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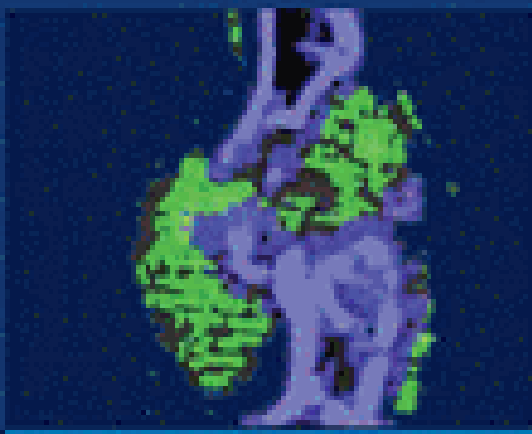

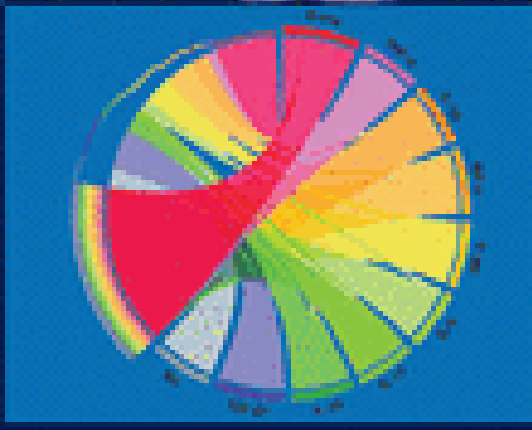

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BMJ Journals, BMA House, Tavistock Square
London WCIH 9JR,UK
E: **ard@bmj.com**

Production Editor
Teresa Jobson
E: **production.ard@bmj.com**

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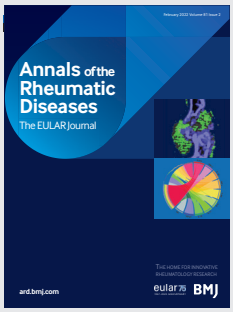

Editorial office
Annals of the Rheumatic Diseases
BMJ Publishing Group Ltd
BMA House
Tavistock Square
London WCIH 9JR,UK
T: **+44 (0)20 3655 5889**
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Editorial

Rheumatoid arthritis

Inflammatory arthritis

Psoriatic arthritis

Imaging

MORE CONTENTS ►

Systemic lupus erythematosus

Systemic sclerosis

Autoimmunity

Letters

Electronic pages

Epigenetic profiling of twins identify repression of KLF4 as a novel pathomechanism in systemic sclerosis

Jörg H W Distler [1], Steven O'Reilly [2]

Systemic sclerosis (SSc) is an idiopathic autoimmune connective tissue disease with vascular abnormalities, inflammation and fibrosis. The fibrosis affects the skin as well as several internal organs. An incomplete understanding of the pathways that govern activation of the cells involved in disease pathogenesis has hampered new therapies. In this issue of Annals of the Rheumatic Diseases, Malaab *et al* (REF to be inserted by ARD) employ a unique cohort of 15 twins disconcordant for the diagnosis of SSc to demonstrate a key role of epigenetics including methylation, alterations of microRNA and ultimately dysregulation of Kruppel-like factor 4 (KLF4) that impacts on fibrosis. This sheds light on underlying epigenetic mechanisms and exposes a new therapeutic target.

Epigenetics is the study of heritable changes in gene expression not mediated by changes in the DNA sequence itself. Three main mechanisms mediate epigenetic changes: non-coding RNAs, DNA methylation and histone modifications.[1] DNA and the cytosine base of DNA can be modified by the addition of a methyl group by a family of three DNA methyltransferases enzymes on the fifth carbon of cytosine. This modification enables binding of DNA methylation proteins such as methyl binding domain proteins, which in turn recruit histone modifying proteins and other epigenetic modulators that ultimately represses gene expression. Non-coding RNAs are defined as a long or short set arbitrarily defined by length. In the case of microRNAs, they regulate gene expression by binding the 3'UTR of mRNA that leads to gene repression. Long non-coding RNAs are usually much longer and regulate gene expression, both positively and negatively, through only partially unknown mechanisms. Finally, alteration in the histone tails of chromatin can also regulate gene expression via many different modifications with complementary functions. Histone acetylation by histone acetyltransferase enzymes has been studied most to date. Other modifications besides acetylation include methylation, ubiquitination, sumoylation and lactylation. All of these modifications can alter chromatin dynamics to promote or repress gene expression and can thus poise cells towards a certain state of activation. These epigenetic modifications are, however, reversible, and the epigenome undergoes continuous modifications to fine-tuning cellular dynamics. Recent evidence has highlighted epigenetic alterations as key-drivers in the pathogenesis of multiple diseases including SSc.[2 3] Multiple studies have provided evidence that different epigenetic alterations promote the persistently activated phenotype of fibroblasts in SSc.[4–9] Epigenetic alterations may also contribute to other features of SSc such as inflammation, autoimmunity and vascular disturbances.

The authors used a cohort of 15 twins discordant for the diagnosis of SSc for an integrated analysis of genome wide methylation patterns and gene expression. Using this combined 'omics' approach, they identified several differentially expressed genes regulated at the level of DNA methylation between controls and patients.

Among those were several homeobox genes including *HOXB3* and *HOXB8*. HOX genes are a family of evolutionarily conserved genes encoding transcription factors with central roles in stem cell differentiation, in the formation of organ symmetry and in topographic memory. First evidence also implicates HOX genes in wound healing and fibrosis,[10] thereby suggesting that dysregulated HOX gene expression may have the potential to contribute to the aberrant activation of fibroblasts in SSc. The authors provide evidence that HOX genes repress the expression of microRNA10a/10b. Mir10a is located upstream of HOXB4 on chromosome 17, while miR10b is located upstream of HOXD4 on chromosome 2. Analysis of SSc patients dermal fibroblast levels of both microRNAs revealed reduced levels of these microRNAs. Reduction of these microRNAs would thus lead to derepression of its cognate targets. Indeed, the microRNA10a/10b targets TFAP2A and TBX5 were indeed upregulated in this twin cohort. Silencing of miR10a and miR10b in normal dermal fibroblasts led to upregulated mRNAs for TFAP2A and TBX5. The functional role of this signalling cascade for fibroblast activation was further highlighted by rescue experiments in fibroblasts derived from patients with SSc. Restoring normal levels of miR10a and 10b in SSc fibroblasts normalised the expression of TFAP2A and TBX5 and reduced the aberrant expression of type I collagen.

The authors went on to examine embryonic stem cell transcription factors including SRY-box 2 (SOX2) and KLF4. They found enhanced SOX2 and decreased KLF4 expression particularly in early stages of SSc. A downregulation of KLF4 was also observed in hepatic stellate cells in human fibrotic liver and carbon tetrachloride induced liver fibrosis,[11] suggesting that impaired KLF4 expression may be a general feature of fibrotic diseases. The downregulation of KLF4 might in part be mediated by TGFβ, which has been shown to inhibit KLF4 expression in a kidney epithelial cell line.[12] KLF4 is a conserved zinc finger transcription factor with pleomorphic functions. It is best known as one of four transcription factors that are required to induce fibroblasts into pluripotent stem cells so-called Yanamaka factors.

Malaab *et al*[13] demonstrated that small interfering RNA mediated depletion of KLF4 increased ACTA2 (encoding α-Smooth muscle actin), CTGF and WNT4A. Conversely, adenoviral overexpression of KLF4 led to reduced collagen, fibronectin and CTGF protein levels suggesting that KLF4 regulates antifibrotic transcriptional programmes in fibroblasts. These findings are consistent with studies in isolated kidney cells, in which KLF4 repressed the expression of key fibrosis markers including α-smooth muscle actin.[12] Finally, the authors demonstrated that KLF4 conditional KO mice spontaneously develop skin fibrosis with upregulation of multiple profibrotic transcripts. The authors also noted a prominent activation of WNT signalling with accumulation of β-catenin, the central integrator of canonical WNT signalling. This is particularly intriguing, as WNT signalling has emerged as a core pathway of fibrosis in SSc.[14–17]

[1]Department of Internal Medicine III, University of Erlangen, Erlangen, Germany
[2]Stipe Therapeutics, Aarhus, Denmark

Correspondence to Dr Steven O'Reilly, Stipe Therapeutics, Aarhus, Denmark; SOR@stipetherapeutics.com

Consistent with the findings of Malaab *et al*, a recent study identified significantly reduced KLF4 in idiopathic pulmonary fibrosis samples, a disease that shares excessive fibroblast activation and fibrotic tissue remodelling with SSc.[18] Overexpression of KLF4 in mice with bleomycin-induced pulmonary fibrosis model significantly retarded fibrosis compared with wildtype mice. Interestingly, siRNA interference of KLF4 potentiated Dishevelled 2 (Dvl2) expression.[18] Dvl2 is a member of the WNT family that integrates and promotes wingless-related Integration site (WNT) signalling by stabilising the key effector molecule β-catenin, thereby preventing its degradation and promoting canonical WNT signalling.[19] Accordingly, the downregulation of KLF4 in SSc, eventually via TGFβ-dependent mechanisms, may upregulate Dvl2 expression to promote stabilisation and nuclear translocation of β-catenin with subsequent activation of WNT-induced profibrotic programmes.

What are the possible ways to use this information for clinical use? No approaches to induce KLF4 expression or activity directly are available to date. However, this study identifies two microRNAs that are regulating homeobox proteins that could regulate KLF4 amounts. One possible way to restore KLF4 levels would be to elevate the diminished levels of miR10a/b. This could be achieved using microRNA mimics. These are chemically modified microRNAs with increased stability to endogenous RNAses in vivo. This approach would ideally enhance the expression of KLF4. However, miRNAs do not regulate individual genes, but rather sets of different genes and miR10a/b mimics may thus modulate other, potentially homoeostatic pathways as well as KLF4.

Alternatively, the WNT pathway as a downstream mediator de-repressed by the downregulation of KLF4 offers therapeutic potential. After long being considered as 'undrugable', several inhibitors of WNT signalling have been developed during the last years. These include among others, inhibitors of porcupine. Porcupine is an acetyltransferase that palmitolyates WNT proteins, which is required for secretion of WNT ligands. In that regard, a porcupine inhibitor was used in different preclinical models of skin fibrosis and in clinically relevant doses was found to be effective.[20] Other inhibitors such as tankyrase inhibitors such as XAV-939 have also shown beneficial effects in models of SSc.[21] These data all suggest targeting WNT signalling could offer therapeutic potential in a disease that currently has no therapy that modifies the fibrosis.

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ORCID iDs
Jörg H W Distler http://orcid.org/0000-0001-7408-9333
Steven O'Reilly http://orcid.org/0000-0002-3416-7330

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CLINICAL SCIENCE

Baseline predictors of different types of treatment success in rheumatoid arthritis

Dafne Capelusnik [1,2] Daniel Aletaha [1]

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[1]Department of Rheumatology, Medical University of Vienna, Wien, Austria
[2]Department of Rheumatology, Instituto de Rehabilitación Psicofísica, CABA, Argentina

Correspondence to
Professor Daniel Aletaha, Department of Rheumatology, Medical University of Vienna, Wien 1090, Austria; daniel.aletaha@meduniwien.ac.at

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ABSTRACT

Objective To perform a comprehensive analysis on predictors of achieving disease activity outcomes by change, response and state measures.

Methods We used data from three rheumatoid arthritis (RA) trials (one for main analysis, two for validation) to analyse the effect of patient and disease characteristics, core set measure and composite indices on the achievement of different outcomes: response outcomes (% of patients achieving a relative response margin); state outcomes (remission or low disease activity, LDA) and change outcomes (numerical change on metric scales).

Results We included patients from the ASPIRE trial (for analysis) and from the ATTRACT and GO-BEFORE trials (for validation). While lower disease activity components at baseline—except acute phase reactants—were associated with achievement of state outcomes (such as LDA by the Simplified Disease Activity Index, SDAI), higher baseline values were associated with change outcomes (such as SDAI absolute change). A multivariate analysis of the identified predictors of state outcomes identified best prediction by a combination of shorter disease duration, male gender and lower disease activity. For prediction of response, no consistently significant predictors were found, again, with exception of C reactive protein, for which higher levels at baseline were associated with better responses.

Conclusion Prediction of treatment success is limited in RA. Particularly in early RA, prediction of state targets can be achieved by lower baseline levels of diseases activity. Gender and disease duration may improve the predictability of state targets. In clinical trials, included populations and choice of outcomes can be coordinated to maximise efficiency from these studies.

Key messages

What is already known about this subject?

- Previous studies confirmed that lower levels of disease activity at baseline predict achievement of favourable disease activity states. Additionally, higher levels of disease activity at baseline demonstrated to be associated with greater absolute change.

What does this study add?

- The present study for the first time performs a direct comparison of all relevant rheumatoid arthritis treatment outcomes used in contemporary research (response margins, states, change scores) and allows to contrast predictive markers across the different outcomes within the same datasets.
- Despite the expected highly significant association of baseline disease activity markers with state and change, no prediction could be confirmed for response margins. A notable exception was C reactive protein, which did not predict state outcomes, but did so with response outcomes.

How might this impact on clinical practice or future developments?

- This data may be useful to improve clinical trials design in order to maximise their efficiency. Those trials may not need to include highly active patients if they continue to use response endpoints, such as the American College of Rheumatology response definition, which may alleviate some pertinent issues in recent trials, such as high regression to the mean.

Rheumatoid arthritis (RA) is a heterogeneous disease leading to specific clinical signs and symptoms as well as to systemic inflammation. If untreated, this active disease process leads to structural damage on cartilage, bone and soft tissue, and to functional impairment. Given this multiplicity of different outcomes, many variables can be monitored during the follow-up of RA over the course of the disease and the course of individual treatments. These measures are referred to as the so called core set variables as defined in the 1990s.[1]

Evaluation of treatment success in RA can be challenging given the large number of these measures, and therefore combined indices were developed, which advanced RA disease activity assessment towards more homogeneity and comparability across different patients and across different assessors of the same patient over time.[2 3] These indices allow to either quantify disease activity with a single number on a continuous scale, such as the Disease Activity Score based on 28 joint counts (DAS28),[4] the Simplified Disease Activity Index (SDAI)[5] and the Clinical Disease Activity Index (CDAI)[6]; or determine a patient's responder status (yes/no), as for example, by the European League Against Rheumatism (EULAR) response criteria[7] and the American College of Rheumatology preliminary response criteria (ACR20/50/70),[8] with the latter being the most commonly used endpoints in clinical trials of RA for over two decades; or to assess whether a state target has been reached (yes/no), typically by remission criteria, as for example, by the ACR/

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EULAR Boolean remission criteria[9] or by placing cut-points on the above mentioned continuous scales of DAS28, SDAI or CDAI.

In recent years, strong interest has emerged in understanding predictors of treatment success, however, the aforementioned complexity and diversity of different approaches to determining 'success' has partly led to inconsistent and even conflicting results. For example, data support that lower levels of disease activity at the initiation of disease-modifying antirheumatic drugs (DMARDs) therapy are predictive of reaching state targets (remission (REM)/low disease activity (LDA)) in RA.[10 11] At the same time, the association between clinical core set variables at baseline and the achievement of a certain level of response is less clear, and it might even be inverse. The objective of the present investigation is to perform a comprehensive and systematic analysis of core set variables and derived indices, and their relation to achievement of disease activity outcomes by change, response and state measures.

MATERIAL AND METHODS

Data sources

Data from three clinical trials were used. For each, we received a random ~80%–90% cut of patients data. The Active-Controlled Study of Patients Receiving Infliximab for the Treatment of Rheumatoid Arthritis of Early Onset (ASPIRE) study, a double-blind randomised controlled trial (RCT), included 1004 patients with early RA methotrexate (MTX) naive who were randomised to receive MTX as monotherapy or MTX in combination with infliximab (IFX).[12] The comparator RCTs were the Anti-TNF Theraphy in Rheumatoid Arthritis with Concomitant Therapy (ATTRACT) study, including 428 patients with established RA and active disease despite MTX therapy, who continued with MTX and were randomised to receive placebo (PL) or IFX[13] and the Golimumab Before Employing Methotrexate as the First-Line Option in the Treatment of Rheumatoid Arthritis of Early Onset (GO-BEFORE) trial,[14] including 637 patients with early RA, MTX naïve who were randomised to receive golimumab (GLM) with and without MTX combination, or MTX monotherapy.

For our analyses, we used as base case the patients with early RA from the ASPIRE trial, who received treatment with MTX+IFX. Subsequently, we modified variables and repeated the analysis in established RA with the same regimen (ATTRACT) as well as in early RA with a different compound (GO-BEFORE).

Study variables

Demographic characteristics and measures of disease activity were extracted from the original data sets, including tender joint counts (TJC28) and swollent joint counts (SJC28), patient global and pain assessment (PGA), evaluator global assessment (EGA) (by Visual Analogue Scale), erythrocyte sedimentation rate (ESR), C reactive protein (CRP) and Health Assessment Questionnaire (HAQ) scores as functional measures. Measures of disease activity and function were obtained at baseline (before the start of the DMARD therapy) and for the 30-week time point, which was consistently available across all datasets; ACR20/50/70, SDAI50/70/85 and CDAI50/70/85[15] responses were estimated based on their criteria fulfilments, composite measures SDAI and CDAI were calculated based on their respective formulas and disease states of REM, LDA, moderate disease activity and high disease activity were evaluated based on the established cut-off points.

Analysis of predictors of response, state and change outcomes

The effects of each individual core set measure at baseline on response were analysed by using quartiles of each core set variable and compare response rates for SDAI/CDAI50/70/85, ACR20/50/70 definitions across these four groups using χ^2 test. The same analysis across baseline quartiles of predictors was done using LDA achievement (by SDAI or CDAI) as the outcome. In addition, we performed a trend analysis across response groups and disease activity levels by Wilcoxon rank sum test. For the assessment of association with change scores of composite indices (from baseline to 30 weeks), Spearman correlation was performed with the mentioned (continuous and categorical) baseline predictors.

In a second step, univariable logistic regressions of these baseline variables on the 30-week achievement of response outcomes and state outcomes were performed in separate models. In the logistic regression, aside from disease activity core set variables and indices at baseline, we also considered sociodemographic (age, gender, race) and disease characteristics (early RA, disease duration and seropositivity for rheumatoid factor (RF)). Results were corrected for multiplicity of testing using Bonferroni correction.

For analyses in which several significant predictors, including core sets, were identified from the logistic regression modelling, we performed an integrative analysis on a pooled dataset from all trials using stepwise logistic regression to identify combinations of baseline predictors that best predict the different outcomes; some variables were forced into the model for their presumed clinical relevance, such as gender and disease duration. A graphical representation of the predictor model was developed as a prediction matrix, in which each cell codes for the probability of achieving the respective outcome for each combination of the categorised significant predictors. To enhance the visual reading, in addition to the numerical value in each cell (include its 95% CI), we colour coded probabilities from green (high probability) to orange (low probability). The continuous factors were presented in tertiles based on clinical utility and relevance.

RESULTS

Patient populations and overall responses

We included 821 patients from the ASPIRE trial (patients with early RA, with mean±SD of disease duration: 0.83±0.7 years; 71.2% women; mean age of 49±13 years; 71.5% RF positive), 330 from the ATTRACT trial (established RA; mean disease duration: 10.3±8.1 years; 77.6% women; mean age: 53±12 years; 78.1% RF positive) and 573 from the GO-BEFORE trial (mean disease duration: 3.5±5.2 years; 83.4% women; mean age: 50±12 years; 94.6% RF positive). However, in the main analysis we only included 532 patients from the MTX+IFX arm in ASPIRE trial. Disease characteristics and outcomes of each trial are presented in online supplemental tables 1 and 2. The patient characteristics of our random sample were similar to the original trial reports.

Descriptive analysis of individual predictors of treatment success

For our main analysis, we focused on SDAI70 for response outcome, and LDA by SDAI for state outcome in the MTX+IFX arm of the ASPIRE trial at week 30; also SDAI change from baseline was used as change outcome. SDAI70 response was predicted by higher baseline CRP: patients in higher baseline CRP quartiles were found to achieve SDAI70 responses more

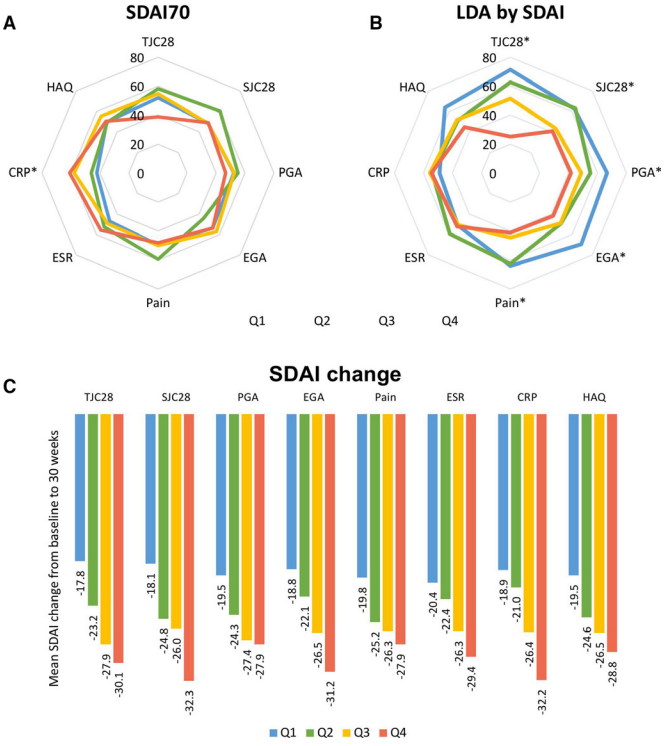

Figure 1 Target outcomes and baseline core set. (A, B) Spider charts represent the association between disease outcome (axis) and core set quartiles (rays). χ^2 test. P value corrected for multiple testing using Bonferroni correction. *P<0.05. (C) Bar graph represents the change from baseline to 30 weeks in mean SDAI based on core set baseline quartiles. Wilcoxon rank sum test. P<0.001 for all variables. CRP, C reactive protein; EGA, evaluator global assessment of disease activity based on a 100 mm visual analogue scale; ESR, erythrocyte sedimentation rate; HAQ, Health Assessment Questionnaire; LDA, low disease activity; PGA, patient global assessment of disease activity based on a 100 mm visual analogue scale; SDAI, Simplified Disease Activity Index; SJC28, swollen joint count based on assessment of 28 joints; TJC28, tender joint count based on assessment of 28 joints.

frequently than in lower quartiles (figure 1A). Conversely, the percentage of patients achieving LDA (including REM) by SDAI showed an increasing tendency with lower baseline values of all composite indices and core set measures, interestingly except acute phase reactants and HAQ (figure 1B). For the change outcome (SDAI change from baseline to 30 weeks), all core set measures showed a significantly higher mean change in higher quartiles (figure 1C, online supplemental table 3).

Additionally, table 1 shows the trend of mean values of the core set/composite measures across SDAI 50/70/85 response groups (table 1, 'Response') and SDAI disease activity states ('Disease activity states'), which are confirmatory of the above analysis using the quartile approach. The change outcomes (table 1, 'Change') also confirmed the results observed in figure 1C with a significant correlation of SDAI and CDAI change with all baseline measures. Trend for ACR response and disease activity state by CDAI are expressed in online supplemental table 4.

Modelled predicted probability of treatment success

Online supplemental table 5 shows the results from the univariable logistic regressions of various demographic, disease and disease activity variables on SDAI70 response or achievement of LDA by SDAI. After correcting for multiple testing, no sociodemographic factor proved to be a significant predictor of either

Table 1 Association between response assessments at 30 weeks and baseline core set factors: trending and correlation analyses

	Response (trend across SDAI response groups by Wilcoxon rank sum)				Disease activity state (trend across SDAI disease activity states by Wilcoxon rank sum)				Change (correlation of changes in SDAI/CDAI by Spearman rho)			
	SDAI-nr	SDAI50	SDAI70	SDAI85	P value	SDAI-HDA	SDAI-MDA	SDAI-LDA	SDAI-REM	P value	SDAI (r)	P value	CDAI (r)	P value
TJC28	14.1 (7.1)	13.7 (6.4)	13.4 (6.0)	12.5 (5.6)	NS	19.3 (6.1)	14.2 (6.1)	11.9 (5.7)	10.7 (4.9)	<0.001	0.402	<0.001	0.420	<0.001
SJC28	8.9 (4.5)	9.6 (5.0)	8.7 (4.2)	9.1 (3.6)	NS	11.5 (5.4)	9.6 (4.5)	8.2 (3.8)	8.3 (3.3)	<0.001	0.432	<0.001	0.440	<0.001
PGA	63.0 (26.1)	60.2 (24.3)	61.4 (22.2)	59.2 (26.4)	NS	75 (20.4)	63.2 (22.6)	57.9 (24.3)	54 (27.8)	<0.001	0.245	<0.001	0.214	<0.001
EGA	63.9 (18.7)	66.6 (17.6)	68.0 (18.0)	65.4 (19.5)	NS	70.8 (17.8)	68.8 (16)	63.7 (19.7)	61.9 (19.7)	<0.001	0.382	<0.001	0.348	<0.001
Pain	65.4 (22.4)	62.6 (21.7)	65.0 (20.5)	62.2 (23.4)	NS	74.8 (18.3)	67 (19.7)	61 (21.2)	57.4 (25.8)	<0.001	0.260	<0.001	0.211	<0.001
ESR	40.2 (28.6)	48.1 (28.8)	49.9 (29.7)	43.8 (27.6)	NS	48 (34.3)	46.7 (29.7)	46.8 (27.7)	39.5 (25.6)	NS	0.287	<0.001	0.155	<0.001
CRP	2.2 (2.8)	2.6 (2.9)	3.5 (3.7)	3.2 (3.5)	<0.001	3.2 (3.9)	2.6 (3)	3.4 (3.7)	2.3 (2.6)	NS	0.397	<0.001	0.228	<0.001
HAQ	1.5 (0.7)	1.5 (0.7)	1.5 (0.7)	1.5 (0.6)	NS	1.8 (0.6)	1.6 (0.6)	1.4 (0.7)	1.3 (0.6)	<0.001	0.275	<0.001	0.201	<0.001
CDAI	35.7 (12.6)	36.1 (11.2)	35.1 (10.5)	34.1 (10.0)	NS	45.4 (11.1)	36.9 (10.1)	32.3 (10.1)	30.5 (9.3)	<0.001	0.520	<0.001	0.523	<0.001
SDAI	37.9 (13.6)	38.7 (12.0)	38.6 (11.8)	37.3 (11.4)	NS	48.6 (11.4)	39.6 (11.3)	35.5 (11.4)	33 (10.3)	<0.001	0.577	<0.001	0.522	<0.001

Wilcoxon rank sum test for SDAI response and disease state (by SDAI) and Spearman correlation composite indices change. Corrected for multiple testing using Bonferroni correction. Values are mean (SD) and Spearman rho.
SDAI response was categorised in exclusive groups: SDAI85 is not included in SDAI70 nor SDAI50; SDAI70 is not included in SDAI50.
CDAI, Clinical Disease Activity Index; CRP, C reactive protein; EGA, evaluator global assessment of disease activity based on a 100 mm visual analogue scale; ESR, erythrocyte sedimentation rate; HAQ, Health Assessment Questionnaire; HDA, high disease activity; LDA, low disease activity; MDA, moderate disease activity; PGA, patient global assessment of disease activity based on a 100 mm visual analogue scale; REM, remission; SDAI, Simplified Disease Activity Index; SJC28, swollen joint count based on assessment of 28 joints; TJC28, tender joint count based on assessment of 28 joints.

