes we defined and the 5 genes comprising IGS5) in RA samples at baseline (RA-MAP cohort, N = 240) with change in DAS28 after MTX treatment in all cell types, PBMC, and WB, and across several time points (3, 6, 9, 12, 15, and 18 months) using data released by the RA-MAP consortium [14] (Fig 5A, Supplementary Table S4). We found that effect directions in the RA-MAP cohort were consistent across time points (3-18 months; Fig 5A), so differences in the timing of recording treatment response were unlikely to be responsible for the contrasting directions between JIA and RA. On the other hand, we did see heterogeneity between cell types

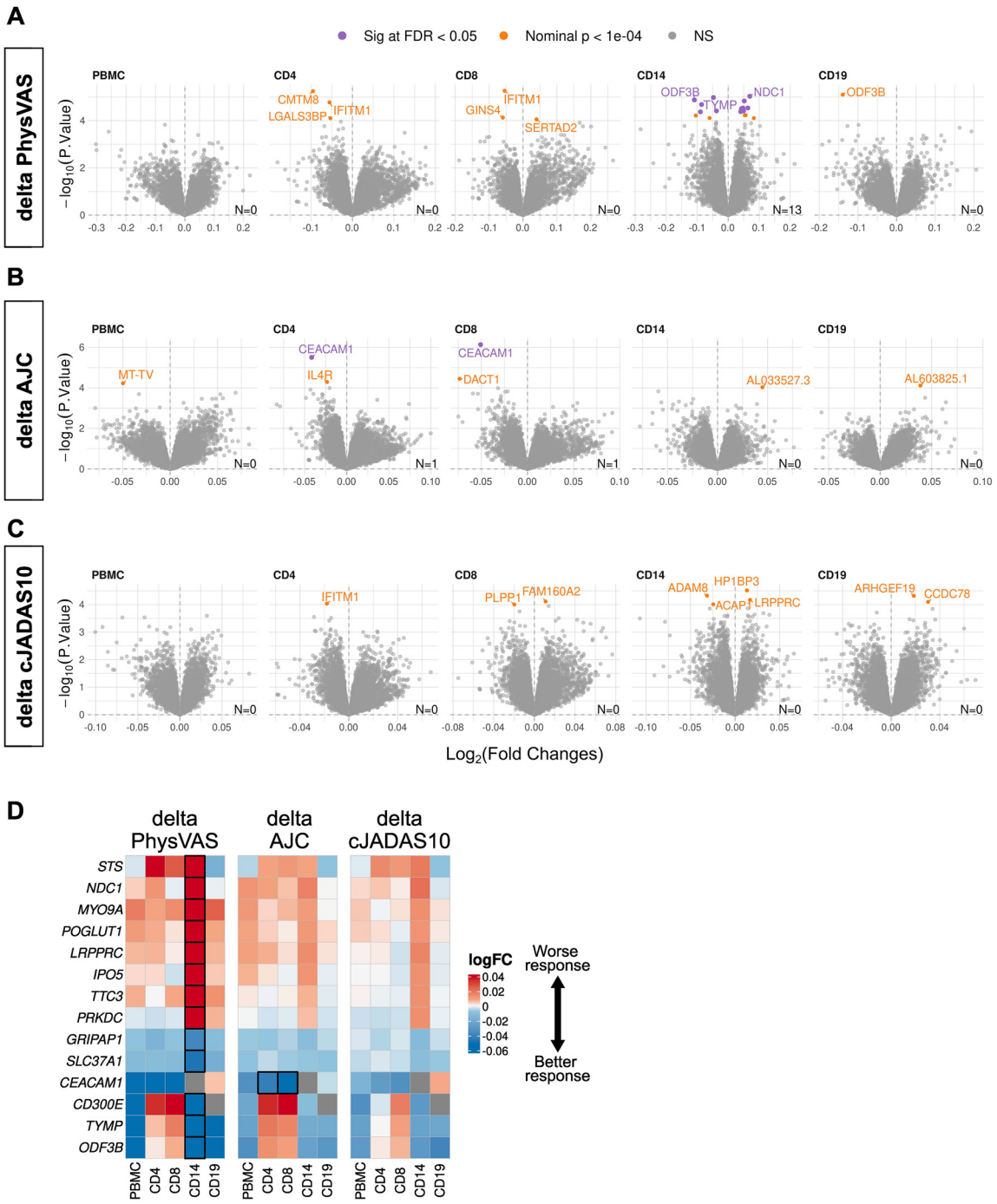

Figure 2. Analysis of gene expression across cell types and outcome measures. Gene expression was measured using RNA sequencing in sorted cell types and total peripheral blood mononuclear cells (PBMC) taken prior to starting methotrexate (MTX). Gene expression was analysed for associations with response to MTX treatment, with outcome assessed by change in Physician Visual Analogue Score (delta-PhysVAS), change in active joint count (delta-AJC), or change in the clinical Juvenile Arthritis Disease Activity Score 10 (delta-cJADAS10) in patients with juvenile idiopathic arthritis. Analysis was conducted in parallel within cell types, and we highlight genes that are significant in any one cell type. Results for (A) delta-PhysVAS, (B) delta-AJC, and (C) delta-cJADAS10 are shown as volcano plots for each cell type with nominally ($P < 10^{-4}$) and false discovery rate (FDR < 0.05) significant genes, which are highlighted in orange and purple, respectively. Gene names are shown for the top 3 genes either by FDR or *P* value significance. (D) Results across all outcomes for significant genes and cell types are summarised in a heatmap showing fold change (FC; colour) and significance (FDR < 0.05, solid box). A negative delta value (calculated as value at timepoint 2 − value at timepoint 1) indicates a decreasing measure of disease activity and, hence, a good response to treatment. NS, not significant.

for RA-MAP data, with the most consistently positive associations seen for WB and the least for PBMC.