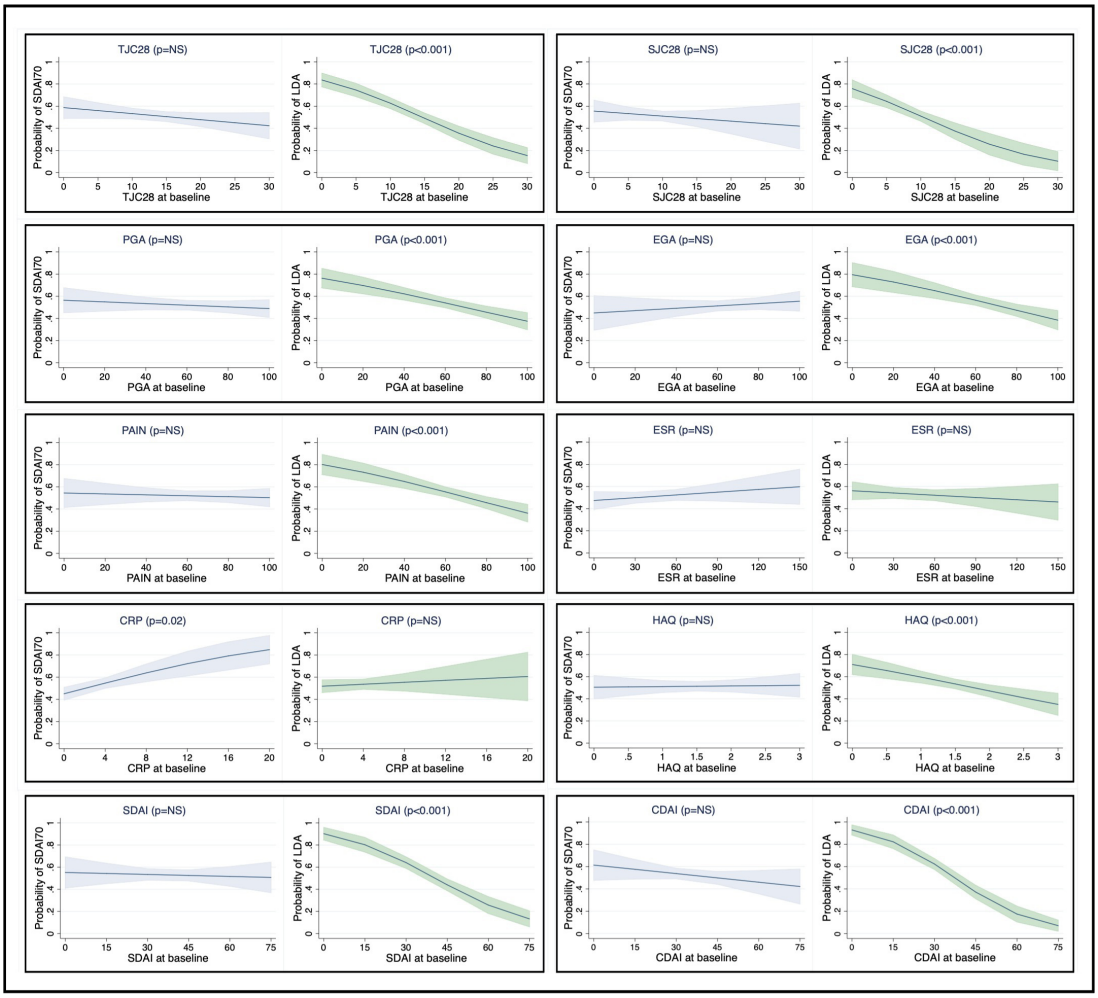

Figure 2 Predicted probability of achieving SDAI70 response (blue) or LDA by SDAI (green) at 30 weeks. Each couple of graphs represent the predicted probabilities of achieving the outcomes (with the 95% CI) for each respective core set variable and index in an early RA population from the ASPIRE trial. P values were corrected by Bonferroni test. CDAI, Clinical Disease Activity Index; CRP, C reactive protein; EGA, evaluator global assessment of disease activity based on a 100 mm visual analogue scale; ESR, erythrocyte sedimentation rate; HAQ, Health Assessment Questionnaire; LDA, low disease activity; PGA, patient global assessment of disease activity based on a 100 mm visual analogue scale; RA, rheumatoid arthritis; SDAI, Simplified Disease Activity Index; SJC28, swollen joint count based on assessment of 28 joints; TJC28, tender joint count based on assessment of 28 joints.

response or state (although age showed a trend, it was not significant). Figure 2 demonstrates that CRP was the only disease activity core set variable associated with treatment response (panels with blue lines); in general, levels of patient-reported outcomes (PGA, pain, TJC28) at baseline were inversely associated with response, while more objective measures (EGA, CRP, ESR) were positively associated. In contrast, achievement of state targets (LDA/REM) was significantly and inversely associated for all core sets with the notable exception of the acute phase response (CRP, ESR) (panels with green lines). Again, the findings are in line with the initial descriptive analysis presented further above, with the exception of HAQ, that despite no being significantly associated with the target state as a categorical variable, it was during the regression analysis when assessed as a continuous variable.

Sensitivity analyses

We subsequently tested the robustness of the findings in using different response targets (ACR20/50/70, SDAI50/70/85 and CDAI50/70/85), study population (established instead of early RA) and different types of treatment (different tumour necrosis factor inhibitor (TNFi) (GLM) in another early RA population, and MTX monotherapy instead of combination therapy of IFX+MTX). In general, results from the analysis on prediction of response were also non-associative as in the main analysis; results of the state analysis were supportive of the association of baseline core set measures with this outcome, but not so in the established RA population (online supplemental table 6A and B).

Integrative multivariate risk profiling (matrix prediction model)

Based on multivariate logistic regression modelling and the OR for each of the predictors, we attempted to create a matrix model for 'risk of achieving target state'; we did not create such model for the 'risk of response', since there, no significant predictors had been identified. For our model, we pooled all datasets and introduced the demographic variable sex, the disease variable 'duration of RA' and baseline disease activity, represented in a combined way using the CDAI (figure 3).

DISCUSSION

In the present study, we aimed to perform a systematic and comprehensive analysis of all core set variables at baseline and

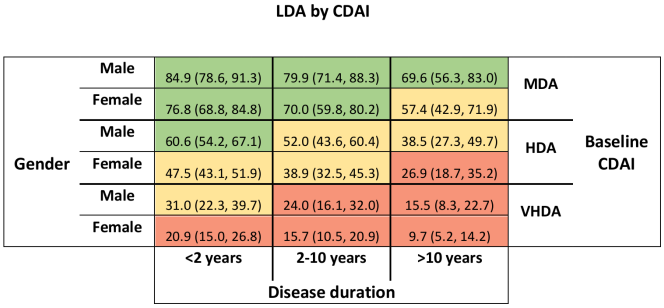

Figure 3 Matrix prediction model. Probability of achieving LDA by CDAI at 30 weeks. The number in the cells represents the percentage (and 95% CI) of achieving the outcome, based on the predictors combination at baseline. Colours represents predictive probability as follow: orange: 0%–30.9%; yellow: 31.0%–59.9%; green: 60.0%–100%. CDAI, Clinical Disease Activity Index: HDA, high disease activity >22 ≤50; LDA, low disease activity; MDA, moderate disease activity ≤22; VHDA, very high disease activity >50.

their predictive value for reaching main target outcomes used in clinical studies and clinical practice: achievement of certain response margins, of favourable disease activity states and of numerical improvement on metric disease activity scales.

The results of this extensive post hoc analysis showed evidence of a discrepancy in predictors depending on what is considered treatment success and the population involved. In early RA, when treatment is targeted towards a state, patients with lower levels of disease activity at baseline have the greatest chance for success; a notable exception of this are CRP and ESR, for which the effect was weaker and not significant. In contrast, when achieving a certain response margin was targeted, there was no association of any of the variable that were predictive in the state analysis; again with the notable exception of CRP, where there was a trend towards higher levels at baseline being associated with a higher chance of response, although significance across this trend was detected only in the main analysis cohort (ASPIRE). However, these two approaches—apart from being continuous versus binary—also differ with respect to their measuring of absolute and relative changes; this may lead to the conclusion that higher levels on a specific metric do allow greater absolute changes to be detected, but that this association is blunted by assessing a binary response relative to a baseline or reference.

For the third and final type of treatment target, namely the change (improvement) observable on metric disease activity scales, higher baseline values of all core set variables were significantly associated with higher mean change.

The strive of understanding simple predictors of treatment success has been part of RA management throughout modern rheumatology. This is particularly relevant when decisions need to be made on inclusion criteria and endpoints in clinical trials. Our finding that lower disease activity at baseline is associated with better achievability of LDA is intuitive and confirmatory of several previous observations.[10 16 17] As most trials are powering for ACR response outcomes—and thus for a proportion of patients achieving a response margin—it was interesting to learn that, using the same data source for the state outcome analysis, none of the identified predictors remained significant. Here, the results of the state analysis serve as the positive control, confirming that an association is indeed found where it is expected, while in another analysis—like the one on response—there is an impressive absence of predictive information at baseline.

Despite the failure to finding predictors of response measures, it may have a highly informative character for trial design, as current inclusion criteria are still leading to very active patients and therefore to recruitment problems in many parts of the world. Over incentivised inclusion or movement to less affluent countries is a natural consequence, and a relevant reason for high PL responses and regression to the mean. Knowing that higher or lower levels do not change the chance of observing a response (or difference in response) is therefore important for sponsors of clinical trials, which currently still have to use ACR response rates in their pivotal trials in order to fulfil regulatory claims.

Several additional details are of interest. First, the CRP associations in both analyses (state and response) contrast with the other disease activity variables: in the state target analysis, CRP and ESR are the only non-significant variables, while in the response target analysis CRP is the variable that has the highest degree of response association compared with all others. In this sense also the analyses in the other cohorts are supportive. Second, in established/late RA the state target analysis did not identify any significant variables at baseline, indicating that in this population these targets are not only more difficult to achieve, but also such achievement is less predictable. Third, the analyses are likely specific to trial settings, as real-life populations will be different. In fact, an analysis from Swedish RA real-world data reported associations of baseline disease activity measures with response targets,[18] which, however, have little significance in usual clinical practice. Furthermore, prediction of short term results may not necessarily be applicable to long term outcomes, and they may not be generalisable to all biologic DMARDs or targeted synthetic DMARDs therapies, with a different molecular target. Fourth, regression to the mean is a well-described problem in contemporary clinical trials.[19] Although it is unclear how it would exactly affect results, it is likely that at least it will add 'noise' to the performed analyses. Thus it may have reduced the power of detecting a signal. In an attempt to prevent from statistically falsely claiming association, we controlled all analyses for multiplicity, which—analogously to the impact of regression to the mean—may have eliminated variables with smaller effects, even in the large patient cohort. And finally, pooled matrix analysis using the state target as outcome showed the potential to combine findings on disease activity levels with other known factors associated with better outcomes that are relevant from a clinical perspective, including sex and disease duration. It allows for an estimation of the response chance based on a patient's profile on these variables.

To the best of our knowledge, this is the first study that assessed predictors of these different types of treatment outcomes 'head-to-head' based on using the same datasets. So in conclusion, we provide a comprehensive analysis across different types of therapeutic outcomes in RA to understand the distinctive implications of baseline markers to predicted treatment success. As a take home message from our report, the achievement of response target remains without accurate predictors. Nevertheless, state targets can be predicted by lower baseline disease activity, along with gender and disease duration, and these predictors may be used to inform the definition of study entry criteria to improve feasibility of recruitment. However, the choice of the right outcomes depends also on the clinical and scientifically purpose of measurement in the respective setting; it will differ, for example, between efficacy trials and strategy trials. The information provided here should hopefully be supporting individual researchers or companies in designing clinical trials in RA.

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ORCID iDs
Dafne Capelusnik http://orcid.org/0000-0001-9336-0416
Daniel Aletaha http://orcid.org/0000-0003-2108-0030

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Perceptions and experiences of individuals at-risk of rheumatoid arthritis (RA) knowing about their risk of developing RA and being offered preventive treatment: systematic review and thematic synthesis of qualitative studies

Heidi J Siddle ,[1] Lara S Chapman ,[1] Kulveer Mankia ,[1,2] Codruța Zăbălan,[3] Marios Kouloumas,[4] Karim Raza ,[5,6] Marie Falahee ,[5] Joel Kerry,[7] Andreas Kerschbaumer ,[8] Daniel Aletaha ,[8] Paul Emery ,[1,2] Suzanne H Richards [9]

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For numbered affiliations see end of article.

Correspondence to
Dr Heidi J Siddle, Leeds Institute of Rheumatic and Musculoskeletal Medicine, University of Leeds, Chapel Allerton Hospital, Chapeltown Road, Leeds, LS7 4SA, UK; h.siddle@leeds.ac.uk

HJS and LSC are joint first authors.

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ABSTRACT

Objectives There is increasing interest in identifying individuals at-risk of rheumatoid arthritis (RA) and initiating early treatment to prevent or delay the onset of arthritis. We aimed to describe the perceptions and experiences of at-risk individuals and to inform the conduct of clinical trials and studies, and clinical practice.

Methods A systematic review and thematic synthesis of qualitative studies was conducted. Two review authors independently screened studies for inclusion, appraised their methodological quality using the Critical Appraisal Skills Programme checklist and assessed confidence in the findings using the Grading of Recommendations Assessment, Development and Evaluation–Confidence in Evidence from Reviews of Qualitative Research approach.

Results Seven studies involving 115 individuals at-risk of developing RA were included. Three major themes (seven subthemes) were identified: understanding the risk of developing RA (knowledge of RA and identification of potential risk factors); preventive interventions to reduce the risk of developing RA (understanding the value and role of preventive interventions, and engagement with preventive interventions); and perceptions of predictive testing for RA (benefits of predictive testing, decision to undertake predictive testing and concerns about predictive testing). Moderate confidence in most review findings was evident.

Conclusion While there are clear benefits in informing individuals at-risk of RA about their risk following predictive testing and offering preventive treatment, there are potential barriers to engagement, intensified by the burden of uncertainty. Identification of the optimum approaches for presenting risk information, including the risks and benefits of engaging with preventive interventions, is urgently needed to support individuals at-risk of RA in their decision making.

PROSPERO registration number CRD42021236034.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory arthritis with a profound impact on quality of life and function. Identifying individuals with early RA and early initiation of treatment has shown to be effective at reducing the long-term damage associated with the erosive and persisting nature of the disease.[1–3] As well as improved clinical outcomes, early identification has also been associated with improved health-related quality of life and work ability.[4]

Research regarding the early diagnosis of RA has resulted in prediction models and international classification criteria,[5–9] and there is an increasing focus on the efficacy of preventive medication in the preclinical phase to prevent or delay the progression of RA.[10–14] However, there is limited insight into how at-risk individuals understand their own risk and on their views regarding predictive testing and engaging with preventive interventions, including lifestyle changes and medication. A metasynthesis of qualitative studies exploring the perceptions of predictive testing for those at-risk of developing a chronic inflammatory disease has previously been conducted.[15] This review identified patients' concerns about confidentiality, lack of motivation for change, poor clinician–patient communication and impact of the test result on emotional well-being as perceived barriers to predictive testing. A patient-centred approach throughout the testing process, including accessible information and support for patients to engage in risk-reducing health behaviours, was recommended. However, this review did not identify any participants at-risk of developing RA; the majority of studies included participants at-risk of diabetes and cardiovascular disease. These diseases are more prevalent than RA, routinely screened for in healthcare, and their risk factors and implications are better understood by the general public, with fewer misconceptions.[16]

In order to identify, recruit, offer intervention and monitor individuals at-risk of developing RA, it is imperative to appreciate potential barriers and facilitators to individuals' understanding of their risk and their motivations for engaging in clinical trials and studies. This is particularly important, given the current uncertainty about whether or when those who have been identified as at-risk of RA will actually develop clinical arthritis.

The aim of the current study was to synthesise qualitative studies exploring the perceptions and

experiences of individuals at-risk of developing RA, to inform the conduct of clinical trials and studies, and clinical practice.

METHODS

We followed the Enhancing Transparency of Reporting the Synthesis of Qualitative Research framework in reporting this review.[17]

Inclusion criteria

We included qualitative studies in which the authors undertook interviews or focus groups with adults (>18 years) at-risk of developing RA to explore the perceptions or experiences of being informed of this risk or being offered preventive treatment. At-risk populations eligible for inclusion were (1) asymptomatic at-risk individuals, which includes first-degree relatives (FDRs) of people with RA and indigenous North Americans; (2) at-risk individuals with musculoskeletal symptoms without clinical arthritis; and (3) at-risk individuals with early clinical arthritis, which includes patients with palindromic rheumatism and undifferentiated arthritis.[18] We included full articles in the English language that were published in peer-reviewed journals. Conference abstracts were excluded. Mixed-methods studies reporting quantitative and qualitative data were only eligible for inclusion if the qualitative data could be extracted separately. Studies including participants other than at-risk individuals (eg, healthcare professionals or patients with a diagnosis of RA) were included only if the data on eligible at-risk participants could be separated from the data on ineligible participants.

Search strategy

A literature search was performed using MEDLINE, Embase, PubMed and the Cochrane Central Register of Controlled Trials from inception to April 2021. The search strategy was conducted with guidance from a health librarian (JK) and is included within online supplemental file 4. An extensive manual search of reference lists and related citations of relevant articles was also conducted, followed by forward citation tracking using Scopus. Finally, we held discussions about the literature with experts in this field, including authors of included articles, to minimise the likelihood of overlooking any additional relevant material.

Study selection and data extraction

All activities were undertaken by researchers trained in qualitative methods (HJS and LSC) and under the supervision of an experienced qualitative methodologist (SHR). Studies retrieved from the searches were recorded on a central database. After excluding duplicate articles, two review authors (HJS and LSC) independently screened all titles and abstracts. Full texts of the studies identified as being potentially eligible for inclusion were then independently assessed against the inclusion and exclusion criteria by the two review authors. The following data were extracted electronically from eligible articles by one review author (LSC) using a standardised data collection form in Microsoft Excel (Microsoft Office Professional Plus 2016): study details (lead author and year of publication); participants (at-risk population, sample size and demographic characteristics); setting (country); data collection method (interview or focus group); recruitment technique; patient involvement; and data analysis method. All data within the results section of each study were extracted, including themes, subthemes, supporting verbatim quotations and the authors' interpretations of the data. Any disputes were settled by discussion between the two review authors or resolved through further discussion with two other members of the review team (SHR and KM) where necessary.

Quality assessment

Two review authors (HJS and LSC) independently assessed the quality of each included study using the Critical Appraisal Skills Programme (CASP) checklist for qualitative studies.[19] Any discrepancies were discussed until consensus was reached or resolved in further discussion with SHR and KM. The CASP checklist consists of 10 items, and each item includes multiple signalling questions to help users interpret the item (29 signalling questions in total). A summary table detailing the frequency of responses to each signalling question was constructed.[20] The CASP checklist has no scoring matrix; therefore, a narrative summary of the quality of the individual included studies is provided.

Data synthesis and analysis

We used the method of thematic synthesis described by Thomas and Harden to identify and develop themes from our included articles.[21] All extracted data from the Results section of each study were considered in the synthesis. Two review authors (HJS and LSC) read each article multiple times to achieve immersion, then independently performed line-by-line coding of the data to search for concepts. Following comparisons of common convergent and divergent concepts within and across studies, codes were organised into related areas to construct descriptive themes and subthemes. This was achieved through an iterative process of translating concepts from one study to another by adding coded text to existing concepts and creating new concepts when deemed necessary. The preliminary coding framework was discussed with a third author (SHR). Descriptive themes were then inductively analysed further to construct analytical themes, to 'go beyond' the findings reported in our included studies and generate additional understanding relating to our research question.[21] Both review authors then reread each included article to ensure themes were represented in the primary data, and illustrative verbatim quotations were incorporated. The proposed descriptive and analytical themes were subsequently presented, discussed and finalised with the entire review team, including two patient research partners (CZ and MK).

Assessment of confidence in the review findings (Grading of Recommendations Assessment, Development and Evaluation–Confidence in Evidence from Reviews of Qualitative Research (GRADE-CERQual))

Two review authors (HJS and LSC) independently assessed the confidence in each individual review finding using the GRADE-CERQual approach.[22] Four components were considered to formulate an overall assessment of confidence in each synthesised qualitative finding: methodological limitations (using CASP), coherence of data, adequacy of data and relevance of the studies.[23–27] Both review authors then independently judged overall confidence in each review finding as high, moderate, low or very low. Full definitions of each GRADE-CERQual component and confidence ratings are presented in online supplemental file 2. Disagreements in confidence ratings were resolved via discussion or through inclusion of a third author (SHR).

Patient and public involvement

International patient research partners (CZ and MK) were engaged throughout each stage, including during the development of the review question and interpretation of the results,

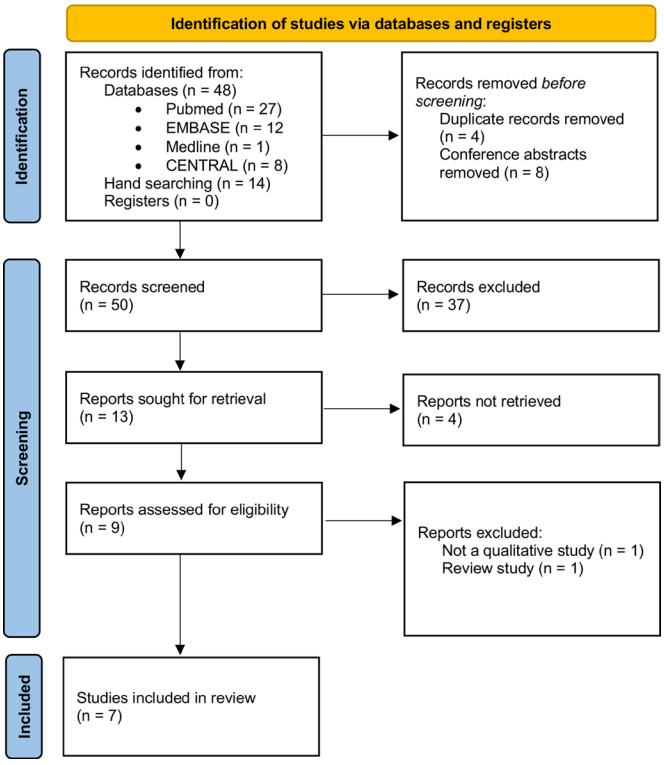

Figure 1 PRISMA flow diagram (adapted from Page *et al*).[44] PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses.

through attendance at study meetings and contributions to ongoing discussions about the findings. Our patient research partners reviewed each manuscript draft; their feedback resulted in changes to the presentation of source data and thematic schema, and to the overall structure of the results.

RESULTS

Study selection

In total, the searches yielded 62 records, of which 9 were retrieved for full-text screening. Seven studies representing six data sets met our inclusion criteria. The full selection process is presented in a Preferred Reporting Items for Systematic Reviews and Meta-Analyses 2020 flow diagram (figure 1). Studies were conducted in the UK, Austria, Germany, Switzerland, the Netherlands and Canada. The sample includes 115 individuals at-risk of RA. Table 1 provides an overview of the study characteristics and participant demographics. Four studies[28–31] used individual semistructured interviews, whereas three studies[32–34] used focus groups.

Quality appraisal

The frequency of responses ('yes' or 'no') to each signalling question in the CASP checklist is detailed in online supplemental file 3. Strengths observed in all studies included clearly stated objectives, appropriate methodology and design, clearly stated and justified data collection methods, and confirmation of ethical approval. The following limitations were identified in at least five studies: no discussions around recruitment (eg, why some people chose not to take part)[29–32 34]; no justification for data collection setting[28 29 32–34]; no discussion of the issues raised by the study[28 29 32–34]; no critical examination of the researchers' own role, potential bias and influence during the formulation of the research question and data collection[28–34]; and no critical examination of the researcher's own role, potential bias and influence during analysis and selection of data for presentation.[28–32 34]

Synthesis of qualitative studies

Our thematic synthesis identified seven descriptive themes describing the perceptions and experiences of study participants. These were organised into three major analytical themes: understanding the risk of developing RA (theme 1), preventive interventions to reduce the risk of developing RA (theme 2) and perceptions of predictive testing for RA (theme 3). Illustrative quotes for each major theme are presented in tables 2–4, and conceptual links among themes are displayed in figure 2.

Theme 1: understanding the risk of developing RA

Within theme 1, two descriptive subthemes were identified relating to understanding the risk of developing RA: knowledge of RA and identification of potential risk factors.

Knowledge of RA

Many participants had witnessed the severity and impact of RA on their relatives.[29–34] Some participants expressed concerns about developing RA, perceiving that it would be painful and unpredictable, restricting daily living.[31–33] Participants with symptoms revealed experiences of unpredictable pain and fatigue, with negative consequences such as reduced ability to participate in hobbies and social outings, and perceived that these issues would worsen if they developed RA.[33]

Some participants recognised they lacked knowledge about RA and their risk as a relative.[31 32 34] Participants felt they needed more information about RA and its related risk factors, particularly to inform their decision making around preventive interventions and undergoing predictive testing.[28 30–32 34]

Identification of potential risk factors

In four studies, participants directly or indirectly identified known risk factors for developing RA, including diet, being overweight, smoking, family history and being female.[30–33] Other participants identified what they considered to be risk factors, such as sports participation and ageing, which have not been identified as predictors of developing RA in empirical research.

Theme 2: preventive interventions to reduce the risk of developing RA

Within theme 2, two descriptive themes related to preventive interventions to reduce the risk of developing RA: understanding the value and role of preventive interventions, and engagement with preventive interventions.

Understanding the value and role of preventive interventions

Five studies discussed the role of preventive interventions in reducing the risk of developing RA.[28–30 32 34] Preventive interventions identified by participants included medications, lifestyle changes, screening and alternative medicines (eg, herbal treatments). Some participants suggested that preventive medication would be similar to that received by their relatives with RA,[30 32] while others thought preventive medication would be 'less strong'.[29] Specific lifestyle changes considered by participants included healthy eating, increased exercise and smoking cessation.[28 30 34] While most studies focused on the role of treatments in preventing the development of RA, participants in one study acknowledged that preventive interventions might at best delay the onset of RA rather than stop it altogether.[29]

Table 1 Study characteristics

Study ID	At-risk population	Sample size	Age	Female	Ethnicity	Setting (country)	Data collection method	Recruitment technique	Patient involvement	Analysis
Mosor *et al* 2020[28]	24 ACPA/RF positive with arthralgia (9 FH RA); 10 asymptomatic ACPA/RF positive (1 FH RA)	34	Mean (SD): symptomatic 48.6 (14.4), asymptomatic 61.7 (9.6), total 52.4 (14.4); range 18–81	26	Not reported	Rheumatology centres (15 Austria, 15 Germany, 4 UK)	Semistructured interviews	Individuals were either referred for testing because of symptoms or had a predictive test for RA as part of an extended medical check-up.	English interview guide co-developed with patient research partners; results reviewed by patient research partners.	Thematic
Munro *et al* 2018[32]	FDRs	5	Mean (SD) 29.4 (12.4)	Not reported	Not reported	Arthritis Consumer Experts/ Joint Health Group and Arthritis Research Canada Arthritis Patient Advisory Board (Canada)	Focus group	Marketing and communications lists of the Arthritis Consumer Experts/Joint Health group and Arthritis Research Canada Arthritis Patient Advisory Board mailing lists, or snowballing sampling through the patient participants	Semistructured interview guide informed by consultation with a patient partner	Framework
Newsum *et al* 2016[33]	4 with CSA (1 ACPA and RF positive; 1 FH RA; 2 seronegative)	4	Mean 37.5, range 24–54	4	Not reported	Secondary care (Netherlands)	Focus group	Randomly selected individuals with arthralgia of <1 year of hand or foot joints without clinical arthritis at physical examination and an increased risk of developing clinical arthritis according to the rheumatologists were approached by telephone and asked to participate in the focus group discussion.	Not reported	IPA
Novotny *et al* 2013[29]	FDRs; 4 siblings, 14 parents; 2 parents/grandparents	20	Mean (SD) 45 (12), range 21–78	18	Not reported	Hospital rheumatology department (Switzerland)	Semistructured interviews	Announcements in the press to invite FDRs of patients with RA to participate in a cohort study; to encourage participation, the main biomarkers predicting RA were assayed free of charge.	Not reported	Thematic
Simons *et al** 2018[30]	FDRs; 6 siblings, 26 parents, 2 siblings/parents	34	Mean (SD) 39 (10.8); Range 21–67	26	32 White, 2 Asian	Secondary care (24 UK, 3 Germany, 7 Austria)	Semi-structured interviews	Patients with RA were approached during their routine secondary care clinic appointments and asked to consider contacting a FDR about participating in an interview study about risk and predictive testing for RA.	Interview schedule was informed by consultation with patient research partners; patient research partners blind coded three transcripts; coding framework discussed with patient research partners.	Thematic
Stack *et al* 2016* [31]	FDRs; 6 siblings, 26 parents, 2 siblings/parents	34	Mean 39, range 21–67	26	32 white, 2 Asian	Secondary care (24 UK, 3 Germany, 7 Austria)	Semistructured interviews	Patients with RA were approached during their routine secondary care clinic appointments and were given a letter to pass on to an FDR of their choosing inviting them to participate in an interview about risk and predictive testing for RA.	Patient research partners reviewed and redrafted the interview schedule and blind coded three transcripts; discussion of the coding framework took place between researchers and patient research partners.	Thematic
van Boheemen *et al* 2021[34]	ACPA or ACPA and RF positive with arthralgia and no history of clinical arthritis	18	Mean (SD) 59 (9)	10	Not reported	Not reported	Focus group	Participants of a prevention trial or individuals who declined trial participation but consented to be contacted	Not reported	Thematic

*Same data set.
ACPA, anticitrullinated protein antibody; CCP, cyclic citrullinated peptide; CSA, clinically suspect arthralgia; FDR, first-degree relative; FH, family history; IPA, interpretive phenomenological analysis; RA, rheumatoid arthritis; RF, rheumatoid factor.

Table 2 Theme 1 illustrative quotes

Theme 1: understanding the risk of developing RA	
Descriptive theme	**Illustrative quotes**
Knowledge of RA	
Individuals at-risk of RA have gained knowledge of RA through experiencing symptoms or witnessed the impact of RA on their relatives.	'However, I do notice that I want to avoid certain situations. For instance, sometimes I put off visitors because I know they won't understand I am in pain. Or because they don't take into account that I have to stand up on my feet quite often. Then I prefer to say 'Well, not today, thank you,' instead of joining them for an outing'.[33] 'She [family member] had a life and then once the disease came and took it from her, she didn't [anything] anymore. She couldn't do things'.[32]
Individuals at-risk of RA identified a need for more knowledge about RA and risk factors.	'Up until now I have never thought about it, what that would be like, whether it might happen'.[31] 'And I've heard theories, everything from it [RA] skips generations to it's immediate, to you know it only affects the women in one side of the family. I've heard a whole bunch of different crazy different things'.[32]
Identification of potential risk factors	
Individuals at-risk of RA perceived that certain factors increase the risk of developing RA.	'Yeah, I looked it [information about RA] up online, and yes, then you see how bad it can get, and I think, well, I'm not that far along yet'.[34] 'So I know it's blood-related…I think if it was your cousin or your aunt there'd be a slim chance…being direct blood-related, I would class myself as, or think of myself that I am at a higher risk than most'.[31] 'I think it probably half depend on what kind of person you are, I know for my sister she was much more worried than I was only because she's a lot older than me and she's overweight and she saw that as kind of, like without reading the letters I could figure she was going to get it more than me'.[30]

RA, rheumatoid arthritis.

Engagement with preventive interventions

Some participants expressed willingness to engage with preventive interventions, including through participation in research.[29 30] However, most participants indicated that their perceived engagement with preventive interventions would depend on a balance of certain factors; primarily the effectiveness of preventive interventions in reducing risk, their experience of symptoms, seeing the impact of RA on a relative, adverse effects of preventive medication and information provided by health professionals.

Some participants expressed that the effectiveness of an intervention in reducing their risk of developing RA would affect their decision to engage.[30 32 34] Participants confirmed that the presence of RA symptoms would make them more likely to engage with preventive interventions.[28–30 34] Understanding of the impact of RA also affected participants' perceived or actual engagement with preventive interventions, with some expressing a willingness to consider medication or participate in a clinical trial involving medication, to prevent symptoms of the disease that they had witnessed in relatives.[29 30 34]

Many participants had concerns about preventive medication specifically.[28–30 34] In some cases, these concerns were based on negative attitudes towards taking medications in general. Other participants had concerns about the side effects of preventive medication, particularly if they currently felt healthy.[29 30 32 34] These included physical side effects, such as infections and liver

Table 3 Theme 2 illustrative quotes

Theme 2: preventive interventions to reduce the risk of developing RA	
Descriptive theme	**Illustrative quotes**
Understanding the role and value of preventive interventions	
Individuals at-risk of RA acknowledged that preventive interventions have a role in modifying risk.	'I think drugs would be involved, drugs that are less strong than those used to treat the disease'.[29] 'ifestyle changes, I'm up for any kind really, yeah. Healthy eating and exercise, although I can't do a lot but I do try and do as much as I can'.[30]
Engagement with preventive interventions	
Individuals at-risk of RA identified that engagement with a preventive intervention would be influenced by its effectiveness in reducing risk.	'I've got to take a medication for how long, the rest of my life? … It's a big commitment when the odds of developing the disease is still fairly high if I've got a 50% risk of still developing it, whereas if you tell me, 'Well, actually, if you take it and based on what we can tell you about your predictability factors, your odds of developing the disease are gonna be down to 5%,' then I might consider it'.[30]
Having symptoms would make individuals at-risk of RA more willing to consider preventive interventions.	'Well, changing lifestyle means changing diet, difficult, because changing your diet, abstaining from certain food that you like to eat, means reducing your quality of life. I personally don't agree with that, I'm definitely not going on a diet because of a disease I don't have at the moment! But I certainly would if I had any symptoms'.[28] 'The chance that I would do it would increase hand over hand if I had severe pain'.[34]
Seeing the impact of RA on a relative would make individuals at-risk of RA more willing to consider preventive interventions.	'RA is in my family unfortunately. My mother, my grandmother, they're both gone (…). And the fact that I participate in the medication trial is just like, yes, I've seen what RA can do'.[34]
Individuals at-risk of RA had concerns about taking preventive medication.	'I prefer a drug that doesn't affect the immune system(…)drugs can make us more vulnerable to infections'.[29] 'You know, I went to Europe last year with my wife. We were gone for, you know, half a year. Now if I wasn't able to do that because I had to go to a specific doctor twice a week to get this thing, no thanks. I'm good'.[32]
Individuals at-risk of RA highlighted a need for more information about their actual risk and preventive interventions before engaging.	'Only under the condition that a person would receive the necessary information to be able to decide whether to take a preventive medicine'.[28] 'From where it would be coming from, Dr.— was like, 'Hey, you know, there's this treatment. You know, I know how badly it effects your mother. I think that you are possibly at-risk for having it,' and he suggested it to me, I would definitely take a look at it'.[32]

RA, rheumatoid arthritis.

Table 4 Theme 3 illustrative quotes

Theme 3: perceptions of predictive testing for RA	
Descriptive theme	**Illustrative quotes**
Benefits of predictive testing	
Individuals at-risk of RA perceived predictive testing as useful.	'I think that with kind of information, I'd be more keen to, sort of, sort out what I needed to do to try and prevent that becoming a problem. If I could take some sort of medication to…head it off before it became a big problem'.[31] Yes, I have pain in the joints regularly and that's why it was interesting to me to find out the results. I think it was just confirmation that my feeling wasn't just made up of thin air'.[28]
Decision to undergo predictive testing	
Presence of symptoms, perceived effectiveness and understanding of the impact of disease affect individuals' decision to undergo predictive testing	'If there were perhaps a treatment that were extremely preventive and very effective at lessening the risk of developing such a disease, I absolutely would take the test because that to me leads to something that is preventive. That leaves me being able to take some action'.[31] 'It's like looking into a crystal ball [of a fortune teller] and saying to you, "Oh, you could potentially get rheumatoid arthritis." And then, always, I have images of people in my mind who have deformities and disabilities'.[28]
Concerns about predictive testing	
Individuals at-risk of RA had concerns about predictive testing.	'Because if told me—it's only how likely, it's not a, 'You will develop it,' and it doesn't tell you when you will develop it. So I think if somebody said to me, 'There's this test out there and it'll tell you whether you might develop it,' I wouldn't want it, because you could just live your life in fear and never actually develop it. So unless it was 100% guaranteed, and somebody could say, 'You will develop it within this time frame,' I don't wanna [want to] spend the next 30 years worrying about something, when I could be enjoying those 30 years. So, no, I'd probably—it depends on the exact details of the test'.[31] 'Statistics like 1 out of 10 really don't mean a thing to me. The way I reason is, I am not 1 out of 10. That's how I feel about it, it won't be me'.[33]

RA, rheumatoid arthritis.

damage, and psychological side effects. In some cases, these concerns were based on participants' experiences of seeing relatives take medication for RA. Two studies also identified concerns relating to the administration of medication[29 32]; participants indicated a preference for tablets over injections due to the perceived impact of having regular injections on their lives, for example, limitations in travelling.

Participants identified the need to weigh up the pros and cons of engaging with a preventive intervention. This involved taking into consideration their risk of developing RA against the effectiveness and adverse effects of preventive medication or the perceived negative consequences of making lifestyle changes.[29 30 34] Participants highlighted a need for more information about their actual risk before engaging with preventive interventions. Additionally, participants recognised a need for more information about preventive medication, including adverse effects and mode of administration, to inform their decision making.[28 30 32 34] Some participants suggested they would be more likely to take medication or participate in a clinical trial involving preventive medication, if a trusted health professional recommended it.[32 34] One study, undertaken in Canada, also acknowledged cost as a potential factor that could affect engagement with preventive medication.[32]

Some participants acknowledged they would be more willing to make lifestyle changes, undergo screening or take alternative medicines than take preventive medication.[28 30 32 34] Participants in one study had already adopted healthy behaviours in an attempt to deal with their arthralgia, including dietary changes, mindfulness and yoga,[33] while other participants acknowledged their attempts to live as healthily as possible regardless of their risk of developing RA.[30]

Theme 3: perceptions of predictive testing for developing RA

Three descriptive subthemes themes contributed to the main theme of perceptions of predictive testing for developing RA: benefits of predictive testing, decision to undergo predictive testing and concerns about predictive testing.

Benefits of predictive testing

Some participants perceived predictive testing as useful to clarify their symptoms or risk status,[28 31 33] prepare mentally and

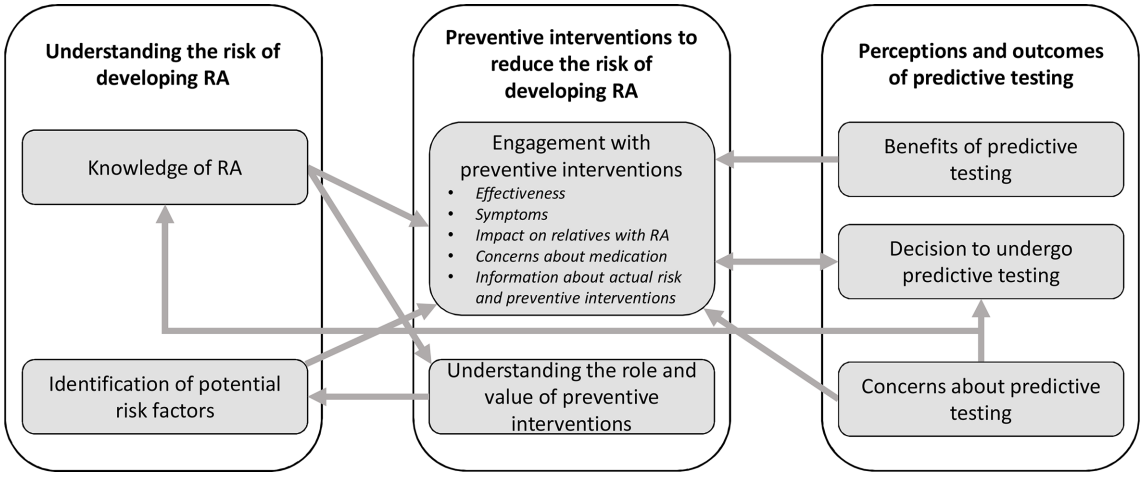

Figure 2 Thematic schema. RA, rheumatoid arthritis.

physically for the future,[31] or to contribute to research to ultimately help other people.[28 31] Other participants recognised that confirmation of their risk of developing RA would allow them to be proactive about their health, prompting them to monitor early symptoms and report changes to a health professional, make lifestyle changes or take preventive medication.[28 30–32]

Decision to undergo predictive testing

Participants' decision to undergo predictive testing was influenced by presence of symptoms. One study identified that symptomatic participants were more likely to undergo predictive testing than asymptomatic participants.[28] However, another study reported that a small number of symptomatic participants were fearful of clarification and had not sought further advice.[31] The perceived effectiveness of preventive interventions may also influence participants' decision to undergo predictive testing, prompting them to take action.[32] For some participants, the decision to undergo predictive testing would be influenced by the ability of a test to provide a definitive result and a confirmed timeline for developing RA, rather than a probability.[31] Some participants suggested they would take the opinion of a trusted health professional into consideration and might be more likely to undergo predictive testing if it was recommended.[32]

Concerns about predictive testing

Participants in two studies described feeling fearful and anxious about the outcome of predictive testing,[31 32] perceiving that confirmation of risk status would reduce their ability to enjoy life.[31] These concerns were compounded by uncertainty around whether or not the disease would actually develop.

Other participants had concerns about the accuracy of the test,[28 31 32] the impact of a false-positive result[31 32] and the potential for predictive testing to trigger further invasive tests, such as biopsies.[28] Some participants described how their relatives with RA also had concerns about predictive testing, especially as both parties may not have considered participants' susceptibility of developing the condition prior to taking part in research.[31]

Two studies discussed communication of test results.[28 31] Some participants had concerns about how their test results would be communicated, based on their previous negative experiences of receiving other test results, and suggested that information should be understandable without the use of medical terms and with accompanying examples to enhance comprehension.[28] Another study revealed that participants felt they were unable to interpret prognostic information in terms of probabilities of their symptoms progressing to RA.[33] Participants recognised a need for support from health professionals throughout the predictive testing process during both the delivery of test results and in the at-risk stage.[28 31]

Participants in two studies had already undergone predictive testing.[28 31] Reactions to the test result varied; most asymptomatic participants reported feeling calm, while symptomatic participants described feeling anxious, shocked, worried about the future (with regards to the potential impact on working, for example), and had difficulties in discussing the outcome with others.[28]

Assessment of confidence in the review findings (GRADE-CERQual)

We had moderate confidence in most of the review findings (table 5; a detailed GRADE-CERQual Qualitative Evidence Profile is also presented in the online supplemental file 1). This was due primarily to concerns regarding methodological limitations, adequacy of the data (due to the limited number of studies meeting our inclusion criteria) and relevance of each contributing study to the review question (given the absence of studies of participants with early clinical arthritis, one study failing to report its setting and all of the remaining studies from high-income countries, with none having recruited from primary care). We had low confidence in two findings: engagement with a preventive intervention would be influenced by its effectiveness in reducing risk, and the presence of symptoms, perceived effectiveness and understanding of the impact of disease affect individuals' decisions to undergo predictive testing.

DISCUSSION

This review informs our understanding of the factors that may influence the willingness of individuals at-risk of RA to undertake

Table 5 GRADE-CERQual summary of review findings

Summary of review finding	Studies contributing to the finding	GRADE-CERQual assessment of confidence in the evidence
Individuals at-risk of RA have gained knowledge of RA through experiencing symptoms or witnessing the impact of RA on their relatives	29–34	Moderate confidence
Individuals at-risk of RA identified a need for more knowledge about RA and risk factors.	30–32 34	High confidence
Individuals at-risk of RA perceived that certain factors increase the risk of developing RA.	29–32	Moderate confidence
Individuals at-risk of RA acknowledged that preventive interventions have a role in modifying risk.	28–30 32 34	Moderate confidence
Individuals at-risk of RA identified that engagement with a preventive intervention would be influenced by its effectiveness in reducing risk.	30 32 34	Low confidence
Having symptoms would make individuals at-risk of RA more willing to consider preventive interventions.	28–30 34	Moderate confidence
Seeing the impact of RA on a relative would make individuals at-risk of RA more willing to consider preventive interventions.	29 30 34	Moderate confidence
Individuals at-risk of RA had concerns about taking preventive medication.	28–30 32 34	Moderate confidence
Individuals at-risk of RA highlighted a need for more information about their actual risk and preventive interventions before engaging.	28–30 32 34	Moderate confidence
Individuals at-risk of RA perceived predictive testing as useful.	28 30–33	Moderate confidence
Presence of symptoms, perceived effectiveness and understanding of the impact of disease affect individuals' decision to undergo predictive testing.	28 31	Low confidence
Individuals at-risk of RA had concerns about predictive testing.	28 31–33	Moderate confidence

GRADE-CERQual, Grading of Recommendations Assessment, Development and Evaluation–Confidence in Evidence from Reviews of Qualitative Research; RA, rheumatoid arthritis.

predictive testing and engage with preventive interventions. We specifically focused on the perceptions and experiences of individuals at-risk of RA, rather than those of health professionals or patients with RA.

Although individuals discussed potential risk factors for developing RA, including smoking and increased weight, in this review, they did not identify certain modifiable risk factors for developing the condition, such as the contribution of poor dental hygiene and periodontal disease.[35] The potential gap in knowledge among individuals at-risk of RA regarding poor dental health as a risk factor for developing the disease is also in concordance with the previous literature.[36] Our review indicates that understanding the risk of developing RA is underpinned by the individual's knowledge of both RA and the risk factors for developing the disease. In a study assessing knowledge of RA risk factors among asymptomatic FDRs, baseline knowledge of this risk factor was low, but increased significantly following personalised RA educational intervention.[36] The effectiveness of providing personalised risk information to FDRs to calculate disease risk[37] and increase motivation to improve RA risk-related behaviours,[38] has previously been demonstrated.

While our synthesis indicated that participants were willing to make lifestyle changes to prevent or delay the onset of RA, we identified some misconceptions relating to risk factors and subsequent lifestyle changes. For example, some participants within our review incorrectly identified ageing as a risk factor. This suggests there is confusion between RA and osteoarthritis, in concurrence with a previous qualitative exploration of illness perceptions of RA in the general public.[16] Our findings reveal that the decision to engage with preventive medication is multifactorial and links closely with knowledge of risk and the resulting disease. Our review identified that at-risk individuals would be more willing to make lifestyle changes than take preventive medication, in concordance with the previous literature.[1 14] This is in contrast to a previous survey involving rheumatologists, where the majority were unlikely to advise lifestyle changes and were more willing than at-risk individuals to start preventive medication, regardless of side effects.[14]

The personal burden of living with RA, including illness uncertainty, has been well established.[39] Our review indicated that individuals at-risk of RA may have concerns about predictive testing and finding out their risk status because of this perceived burden. Illness uncertainty has been identified as a cognitive stressor that impacts on treatment adherence.[40] Patients with RA have expressed uncertainty about symptoms and prognosis, treatment effectiveness and toxicity, and potential consequences of the disease on their lives.[41] Our findings suggest that individuals at-risk of RA have similar experiences but must also manage the additional uncertainty of future disease progression. A common finding across all themes was individuals' need for further information, which is accurate and personalised to the individual at-risk of RA, to inform decision making around preventive interventions and predictive testing. However, our review has established that while information provided by health professionals can be influential, many individuals at-risk of RA also draw on their experiences of relatives living with RA to inform their decision making.

Perceptions of predictive testing among individuals at-risk of RA identified in our review are similar to those identified in a previous meta-synthesis of qualitative studies involving participants with other chronic inflammatory diseases (diabetes, cardiovascular disease and inflammatory bowel disease).[15] This meta-synthesis identified the benefits of predictive testing to motivate lifestyle changes, but also revealed the potential negative emotional impact of testing. In congruence with our synthesis, previous surveys conducted with individuals at-risk of RA[14 42] and spondyloarthropathy[43] highlighted participants' concerns about preventive medication, particularly with regard to side effects. For example, in one such study, willingness to take preventive medication decreased by approximately half with the possibility of mild side effects.[43] Synonymous with our review findings, previous studies have also revealed that the decision to engage with preventive interventions depends on the effectiveness of these treatments,[14 42] the opinions of trusted healthcare professionals,[42] individuals' perceptions of how severe the disease is[43] and when their risk of developing the disease is increased.[14 43] However, in contrast to our findings, one previous study found that mode of drug administration did not influence at-risk individuals' decisions to take preventive treatment.[43] Further understanding of how the delivery of preventive medication affects people's perceptions and decision making is required.