We performed univariate comparisons of DAS28 in RA and PhysVAS in JIA at baseline (ie, t1), 6 months (ie, t2), and the change in each measure (delta-DAS28 and delta-PhysVAS, respectively) against the IGS5 previously defined in RA studies [24]. We saw that disease activity tends to correlate positively with the IGS5 in PBMC at baseline in both RA and JIA, but while this correlation was maintained in RA at 6 months, the IGS5 in PBMC at baseline was negatively correlated with disease activity at 6 months in JIA. This led to positive or negligible correlations of the IGS5 at baseline with increasing DAS28 at 6 months in

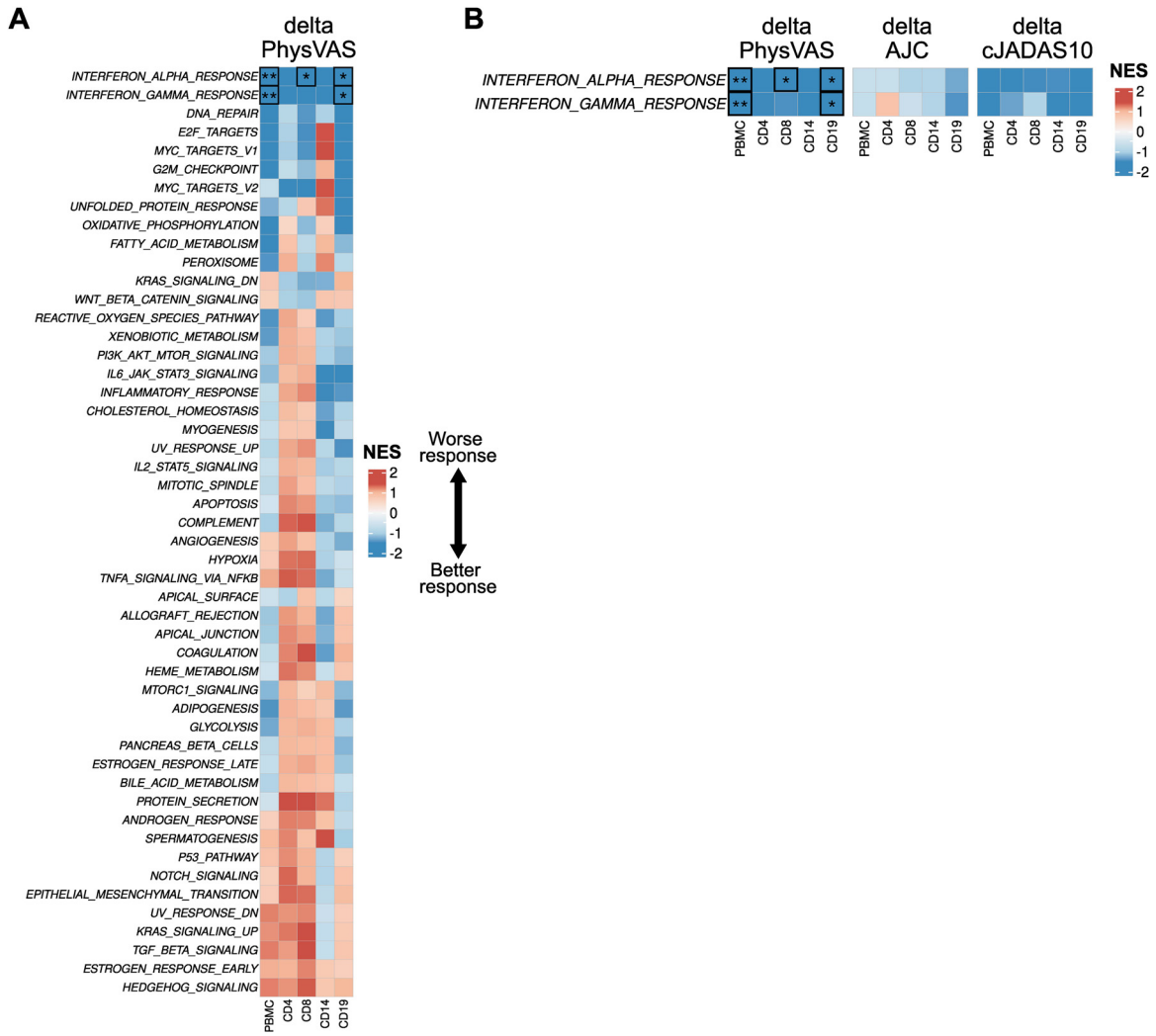

Figure 3. Gene Set Enrichment Analysis (GSEA) identifies the association of interferon (IFN) response gene pathways with response to methotrexate. (A) GSEA analysis in the CLUSTER JIA dataset identified type-I (IFNα) and type-II (IFNγ) response gene pathways as robustly associated with a change in Physician Visual Analogue Score (delta-PhysVAS). The heat map shows the enrichment score (colour) and significance (*false discovery rate [FDR] < 0.05, **FDR < 0.01) of Molecular Signatures Database (MSigDB) Hallmark pathways across delta-PhysVAS over 5 cell types. (B) Both type-I (IFNα) and type-II (IFNγ) response pathways showed consistent directions of effect with other outcomes, change in active joint count (delta-AJC), or change in the clinical Juvenile Arthritis Disease Activity Score 10 (delta-cJADAS10). JIA, juvenile idiopathic arthritis; NES, normalised enrichment score; PBMC, peripheral blood mononuclear cells.