To our knowledge, this is the first study that synthesises existing qualitative literature on the perceptions and experiences of individuals at-risk of developing RA. We systematically assessed and coded all relevant data using established and prespecified methodology. At least two review authors were involved in study selection, data extraction, CASP assessments and coding of data, reducing the potential for errors, and we formally assessed our confidence in each review finding using the GRADE-CERQual approach. Our review findings should be considered in light of some limitations. First, only seven studies met our inclusion criteria, and two of these studies were from the same data set. We carried out an extensive search of the literature to ensure no relevant articles were missed. The small number of included studies reflects the lack of qualitative studies undertaken in this evolving area of RA research, highlighting the need for further studies in this area. Although our review included 115 participants from six countries, we recognise that some of the original studies were conducted in their native language and then translated into English for publication. This has the potential for cultural meanings to be modified in our thematic synthesis and theme development. Second, overall confidence in each finding was hindered somewhat by methodological limitations of the studies, particularly lack of reporting around non-participation characteristics and rates. It is possible that individuals who were more engaged with the idea of predictive testing and preventive interventions were willing to participate. Future research in this area should aim to minimise the impact of this limitation, for example, by asking individuals who decline participation to detail their reasons for this decision. Third, the participants included within our review were either individuals with musculoskeletal symptoms but without clinical arthritis or FDRs of individuals with RA recruited through secondary care; therefore, our findings provide limited insights into the perceptions and experiences of other individuals at-risk of developing the condition, such as other asymptomatic at-risk individuals (eg, indigenous North Americans who are at increased genetic risk) and at-risk individuals with early clinical arthritis, including patients with palindromic rheumatism and undifferentiated arthritis.[18] We acknowledge that the themes we have identified might potentially differ between the different groups of individuals at-risk included within this review (eg, in terms of their knowledge of RA), as well as groups of at-risk individuals not represented in study samples. Additionally, while our search strategy included the term 'inflammatory arthritis', no qualitative studies relating to patients at-risk of inflammatory arthritides other than RA were found. Therefore, our review specifically focused on

RA and our findings may not be transferable to other forms of inflammatory arthritis, although surveys have demonstrated similar perspectives of risk and preventive interventions among individuals at-risk of spondyloarthropathy.[14 43] Finally, only two studies explicitly reported the ethnic background of participants, and all studies were conducted in high-income countries; therefore, findings may not be transferable to individuals from ethnic minority groups or to different healthcare settings. Other factors, such as gender, cultural background, socioeconomic status and health literacy levels, may also influence the decision to undertake predictive testing and engage with preventive interventions. Future research should focus on these gaps.

Several implications arise from our review. Our findings suggest that while there are benefits in informing individuals at-risk of developing RA about their risk and offering preventive treatment, there are potential barriers to engagement among these individuals. We propose that individuals' knowledge about their risk of developing RA may inform their decision to engage with preventive interventions, including medication and lifestyle changes. We recommend that individuals be informed about their risk of developing RA using a personalised approach, ensuring they understand risk factors, their personal risk and how to reduce this risk, and addressing any misconceptions. A previous randomised controlled trial has demonstrated that personalised educational tools can support communication of risk in this population,[37] but prognostic information based on risk percentages may not be considered as useful to individuals at-risk of RA.[33] We propose that communication should be tailored to the individual, with accessible, patient-understandable information on the impact of preventive interventions provided. Participants' educational status and literacy levels should also be considered, as these may affect their decisions and needs. Information should include the nature and likelihood of immediate and long-term physical and psychological side effects, medication administration and the anticipated effectiveness of the intervention. Fundamental to this tailored communication is wider exploration of the concerns individuals may have based on their own experiences. Further support from trusted health professionals should be available for at-risk individuals, particularly taking into consideration the potential negative emotional impact of testing and the additional burden of uncertainty that testing may produce. This will become increasingly important in clinical practice as the focus of rheumatology care shifts to prevention of disease in individuals at-risk of RA as opposed to intervention in early RA. Future studies should establish the optimum approaches for conveying the risk of developing RA to at-risk individuals, and determine how at-risk individuals assess risk versus benefit when deciding whether to engage with preventive interventions. Our recommendations primarily aim to inform the conduct of future clinical trials and observational studies, but are also applicable to broader clinical practice.

Author affiliations
[1]Leeds Institute of Rheumatic and Musculoskeletal Medicine, University of Leeds, Leeds, UK
[2]NIHR Leeds Biomedical Research Centre, Leeds, UK
[3]Romanian League against Rheumatism, Bucharest, Romania
[4]Federation of Patient Associations of Cyprus, Nikosia, Cyprus
[5]Rheumatology Research Group, Institute of Inflammation and Ageing, College of Medical and Dental Sciences, University of Birmingham, Birmingham, UK
[6]Department of Rheumatology, Sandwell and West Birmingham NHS Trust, Birmingham, UK
[7]Library and Information Service, Leeds Teaching Hospitals NHS Trust, Leeds, UK
[8]Department of Rheumatology, Medical University of Vienna, Vienna, Austria
[9]Leeds Institute of Health Sciences, University of Leeds, Leeds, UK

Twitter Heidi J Siddle @HeidiSiddle

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ORCID iDs
Heidi J Siddle http://orcid.org/0000-0002-6015-332X
Lara S Chapman http://orcid.org/0000-0002-4207-2809
Kulveer Mankia http://orcid.org/0000-0002-7945-6582
Karim Raza http://orcid.org/0000-0003-1570-1232
Marie Falahee http://orcid.org/0000-0001-5928-486X
Andreas Kerschbaumer http://orcid.org/0000-0002-6685-8873
Daniel Aletaha http://orcid.org/0000-0003-2108-0030
Paul Emery http://orcid.org/0000-0002-7429-8482
Suzanne H Richards http://orcid.org/0000-0003-1416-0569

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CLINICAL SCIENCE

Joint inflammation tends to recur in the same joints during the rheumatoid arthritis disease course

Sascha L Heckert ,[1] Sytske Anne Bergstra ,[1] Xanthe M E Matthijssen ,[1] Yvonne P M Goekoop-Ruiterman,[2] Faouzia Fodili,[3] Saskia ten Wolde,[4] Cornelia F Allaart,[1] Tom W J Huizinga [1]

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For numbered affiliations see end of article.

Correspondence to
Sascha L Heckert, Rheumatology, Leiden University Medical Center, Leiden, Netherlands; s.l.heckert@lumc.nl

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ABSTRACT

Objectives We investigated whether local joint swelling recurs in the same joints over time in patients with rheumatoid arthritis (RA) who are treated to target.

Methods Patients with newly diagnosed RA participating in the Behandel-Strategieën, "treatment strategies" (BeSt) study (n=508) were followed for median 10 years while receiving Disease Activity Score (DAS) ≤2.4 steered treatment. Every 3 months 68 joints were assessed for the presence of swelling. We evaluated whether baseline local joint swelling was predictive for swelling in the same joint during follow-up using a multilevel mixed-effect logistic regression model. Different strategies were used to account for missing data. A permutation test was performed to assess if joint swelling was better predicted by baseline swelling of the joint itself than by baseline swelling of randomly selected other joints.

Results In 46% of the joints that were swollen at baseline, joint swelling later recurred at least once during follow-up. Joint swelling at baseline was statistically significantly associated with swelling in the same joint during follow-up (OR 2.37, 95% CI 2.30 to 2.43, p<0.001), and also specifically with recurrent swelling in the same joint (OR 1.73, 95% CI 1.37 to 1.59, p<0.001). Local joint swelling was better predicted by baseline swelling of that particular joint than by baseline swelling of other joints (p<0.001).

Conclusion Joint swelling tends to recur locally in the joints swollen at RA onset. This suggests that local factors influence the manifestation of joint inflammation over time.

Key messages

What is already known about this subject?

- Several local factors have been assumed to play a role in joint inflammation in rheumatoid arthritis (RA), but it is not known whether patterns of clinical joint inflammation during the disease course show a predilection for local recurrence.

What does this study add?

- This first study in which joint involvement was longitudinally assessed at joint level shows that joint swelling in RA tends to recur in the same joints over time (OR for the association between joint swelling at baseline with swelling in the same joint during follow-up: 2.37).
- This suggests that local factors play a role in the occurrence of joint inflammation during the RA disease course.

How might this impact on clinical practice or future developments?

- More intensive monitoring over time and more intensive treatment of local joint inflammation may be needed
- Future studies in which the underlying mechanisms of local joint recurrence are investigated might yield new treatment targets for RA.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease that is primarily characterised by pain, swelling and functional limitations of synovial joints. In addition to systemic inflammatory processes, several local factors have been assumed to play a role in joint inflammation. Location-specific differentiation of fibroblasts, difference in vascularisation and innervation and local differences in exposure to mechanical stress between different types of joints are thought to make individual joints more susceptible for inflammation in RA.[1] For example, different synovial fibroblast phenotypes have been found in the small hand joints compared with other joints of patients with RA.[2] It has also been hypothesised that autoreactive B cells migrate to joints and initiate arthritis locally.[3] Results of in vitro experiments suggest that B cells can survive for months in the synovial compartment and might contribute to joint inflammation becoming chronic.[4]

Insight in joint involvement patterns might provide important clues about local underlying mechanisms of the development of inflammation in RA over time. Therefore, our aim is to investigate whether in RA, despite systemic treatment aimed at suppression of overall disease activity, joint inflammation is more likely to recur in the same joints.

METHODS

Patients

This study is a subanalysis of data from the BeSt study. The BeSt study is a multicentre randomised treat-to-target trial, starting in 2000 with a follow-up period of 10 years, in 508 patients with newly diagnosed active RA. All patients fulfilled the

American College of Rheumatology 1987 RA criteria[5] and had a symptom duration ≤2 years. Patients were randomised into four different treatment strategy arms ((1) sequential monotherapy, (2) step-up combination therapy starting with methotrexate, (3) initial combination therapy with methotrexate, sulfasalazine and prednisone or (4) initial combination therapy with methotrexate and infliximab). Treatment adjustments were based on 3 monthly study visits with a treatment target of DAS≤2.4. Details of the BeSt study have been described previously.[6 7]

Patient and public involvement

Patient and public were not involved in design, conduct, reporting or dissemination of the research, initiation of the BeSt study being at a time when this was not common practice.

Joint assessment

At each study visit, 66/68 joint counts were performed, with additional evaluation of the metatarsal joints, totalling 68 individual joints assessed for swelling. For the current analysis, we only used swollen joint assessments as representation of joint inflammation. Joints were assessed by trained nurses who were blinded for treatment.

A joint swelling episode was defined as a period of one or multiple subsequent study visits at which a joint was persistently swollen. A joint swelling episode starts with swelling present at baseline, or joint swelling following a study visit without swelling in that joint. Missing assessments were regarded as absence of joint swelling. Joint swelling was considered recurrent if, after local absence of swelling, a second (or third, etc) joint swelling episode occurred in the same joint. Persistent joint swelling was defined as local joint swelling at two or more consecutive study visits, either from baseline or from a later timepoint. For the statistical analyses, baseline joint swelling was used as a reference point for assessment of joint swelling during follow-up.

Statistical analyses

Baseline characteristics were described for all study participants and baseline joint swelling was described at joint level. To study the association between local joint swelling at baseline and later swelling of the same joint, we used a multilevel mixed-effect logistic regression model. The model was adjusted for joint location and for time point (study visit) during follow-up, with joints clustered within patients to take into account a possible correlation between multiple joints of the same patient.

A permutation test[8] was performed for this model to evaluate whether joint swelling during follow-up was specifically predicted by baseline swelling of that particular joint, rather than by baseline swelling in other joints as a representation of general disease activity. Within patient and visit strata, 1000 random permutations were performed, that is, the model was repeated 1000 times with random shuffling of joint swelling scores between joints. In this analysis, a p value<0.05 indicates that joint swelling is better predicted by baseline swelling of that specific joint than by baseline swelling of randomly selected other joints. A 95% CI for the p value is provided to address the uncertainty of this estimated p value since only a selection (that is, 1000 permutations) of all possible permutations is tested.[9]

To investigate whether treatment affected the association between baseline and later joint swelling, the analysis was subsequently stratified for treatment arm.

In a separate model, we added an interaction term between each joint and its baseline swelling status to determine whether an observed association between baseline swelling and later local swelling was similar for all individual joints. Metacarpophalangeal (MCP)-2 (right side) was chosen as the reference joint, since it was the joint that was most often affected in the study population.

Since joint swelling during follow-up can either be recurrent (the joint was not swollen at the visit prior to the visit of interest, that is, the start of a new episode) or persistent (the joint was swollen at the visit prior to the visit of interest, that is, continuous swelling within an episode), we subsequently stratified the analysis for recurrent and persistent swelling to assess whether baseline joint swelling was also predictive for recurrent swelling specifically.

The association between baseline joint swelling and the number of joint swelling episodes during follow-up was evaluated using a multilevel Poisson regression model. The model was adjusted for joint location and follow-up duration, with joints clustered within patients to take into account a possible correlation between multiple joints of the same patient. For this model, another permutation test with 1000 permutations within patient strata was performed, to assess whether the number of joint swelling episodes was predicted by baseline swelling of that particular joint specifically.

In addition, we assessed the effect of the duration of baseline swelling on swelling during follow-up. For this, we used a multilevel mixed-effect logistic regression model as described before, with the number of subsequent visits at which the joint was swollen from baseline as a predictor.

A sensitivity analysis was done for the 25% joints that were most often scored as swollen (MCP joints 1–3, proximal interphalangeal (PIP) joints 2 and 3, the wrists and metatarsophalangeal (MTP) joints 2–4), to rule out a strong contribution of joints that were rarely inflamed to the total observed effect. To assess whether an association between baseline joint swelling and joint swelling during follow-up was more likely to be a result of previous swelling in the same joint rather than a higher susceptibility of joint swelling in general, we performed a permutation test on this model (with only the most susceptible joints included) as well.

Another sensitivity analysis was performed excluding early dropouts (no information available on individual joints after the first 2 years of the BeSt study), since dropout might have been related to the number of times the patients experienced inflammation in the same joints.

All models were repeated to account for missing data in two ways. First, all missing joint evaluations until end of follow-up were regarded as not swollen. Second, last observation was carried forward for one missing time point if the joint evaluation (swelling yes/no) at the time point before a missing evaluation was the same as at a subsequent time point after the missing evaluation.

All analyses were performed in Stata SE16 (StataCorp).

RESULTS

The 508 patients had a median (IQR) follow-up duration of 40 (24–40) study visits, that is, 10 (6–10) years. At baseline, the mean (SD) age was 54 (14) years, median (IQR) symptom duration was 23 (14–53) weeks. Mean Disease Activity Score (DAS) (SD) was 4.42 (0.86) and the mean (SD) number of swollen joints was 16 (8) joints.

At baseline, 8137/34 423 (24%) assessed joints were scored as swollen. Joint swelling was subsequently persistent in 30% of the joints that were swollen at baseline with a median (IQR) duration of 1 (1–2) visit (±3 months after baseline). In addition,

Table 1 Joint swelling at baseline versus joint swelling during follow-up (persistent joint swelling from baseline disregarded) in all joints assessed at baseline

	Joints with no joint swelling at baseline (n=26 286)	Joints with joint swelling at baseline (n=8137)
No joint swelling during follow-up	21 189 (81%)	4420 (54%)
Joint swelling during follow-up (at least once)	5097 (19%)	3717 (46%)

in 46% of the joints that were swollen at baseline, local swelling recurred at least once during follow-up (table 1; figure 1).

The mixed model analysis showed that baseline swelling was predictive for swelling during follow-up with an OR of 2.37 (95% CI 2.30 to 2.43, p<0.001) for swelling during follow-up if the joint was swollen at baseline. These results were comparable between the treatment arms (OR 2.13, 2.56, 2.25 and 2.52 for treatment arms 1–4, respectively).

The statistically significant result of the permutation test (p<0.001, 95% CI 0 to 0.004) indicated that joint swelling is better predicted by baseline swelling of that same joint than by baseline swelling of randomly selected other joints. The association between baseline joint swelling and joint swelling during follow-up was variable between joints (ORs and p values for the interaction terms varied, relative to MCP-2 right, online supplemental figure 1). The association between baseline and later joint swelling was not affected by whether a joint was weight bearing (as a proxy for mechanical stress) or by symptom duration at baseline (online supplemental table 1).

Baseline joint swelling was not only predictive for joint swelling during follow-up in general, but also for recurrent joint swelling in particular, as was shown by the stratified analysis for recurrent and persistent joint swelling (OR in joints that were swollen at the previous visit: 1.52, 95% CI 1.42 to 1.61, p<0.001, OR in joints that were not swollen at the previous visit (recurrent swelling): 1.73, 95% CI 1.67 to 1.80, p<0.001). Moreover, the number of joint swelling episodes was predicted by the presence of baseline joint swelling in that joint (incidence rate ratio 1.48, 95% CI 1.37 to 1.59, p<0.001). A permutation test showed that the number of swelling episodes in a joint was better predicted by baseline swelling status of that same joint than by baseline swelling status of randomly selected other joints (p<0.001, 95% CI 0 to 0.004).

Not only presence of baseline joint swelling, but also the duration of baseline joint swelling was statistically significantly associated with joint swelling during follow-up (OR 1.20 per 3 months, 95% CI 1.19 to 1.21, p<0.001).

A sensitivity analysis of only the most affected joints (MCP joints 1–3, PIP joints 2–3, the wrists and MTP joints 2–4) showed a similar association between baseline and later joint swelling as in the complete analysis (OR 2.11, 95% CI 2.03 to 2.19, p<0.001). The permutation test showed that also in the most susceptible joints, joint swelling was best predicted by baseline swelling of that particular joint, as opposed to baseline swelling of other joints.

The association was also similar in a sensitivity analysis in which early dropouts were excluded (OR 2.32, 95% CI 2.26 to 2.39, p<0.001). For the other models, these sensitivity analyses showed comparable results too (online supplemental table 2).

Within the available follow-up period for all patients 18% (209 247/1 137 508) of the data points was missing. When accounted for missing data, using the two different methods described before, all models showed similar results (online supplemental table 2).

DISCUSSION

In this subanalysis of the BeSt study, we evaluated if joint inflammation tends to recur locally in the same joints over time. Joint swelling was assessed in 508 patients with newly diagnosed active RA who were treated to target (DAS ≤2.4) during up till 10 years. We observed that local joint swelling during the follow-up period recurred at least once in 46% of the joints that were swollen at baseline. We found that baseline local joint swelling was predictive for both the occurrence of swelling in that same joint during follow-up and the number of recurrent swelling episodes in that joint. This effect was stronger for joints with a longer duration of swelling. Moreover, the association between baseline and later local joint swelling was stronger within the same joint than between different joints. This joint-specific association may suggest that apart from systemic effects of systemic inflammatory processes in RA, local conditions in individual joints affect the course of inflammation in these joints.

To our knowledge, this is the first study in which location-specific recurrence of joint involvement in RA was investigated. Previous studies have compared patterns of joint involvement in an RA study population at several time points, but no intraindividual comparisons were made.[10 11]

We also found that the strength of the association between baseline joint swelling and joint swelling during follow-up was different for individual joints. This difference is probably related to variable susceptibility for swelling between joints. Therefore, we performed a sensitivity analysis in the 25% joints that were most often scored as swollen. In these joints too, we observed an association between baseline swelling and later swelling in the same joint. This finding supports the idea that local factors, rather than systemic inflammation only, play a role in joint swelling.

The major strength of our study is that joint assessment was performed systematically over a long time period of 10 years. All 68 joints were assessed for swelling every 3 months in a large patient cohort (508 patients). Joint assessment was done according to the European League Against Rheumatism handbook by professionals who were trained and retrained by the same rheumatologist, and most assessors followed up the same patients for many years. This allowed us to do a longitudinal analysis within an extensive and reliable data set. Because of the treat-to-target design of the BeSt study, we were able to investigate recurrence of joint swelling in patients who are intensively treated. Furthermore, the various sensitivity analyses and permutation tests substantiate the robustness of the obtained results.

Nevertheless, there are also some limitations to our study. As swelling of joints is more strongly associated with inflammation than tenderness, for our analysis we only considered scores for joint swelling.[12 13] However, inflammation may have been present in tender joints that were not swollen.[12 13] Thus, inflammation may have been persistent where we have called swelling 'recurrent'. Joint swelling assessments were also not always available. During the 10 years follow-up of the BeSt study, patients missed study visits and we found that 18% of the data for our analyses was missing. It is possible that both loss to follow-up and missing joint assessments within the follow-up time are non-random. Nevertheless, different methods of dealing with missing data yielded comparable results. Both conservative analyses in which a joint was regarded as not swollen if the joint swelling status was missing and analyses in which presence or absence of

Joint	Percentage of patients in whom the joint was swollen at baseline	Percentage of patients in whom the joint was recurrently swollen after baseline swelling
Jaw right	2%	0%
Jaw left	1%	14%
Sternoclavicular right	11%	41%
Sternoclavicular left	6%	13%
Acromioclavicular right	5%	8%
Acromioclavicular left	4%	14%
Shoulder right	12%	25%
Shoulder left	11%	14%
Elbow right	19%	41%
Elbow left	19%	48%
Wrist right	61%	60%
Wrist left	60%	61%
MCP 1 right	46%	53%
MCP 1 left	46%	52%
MCP 2 right	64%	63%
MCP 2 left	54%	57%
MCP 3 right	45%	56%
MCP 3 left	43%	44%
MCP 4 right	20%	25%
MCP 4 left	19%	15%
MCP 5 right	29%	34%
MCP 5 left	22%	20%
IP right	33%	35%
IP left	30%	33%
PIP 2 right	63%	53%
PIP 2 left	55%	50%
PIP 3 right	67%	56%
PIP 3 left	57%	50%
PIP 4 right	33%	39%
PIP 4 left	30%	32%
PIP 5 right	33%	32%
PIP 5 left	29%	33%
DIP 2 right	10%	15%
DIP 2 left	11%	13%
DIP 3 right	9%	11%
DIP 3 left	10%	20%
DIP 4 right	4%	11%
DIP 4 left	4%	10%
DIP 5 right	5%	9%
DIP 5 left	4%	5%
Knee right	35%	58%
Knee left	27%	49%
Ankle right	32%	50%
Ankle left	31%	46%
Subtalar right	23%	39%
Subtalar left	23%	41%
Midtarsal right	7%	16%
Midtarsal left	8%	13%
MTP 1 right	21%	32%
MTP 1 left	21%	29%
MTP 2 right	42%	59%
MTP 2 left	40%	58%
MTP 3 right	45%	62%
MTP 3 left	46%	60%
MTP 4 right	32%	55%
MTP 4 left	34%	53%
MTP 5 right	12%	27%
MTP 5 left	14%	40%
Feet IP right	3%	13%
Feet IP left	5%	4%
Feet IP 2 right	2%	0%
Feet IP 2 left	4%	6%
Feet IP 3 right	2%	0%
Feet IP 3 left	2%	10%
Feet IP 4 right	2%	22%
Feet IP 4 left	2%	0%
Feet IP 5 right	1%	0%
Feet IP 5 left	1%	0%

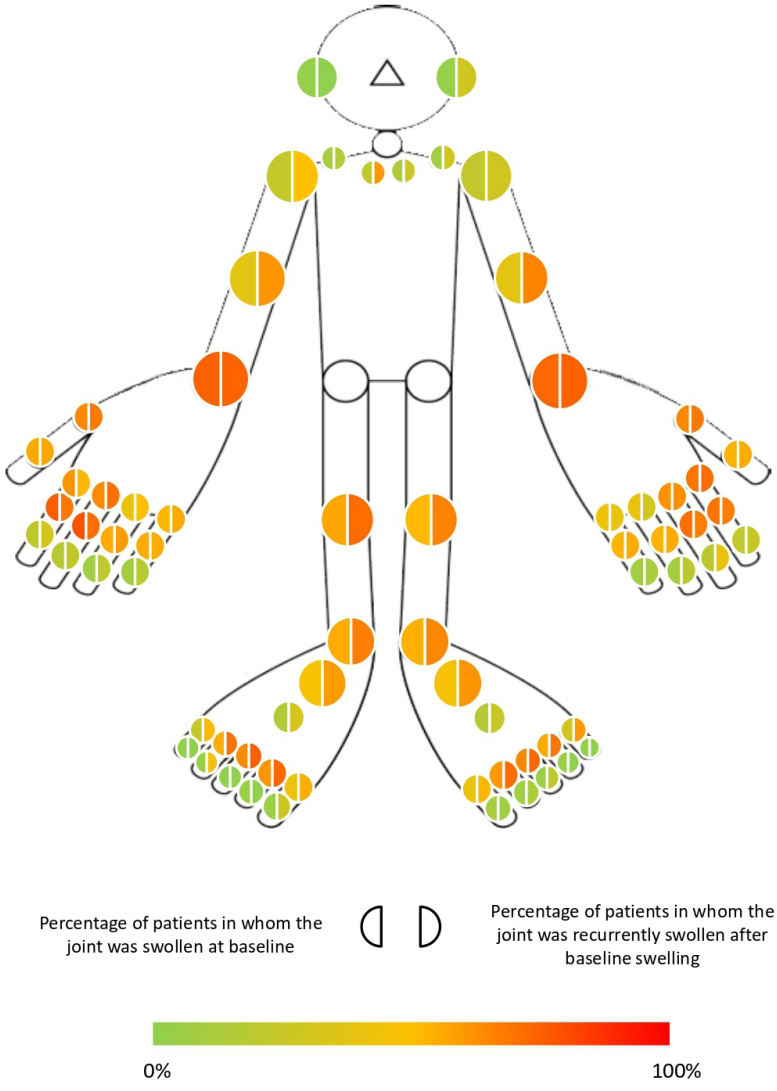

Figure 1 Joint swelling at baseline and local recurrence of swelling following baseline joint swelling, for each assessed joint.

swelling was assumed based on the previous and following study visit showed similar outcomes as the analyses based on complete data. We did not have information on joint swelling between two consecutive study visits and assumed that the swelling status of a joint did not change in between two consecutive study visits. Although this assumption might not always be true, the time between two study visits was relatively short (3 months) and it is probably as unlikely that joints were swollen in between two visits in which no swelling was observed as it is that joints are not swollen in between two visits when swelling was observed.

In addition, since radiographic data were not available for each joint included in the analysis, we cannot exclude that some local joint swelling was due to local joint damage caused by either RA or other diseases such as osteoarthritis. However, this misclassification would probably lead to joints consistently being assessed as swollen at every study visit, which would not affect the analyses of recurrent swelling. Another limitation of this study is that, since we chose to analyse only baseline swelling as a predictor for later swelling, we were not able to show an association between swelling after baseline and later swelling.

Local recurrence of joint swelling might be a result of inflammatory tissue priming as described in rodent models, in which it was shown that synovial fibroblasts of joints that were previously exposed to inflammatory triggers were sensitised, leading to a higher susceptibility to inflammation of the joint tissue.[14] More research is needed to uncover the underlying local mechanisms of recurrence of joint swelling.

The finding that joint swelling tends to recur in the same joints might support more intensive local monitoring, including imaging techniques if joints appear clinically no longer swollen. Subclinical inflammation has been found to be associated with radiographic progression.[15] Moreover, it has been shown that, despite treatment to target, the risk of radiographic joint damage progression is higher in joints with previous clinical signs of synovitis.[16 17] Therefore, local treatment, combined with systemic treatment, may be necessary to avoid local damage progression. However, in previous studies the effects of intra-articular corticosteroids on radiographic damage remain uncertain, although they can provide short-term and sometimes long-term reduction of signs and symptoms of local arthritis.[18–20] The recurrence of signs and symptoms after injections may indicate that more effective local therapies need to be investigated or developed. So far, previously investigated local therapies, potentially with the exception of surgical synovectomy for refractory symptoms, did not show convenient results.[21–26]

To conclude, this is to our knowledge the first study to investigate joint-specific recurrence of swelling in RA. We found that, even in patients who are intensively treated, joint swelling tends to recur in the same joints, suggesting that local factors play a role in the occurrence of clinical joint inflammation during the disease course. More research is needed to investigate the consequences of recurrence of joint swelling, and potentially find the mechanisms behind it. This might lead to advances in personalised monitoring and treatment of patients with RA.

Author affiliations
[1]Rheumatology, Leiden University Medical Center, Leiden, Zuid-Holland, The Netherlands
[2]Rheumatology, HagaZiekenhuis, Den Haag, Zuid-Holland, The Netherlands
[3]Rheumatology, Reumazorg Zuid West Nederland, Roosendaal, The Netherlands
[4]Rheumatology, Spaarne Hospital, Haarlem, Noord-Holland, The Netherlands

Contributors Concept and design: SLH, SAB and CFA. Acquisition of data: YPMG-R, FF, StW, CFA and TWJH. Analysis and interpretation: SLH, SAB, XMEM, CFA and TWJH. Drafting the manuscript: SLH, SAB and CFA. Revising the manuscript critically: all authors. All authors read and approved the final manuscript.

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ORCID iDs
Sascha L Heckert http://orcid.org/0000-0002-3320-905X
Sytske Anne Bergstra http://orcid.org/0000-0002-7136-5248
Xanthe M E Matthijssen http://orcid.org/0000-0001-7332-8072
Tom W J Huizinga http://orcid.org/0000-0001-7033-7520

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CLINICAL SCIENCE

Trajectory clusters of radiographic progression in patients with rheumatoid arthritis: associations with clinical variables

Alexander Platzer, Farideh Alasti, Josef S Smolen, Daniel Aletaha, Helga Radner, Stephan Blüml

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Department of Rheumatology, Medical University of Vienna, Wien, Vienna, Austria

Correspondence to
Dr Stephan Blüml, Rheumatology, Medical University of Vienna, Wien 1090, Austria;
stephan.bluemel@meduniwien.ac.at

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ABSTRACT

Objectives Identification of trajectories of radiographic damage in rheumatoid arthritis (RA) by clustering patients according to the shape of their curve of Sharp-van der Heijde scores (SHSs) over time. Developing models to predict their progression cluster from baseline characteristics.

Methods Patient-level data over a 2-year period from five large randomised controlled trials on tumour necrosis factor inhibitors in RA were used. SHSs were clustered in a shape-respecting manner to identify distinct clusters of radiographic progression. Characteristics of patients within different progression clusters were compared at baseline and over time. Logistic regression models were developed to predict trajectory of radiographic progression using information at baseline.

Results In total, 1887 patients with 7738 X-rays were used for cluster analyses. We identified four distinct clusters with characteristic shapes of radiographic progression: one with a stable SHS over the whole 2-year period (C0/lowChange; 86%); one with relentless progression (C1/rise; 5.8%); one with decreasing SHS (C2/improvement; 6.9%); one going up and down (C3/bothWays; 1.4%) of the SHS. Robustness of clusters were confirmed using different clustering methods. Regression models identified disease duration, baseline C-reactive protein (CRP) and SHS and treatment status as predictors for cluster assignment.

Conclusions We were able to identify and partly characterise four different clusters of radiographic progression over time in patients with RA, most remarkably one with relentless progression and another one with amelioration of joint damage over time, suggesting the existence of distinct patterns of joint damage accrual in RA.

Key messages

What is already known about this subject?

► Joint damage is a defining feature of rheumatoid arthritis, known to be a major driver of functional impairment, reduction of quality of life and mortality. Many factors have been shown to be associated with damage accrual, such as disease activity, serostatus or response to treatment.

What does this study add?

► The temporal evolution of structural damage in individual patients over time have not been investigated. Using a large data set of patient level data, this study identifies distinct trajectories of joint damage, remarkably one with relentless, almost linear decline of structural damage and one with amelioration of radiographic progression over time. The classification of patients into the identified trajectories is influenced by inflammatory load, disease duration and especially type of treatment.

How might this impact on clinical practice or future developments?

► Radiographic progression over time follows certain trajectories. Analysing known factors as well as other parameters typically assessed in daily routine, we cannot satisfactorily explain the risk of individual patients following certain radiographic trajectories. It is therefore important to uncover potential new contributors to the development of structural joint damage and to better characterise factors allowing for possible amelioration of joint damage, as repair of existing joint damage is one of the most unmet needs in rheumatology.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterised by polyarticular synovitis, which, over time, leads to joint destruction. Joint damage is characterised by bone erosions and joint space narrowing, which can be quantified by X-ray scoring methods such as the Sharp score or its modified version, the Sharp-van der Heijde score (SHS).[1] Joint damage is correlated with important outcomes such as impairment of physical function and productivity.[2] These correlations are not strict since halt of radiographic progression may lag behind achievement of stringent remission.[3] Radiographic progression is assessed by comparing the difference between scores at two time points, such as baseline to year 1,[4–6] 2 years,[7] 5 years[8] or similar. A common finding, however, is that the majority of patients treated with disease modifying anti-rheumatic drugs (DMARDs), even with conventional synthetic ones, in clinical trials have little radiographic progression over time and significant progression occurs only in a minority of patients.[9–12]

While only a small proportion of patients are subject to major damage progression, these patients

may experience rapid loss of physical function[13] and social participation and, therefore, require special attention to prevent this bad outcome. On the other hand, if damage progression is variable over time with some patients having only short-term risk of major progression followed by arrest of joint destruction, while damage accrues relentlessly rapidly in others, then a delineation of such different trajectories is of great importance for risk assessment and therapeutic planning. To this end, however, one needs to investigate more than one point in time. Likewise, if radiographic progression is always linear and always related to high disease activity, there would be little gain to be obtained in our clinical and pathogenetic understanding by trajectory analyses when compared with current knowledge. Importantly, a number of predictors of progression have repeatedly been defined, such as acute phase reactants (measured, eg, as CRP,[5 14] erythrocyte sedimentation rate (ESR),[15] interleukin 6),[16] as well as disease activity as measured by swollen joint counts (SJC), or composite disease activity scores,[17 18] magnitude of treatment response,[19–21] seropositivity (rheumatoid factor[22] and anti citrullinated peptide antibodies (ACPA)[23]), initial joint damage,[6 24] smoking,[25] body mass index (BMI) and human leukocyte antigen DRB1 (HLA-DRB1).[26 27] However, the performance of models investigating factors associated with radiographic progression is rather low, with a (pseudo-)R^2 from 0.06 to 0.39.[6 24 28–30]

In this study, we attempt to identify trajectory clusters of radiographic progression in RA over time and to develop models to predict assignment to specific clusters using patient characteristic at baseline and over time.

METHODS

Data source

We used patient-level data of pivotal clinical randomised controlled trials in patients with RA and at least four SHS values, with the last one at week 100 or beyond. All trials assessed tumour necrosis factor inhibitors (TNFi) with or without methotrexate (MTX) including the following: PREMIER,[7] Early RA (ERA),[31] GO-Before,[32] TEMPO[33] and GO-Forward.[34] Ethical approval was obtained for each individual study, as referenced in the respective publications.

Clustering

We used patients with a minimal difference of 10 SHS units between the minimal and maximal SHS value within 100 weeks for initial cluster analyses. This cutpoint was chosen as the smallest detectable difference of change in SHS has been estimated to be between 2 and 8,[35–38] and rapid radiographic progression was conventionally defined as 5/year, corresponding to 10/2 years.[4 24 39]

We normalised SHSs over 2 years to a scale from 0 to 1 per patient to avoid averaging effects of using absolute values (see online supplemental figure 1). These curves were then clustered with the tool kmlShape (K-Means for Longitudinal Data using Shape-Respecting Distance),[40] with the time scale set to 0.01 and the number of 'Senators' (trajectories) to 100. Based on the main cluster themes identified, we then manually established rules that could be used to identify patients fitting into each of these clusters.

Sensitivity analyses

To investigate the stability of the trajectories, we used different cut-offs for SHS change (10, 8, 6, 4 and 2 in 2 years) and re-ran kmlShape analyses. We validated the derived mathematical rules to classify patients in different clusters using different cut-offs of SHS change as well as different treatment groups. We further used two other clustering approaches for comparison, hierarchical clustering with Fréchet distance and SAS procedure TRAJ,[41] and calculated agreement of cluster assignments.

Table 1 Baseline patient characteristics for overall cohort and divided into clusters of X-ray progression

Age	51 (42–59)
Gender (female, %)	77.5
Rheumatoid factor pos (%)	77.3
Disease duration	1.3 (0.5–4.0)
Sharp-van der Heijde score	9.5 (3–26.5)
Erosion	5 (1.5–15)
Joint space narrowing	3.5 (0–12)
Swollen joint count 28	11 (7–17)
Tender joint count 28	14 (9–20)
VAS pain (mm)	59 (41–75)
VAS patient global (mm)	60 (44.5–78)
VAS evaluator global (mm)	60 (48–73)
C reactive protein	1.4 (0.5–3.7)
ESR	35 (22–54)
SDAI	40.4 (30.3–52.6)
CDAI	37.9 (28.3–49)
DAS28	6.4 (5.6–7.2)
HAQ	1.38 (1–1.88)
Treatment:	29%
► csDMARD	36.5%
► bDMARD mono	34.6%
► Combination	

Values are median, quartiles and count for continuous variables, and count and per cent for nominal/dichotomous variables.
bDMARD, biological DMARD; CDAI, clinical disease activity index; csDMARD, conventional synthetic DMARD; DAS28, disease activity score 28; DMARD, disease modifying anti-rheumatic drug; ESR, erythrocyte sedimentation rate; HAQ, health assessment questionnaire; SDAI, simplified disease activity index; VAS, visual analogue scale.

Characterisation of patients assigned to clusters of X-ray progression

To identify differences of patient characteristics assigned to different clusters or radiographic progression, we performed analyses of variance or Wilcoxon test on baseline variables as well as disease activity measures over time. Since in the underlying trials study visits were conducted at different time points, values in between were interpolated linearly.

To predict assignment of patients to different clusters of X-ray progression, we generated several multivariate models: a decision tree model (C4.5 algorithm) using a learning model from the data sample to create a decision tree including variables with the highest information gain[42]; second, multivariate logistic regression models[43 44] using a stepwise approach for variable selection. Performance of the models was assessed using Percent Correctly Classified (PCC), Nagelkerke's and adjusted McFadden Pseudo-R^2.[45 46]

Analyses were run using the tool Weka V.3.8,[47] and the languages SAS V.9.4 and R V.3.5.0.[48]

RESULTS

In total, 1887 patients with 7738 X-rays were included in our study. Baseline characteristics are shown in table 1. Probability plots of radiographic data at weeks 26, 52 and 100 show increasing proportions of patients with radiographic change,

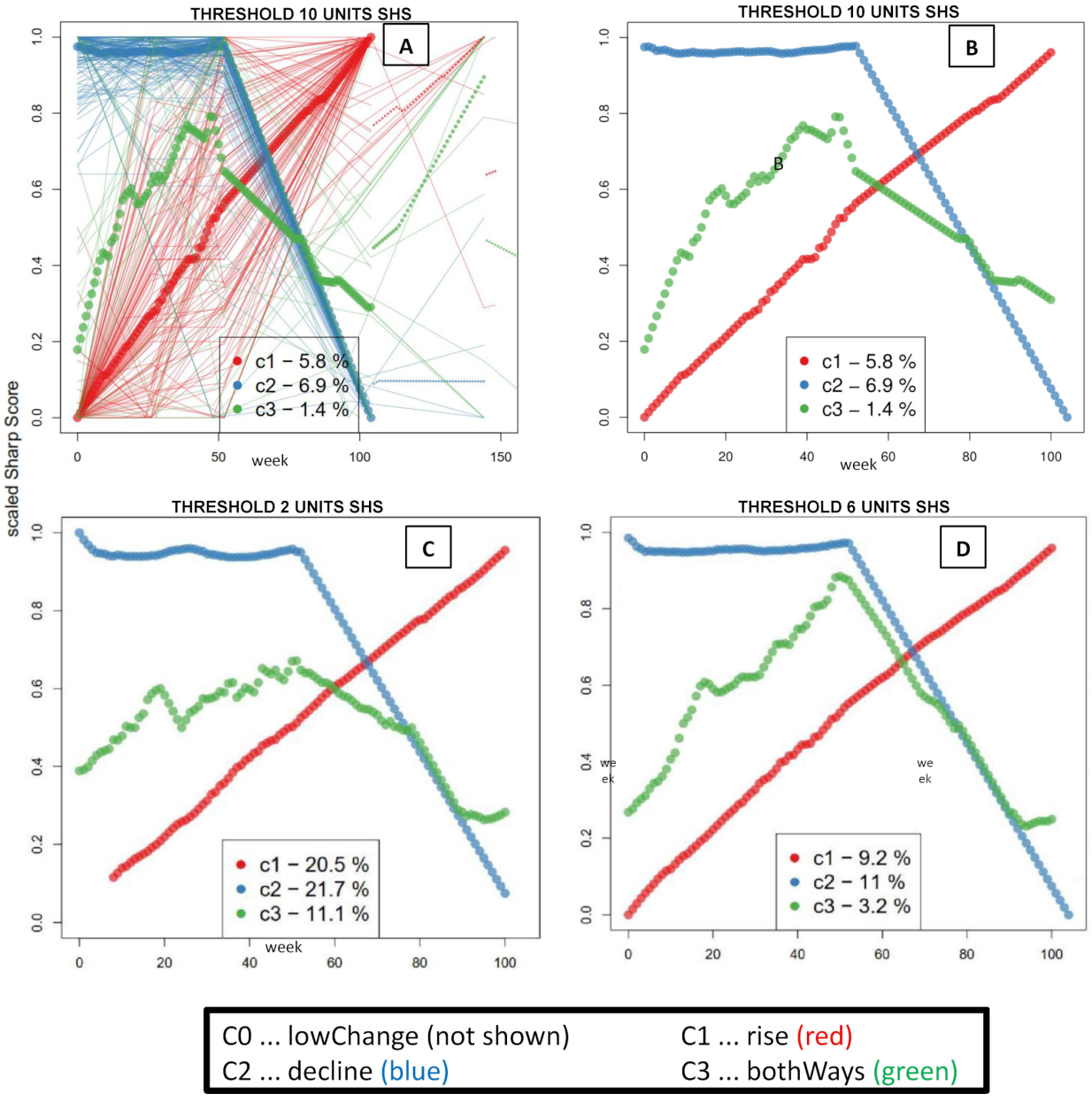

Figure 1 Representations of the three major clusters of radiological progression curves. (A) The curves of all patients with a Sharp-van der Heijde score (SHS) range ≥10, coloured by their cluster. Values between visits were linearly interpolated. Big dotted line is the median of particular cluster, thinner lines represent single patient values. Cluster C0/lowChange are the patients which do not have enough variation in SHS to be seen as a curve and are not shown in the diagrams but added to obtain 100%. (B–D) Lines represent the median of the generated clusters using different SHS thresholds: 10 or more (B), 2 or more (C) and 6 or more (D).

especially radiographic improvement, between baseline and endpoint with increasing time of observation (online supplemental figure 2).

Clustering

We identified 1621 out of 1887 patients (86%) who did not fulfil the minimal change requirement of >10 units SHS between minimal (SHS_{min}) and maximal scores (SHS_{max}). Those were not subjected to the subsequent cluster analysis and were a priori categorised as cluster 'c0/low-change'; in the remaining 266 patients (14%), using kmlShape, we were able to identify three main distinct trajectories: one cluster with an almost linear increase of SHS over the course of the 2 years (c1/rise cluster; 5.8%), one with a net decrease of the SHS (c2/improve cluster; 6.9%) and one with an initial increase followed by decrease of SHS (c3/bothWays cluster; 1.4%) (figure 1A,B). Estimated CIs of the median as well as the 95% ranges of these curves are shown in online supplemental figure 3A,B. The number of clusters is not optimised by the kmlShape procedure and thus needs to be determined manually. Changing the number of clusters did not affect the c1/rise and the c2/improve cluster, with additional clusters varying only slightly in shape (online supplemental figure 4).

We then tested whether the results would be robust against the use of other clustering approaches (hierarchical clustering with Fréchet distance and SAS procedure TRAJ).[41] The agreement of cluster assignment between kmlShape and different approaches was high: adjusted Rand index=0.971, Kappa=0.975 for SAS procedure TRAJ; and adjusted Rand index=0.998, Kappa=0.936 for hierarchical clustering of Fréchet distance (online supplemental figure 5).

In addition, we tested the stability of the identified clusters by reducing the threshold of change SHS from 10 to 8, 6, 4 or 2, respectively, and re-ran kmlShape analyses. In all instances, we found similar clusters of almost identical curve shapes; only, as expected, the percentage of patients increased in each cluster (figure 1C and D and online supplemental figure 6).

To simplify cluster assignment for individual patients to one of the clusters defined by kmlShape, we established the following mathematical rules to allow assignment of patients to one of the clusters:

1. $SHS_{max} - SHS_{min} \leq 10$ (absolute) →c0/lowChange (*a priori rule*)
2. $SHS_{baseline} \leq 0.5$ (rescaled*) AND $SHS_{week100} \geq 0.6$ (rescaled*) →c1/rise
3. $SHS_{baseline} \geq 0.6$ (rescaled*) AND $SHS_{week100} \leq 0.2$ (rescaled*) →c2/improve
4. Otherwise: c3/bothWays

*Rescale change $SHS_{max} - SHS_{min}$ on a scale between 0 and 1.

By applying these rules and again using different thresholds of SHS change, we found almost identical curve shapes as with kmlShape as well as similar proportions of patients assigned to the different clusters (compare online supplemental figures 6 and 7). To rule out that cluster trajectories are influenced by DMARD switch during observational period, we re-run analyses excluding all patients with treatment changes (rescue arms, total n=360) showing almost identical cluster shapes (online supplemental figure 8A). Furthermore, when looking at MTX-naïve and experienced patients separately, we identified similar shaped trajectories (online supplemental figure 8 B,C).

When we plotted absolute values of SHSs of the identified clusters, we could show similar trajectories of median SHSs with narrow CIs, again demonstrating the robustness of the identified clusters (figure 2).

Characterisation of patients assigned to clusters of X-ray progression

As expected, the percentage of patients assigned to the c1/rise cluster was highest in patients receiving csDMARDs (14.4%), lowest in the group that had received a combination therapy (2%) and bDMARD monotherapy in between (6.1%) (online supplemental table S1). In contrast, the distribution of patients assigned to the c2/improve cluster was 0.6%, 2.8% and 6.9% in these groups, for csDMARD, bDMARD monotherapy and combination therapy, respectively. The shape and the relative proportions of c1/rise and c2/improve clusters are consistent at different thresholds of SHS change and within the treatment groups (online supplemental table S1), demonstrating again the robustness of the cluster shapes. Of note, the shape of the c1/rise cluster is stable across all treatment groups, suggesting that even in patients receiving combination therapy, there is a proportion of patients with almost linear progression of radiographic joint damage.

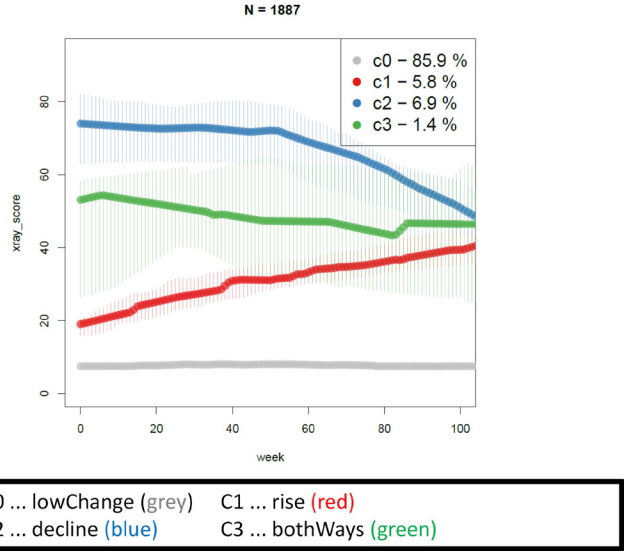

Figure 2 Absolute values of Sharp-van der Heijde score (SHS) over time of the clusters of radiological progression. Median absolute SHS values of all patients with an SHS range ≥10, coloured by their cluster with CI of the mean.

We then compared patient characteristics within different clusters at baseline. Composite measures such as simplified disease activity index (SDAI) or clinical disease activity index (CDAI) as well as their individual components, especially CRP and SJC, were significantly higher in patients assigned to the c1/rise cluster compared with the c2/improve cluster as well as the c0/lowChange, whereas SHS and duration are significantly higher in the c2/improve cluster compared with the c1/rise and c0/lowChange cluster (figure 3, online supplemental table S2). All comparisons are visualised in online supplemental figure 9. This is in line with previous studies identifying risk factors of radiographic progression in general.[6 22 49]

In additional longitudinal analyses of distinct clinical variables over the 2-year observation period, we found that the clear separation of the c1/rise cluster versus the other clusters observed at baseline was maintained throughout the whole period of follow-up when looking at objective markers of inflammation—CRP and, to a lesser extent, SJC (figure 4). Interestingly, tender joint count (TJC), patient global assessment (PGA) as well as visual analogue scale (VAS) pain did not allow discrimination between the clusters, the latter even being lower in the c1/rise cluster than in c2/improve or c0/low change (figure 4). The composite measures SDAI, CDAI and disease activity score 28 (DAS28) hardly discriminate at early time points (within first year), but rather later between clusters of radiographic progression.

Using disease characteristics over time in clusters derived from different thresholds of SHS change we found consistent discrimination of c1/rise cluster and both, c0/lowChange and c2/improve for CRP and SJC suggesting that the c1/rise cluster is associated with a high and persistent inflammatory load irrespective of cut-off used to define progression (online supplemental figure 10–13).

Multivariable models to predict cluster assignment

To predict assignment of patients to different progression clusters we developed a decision tree model using information at baseline. Cluster c0/lowChange has been randomly down sampled to be equal to the second largest cluster to avoid a bias towards classification to this cluster because of its large size. The decision tree learner selected the following variables to be included in the model: SHS, treatment, age, sex, disease duration, and CRP (figure 5). For this model, baseline SHS was identified to be most relevant to predict assignment to different progression cluster, followed by CRP. Using leave-one out cross-validation, the overall performance of the model was PCC 66.4%.

A stepwise, multivariate logistic model selected following variables to be significantly associated with cluster assignment: SHS at baseline, CRP, treatment and disease duration. The association

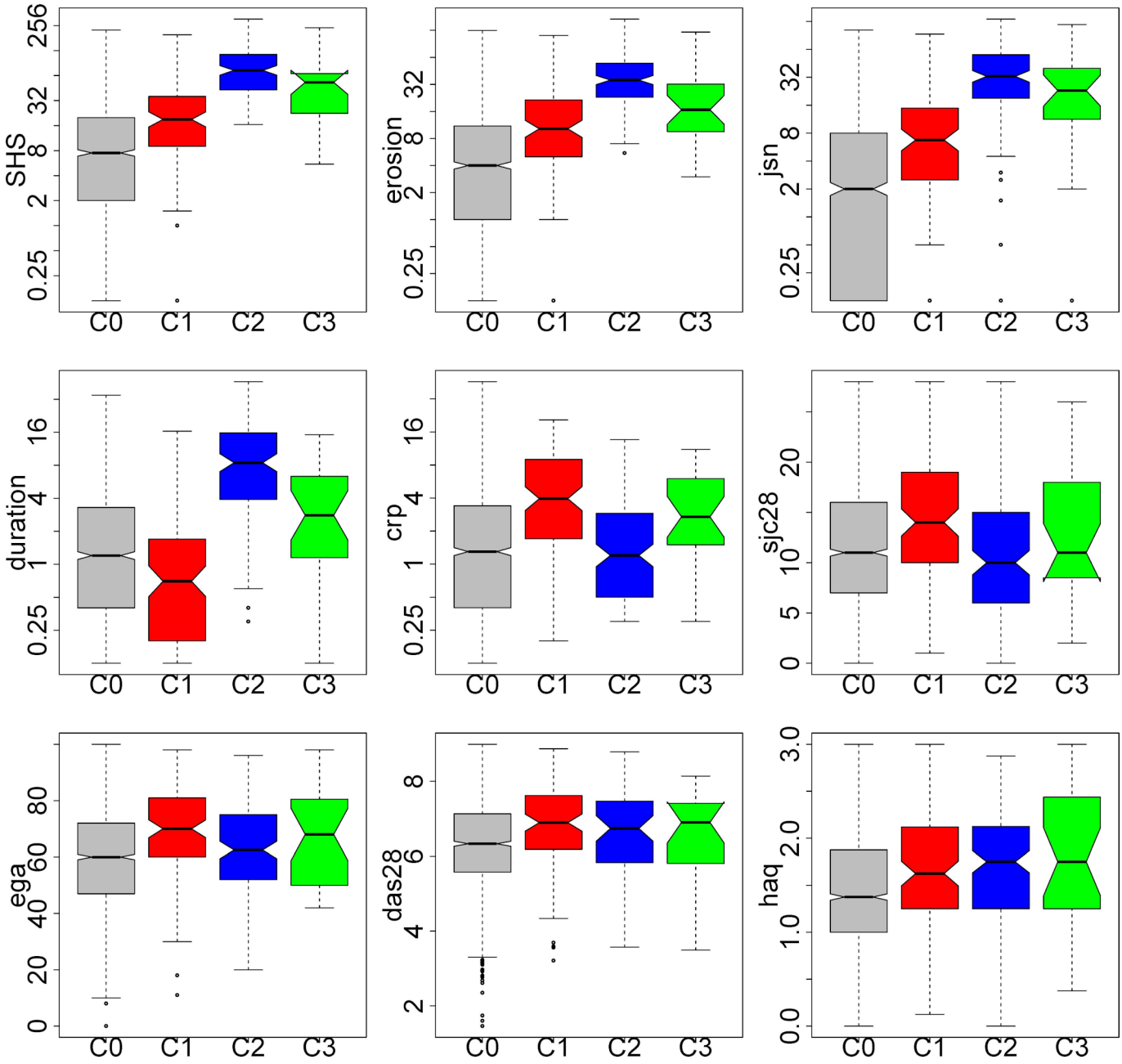

Figure 3 Univariable associations between radiological progression clusters and patient characteristics at baseline. Boxplot displays median and its 95% CI (notches), quartiles and range of values. Overlap of notches indicates non-significant difference of median values between progression clusters.

of variables with cluster assignment was similar to the one identified by the tree model, namely higher CRP values (OR 1.1), lower disease duration (OR 0.87) and csDMARD therapy versus combination treatment (OR 5.0) are associated with an increased risk of c1/rise cluster whereas csDMARD, when compared with combination therapy, decreases the chance of c2/improve cluster assignment (OR 0.05; table 2). Performance of the model was moderate (PCC overall 87.4%; Nagelkerke R^2=0.36; McFadden R^2=0.26).