RA. In JIA, this gave a positive correlation of the IGS5 with PhysVAS at baseline and with response, as assessed by a decrease in PhysVAS (Fig 5B). We also saw the same direction of effect when we analysed these measures against the 51-gene score in JIA (Supplementary Fig S4).

The pattern in Figure 5B could suggest that the effect on treatment response, as marked by IFN response gene expression at baseline, is mediated by disease severity at baseline, although this would be in contrast to the pathway analyses conducted independently at baseline and t2. To examine this in more detail, we compared models for delta-PhysVAS with and without adjustment for baseline measures in the CLUSTER and CHARMS cohorts. We found that association with treatment response was maintained even after adjustment for disease severity at baseline and saw no significant interaction effects with baseline measures (Fig 6A-D), suggesting that the effect marked by IFN response gene expression at baseline is not mediated by disease severity at baseline. We found a similar pattern using AJC in CLUSTER, CHARMS, and a second validation cohort, US JIA (Supplementary Fig S5); note that PhysVAS was not available in the US cohort. These results suggest that even though there is an association between IFN-driven gene expression and disease activity prior to treatment, after adjusting for this effect, there remains an association between response and the baseline expression of the IFN-driven signature.

In these analyses, both gene scores were nominally associated with delta-PhysVAS and AJC in the validation cohorts, where they were measured in the baseline-adjusted models, providing further support for the validation of our results in these independent cohorts.

IFN response genes show a consistent direction of association with the ACR-DOI in JIA

The majority of clinical trials in JIA have used the ACR paediatric DOI tool to define response to treatment [16]. Therefore, we wished to confirm that our findings were consistent when the treatment response outcome was defined by ACR-DOI. The distribution of delta-PhysVAS by the ACR group in the CLUSTER cohort is shown in Figure 7A. As expected, children with a good response to MTX (here defined as reaching American College Rheumatology (ACR) 50 (ACR50) or above) have a more