DISCUSSION

In this large study using patient-level data from five pivotal randomised controlled trials of more than 1800 patients with RA, we were able to describe four distinct clusters of radiographic progression. Irrespective of the threshold used to define radiographic change, trajectories remained stable with a cluster of almost linear progression (C1/rise) and one with a decrease of the SHS (C2/improve) over time. In the majority of patients, however, radiographic changes were not pronounced enough to be included in the analyses of the trajectories. When we lowered the threshold of SHS for inclusion into the analysis of radiographic trajectories, still almost 50% of patients remained stable radiographically, despite fairly high disease activity at the beginning of the observation period of the clinical trial. Concerning the c1/rise cluster, the existence and regularity of the trajectory suggest that the rate of radiographic progression in individual patients with RA is predetermined at the start of a treatment cycle. As expected, and in line with numerous descriptions in the past, patients categorised into the c1/rise cluster comprised

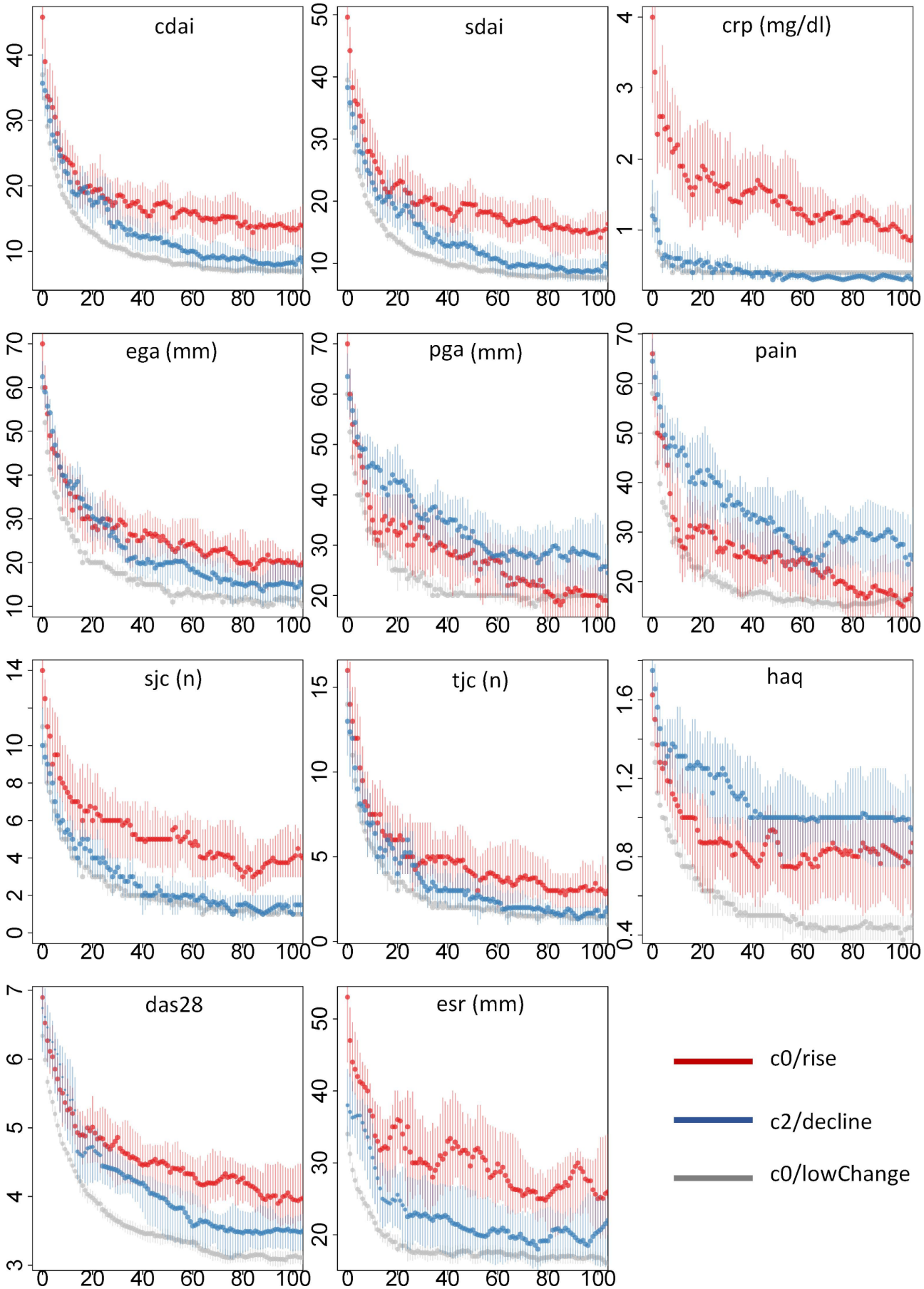

Figure 4 Trajectories of patient characteristics over time, split by cluster of radiographic progression. The dots represent median values, the vertical lines indicate the 95% ranges of the median. The x-axis displays time in weeks. CRP is expressed in mg/dL. c3/bothWays cluster is not displayed.

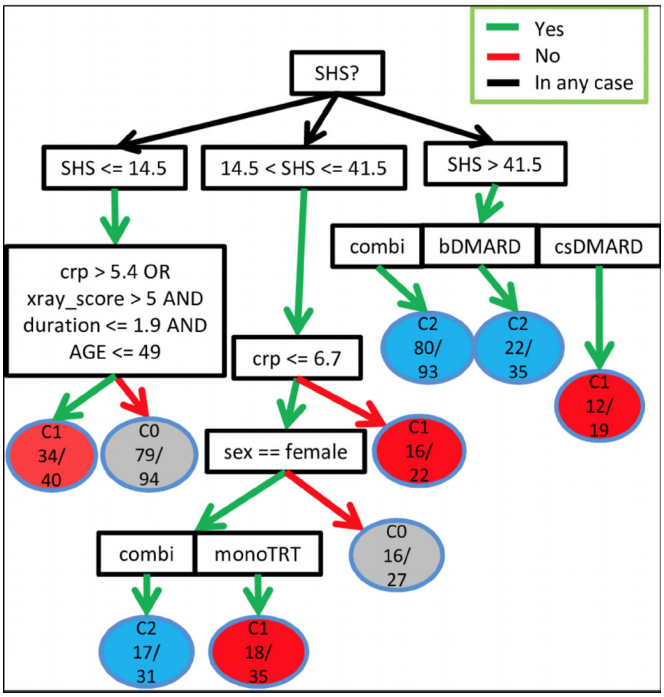

Figure 5 Decision tree model to predict assignment to clusters of radiological progression using baseline information of all five studies. The numbers in the leaves represent the number of patients correctly classified in relation to all patients assigned to this leaf. CRP is expressed in mg/dL, age and duration in years, 'monoTRT' means either csDMARD or bDMARD only. The overall per cent correctly classified (PCC) of the model is 66.4% at a leave-one-out cross-validation. Cluster labels: C0 … lowChange, C1 … 'rise', C2 … 'improve', C3 … 'bothWays'. bDMARD biological DMARDcombi combination treatment; csDMARD conventional synthetic DMARD; CRP C-reactive protein; monoTRT, mono treatment; SHS Sharp van der Heijde Score.

the subgroup with damage already at baseline, a high inflammatory load and short disease duration.[5 8 14–16 27] However, even as CRP is one of the most potent discriminators between the c0/low change and the c1/rise cluster, there is a large overlap between the two groups, suggesting that many patients with high inflammation still do not progress. The percentage of the C1/rise cluster is highest in patients receiving csDMARD monotherapy, while patients receiving combination therapy of csDMARDs and bDMARDs are least likely to linearly progress, with patients on bDMARD monotherapy being in between, demonstrating, as it has been shown before,[19 21 50] that TNFi are especially capable of preventing joint damage independent of disease activity. This effect was also found in animal models of arthritis, where gradual inhibition of TNF lead to decreased bone destruction and erosion formation before an overall effect on joint swelling was observed.[51]

Table 2 Simple logistic regression models to predict assignment to clusters of radiological progression using information at baseline

c0/low change vs:	C1/rise cluster		C2/improve cluster	
Parameters	OR	95% CI	OR	95% CI
X-ray score	1.02	1.01 to 1.03	1.03	1.02 to 1.03
CRP (mg/dL)	1.12	1.07 to 1.16	0.98	0.90 to 1.07
Duration (years)	0.87	0.80 to 0.95	1.09	1.05 to 1.12
Treatment	REF	REF	REF	REF
► Combination	4.98	2.88 to 8.63	0.05	0.01 to 0.23
► csDMARD	2.28	1.29 to 4.04	0.64	0.38 to 1.08
► bDMARD mono				

Performance of the model calculated by per cent correctly classified was 87.5% overall, 2.8 C1/rise cluster and 30.2% C2/improve cluster; Nagelkerke R^2=0.36; McFadden R^2=0.26. Variables entered but not selected by a stepwise approach were rheumatoid factor, age, gender and tender joint count.

In our study, we were also able to identify a cluster comprising about 7% of our patients analysed with a decrease in their SHS over time (C2/improve). Also for this cluster, decreasing the threshold of absolute change in SHS increases the percentage of patients assigned to the C2/improve cluster, with the shapes of the trajectories remaining stable. In line with the C1/rise cluster, the percentage of patients categorised into this cluster is associated with treatment. While hardly any patients receiving csDMARDs therapy were assigned to the c2/improve cluster, increasing proportions of patients receiving bDMARDs and combination therapy, respectively, were. In our analysis, a decrease of SHS was associated with an early and profound drop in objective measures of inflammation such as CRP and SJC.

While it is still under discussion whether a decrease in SHS in RA actually exists and is not a reading error,[11 52] the fact that the type of treatment, especially a combination treatment with MTX and a TNFi, and the absence of inflammation are the main drivers of significant amelioration argues for its existence.[53 54] Previous reports have also demonstrated an influence of treatment on repair of bone erosions in RA.[55 56] However, the extent of actual radiographic improvement in most of the studies is minimal. As we found a significant decrease of the SHS only after 1 year of treatment initiation, we propose that longer periods of longitudinal follow-up are necessary to fully explore the existence and potential of radiographic improvement in RA.

Most importantly, it is currently unclear whether a decrease in the SHS possibly could be translated into an actual improvement of joint function. We do not have any data to suggest that a decrease in the SHS leads to amelioration of joint function, and earlier studies indicated that repair of bone erosions analysed in detail by µCT does not lead to a disappearance of existing erosions, only to a partial filling of the lesion.[56] Further studies will be needed to investigate the functional consequences of a decrease of SHS in the future, which of course would be most relevant for patients with RA.

Interestingly, patients in the improve cluster had the longest disease duration, whereas a short disease duration was significantly associated with relentless progression. It is therefore tempting to speculate about different vulnerability in different phases of disease to the development of structural damage. Early disease with a high inflammatory load seems to be most vulnerable for relentless progression, whereas at later stage the risk decreases and in patients with excellent control of systemic inflammation even the chance of repair becomes possible. One could argue that longer disease duration might be a surrogate for higher SHSs, but the fact that both SHS at baseline as well as disease duration are associated with the chance of assignment to the C1/rise and C2/improve cluster in multivariable analyses calls for an independent effect. Furthermore, the estimated mean annual progression at baseline in patients of the C1/rise cluster is significantly higher compared with patients assigned to the C0/low change and the C2/improve cluster, even though mean progression rates are quite comparable in early and established disease.[57]

Of note, all clusters with significant change in their SHS have a higher HAQ compared with those with no or only minimal progression (c0/lowChange). While in all groups the HAQ drops significantly over time, reflecting a decrease of disease

activity, only in the c0/low change cluster the HAQ returns to almost zero, while in all other groups the HAQ remains elevated (figure 3). These data strongly support the concept of a reversible and irreversible component of the HAQ and underline the functional importance of the clusters we found.

Several limitations need to be addressed: first, in our study we combined data from different trials comprising patients with different disease durations and pre-treatments, and slightly different inclusion criteria. However, this would reflect a real-world setting of heterogeneous patient cohorts. Furthermore, using different sensitivity analyses, we could demonstrate robustness of identified clusters. Second, as our study only reflects a snapshot of time during the course of a patient's disease, we cannot draw a comprehensive picture of radiographic progression over longer terms. Third, having an unbalanced sample in terms of proportion of patients within different clusters, prediction models only have a limited ability to predict cluster assignment with a high chance of predicting C0/lowChange. We tried to overcome this issue in our tree model by iterative and random downsampling of this cluster. While statistically correct, this method distorts reality with a high proportion of patients with little or no radiographic progression and therefore limits the applicability of the model in clinical practice.

With baseline information as well as with additional time-integrated information (data not shown), prediction of cluster assignment was rather poor, which is in line with other reports analysing radiographic progression.[4–6] Therefore, about 30%–60% of the chance of becoming a relentless progressor versus having only minor to no or even improved radiographic change cannot be explained and therefore seems to be determined by factors that are not collected in routine settings of clinical trials. It is therefore important to uncover potential new contributors to the development of structural joint damage in order to identify people with high risk for joint damage accrual who would be eligible to more intensive treatment options. It would be also advantageous to better characterise factors allowing for possible amelioration of joint damage, as repair of existing joint damage is one of the most unmet needs in rheumatology.

In conclusion, the development of radiographic damage in RA follows definable trajectories. The nature of the trajectory is influenced by disease activity, treatment, and disease duration.

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ORCID iDs
Daniel Aletaha http://orcid.org/0000-0003-2108-0030
Stephan Blüml http://orcid.org/0000-0002-2758-4400

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CLINICAL SCIENCE

Integrated safety analysis of filgotinib in patients with moderately to severely active rheumatoid arthritis receiving treatment over a median of 1.6 years

Kevin L Winthrop ,[1] Yoshiya Tanaka ,[2] Tsutomu Takeuchi ,[3] Alan Kivitz,[4] Franziska Matzkies,[5] Mark C Genovese ,[5] Deyuan Jiang,[5] Kun Chen,[5] Beatrix Bartok,[5] Angelika Jahreis,[5] Robin Besuyen,[6] Gerd R Burmester ,[7] Jacques-Eric Gottenberg [8]

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For numbered affiliations see end of article.

Correspondence to
Dr Kevin L Winthrop, Oregon Health & Science University, Portland, OR 97239, USA; winthrop@ohsu.edu

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ABSTRACT

Objective To characterise safety of the Janus kinase-1 preferential inhibitor filgotinib in patients with moderately to severely active rheumatoid arthritis.

Methods Data were integrated from seven trials (NCT01668641, NCT01894516, NCT02889796, NCT02873936, NCT02886728, NCT02065700, NCT03025308). Results are from placebo (PBO)-controlled (through week (W)12) and long-term, as-treated (all available data for patients receiving ≥1 dose filgotinib 200 (FIL200) or 100 mg (FIL100) daily) datasets. We calculated exposure-adjusted incidence rates (EAIRs)/100 patient-years filgotinib exposure (100PYE) for treatment-emergent adverse events (TEAEs).

Results 3691 patients received filgotinib for 6080.7 PYE (median 1.6, maximum 5.6 years). During the PBO-controlled period, TEAEs, including those of grade ≥3, occurred at comparable rates with filgotinib or PBO; long-term EAIRs of TEAEs grade ≥3 were 6.4 and 7.6/100PYE for FIL200 and FIL100. EAIRs for deaths were 0.6/100PYE for FIL200, FIL100 and PBO; long-term EAIRs were 0.5 and 0.3/100PYE for FIL200 and FIL100. EAIRs for serious infection were 3.9, 3.3 and 2.4/100PYE for FIL200, FIL100 and PBO; long-term EAIRs were 1.6 and 3.1/100PYE for FIL200 and FIL100. EAIRs for herpes zoster were 0.6, 1.1, and 1.1/100PYE for FIL200, FIL100 and PBO; long-term EAIRs were 1.8 and 1.1/100PYE for FIL200 and FIL100. EAIRs for major adverse cardiovascular events were 0, 1.7 and 1.1/100PYE for FIL200, FIL100 and PBO; long-term EAIRs were 0.4 and 0.6/100PYE for FIL200 and FIL100. No venous thromboembolism occurred during the PBO-controlled period; long-term EAIRs were 0.2 and 0/100PYE for FIL200 and FIL100.

Conclusions Over a median of 1.6 and maximum of 5.6 years of exposure, safety/tolerability of FIL200 and FIL100 were similar, with a lower incidence of infections with FIL200 among the long-term, as-treated dataset.

Key messages

What is already known about this subject?

- Filgotinib is an oral, preferential Janus kinase-1 inhibitor approved in Europe and Japan for treatment of rheumatoid arthritis (RA).
- In previous clinical trials, filgotinib treatment resulted in improvement in RA signs and symptoms, improvement in physical function, reduced radiographic progression, and improvement in quality of life for patients across the spectrum, from methotrexate-naïve to biologic-refractory RA.
- Filgotinib was generally well tolerated in previous trials and had safety similar to active comparators methotrexate and adalimumab up to 52 weeks.

What does this study add?

- This integrated analysis of safety data from seven clinical trials characterises both the short-term safety compared with placebo (PBO) for 777 and 788 patients receiving filgotinib 200 and 100 mg and long-term safety of filgotinib 200 and 100 mg in patients with RA exposed for 4047.7 and 2032.9 patient-years (median 1.6 and 1.3 years; maximum 5.6 and 4.7 years).
- Overall, both filgotinib 200 and 100 mg were generally well tolerated. Proportions of patients treated with filgotinib 200 and 100 mg who developed infections and serious infections were higher versus PBO. Opportunistic infections, herpes zoster infections, major adverse cardiac events, and venous thromboembolism were infrequently reported. Longer-term study of filgotinib will further elucidate this safety profile.

INTRODUCTION

The oral, Janus kinase-1 (JAK1) preferential inhibitor filgotinib has demonstrated efficacy in rheumatoid arthritis (RA) in phase 2 and 3 trials up to 52 weeks.[1–5] Treatment with filgotinib 200 and 100 mg once daily improved RA signs and symptoms, improved physical function, reduced radiographic progression and improved health-related quality of life across patient populations.[1–5] Filgotinib safety up to 52 weeks was comparable to active comparators (methotrexate, adalimumab) in phase

Key messages

How might this impact on clinical practice or future developments?

- This integrated analysis of PBO-controlled and as-treated extension-study datasets describes the safety of filgotinib as treatment of RA.
- Over a median of 1.6 and maximum of 5.6 years of exposure, safety/tolerability of filgotinib 200 and 100 mg were similar, with a lower incidence of infections with filgotinib 200 mg among the long-term, as-treated dataset.

3 trials.[4 5] Most events occurred in similar proportions across treatments.[1–5]

It has been hypothesised that selectivity for JAK1 may preserve the efficacy benefit seen with less selective JAK inhibitors, while limiting the JAK2- and JAK3-mediated safety and tolerability concerns.[6] Based on a study of in vitro cellular assays and clinical pharmacokinetics of filgotinib, baricitinib, tofacitinib and upadacitinib, filgotinib demonstrated reduced JAK2 and JAK3 activity while maintaining comparable inhibition of JAK1. However, the clinical relevance of JAK selectivity remains unclear.[6]

Here, we use an integrated analysis across seven trials, including long-term extensions (LTEs), to evaluate the safety of filgotinib among patients with RA treated for a median of 1.6 (and up to 5.6) years, with attention to adverse events of special interest (AESIs) with JAK inhibition.

METHODS

Study designs

Patient-level data were integrated from two phase 2 (NCT01668641, NCT01894516), three phase 3 (NCT02889796, NCT02873936, NCT02886728) and phases 2 and 3 LTE trials (NCT02065700, NCT03025308) (table 1). Data from patients receiving monotherapy and concomitant conventional synthetic disease-modifying antirheumatic drugs (csDMARDs) were combined per filgotinib dose. Trials are summarised in online supplemental methods, and the phase 3 LTE protocol is available as supplemental file 2.[1–5 7] All data from patients receiving filgotinib 200 or 100 mg once a day or placebo from completed trials were included. Data from ongoing phase 2 and 3 LTEs were included through 26 April 2019 and 16 September 2019.

Eligible patients were aged ≥18 years with a diagnosis of RA per European League Against Rheumatism/American College of Rheumatology 2010 criteria.[8] Eligible patients were to have swollen and tender joint counts ≥6 and, depending on the study, either documented erosions or elevated serum C reactive protein (CRP).[1–5] Exclusion criteria included recent or active infections, major adverse cardiovascular events (MACE) within 6 months prior to screening, and specified abnormal laboratory results at screening.[1–5] Interruption of study drug was to be considered for any patient who developed an infection during the studies; those with specific laboratory abnormalities (eg, sequential elevations of aspartase aminotransferase or alanine aminotransferase >3× the upper limit of normal with either elevated bilirubin or with symptoms of hepatic injury) were to have study drug discontinued. The protocols for the phase 2 studies required study drug discontinuation for any QuantiFERON (QF) tuberculosis (TB) test positivity during the study, independent of clinical diagnosis. Also, in the phase 2 trials only, lymphopaenia (two sequential lymphocyte counts <500/mm^3) and elevated creatinine (two sequential increases in serum creatinine >50% over the average of screening and baseline values) were criteria for study drug discontinuation. In the phase 3 studies, drug interruption was required per local standard of care for QF TB-positive tests and newly diagnosed latent TB.

Patient and public involvement

Patients were not involved in research design, conduct or reporting. Patients were recruited by individual sites and provided written, informed consent.

Table 1 Key features of filgotinib RA phases 2 and 3 studies

	Required background medication			Control		Protocol-defined rerandomisation to FIL
	None	MTX	csDMARD(s)	PBO	Active comparator	
Phase 3 studies						
FINCH 1 NCT02889796		X		24 weeks	52 weeks (ADA)	PBO patients at week 24
FINCH 2 NCT02873936			X	24 weeks		
FINCH 3* NCT02886728	X			NA	52 weeks (MTX)	
FINCH 4 (LTE) NCT03025308	X†			NA		At study entry‡
Phase 2 Studies						
DARWIN 1 NCT01668641		X		24 weeks		Non-responders at week 12
DARWIN 2 NCT01894516	X			12 weeks		PBO patients and nonresponders at week 12
DARWIN 3 (LTE) NCT02065700	X§			NA		At study entry¶

*In addition to filgotinib 200 mg +MTX and filgotinib 100 mg +MTX, this trial included a filgotinib 200 mg monotherapy treatment arm.
†Patients continued to receive parent study protocol-approved background medication; patients in FINCH 3 receiving MTX discontinued on enrolment in FINCH 4.
‡All patients who received FIL at the time of completion of parent study continued to receive blinded FIL dose (100 mg once a day or 200 mg once a day). Patients who received ADA, PBO or MTX monotherapy, or who completed FINCH 2 on standard of care, were rerandomised at LTE entry to receive either FIL 100 mg or FIL 200 mg. Patients from FINCH 1 and FINCH 3 who completed parent study on standard of care were not eligible.
§Patients were permitted to restart background MTX therapy if deemed necessary by the investigator.
¶Patients who received FIL 200 mg once a day or FIL 100 mg two times per day at the time of completion of parent study continued to receive the same FIL dose in the LTE study. Patients who received FIL 25 mg two times per day, FIL 50 mg once a day, FIL 50 mg two times per day or FIL 100 mg at the time of completion of parent study were assigned either FIL 200 mg once a day or FIL 100 mg two times per day at LTE entry. Patients who received PBO at the time of completion of parent study were rerandomised at LTE entry to receive either FIL 200 mg once a day or FIL 100 mg two times per day. In the USA, dosing in male subjects was restricted to FIL 100 mg once a day.
ADA, adalimumab; csDMARD, conventional synthetic disease-modifying antirheumatic drug; FIL, filgotinib; LTE, long-term extension; MTX, methotrexate; NA, not applicable; PBO, placebo; RA, rheumatoid arthritis.

Analysis sets

The placebo-controlled safety analysis dataset included patients in four placebo-controlled trials randomised to filgotinib 200 or 100 mg once a day or placebo up to 12 weeks (online supplemental figure S1). Treatment-emergent AEs (TEAEs) were defined as any AE with an onset date on or after the first dose of study drug and no later than the earliest date of either 30 days after the last dose of study drug or the first dose date of the switched treatment minus 1 day. Safety of filgotinib relative to active comparators adalimumab and methotrexate was reported by Combe *et al*[4] and Westhovens *et al*[5] and is not presented as part of this analysis.

The long-term, as-treated analysis dataset included all available data from patients in all seven trials who received ≥1 dose of filgotinib 200 or 100 mg once a day. Data were included from the original assigned treatment and after rerandomisation/reassignment to filgotinib. Therefore, patients may have contributed exposure time to more than one treatment group. Events were assigned to treatment received at time of event, with a 30-day window after last dose. The long-term, as-treated analysis dataset was the largest. It included patients with the longest exposure and was used to describe long-term, exposure-adjusted incidence rates (EAIRs). Data are presented through 96 weeks; beyond 96 weeks, the numbers of events and the numbers of patients still exposed to study drug were small, rendering interpretation difficult.

Safety was assessed through TEAEs, TEAEs leading to treatment discontinuation, serious AEs (SAEs), deaths, AE severity, AESIs and laboratory abnormalities coded according to Medical Dictionary for Regulatory Activities.

AESIs included infections, serious infections, opportunistic infections (OIs), active TB, herpes zoster (HZ) reactivation, MACE, venous thromboembolism (VTE), arterial thrombotic events (ATE; not including stroke or myocardial infarctions (MI)), malignancies, non-melanoma skin cancers (NMSC) and gastrointestinal perforation. Serious infections were infections meeting SAE criteria. Mucocutaneous candidiasis and superficial fungal infections were not considered OIs; TB and genital, disseminated and ophthalmic HZ were considered OIs. Herpes simplex virus (HSV) infection was also monitored. MACE, VTE and ATE positively adjudicated by an independent committee were included. MACE included cardiovascular (CV) death, MI and stroke, while ATEs were defined as all arterial events other than MI or stroke. VTE included pulmonary embolism and deep vein thrombosis (DVT). All deaths, including those that occurred off study drug, are reported.

Statistical methods

Baseline demographics and disease characteristics were summarised by descriptive statistics. For the placebo-controlled dataset, the proportion of patients with an event was described (n (%)) during the 12-week, placebo-controlled period.

For each exposure-group in the as-treated dataset, filgotinib patient-years of exposure (PYE) were calculated as (last dose date − first dose date +1)/365.25. If patients had multiple events, only the first event was counted. EAIRs per 100 PYE (100PYE) and 95% CIs were calculated using Poisson regression model including treatment and study as covariates to account for different study sizes and log of PYE as offset. If a specific event was not observed in any study, data were integrated across all studies, and the Poisson regression model only included treatment without adjusting by study. If a specific event was not observed in a treatment after studies were integrated, crude EAIRs and their differences were calculated. For crude EAIRs, 95% CIs were derived using an exact method[9] and based on confidence limits of individual point estimates.[10]

RESULTS

Patient population and exposure

Demographics and disease characteristics at baseline were well balanced and similar across treatment groups. Nineteen percent of patients were aged ≥65 years. At baseline, 91% and 88% of patients received csDMARDs concomitantly with treatment in the placebo-controlled and as-treated datasets; 39% and 38% of patients in the placebo-controlled and as-treated datasets received corticosteroids concomitantly with treatment (table 2). Across datasets, mean baseline Disease Activity Score with 28 joints using CRP was 5.7–5.9. Forty percent to 45% of patients had ≥1 traditional CV risk factor.

The placebo-controlled dataset included 777, 788 and 781 patients receiving filgotinib 200, 100 mg and placebo. In the as-treated dataset, 2267 patients received filgotinib 200 mg for 4047.7 PYE, 1647 patients received filgotinib 100 mg for 2032.9 PYE. Median filgotinib treatment duration was 1.6 years; 2740 (74.2%) patients received treatment for ≥1 year (table 3). As of the data cut-off, 16 September 2019, the longest individual exposure to filgotinib was up to 5.6 years.

Overall AEs

During the 12-week, placebo-controlled period, rates of TEAEs, grade ≥3 TEAEs, serious TEAEs and TEAEs leading to study drug discontinuation were comparable for filgotinib and placebo (table 3). Most common TEAEs were nasopharyngitis, upper respiratory tract infection (URTI) and nausea (table 4). Most common TEAE leading to discontinuation was pneumonia (n=3 (0.4%) filgotinib 200 mg, n=2 (0.3%) filgotinib 100 mg, n=2 (0.3%) placebo), followed by RA flare (among placebo patients only, n=5 (0.6%)) and gamma-glutamyltransferase increased (n=1 (0.1%) filgotinib 200 mg, n=2 (0.3%) filgotinib 100 mg). EAIRs of grade ≥3 TEAEs, SAEs and TEAEs leading to discontinuation were comparable between doses.

Twenty-five deaths were reported in filgotinib groups (table 3). During the placebo-controlled period, four patients died (figure 1A). Long-term, more deaths occurred in the filgotinib 200 mg group than the 100 mg group; EAIRs (95% CI) of all deaths did not change over 96 weeks (figure 1B). Most deaths in the long-term analysis were due to CV events, serious infection, and malignancies (online supplemental table S1); all fatal MI (n=2; one each in filgotinib 200 and 100 groups) and strokes (n=3; 2 with filgotinib 200 and 1 with filgotinib 100 mg) occurred in patients with ≥1 CV risk factor. Acute DVT was the cause of death for one patient receiving filgotinib 200.

AEs of special interest

During the placebo-controlled period, infections were more frequent in both filgotinib groups versus placebo (figure 1C, table 3). Long-term, EAIRs of infections decreased over time (figure 1D). Overall, the most commonly reported infections were URTI, nasopharyngitis and urinary tract infection (UTI). EAIRs were similar between the two doses. During the placebo-controlled period, serious infections occurred in 20 patients (figure 1E). Long-term, EAIRs for serious infections did not vary over time (figure 1F). The most common serious infections were pneumonia, cellulitis and bronchitis; each occurred at similar rates between the filgotinib 200 and 100 groups.

Nine OIs were reported with filgotinib. No OIs or active TB occurred during the placebo-controlled period (table 3). Long term, EAIRs for OIs were 0.1 (0.1–0.3) and 0.2 (0.1–0.5)/100PYE for filgotinib 200 and 100 mg. Active TB was reported in three patients receiving filgotinib 100 mg from endemic areas (Hong Kong, Poland, India).

Table 2 Baseline demographics, disease characteristics and cardiovascular risk factors

	PBO controlled			Long term, as-treated	
	FIL 200 mg N=777	FIL 100 mg N=788	PBO N=781	FIL 200 mg N=2267	FIL 100 mg N=1647
Age, mean±SD years	53±12.6	53±12.4	54±12.6	53±12.8	53±12.8
≥65 years	135 (17.4)	151 (19.2)	158 (20.2)	410 (18.1)	327 (19.9)
≥75 years	26 (3.3)	27 (3.4)	25 (3.2)	76 (3.4)	67 (4.1)
Female	633 (81.5)	636 (80.7)	638 (81.7)	1828 (80.6)	1319 (80.1)
Race					
Asian	137 (17.6)	136 (17.3)	124 (15.9)	372 (16.4)	286 (17.4)
Black or African American	21 (2.7)	20 (2.5)	35 (4.5)	63 (2.8)	53 (3.2)
White	543 (69.9)	548 (69.5)	528 (67.6)	1568 (69.2)	1137 (69.0)
Other	76 (9.8)	83 (10.5)	90 (11.5)	262 (11.6)	170 (10.3)
Hispanic or Latino	151 (19.4)	169 (21.4)	173 (22.2)	525 (23.2)	355 (21.6)
BMI, mean±SD kg/m^2	27.6±6.25	27.4±6.28	27.7±6.28	27.6±6.20	27.6±6.20
≥25 kg/m^2	472 (60.7)	496 (62.9)	482 (61.7)	1402 (61.8)	1034 (62.8)
≥30 kg/m^2	229 (29.5)	234 (29.7)	235 (30.1)	668 (29.5)	498 (30.2)
Duration of RA from diagnosis, mean±SD years	8.6±8.2	9.1±8.0	8.6±8.1	6.3±7.6	7.4±7.8
Range, years	0.3–49.7	0.1–41.8	0.1–51.4	0.0–52.3	0.0–51.4
hsCRP, mean±SD mg/L	18.2±21.4	19.3±25.9	18.0±24.4	18.9±24.5	18.6±25.6
DAS28 (CRP), mean±SD	5.9±0.9	5.8±1.0	5.9±0.9	5.8±0.9	5.8±1.0
CDAI, mean±SD	40.5±12.38	39.9±12.59	40.4±11.69	40.4±12.26	39.7±12.23
HAQ-DI, mean±SD	1.65±0.611	1.61±0.637	1.66±0.600	1.62±0.623	1.62±0.618
Concurrent oral corticosteroids*	300 (38.6)	305 (38.7)	297 (38.0)	781 (34.5)	631 (38.3)
Mean±SD mg/day				6.3±2.69	6.3±2.57
Concurrent csDMARDs*	710 (91.4)	721 (91.5)	712 (91.2)	1843 (81.3)	1500 (91.1)
Concurrent MTX*	685 (88.2)	692 (87.8)	678 (86.8)	1219 (53.8)	1100 (66.8)
Prior exposure to bDMARDs	181 (23.3)	179 (22.7)	164 (21.0)	276 (12.2)	255 (15.5)
Concurrent oral contraceptive*	51 (6.6)	53 (6.7)	31 (4.0)	127 (5.7)†	97 (6.1)†
Concurrent statin*	68 (8.8)	95 (12.1)	93 (11.9)	213 (9.4)	186 (11.3)
Nicotine use‡					
Current	84 (13.5)	95 (15.0)	88 (14.1)	244	193
Former	85 (13.7)	81 (12.8)	80 (12.8)	236	174
Medical history	310 (39.9)§	349 (44.3)§	331 (42.4)§	950 (41.9)¶	740 (44.9)¶
Diabetes‡	76 (12.2)	61 (9.6)	69 (11.1)	221 (9.7)	158 (9.6)
Hypertension	263 (33.8)	276 (35.0)	274 (35.1)	766 (33.8)	560 (34.0)
Dyslipidaemia	118 (15.2)	130 (16.5)	147 (18.8)	336 (14.8)	280 (17.0)
CVD	47 (6.0)	30 (3.8)	44 (5.6)	111 (4.9)	69 (4.2)
Ischaemic CNS vascular conditions	17 (2.2)	17 (2.2)	19 (2.4)	51 (2.2)	34 (2.1)
Peripheral vascular disease	–	–	–	5 (0.2)	6 (0.4)
DVT/PE	6 (0.8)	7 (0.9)	11 (1.4)	18 (0.8)†	14 (0.9)†

Data are n (%) unless otherwise indicated. DVT/PE were unadjudicated.
*On first dosing date in parent study.
†For FIL 200 mg and FIL 100 mg, n=2227 and 1600.
‡PBO-controlled group includes patients enrolled in phase 3 trials only. For FIL 200 mg, FIL 100 mg, and PBO, n=622, 633 and 623.
§Excluding diabetes and peripheral vascular disease.
¶Excluding DVT/PE.
bDMARD, biological DMARD; BMI, body mass index; CDAI, Clinical Disease Activity Index; CNS, central nervous system; CRP, C reactive protein; csDMARD, conventional synthetic DMARD; CVD, cardiovascular disease; DAS28(CRP), Disease Activity Score with 28 joints using CRP; DMARD, disease-modifying antirheumatic drug; DVT, deep vein thrombosis; FIL, filgotinib; HAQ-DI, Health Assessment Questionnaire Disability Index; hsCRP, high sensitivity CRP; MTX, methotrexate; PBO, placebo; PE, pulmonary embolism; RA, rheumatoid arthritis.

In the placebo-controlled period, HZ occurred in 5 patients (figure 1G; table 3). Long term, EAIRs of HZ were higher for filgotinib 200 vs 100 mg and remained stable over time (figure 1H). EAIRs of HZ infection/reactivation were generally higher among Asian patients than among the overall population based on the long-term, as-treated analysis set (online supplemental figure S2). Most HZ infections were mild to moderate, monodermatomal or adjacent dermatomal and nonvisceral. Most patients recovered after treatment interruption and could continue treatment on recovery. Six SAEs of HZ were reported by five patients receiving filgotinib 200 mg and one receiving filgotinib 100 mg. All six patients were aged ≥53 years, four of six were of Asian descent, three of six were taking concomitant corticosteroids and methotrexate, while one of six was taking only concomitant corticosteroids; one of six was known to have been vaccinated against HZ. All six were hospitalised for their HZ event, and all events resolved. One of these cases was cutaneous disseminated HZ in a patient receiving filgotinib 200 mg who was hospitalised and discontinued from the study.

During the placebo-controlled period, four patients reported HSV (table 3). Long term, EAIRs of HSV were 0.6 (0.4–1.1) and 0.9 (0.6–1.4)/100PYE for filgotinib 200 and 100 mg.

During the placebo-controlled period, five patients reported MACE; patients who had MI or stroke all had ≥1 CV risk factor (figure 1I; table 3). EAIRs of MACE for filgotinib 200 and 100 mg remained stable over time (figure 1J). One ATE, a grade 4 SAE of peripheral artery thrombosis, was reported in a 64-year-old patient with hypertension and body mass index of 29.7 who was receiving filgotinib 200 mg.

Nine patients experienced VTEs; none occurred in the placebo-controlled period (figure 1K; table 3). EAIRs remained stable over time (figure 1L). All patients reporting VTEs had ≥1 traditional risk factor.

Table 3 Exposure to study drug and rates of safety events

	PBO-controlled analysis set			Long term, as-treated analysis set	
	FIL 200 mg N=777	FIL 100 mg N=788	PBO N=781	FIL 200 mg N=2267 PYE=4047.7	FIL 100 mg N=1647 PYE=2032.9
Exposure to study drug, years					
Mean±SD	0.4±0.1	0.4±0.1	0.4±0.1	1.8±1.2	1.2±0.7
Median (Q1, Q3)	0.5 (0.5, 0.5)	0.5 (0.5, 0.5)	0.5 (0.3, 0.5)	1.6 (1.0, 2.1)	1.3 (0.5, 1.8)
Cumulative n (%) exposed to study drug					
Week 12	748 (96.3)	754 (95.7)	649 (83.1)	2165 (95.5)	1547 (93.9)
Week 52	NA	NA	NA	1731 (76.4)	1001 (60.8)
Week 96	NA	NA	NA	799 (35.2)	360 (21.9)
Rates of safety events	EAIR (95% CI) per 100 PYE			EAIR (95% CI) per 100 PYE	
TEAE	195.4 (173.7 to 219.8) n=354	176.3 (156.0 to 199.2) n=323	175.9 (155.5 to 198.9) n=316	40.4 (38.3 to 42.7) n=1771	64.2 (58.9 to 69.9) n=1140
TEAE Grade ≥3	12.0 (7.4 to 19.5) n=31	11.5 (7.0 to 18.7) n=30	10.6 (6.4 to 17.5) n=27	6.4 (5.6 to 7.4) n=309	7.6 (5.3 to 10.8) n=206
TE SAE	10.9 (6.7 to 17.8) n=21	12.8 (8.1 to 20.4) n=25	8.9 (5.2 to 15.2) n=17	6.1 (5.4 to 7.0) n=254	7.5 (5.6 to 10.1) n=166
TEAE leading to premature discontinuation	8.7 (4.9 to 15.3) n=15	6.3 (3.3 to 12.0) n=11	8.8 (5.0 to 15.4) n=15	6.0 (5.3 to 6.9) n=239	6.8 (5.4 to 8.6) n=93
TEAE leading to temporary interruption	27.3 (19.7 to 37.8) n=58	25.6 (18.3 to 35.6) n=55	21.9 (15.4 to 31.1) n=46	12.5 (11.3 to 13.8) n=576	14.8 (11.9 to 18.5) n=364
All deaths	0.6 (0.1 to 3.9) n=1	0.6 (0.1 to 3.9) n=1	0.6 (0.1 to 4.0) n=2	0.5 (0.3 to 0.7) n=19	0.3 (0.1 to 0.7) n=6
Infectious AEs	76.9 (63.7 to 92.9) n=139	67.3 (55.2 to 82.1) n=123	58.0 (47.0 to 71.7) n=104	24.8 (23.1 to 26.5) n=1074	34.4 (30.4 to 38.8) n=648
Serious infectious AEs	3.9 (1.6 to 9.1) n=8	3.3 (1.4 to 8.2) n=7	2.4 (0.9 to 6.7) n=5	1.6 (1.2 to 2.1) n=67	3.1 (2.1 to 4.5) n=51
Opportunistic infections	0	0	0	0.1 (0.1 to 0.3) n=5*	0.2 (0.1 to 0.5) n=4†
Active TB	0	0	0	0	0.1 (0.0 to 0.5) n=3
Herpes zoster	0.6 (0.1 to 3.9) n=1	1.1 (0.3 to 4.4) n=2	1.1 (0.3 to 4.5) n=2	1.8 (1.4 to 2.3) n=74	1.1 (0.8 to 1.7) n=23
Herpes simplex virus	1 (0.1)	1 (0.1)	2 (0.3)	0.6 (0.4 to 1.1) n=33	0.9 (0.6 to 1.4) n=18
Adjudicated MACE	0	1.7 (0.3 to 4.8) n=3	1.1 (0.1 to 4.0) n=2	0.4 (0.2 to 0.7) n=19	0.6 (0.4 to 1.1) n=13
CV death	0	0.6 (0.0 to 3.1) n=1	0	0.1 (0.1 to 0.3) n=6	0.2 (0.1 to 0.5) n=4
Nonfatal MI	0	1.1 (0.1 to 4.0) n=2	0.6 (0.0 to 3.1) n=1	0.1 (0.0 to 0.3) n=4	0.2 (0.1 to 0.6) n=5
Nonfatal stroke	0	0	0.6 (0.0 to 3.1) n=1	0.2 (0.1 to 0.5) n=10	0.2 (0.1 to 0.5) n=4
Adjudicated ATE	0	0	0	0.0 (0.0 to 0.2) n=1	0
Adjudicated VTE	0	0	0	0.2 (0.1 to 0.4) n=8	0.0 (0.0 to 0.3) n=1
PE	0	0	0	0.1 (0.1 to 0.3) n=6	0.0 (0.0 to 0.3) n=1
DVT	0	0	0	0.1 (0.1 to 0.3) n=6	0
Malignancy excluding NMSC	0	0.6 (0.0 to 3.1) n=1	0.6 (0.0 to 3.1) n=1	0.6 (0.4 to 0.9) n=22	0.5 (0.3 to 1.0) n=11
NMSC	0	0	0	0.2 (0.1 to 0.4) n=9	0.1 (0.0 to 0.5) n=3
Gastrointestinal perforations	0	0	0	0.1 (0.0 to 0.2) n=3	0

The PBO-controlled analysis set only included data up to 12 weeks.
*Two patients reported oesophageal candidiasis, one patient reported pneumonia cryptococcal, one patient reported herpes zoster disseminated and one patient reported both oesophageal candidiasis and pneumonia cryptococcal.
†One patient reported oesophageal candidiasis, one patient reported TB, one patient reported lymph node TB, one patient reported meningitis TB and one patient reported pulmonary TB.
AE, adverse event; ATE, arterial thrombotic event; CV, cardiovascular; DVT, deep vein thrombosis; EAIR, exposure-adjusted incidence rate; FIL, filgotinib; MACE, major adverse cardiovascular event; MI, myocardial infarction; NA, not applicable; NMSC, non-melanoma skin cancer; PBO, placebo; PE, pulmonary embolism; PYE, patient-years exposure; SAE, serious adverse event; TB, tuberculosis; TE, treatment-emergent; TEAE, treatment-emergent adverse event; VTE, venous thromboembolism.

During the placebo-controlled period, one malignancy each was reported with filgotinib 100 mg (cervix carcinoma) and placebo (malignant glioma) (figure 1M; table 3). Long term, EAIR of all non-NMSC malignancies for filgotinib 200 and 100 mg remained stable over time (figure 1N). In patients receiving filgotinib 200 mg, one diffuse large B-cell lymphoma and three non-Hodgkin's lymphomas were reported; 1 T-cell lymphoma and one central nervous system lymphoma were reported with filgotinib 100 mg. During the placebo-controlled period, no NMSCs were reported (figure 1O, table 3). EAIRs for NMSC were 0.2 (0.1–0.4) and 0.1 (0–0.5)/100PYE for filgotinib 200 and 100 mg (figure 1P).

No gastrointestinal perforations occurred during the placebo-controlled period (table 3). Gastrointestinal perforations were reported for three patients receiving filgotinib 200 mg with risk factors of concomitant non-steroidal anti-inflammatory (one

Table 4 Common TEAEs (≥3% in any treatment group) in the PBO-controlled period up to week 12

	FIL 200 mg N=777	FIL 100 mg N=788	PBO N=781
Nasopharyngitis, n (%)	27 (3.5)	19 (2.4)	19 (2.4)
Upper respiratory tract infection, n (%)	26 (3.3)	20 (2.5)	14 (1.8)
Nausea, n (%)	27 (3.5)	18 (2.3)	13 (1.7)

FIL, filgotinib; PBO, placebo; TEAE, treatment-emergent adverse event.

patient) and corticosteroid (one patient) use; EAIR was 0.1 (0–0.2)/100PYE.

Graded laboratory abnormalities occurring during the placebo-controlled period are reported in online supplemental table S2.

DISCUSSION

We evaluated the safety of filgotinib as treatment for RA with an integrated analysis encompassing seven trials that included 3691 patients, treated for a median of 1.6 years (maximum exposure, 5.6 years in <3% of patients). In the placebo-controlled analysis dataset, proportions of patients with TEAEs, SAEs and AESIs were similar between those receiving filgotinib 200, 100 mg or placebo. The long-term, as-treated dataset revealed similar incidence between doses for most AESIs, with the exception of numeric differences in serious infection (higher incidence with filgotinib 100 vs 200 mg) and in VTE and HZ (higher incidence with filgotinib 200 vs 100 mg). Incidence of malignancy, MACE, and other serious events were similar between doses.

A numeric increase in mortality among the long-term, as-treated dataset was observed for filgotinib 200 (0.5/100PYE) vs 100 mg (0.3/100PYE) and appeared to remain stable over time; however, rates were similar overall with overlapping CIs. Mortality rates were not adjusted for demographic factors or ageing over the study period, but they appear to fall within reported RA population rates and were consistent with those observed with other RA therapeutics.[11–13] The leading causes of death for patients receiving filgotinib were those most frequently reported in patients with RA: CV death, infections and malignancies.[12 14–16] All fatal MI and strokes occurred in patients with ≥1 CV risk factor.[17]

Patients with RA have increased risk for infection due to underlying disease and many of the immunosuppressive therapies used to treat it.[18 19] Compared with csDMARDs, JAKi are associated with greater risk of serious infection, with observed incidence rates from RA clinical trials (generally 3–5/100PYE) similar between JAKi and biological DMARDs.[19 20] The most common serious infections observed were those common among patients with RA (eg, pneumonia, skin and soft tissue infection, UTI).[21] Though incidence of serious infection for the placebo-controlled dataset was 1.0% with filgotinib 200 mg and 0.9% with filgotinib 100 mg, serious infection EAIRs were higher for filgotinib 100 mg (3.1/100PYE) vs 200 mg (1.6/100PYE). Overall infection rates decreased over time, while rates of serious infections appeared to remain stable. Though cross-trial comparisons are fraught with limitations and potential bias, the EAIR for serious infections with filgotinib 100 mg was similar to those reported for other JAKi (including over LTE periods), which range from 2.7 to 6.2/100PYE, while the EAIR with filgotinib 200 mg was slightly lower.[13 22–24] It is possible this lower EAIR with filgotinib 200 mg may be explained, at least partially, by reduced inhibition of JAK2 and JAK3 relative to other JAKi.[6]

RA confers elevated risk for HZ, and corticosteroids and JAKi can further increase this risk.[25] Reactivation of latent varicella zoster virus by tofacitinib, baricitinib and upadacitinib has been described.[13 23 24 26] From the placebo-controlled dataset, there were five cases of HZ: 1 (0.1%) with filgotinib 200 mg and 2 (0.3%) with filgotinib 100 mg and with placebo. For filgotinib 200 and 100 mg, EAIRs of HZ were 1.8 and 1.1/100PYE, and EAIRs were higher among Asian populations than among the overall population. As in other JAKi programmes, most HZ cases were monodermatomal and not serious.[13 23 24] Among the six patients who had SAEs of HZ, three were receiving concomitant corticosteroids and methotrexate, while one was receiving concomitant corticosteroid alone.

In the filgotinib programme, OIs—including active TB—were infrequent; however, QF status was carefully monitored, and patients with changes in QF status discontinued the phase 2 LTE or had to pause study drug and start treatment for latent TB if applicable in the phase 3 LTE. Longer-term, real-world and population-based data are needed to better understand the potential TB risk of filgotinib and other JAKi.

VTE risk is elevated for patients with RA compared with the general population[27 28]; risk with JAKi is incompletely understood,[29 30] as is a potential mechanism for JAKi to cause VTE. Here, VTEs were infrequently reported (none from the placebo-controlled data set and EAIRs of 0.2/100PYE and 0.0/100PYE for filgotinib 200 and 100 mg), and their incidence did not increase over time. EAIR of VTEs was 0.5/100PYE for all doses in an integrated safety analysis of baricitinib[22] and 0.6/100PYE for upadacitinib 15 mg.[13] While real-world and population-based data are needed to better understand the potential risk of VTE associated with JAKi, our findings with filgotinib suggest a risk no greater than that reported from real-world studies showing background rates of VTE in RA of 0.3–1.0/100PYE.[27 28]

Patients with RA are also at increased risk for MACE compared with the general population.[31 32] As expected, 40%–45% patients had a medical history of CV risk factors at baseline. MACE were infrequent, and EAIRs (0.4/100PYE and 0.6/100PYE for filgotinib 200 and 100 mg) remained stable over time.