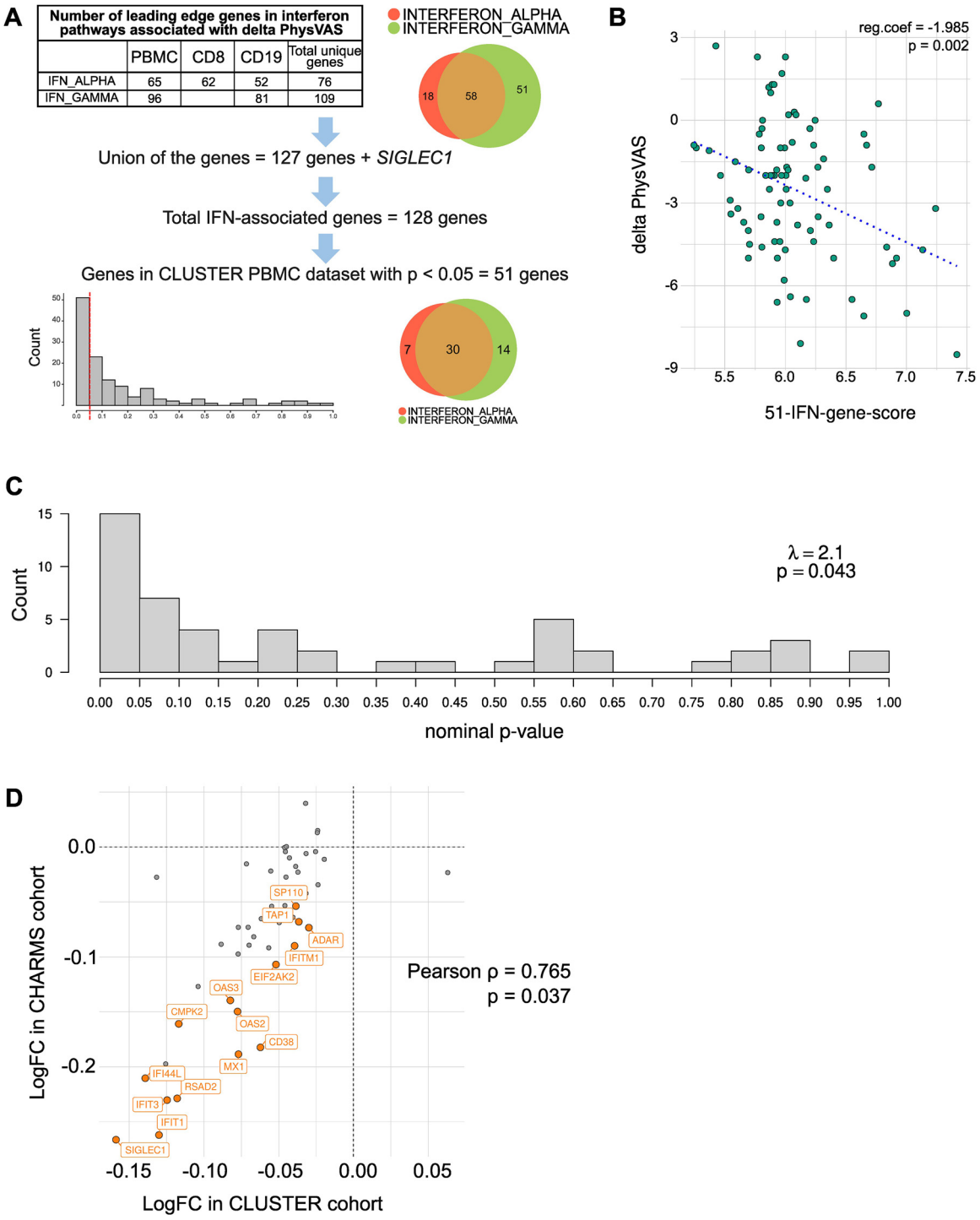

Figure 4. Validation of the association between interferon (IFN) response gene expression and treatment response. (A) The table shows the number of leading-edge genes in each cell type (peripheral blood mononuclear cells [PBMC], CD8, and CD19) where response was significantly associated with type-I and type-II IFN pathways. The union of leading-edge genes (127 genes) from both pathways across different cell types showed a substantial overlap (n = 58 genes) in gene membership between type-I and type-II pathways (upper Venn diagram). Including *SIGLEC1*, a total of 128 interferon-related genes were considered as marking the interferon response pathways, of which 51 were found to be nominally significant ($P < .05$) in the PBMC dataset. The bottom Venn diagram shows overlapped gene membership between the 2 IFN pathways for those 51 genes (lower plot). (B) A 51-IFN-gene score was generated using the mean log2 expression of the 51 significant genes. The dot plot shows the correlation of the 51-IFN-gene score in PBMC with a change in Physician Visual Analogue Score (delta-PhysVAS) in the CLUSTER dataset ($P = .002$). (C) Regression of delta-PhysVAS on PBMC gene expression in the independent CHARMS dataset of 26 children showed significant enrichment of small P values among the 51 IFN response genes ($\lambda = 2.1$, $P = .043$). (D) Estimated effect sizes for 51 IFN response genes in the CHARMS replication dataset were significantly correlated with those estimated in the CLUSTER dataset ($\rho = 0.765$, $P = .037$). Genes highlighted in orange represent the 15 genes reaching a nominal P value of <.05 in the CHARMS JIA cohort. CHARMS, Childhood Arthritis Response to Medication Study; FC, fold change; JIA, juvenile idiopathic arthritis.

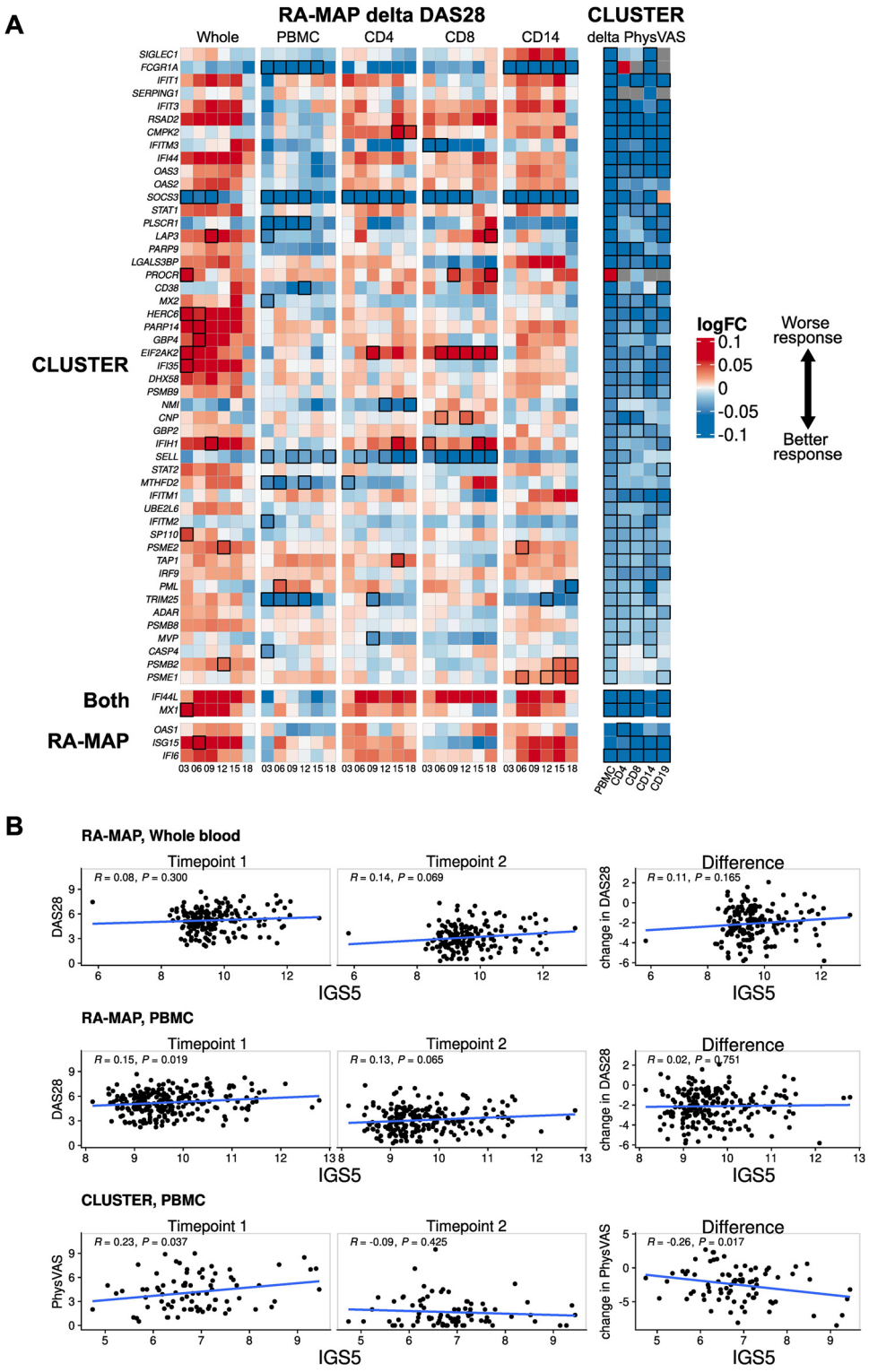

Figure 5. Comparative analysis between juvenile idiopathic arthritis (JIA) and adult Rheumatoid Arthritis (RA) for association of gene expression with response to methotrexate. (A) Heatmap of log-fold changes (FCs) estimated in expression analysis of the 54 interferon response genes (51 from our study and 5 from the RA-MAP study, including 2 in both RA samples [RA-MAP] and JIA samples [CLUSTER]) across all available time points and cell types for delta-DAS28 (RA) and a change in Physician Visual Analogue Score (delta-PhysVAS, in JIA). For all plots, genes are shown in rows. Columns in RA show the time point at which delta-DAS28 was calculated, with sets of columns split by cell type. In CLUSTER, columns show different cell types. The value of logFC is represented by colour, and a black square indicates nominal significance ($P < .05$). (B) Comparison of the 5-gene score (IGS5) with measures of disease activity (DAS28 in RA and PhysVAS in JIA) at baseline (timepoint 1), 6 months (timepoint 2), and differences between the time points. For RA, the IGS5 is shown for whole blood and peripheral blood mononuclear cells (PBMC), and in JIA, only in PBMC.

negative value for delta-PhysVAS. We conducted further analysis of the baseline expression of the 51 IFN-driven genes against ACR status at follow-up in both the CLUSTER and US JIA cohorts. Note that only 3 children in CHARMS had a response of American College Rheumatology (ACR) 30 (ACR30) or below, so we were not powered to perform this analysis in the CHARMS cohort. We again saw a significant positive correlation between the effect size (log-fold change) for delta-PhysVAS and the effect

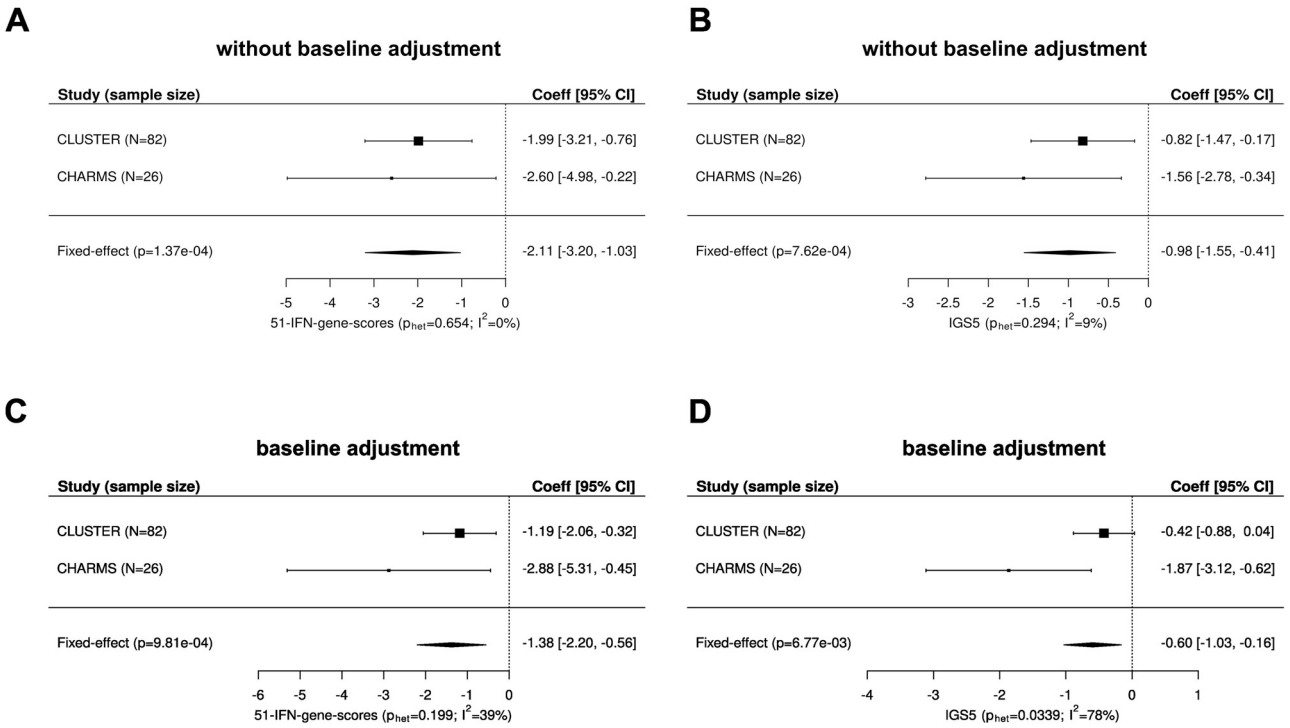

Figure 6. The association of change in Physician Visual Analogue Score (delta-PhysVAS) with both gene scores remains after adjustment for baseline. (A) Meta-analysis of the estimated effect of the 51 gene score on delta-PhysVAS across the CLUSTER and CHARMS cohorts. The bottom row shows the fixed effects meta-analysis result. (B) Meta-analysis of the estimated effect of the 5-gene score (IGS5) on delta-PhysVAS across the CLUSTER and CHARMS cohorts. (C) Same model as A, adjusted for baseline PhysVAS at timepoint 1. (D) Same model as B, adjusted for baseline PhysVAS at timepoint 1. Across all plots, phet is the *P* value for testing the significance of Cochran's Q statistic, and heterogeneity index, I2 (0-100), is the proportion of the total variation due to heterogeneity. CHARMS, Childhood Arthritis Response to Medication Study; IFN, interferon.

size for ACR across the 51 IFN genes in the CLUSTER JIA dataset ($\rho = 0.853$, $P = .019$; Supplementary Fig S6A). The same direction of correlation was observed with the US JIA dataset compared with the CLUSTER cohort, although this did not reach significance ($\rho = 0.40$, $P = .298$; Supplementary Fig S6B).

We also tested the 51-IFN-gene score as a univariate measure for association with response at 6 months in the CLUSTER cohort using the ACR-DOI outcome, with cases dichotomised into responders (defined as reaching ACR50 or above) and non-responders (those reaching ACR30 or below). Figure 7B shows that the 51-IFN-gene score, calculated from baseline PBMC gene expression (n = 118 cases), was significantly higher ($P = .00556$) in responders than in non-responders.

Next, we chose to use the IGS5 as a further univariate summary of IFN response, defined independently of our data, to support the most robust analyses. We compared the IGS5 between ACR outcome groups in the CLUSTER cohort and saw a nominally significant difference across all cell types ($P < .03$) between responders (defined as reaching ACR50 or above) and nonresponders (those reaching ACR30 or below), with a higher IGS5 in responders in all cell types (Fig 7C).

Finally, as we have previously observed that the serum concentration of the protein MRP8/14 (also known as S100A8/9), measured before treatment with MTX, in nonsystemic cases of JIA [27] correlates with response to MTX, we asked how this protein relates to the IFN score measured here. We found that serum MRP8/14 concentration correlates weakly with the 51-IFN-gene score ($\rho = 0.27$, $P < .09$; Supplementary Figs S7 and S8). The weak association is perhaps unsurprising given that cell sources of this serum protein include blood monocytes and neutrophils, as well as synovial tissue itself, while the 51-IFN-gene score derives from PBMC. The direction of the association, however, is consistent, meaning that in this study, children with a higher inflammatory signal by both measures were more likely to respond to MTX.

DISCUSSION

Reliable biomarkers that predict response to medication in inflammatory arthritis would offer a major step towards personalised treatment. In this large study of JIA, we took an unbiased approach to define genes and pathways whose expression in blood mononuclear cells prior to the start of treatment is associated with response to MTX. Within our discovery cohort, we found that genes whose transcription is driven by IFNα or IFNγ are expressed at higher levels prior to treatment in those who then achieve a good response to MTX. We validated this finding in 2 further JIA cohorts, one from the same centre (n = 26) and a second independent cohort (n = 47). To our knowledge, this is the first study to analyse how the pre-treatment transcriptome of both PBMC and separated major mononuclear cell types (T cells, B cells, and monocytes) are associated with response in JIA.

Our initial analyses to identify genes that were significantly associated with treatment response in each specific cell type or total PBMC yielded relatively few associated genes with an FDR < 0.05. This concurs with previous studies, all of which were on total PBMC, where few individual genes were identified as predictive of response to MTX [13,28]. However, GSEA analysis demonstrated a significant association of both IFNα (type-I) and IFNγ (type-II) pathways with response to MTX. Notably, we used the original GSEA method, including performing permutations on raw data to generate *P* values rather than gene labels in summary data, to minimise the risk of false positives and provide the greatest confidence in our results. The observation that the direction of this effect was the same across all cell types

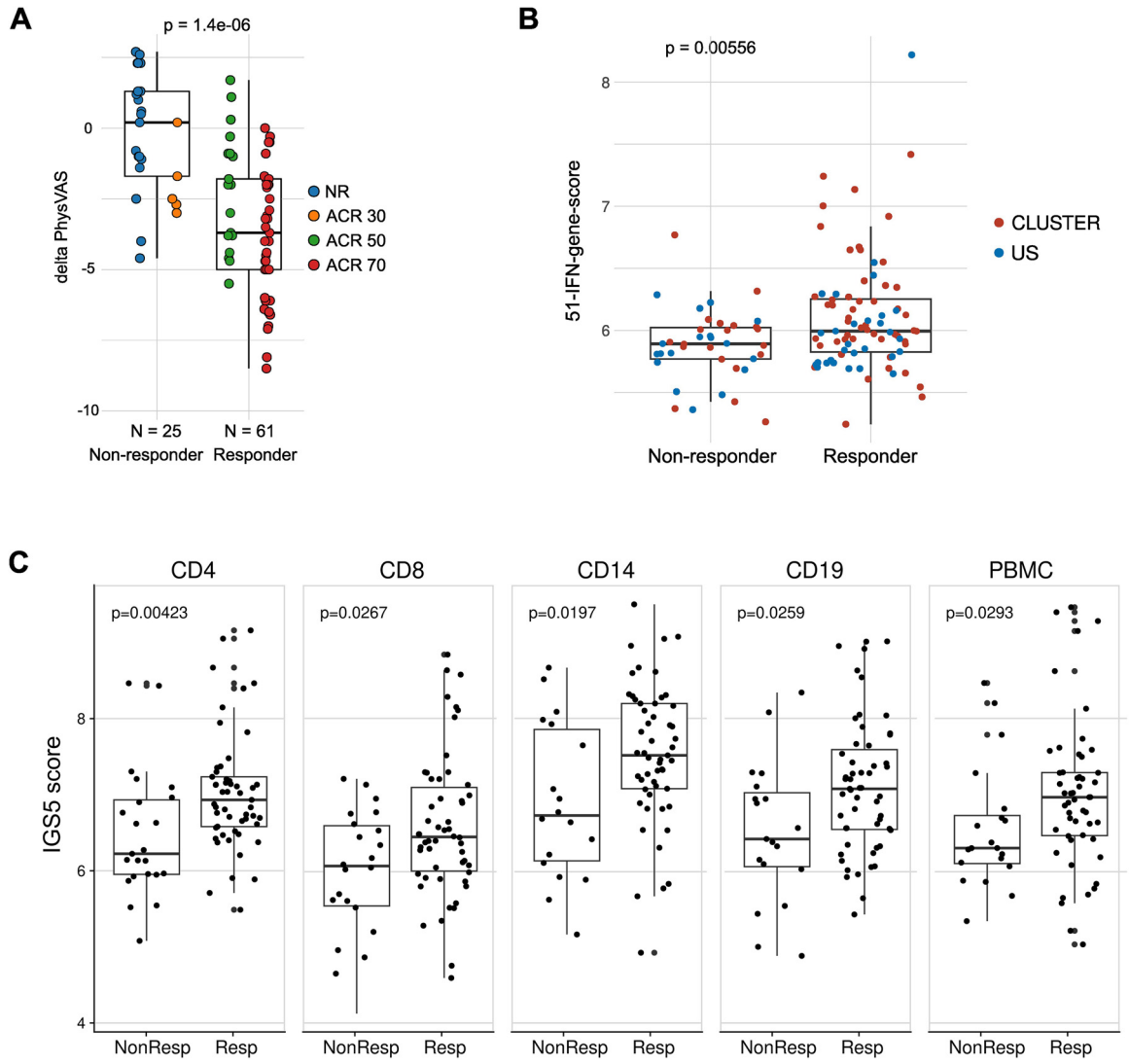

Figure 7. The association of treatment response with interferon (IFN) genes is recapitulated using American College Rheumatology (ACR) status. (A) Measures of change in Physician Visual Analogue Score (delta-PhysVAS) were higher in non-responders (Non-responder; NR [Non-responder] and ACR30) than in responders (Responder; ACR ≥ 50), $P = 1.4e10^{-6}$. N = 86 cases had full ACR status determined. (B) The 51-IFN-gene score calculated in peripheral blood mononuclear cells (PBMC) was higher in Responder (ACR ≥ 50) than in Non-responder (NR and ACR30). N = 71 cases of the CLUSTER cohort (blue dots) and n = 47 cases of the US cohort (red dots) had full ACR status determined and 51-IFN-gene scores calculated. (C) Mean log2 expression of the 5 genes, *MX1, IFI44L, OAS1, ISG15, and IFI6,* were used to provide a univariate summary (5-gene score [IGS5]) of the IFN response gene signature. The IGS5 was calculated in all 5 cell types (from left to right: CD4, CD8, CD14, CD19, and PBMC, as shown) and was significantly higher in Resp (ACR ≥ 50) than in NonResp (NR and ACR30) in all cell types. All data in this figure are from the CLUSTER cohort except for B, which includes data from the US cohort as shown.

suggests that this could be translatable to a meaningful clinical biomarker test in the future. In another study of response to MTX in JIA, 5 (*SOCS3, B2M, CASP3, IL4R,* and *CD40*) of 94 genes implicated in the association with response [28] overlapped with our list of 128 leading genes. Interestingly, other pathways that have previously been implicated in response to MTX, such as interleukin-6/JAK/STAT3 signalling, showed opposite directionality of effect in T cells to those in monocytes or B cells, although these did not reach significance. This cell-specific discrepancy is perhaps unsurprising given the known complexity of mechanisms of MTX, which affect many immune cell types [29] and may contribute to the challenge of finding robust biomarkers of response using PBMC or WB samples.

Given our ultimate goal to develop biomarker tests with clinical utility, we focused on significant pathways that were consistently associated across all cell types and PBMC. By selecting the IFN-pathway genes from cell types that reached significance, we developed a robust gene score of 51 IFN-driven genes to take forward for validation. We confirmed that this score correlated with change in 3 individual outcome measures used and the ACR-DOI outcome, and we then validated our results in an independent cohort of patients ($P = .037$). We also replicated this result using the ACR-DOI tool as a dichotomous outcome in a separate independent cohort across all the cell types.

Previous studies have shown raised IFNα- and IFNγ-driven signatures in PBMC, T cells, and monocytes from treatment-naive JIA patients, and enhanced IFN signalling in these cells taken prior to treatment, as well as greater development of Th1 or Th17 cells *in vitro*, but this signal varies between patients [30–32]. It is possible that MTX treatment is more effective in a distinct immunophenotype (present across many International League of Associations for Rheumatology subtypes), where IFN-driven processes are dominant early in the disease. New studies where the single-cell transcriptome of JIA synovial tissue is analysed and compared with matched blood will reveal whether this holds true for the tissue site itself [8,33].

We were intrigued to compare JIA with adult RA because an IFN IGS5 has been proposed as a predictor of response to DMARD treatment in RA [24]. In contrast to our study, a high baseline IFN score in RA has been shown to predict non-response to MTX [24,26] and rituximab [25]. Importantly, these RA studies were performed on WB samples; in RA, the greatest contributor to the baseline IFN score in WB prior to treatment has been shown to come from the neutrophils [34], while WB or neutrophils were not examined in our JIA cohorts. Our comparative analysis of expression of the 51-IFN-gene score and the RA IGS5 across all cell types revealed that certain genes showed the same direction of effect across time and cell types in RA as in JIA, including *FCGR1A, SOCS3,* and *SELL* (CD62L and L-selectin). However, for other genes, differences in the direction of association were observed in adult arthritis, even in the parallel cell type. These differences between adult and child arthritis may relate to underlying pathological differences between the 2 conditions, different disease duration at the start of treatment (itself a known predictor of response in JIA [35]), different magnitude of disease burden at the time of treatment start, the fact that adult RA patients frequently have a combination of DMARD therapies (MTX with hydroxychloroquine or other medications), or technical differences between these studies. This highlights the need for further investigations of treatment efficacy for children rather than extrapolation of data from adults to children with inflammatory arthritis.

Despite these differences, our findings in JIA were further confirmed when using the ACR-DOI measure to dichotomise JIA patients into responders (≥ACR50) and non-responders (Non-responder [NR] and ACR30), both for the 51 genes and the independent IGS5. For both tools, a higher score was associated with JIA responders (≥ACR50) in the discovery and validation cohorts.

Our study has some limitations. Despite the large discovery cohort and 2 replication cohorts, the analyses and necessary corrections for multiple testing mean that the study may have been underpowered to detect pathways that have a small but biologically important impact on response. Power considerations also precluded subanalyses based on the JIA subtype. In addition, WB RNA samples were not available to test whether the 51-IFN-gene score in WB is associated with response. Given the differences seen in the expression of these 51 genes between WB and PBMC in adult RA samples and prior evidence of a dysregulated neutrophil transcriptome in both pre-treatment and on-treatment JIA [36], this is an important limitation that will be key to consideration in future studies. Future studies will include not only WB and neutrophil analysis but also analysis from the tissue site, the inflamed synovium. Finally, we have shown that response to treatment can fluctuate over time [7], while this study analysed outcomes only at 1 time point (6 months).

Since we took a hypothesis-free approach at the start of this analysis, we have not yet interrogated these data for signals related to pathways known to be mechanistically impacted by MTX or those involved in the metabolism of MTX. Our previous studies [37,38] have shown that these may have effects on treatment response to MTX in JIA, meaning that such ‘candidate pathway’ analyses will be valuable to explore within these data.

Here, we have systematically interrogated the transcriptional profile of PBMC and their major constituent mononuclear cell populations for genes or pathways that may be useful in building treatment-response predictive biomarkers. Given that parents and patients report uncertainty about treatment response as a major burden and that MTX intolerance is very prevalent in JIA [4,39], the clinical value of a biomarker measured in a small blood sample that could predict response to MTX and aid clinical choices of medication would be high. Further studies are warranted to define the most cost-effective assay, using a small non-redundant gene set derived from our 51 genes, investigate if this test has utility if measured in WB, and then test such a tool in new prospective studies of JIA patients treated with MTX. Our study provides proof of principle that carefully designed analyses can yield hope for a more precision-based approach to treatment in the future for children and families living with arthritis.

Competing interests

LRW declares consultancies with Pfizer Inc and Cabaletta unrelated to this work and research funding from Pfizer Inc for a completely separate project. The CLUSTER Consortium has had support through contributions-in-kind from GSK, Pfizer, and UCB, as well as research funding from AbbVie Inc, Lilly, and SOBI. CW receives funding from MSD and GSK and is a part-time employee of GSK. These organisations did not contribute to the planning or analysis of this work. All the other authors declare no competing interests.

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The data presented here have not been published before; however, some of the data formed part of a recent conference abstract at the Paediatric Rheumatology European Society Congress, 2024 [40].

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Contributors

MK, W-YL, LRW, and CW conceptualised the study, analysed data, and co-wrote the manuscript. MK and W-YL contributed equally to this paper. LRW and CW led and supervised the study under the CLUSTER Consortium and obtained funding. MK, BJ, KOB, EW, and ER performed the wet laboratory experiments. MK, BJ, and EW curated the clinical data. W-YL and RR established the RNAseq pipeline. W-YL, MK, and RR performed data quality control. W-YL and CW led the statistical analyses. ECR and CTD designed and supervised parts of the study. All authors critically reviewed the manuscript and approved the final draft for submission.

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Patient consent for publication

Not applicable, consent provided through recruitment through ethically proved study.

Ethics approval

This study involves human participant and ethics were approved by London-Bloomsbury Research Ethics Committee 05/Q0508/95. All participants gave informed consent and age-appropriate assent, in accordance with the Declaration of Helsinki.

Provenance and peer review

Not commissioned; externally peer reviewed.

Data availability statement

Data are available upon reasonable request. RNAseq FASTQ files and full core set clinical variables at t1 and t2 are available by request for data access through EGA. EGAD50000001457 is for PBMC dataset, EGAD50000001455 is for CD4 dataset, EGAD50000001456 is for CD8 dataset, EGAD50000001458 is for CD14 dataset, and EGAD50000001459 is for CD19 dataset.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ard.2025.03.007.

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