Patients with RA experience higher rates of malignancies, due to underlying disease and immunosuppressive treatments, compared with the general population.[33] In this analysis, malignancies were uncommon, and EAIRs did not increase with time exposed to filgotinib. Rates of malignancy excluding NMSC with filgotinib treatment (0.6 and 0.5/100PYE for filgotinib 200 and 100 mg) appeared to fall within the range reported from large registries of patients with RA.[34] In integrated analyses of baricitinib and tofacitinib, EAIRs of malignancy excluding NMSC were 0.8 and 0.9/100PYE.[22 23]

Filgotinib was associated with decreases in mean neutrophil, lymphocyte and platelet counts and increases in mean lipid, CK and creatinine levels, as previously reported.[1–5 7] There were small numerical differences in frequencies of Grade 3/4 neutropenia and lymphopenia in patients treated with filgotinib versus placebo.

Limitations of this analysis include comparatively short follow-up for rare and long-latency events, especially malignancies. Relative to other JAKi, the filgotinib RA programme has included fewer patients. The short placebo-controlled period limited the assessment of filgotinib against the background of rare events, such as VTE. Filgotinib was also evaluated against active comparators for only 52 weeks.[4 5] The LTE trials did not have a control group, and clinicians were permitted to modify background therapy per clinical judgement, as they would in real-world treatment plans. Another limitation is survival bias: patients who had intolerable AEs or lack of efficacy were discontinued from their studies. Longer-term, adequately powered studies with greater numbers of patients and events are needed to better understand the safety of filgotinib, describe the incidence of uncommon events over time, and assess its safety relative to other JAKi.

AESI incidence is generally similar between filgotinib 200 and 100 mg. Serious infection risk is likely elevated with filgotinib vs

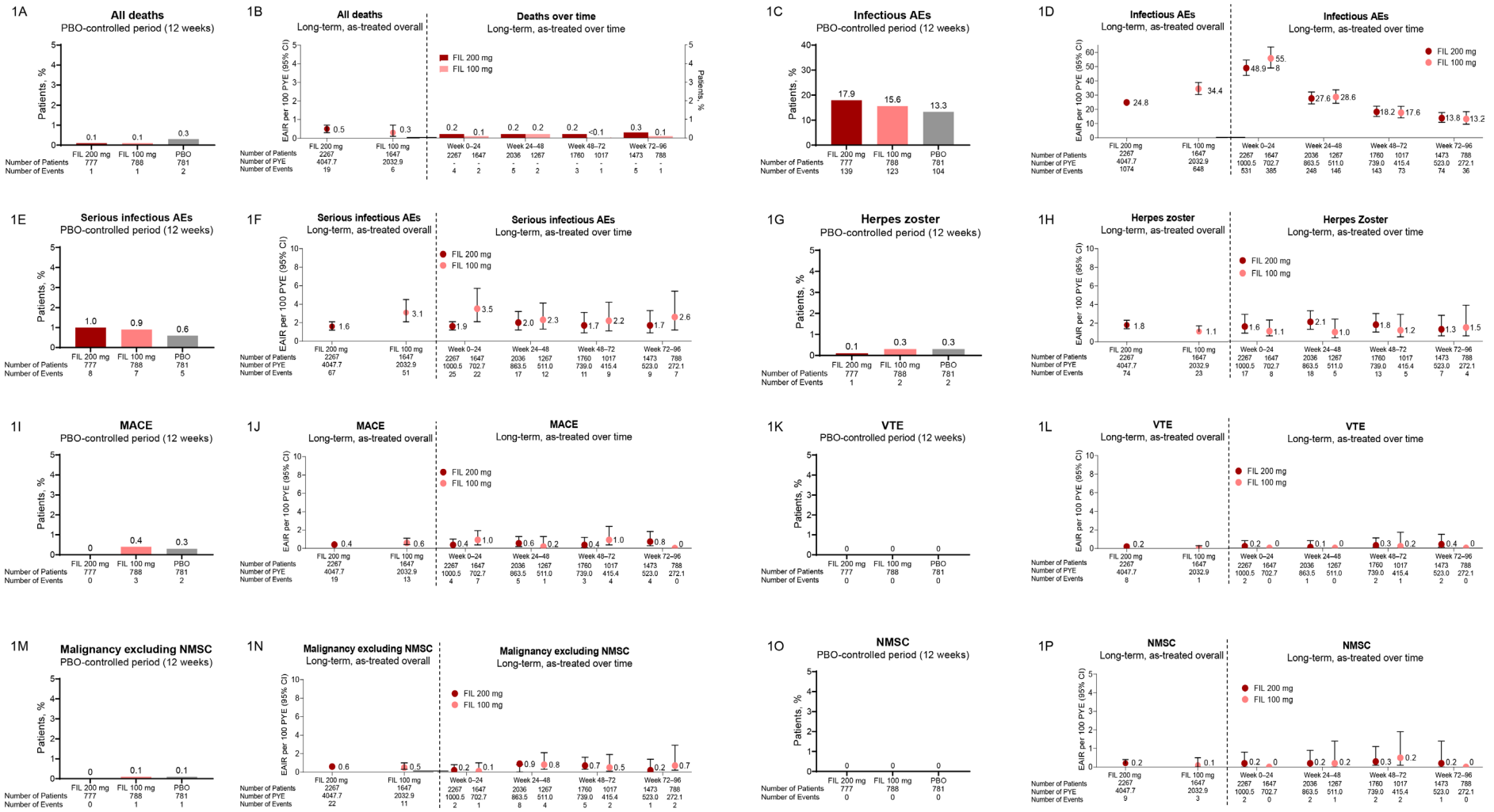

Figure 1 Summary safety event rates. (A) All deaths during the PBO-controlled period; (B) all deaths in the long-term, as-treated set and over time; (C) infectious AEs during the PBO-controlled period; (D) infectious AEs in the long-term, as-treated set and over time; (E) serious infectious AEs in the PBO-controlled period; (F) serious infectious AEs in the long-term, as-treated set and over time; (G) herpes zoster during the PBO-controlled period; (H) herpes zoster in the long-term, as-treated set and over time; (I) MACE during the PBO-controlled period; (J) MACE in the long-term, as-treated set and over time; (K) VTEs during the PBO-controlled period; (L) VTEs in the long-term, as-treated set and over time; (M) non-NMSC malignancy during the PBO-controlled period; (N) non-NMSC malignancy in the long-term, as-treated set and over time; (O) NMSC malignancy during the PBO-controlled period; (P) NMSC malignancy in the long-term, as-treated set and over time. MACE and VTEs were positively adjudicated. AE, adverse event; EAIR, exposure-adjusted incidence rate; FIL, filgotinib; MACE, major adverse cardiovascular events; NMSC, nonmelanoma skin cancer; PBO, placebo; PYE, patient-years exposure; VTE, venous thromboembolism.

placebo, as is risk of HZ. Rates of VTE were low; malignancy and MACE were low and similar to that reported in population-based studies of RA. Over a median of 1.6 and maximum of 5.6 years of exposure, safety/tolerability of FIL200 and FIL100 were similar, with a lower incidence of infections with FIL200 among the long-term, as-treated dataset.

Author affiliations
[1]Oregon Health & Science University, Portland, Oregon, USA
[2]The First Department of Internal Medicine, University of Occupational and Environmental Health, Japan, Kitakyushu, Japan
[3]Keio University School of Medicine, Shinjuku-ku, Tokyo, Japan
[4]Altoona Research, Duncansville, Pennsylvania, USA
[5]Gilead Sciences, Inc, Foster City, California, USA
[6]Galapagos BV, Leiden, The Netherlands
[7]Charité University Hospital Berlin, Berlin, Germany
[8]Strasbourg University Hospital, Strasbourg, France

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ORCID iDs
Kevin L Winthrop http://orcid.org/0000-0002-3892-6947
Yoshiya Tanaka http://orcid.org/0000-0002-0807-7139
Tsutomu Takeuchi http://orcid.org/0000-0003-1111-8218
Mark C Genovese http://orcid.org/0000-0001-5294-4503
Gerd R Burmester http://orcid.org/0000-0001-7518-1131
Jacques-Eric Gottenberg http://orcid.org/0000-0002-9469-946X

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TRANSLATIONAL SCIENCE

Loss of balance between protective and pro-inflammatory synovial tissue T-cell polyfunctionality predates clinical onset of rheumatoid arthritis

Achilleas Floudas ,[1] Nuno Neto,[2,2] Carl Orr,[3] Mary Canavan,[1] Phil Gallagher,[4] Conor Hurson,[4] Michael G Monaghan ,[2] Sunil Nagpar,[5] Ronan H Mullan,[6] Douglas J Veale ,[3] Ursula Fearon [1]

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[1]Department of Molecular Rheumatology, Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin, Ireland
[2]Department of Mechanical and Manufacturing Engineering, Trinity College Dublin, Dublin, Ireland
[3]Department of Rheumatology, EULAR Centre of excellence, Centre for Arthritis and Rheumatic Diseases, St Vincent's University Hospital, UCD, Dublin, Ireland
[4]Department of Orthopaedics, St Vincent's University Hospital, Dublin, Ireland
[5]Department of Immunology, Janssen Research & Development, Immunology, Philadelphia, Pennsylvania, USA
[6]Department of Rheumatology, Tallaght University Hospital, Dublin, Dublin, Ireland

Correspondence to
Professor Ursula Fearon, Department of Molecular Rheumatology, Trinity College Dublin, Dublin, Ireland; Fearonu@tcd.ie

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ABSTRACT

Objectives This study investigates pathogenic and protective polyfunctional T-cell responses in patient with rheumatoid arthritis (RA), individuals at risk (IAR) and healthy control (HC) synovial-tissue biopsies and identifies the presence of a novel population of pathogenic polyfunctional T-cells that are enriched in the RA joint prior to the development of clinical inflammation.

Methods Pathway enrichment analysis of previously obtained RNAseq data of synovial biopsies from RA (n=118), IAR (n=20) and HC (n=44) was performed. Single-cell synovial tissue suspensions from RA (n=10), IAR (n=7) and HC (n=7) and paired peripheral blood mononuclear cells (PBMC) were stimulated in vitro and polyfunctional synovial T-cell subsets examined by flow cytometric analysis, simplified presentation of incredibly complex evaluations (SPICE) and FlowSom clustering. Flow-imaging was utilised to confirm specific T-cell cluster identification. Fluorescent lifetime imaging microscopy (FLIM) was used to visualise metabolic status of sorted T-cell populations.

Results Increased plasticity of Tfh cells and CD4 T-cell polyfunctionality with enriched memory Treg cell responses was demonstrated in RA patient synovial tissue. Synovial-tissue RNAseq analysis reveals that enrichment in T-cell activation and differentiation pathways pre-dates the onset of RA. Switch from potentially protective IL-4 and granulocyte macrophage colony stimulating factor (GMCSF) dominated polyfunctional CD4 T-cell responses towards pathogenic polyfunctionality is evident in patient with IAR and RA synovial tissue. Cluster analysis reveals the accumulation of highly polyfunctional $CD4^{+}$ $CD8^{dim}$ T-cells in IAR and RA but not HC synovial tissue. $CD4^{+}$ $CD8^{dim}$ T-cells show increased utilisation of oxidative phosphorylation, a characteristic of metabolically primed memory T-cells. Frequency of synovial $CD4^{+}$ $CD8^{dim}$ T-cells correlates with RA disease activity.

Conclusion Switch from potentially protective to pathogenic T-cell polyfunctionality pre-dates the onset of clinical inflammation and constitutes an opportunity for therapeutic intervention in RA.

Key messages

What is already known about this subject?
- T-cells infiltrate the inflamed rheumatoid arthritis (RA) synovial tissue.
- Presence of polyfunctional ex-Th17 cells in the synovial fluid of patients with RA.
- Synovial fluid ex-Th17 T-cells are resistant to regulation by regulatory Treg cells.

What does this study add?
- First study to show that synovial tissue T-cell dysregulation towards pathogenic polyfunctionality pre-dates the clinical onset of arthritis.
- Identification of a novel highly polyfunctional synovial T-cell population with potential as a prognostic and therapeutic target for RA.

How might this impact on clinical practice or future developments?
- The data presented in this study will impact therapeutic strategy development and form the basis for more refined T-cell directed therapies in RA.

INTRODUCTION

The window for effective therapeutic intervention in rheumatoid arthritis (RA) is limited. Additionally, current T cell-specific therapies for the treatment of RA are broad and affect all T cells irrespective of their contribution to disease pathogenesis and progression, therefore, not differentiating between protective and pathogenic T cell responses.[1 2] More refined and targeted therapeutic approaches will improve disease progression while limiting unwanted side effects and toxicity, while characterisation of T cell responses in the synovial tissue of at-risk individuals will help identify prognostic markers and extend the window for effective therapeutic intervention. Ex-Th17 T cells with the capacity to produce multiple proinflammatory cytokines simultaneously have previously been identified in the synovial fluid of patients with RA, these polyfunctional T cells are also resistant to inhibition by regulatory T cells (Treg).[3 4]

While, the implications of polyfunctional T cell responses extend beyond autoimmunity, response to pathogens, anti-cancer responses and encompass vaccine development, characterisation of polyfunctional T cells is technically challenging and requires new data exploration, visualisation and analytical

approaches.[5] Polyfunctional CD4 T cells are molecularly distinct from monofunctional cells; however, several questions regarding the temporal cytokine production dynamics, transcriptional control and stimulation requirements of polyfunctional T cells remain unanswered.[6] Early studies suggest that T cell polyfunctionality is a result of sequential cytokine release with T cells obtaining polyfunctionality status gradually and maintaining simultaneous cytokine secretion for a short period of time.[7] SARS-CoV-2-specific T cell IL-2, tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) cytokine polyfunctionality is dependent on viral antigen specificity and contributes to viral clearance.[8] Importantly, T cell receptor (TCR)-dependent and TCR-independent stimulation resulted in similar cytokine secretion patterns over time, suggesting that both TCR-mediated signals and bystander T cell activation could be important for the emergence of polyfunctional T cell states.[7]

Recent studies have highlighted the importance of deleterious polyfunctionality in autoimmune inflammation. Highly polyfunctional T cell responses have been identified in the synovial tissue of psoriatic arthritis patients and their frequency correlates strongly with disease severity.[9] Increased T cell plasticity across multiple T cell subpopulations and polyfunctionality of Th cells have recently been identified in children with Down syndrome-associated arthritis, an aggressive form of erosive polyarticular inflammatory arthritis.[10]

Despite accumulating evidence regarding the importance of T cell polyfunctionality in autoimmunity, little is known about the potential contribution of polyfunctional T cells to RA disease progression and the timing of their emergence in relation to the onset of clinical inflammation. Additionally, polyfunctional T cell responses can be highly heterogeneous raising the potential for immunoregulatory as well as proinflammatory states of polyfunctionality.[11] In this study, we have performed extensive characterisation of peripheral blood and synovial tissue T cell subpopulations with emphasis on polyfunctional T cell responses in patient with RA, individuals at risk (IAR) and healthy control (HC) synovial tissue biopsies. Synovial tissue RNAseq analysis with pathway enrichment analysis revealed an enrichment in T cell activation and differentiation pathways prior to clinical inflammation. Importantly, we have identified synovial polyfunctional T cell responses that pre-date the clinical onset of RA with distinct and potentially protective polyfunctionality in HC synovial biopsies. In addition, we report the identification of highly polyfunctional synovial $CD4^+$ $CD8^{dim}$ T cells that correlate with disease severity in patients with RA.

The study of polyfunctional synovial tissue T cell responses and the characterisation of specific highly polyfunctional T cell clusters that do not require intracellular cytokine staining for their identification raises the potential for targeted therapeutic intervention at early stages of disease onset in RA.

MATERIALS AND METHODS

Synovial tissue single-cell suspensions

RA patient arthroscopies were performed under local anaesthetic using Wolf 2.7 mm needle arthroscopy or ultrasound-guided biopsy as previously described.[12] Clinical characteristics of patients with RA who donated synovial fluid or synovial tissue samples are included in online supplemental table S1 and online supplemental table S2. HC synovial tissue was obtained at the time of anterior cruciate ligament surgery. Synovial tissue isolation and generation of single-cell suspension for further analysis were performed as described previously.[13] Briefly, synovial biopsies~15 were enzymatically and mechanically digested using the GentleMACS Tumor Dissociation Kit, human (Miltenyi Biotech) as per manufacturers' instructions. Biopsies are placed in 4.7 mL of Roswell Park Memorial Institute(RPMI) supplemented with 200 µl of enzyme H, 100 µl enzyme R and 25 µl enzyme A in a gentleMACS C Tube followed by initial mechanical disruption of the tissue using programme h_tumor_01 on a gentleMACS Dissociator. Samples are then incubated for a total of 1 hour at 37°C under continuous rotation using the MACSmix Tube Rotator with further applications of the gentleMACS Dissociator at the halfway point and the end of the 1 hour incubation according to manufacturer's instructions. A single synovial cell suspension was generated by filtration through a 70 µM cell strainer.

Flow cytometric analysis

Patient with RAperipheral blood mononuclear cells (PBMC), synovial fluid mononuclear cells (SFMC) and synovial tissue single-cell suspensions were used for flow cytometric analysis. For intracellular cytokine staining, cells were plated at 3×10^6 cells/mL in a flat-bottom 96-well plate and were stimulated in cRPMI supplemented with cell stimulation cocktail (photomultiplier detector assembly (PMA)/Ionomycin) (Biosciences, according to the manufacturer's instructions). 1 hour later, cells were treated with brefeldin-A (BFA_ and monensin (both from Biosciences) and left to incubate for a further 4 hours prior to flow cytometric analysis. Single-cell suspensions were washed with PBS followed by incubation with LIVE/DEAD fixable NIR (Thermofisher) viability dye according to the manufacturer's instructions. Cells were then washed with PBS and subjected to an Fc receptor block step (TruStain FcX Receptor blocking solution (Biolegend)) immediately followed by extracellular staining with antibody combinations for 30 min at 4°C (online supplemental table S1). If multiple Brilliant Violet fluorochrome conjugated antibodies were used, cells were stained in Briliant Stain buffer (BD). Following incubation, cells were washed x2 in FACS buffer (PBS with 2% FBS and 0.002% w/v sodium azide, made in house, all reagents from Sigma) and fixed with 1% PFA for 10 min at 4°C. If intracellular staining was required, cells were not fixed with PFA and instead were fixed and permeabilised using the intracellular Foxp3 staining kit (eBiosciences) as per the manufacturer's instructions. Following the fixation and permeabilisation step, cells were washed in perm buffer and incubated with antibodies against intracellular targets for 30 min at 4°C (online supplemental table S1). Cells were then washed x1 with perm buffer and x1 with FACS buffer prior to acquisition on a four laser LSR Fortessa cytometer (BD). Analysis was performed on FlowJo (v10) (BD) using additional software plugins t-SNE and FlowSOM. Key gating steps followed prior to specific population subgating included gating of cells based on forward and side scatter characteristics, two independent doublet cell exclusion steps, followed by gating of live cells. Fluorescent minus one gating controls used were appropriate.

Flow cell sorting

T cell subpopulations were sorted from patient with RA PBMC. Briefly, cells were washed with PBS followed by incubation with TruStain FcX Receptor blocking solution (Biolegend) for 10 min at 4°C. Cells were then incubated with antibodies against CD45, CD3, CD4 and CD8 in 1% BSA/PBS (sterile filtered, Sigma) for 30 min at 4°C. Following incubation, cells were washed two times in 1% BSA/PBS prior to flow cell sorting on a four-laser BD FACSAria Fusion cell sorter, housed in a biosafety level two

cabinet. Propidium iodide (RnD) was added immediately before sorting for dead cell exclusion.

Flow imaging

Patient with RA PBMC and synovial fluid mononuclear cells (SFMC) were stained with antibodies against CD3, CD4 and CD8 as per flow cytometric analysis staining protocol described herein with propidium iodide for dead cell exclusion. Cells were then acquired on a four laser, 12 parameter Image Stream MKII analyzer (Amnis) at low speed (high sensitivity) and 40× magnification. Data were analysed with image data exploration and analysis sotware (IDEAS)(Amnis).

RNAseq

Analysis was performed on previously obtained RNAseq data of RA (n=118), IAR (n=20) and HC (n=44) synovial tissue biopsies.[14] Briefly, quality of RNA was evaluated using an Agilent bioanalyzer followed by RNAseq by Q2 Solutions (Morrisville, NC). Sequencing libraries were prepared on Truseq stranded total RNA using the Illumina Ribo-Zero protocol. Sequencing of pooled libraries was performed on an Illumina HiSeq 2000, and raw read quality was evaluated using FastQC. Raw reads were trimmed based on sequence quality and adaptors leading to an average number of clusters per sample of 8.9×10^7. Reads were then aligned to the human reference genome b37.3 using STAR V.2.4.[15] Quantification of aligned reads was performed using RSEM V.1.2.14 with the University of California Santa Cruz transcriptome model (accessed on 17 March 2014) that included lincRNAs from Ensembl V.75. Aligned data were subjected to evaluation of quality using several metrics including mapping rate, coverage and deviation from principal component analysis (PCA).

Pathway enrichment analysis

Raw counts were analysed using the *DESeq2* (V.1.28.1) pipeline in R for the identification of differentially expressed genes for the binary comparisons as indicated. Pathway analysis was then performed using package *pathfindeR* (V.1.6.1) in R with the most recently update KEGG database and a stricter to default adjusted p value enrichment threshold of 0.01, gene sets with 5–500 genes were considered and an enrichment threshold of 0.01.[16] MA plots were generated using package *DESeq2* with *ashr* shrinkage of the data in R. The effect of transformation on variance was assessed by generating SD to mean plots by using function *meanSdPlot(assay(ntd))* as part of package *DESeq2* (online supplemental figure S1). Term and UpSetplots were generated in R, package *pathfinder*.

Two-photon fluorescence lifetime imaging microscopy

Two-photon fluorescence lifetime imaging microscopy (2P-FLIM) relies on endogenous fluorophores such as nicotinamide adenine nucleotide (NAD(P)H) in order to obtain an image and infer on the cellular metabolic state.[17–19] Its potential is correlated with the ability to distinguish between protein-bound and free NAD(P)H due to NAD(P)H self-quenching process.[20] This results in a longer fluorescence lifetime of ~2.5 ns for protein-bound NAD(P)H and a short lifetime of ~0.4 ns for free NAD(P)H. It has been shown that an increase in the fraction of protein-bound NAD(P)H results from an increase in oxidative phosphorylation (OxPhos) metabolic dependence.[21–23] Flow-sorted T cells were immediately transferred to a 18-well 15-µ-Slide (Ibidi). 2P-FLIM was performed using an upright Olympus BX61W1 multiphoton microscopy system equipped with a Titanium: sapphire laser (Chameleon Ultra, Coherent), a water-immersion objective (25× Olympus 1.05NA) and a temperature-controlled stage (37°C). NAD(P)H excitation was performed at a wavelength of 760 nm and fluorescence emission was isolated with a 455/90 nm bandpass filter. Fluorescence lifetime decay measurements were obtained using a PicoHarp 300 TCSPC system operating in the time-tagged mode coupled with a PMA hybrid detector (PicoQuanT GmbH, Germany) at 256 time bins per pixel, at least three images were acquired for each sample. The overall decay curves were generated and fitted using a two-component fitting to differentiate between the protein-bound (τ_1) and free (τ_2) NAD(P)H (Eq.1):

$$I(t) = \alpha_1 e^{-\frac{t}{\tau_1}} + \alpha_2 e^{-\frac{t}{\tau_2}} + c(1)$$

$I(t)$ corresponds to the fluorescence intensity measured at time t after laser excitation; α_1 and α_2 represent the fraction of the overall signal comprise of a long and short lifetime components, respectively; C corresponds to background light. To easier understand the change of both fluorescence lifetime components, we calculated the average lifetime (τ_{avg}) of NAD(P)H (Eq.1).

$$t_{avg} = \frac{(\alpha_1\times\tau_1)+(\alpha_2\times\tau_2)}{(\alpha_1+\alpha_2)}$$

Treg suppression assay

RA patient's T cells were enriched with the untouched T cell isolation kit (Milteniy) according to the manufacturer's instructions. Following enrichment, T cells were stained with the following panel CD3, CD4, CD25, CD127, CD8 and flow sorted for $CD4^+$, $CD4^+$ $CD8^+$ DP T cells and $CD127^-CD25^+$ Treg cells. Propidium iodide (RnD) was added immediately before sorting for dead cell exclusion. $CD4^+$ and $CD4^+$ $CD8^+$ DP T cells were stained with differential cell trackers (FarRed and cell trace violet (CTV), respectively, both from ThermoFisher) according to manufacturer's instructions. $2\text{^}10^4$ $CD4^+$ and $2\text{^}10^4$ $CD4^+$ $CD8^+$ DP T cells were placed per well in a round-bottom 96-well plate in cRPMI with halving number of autologous $CD127^-$ $CD25^+$ Treg cells and aCD3/aCD28/aCD2 activation beads (Treg suppression inspector, Milteniy) according to the manufacturer's instructions. T cell proliferation was assessed 5 days postinitial activation, the cell proliferation plugin of FlowJo v10 was used for analysis of proliferating cells.

Assessment of T cell cytokine expression following metabolic pathway inhibition

PBMC or RA patient's T cells, which were enriched with the untouched T cell isolation kit (Milteniy), according to the manufacturer's instructions, were used as indicated. Following enrichment, T cells were stained with the following panel CD3, CD4, CD8 and flow sorted for $CD4^+$ and $CD4^+$ $CD8^+$ DP T cells. Propidium iodide (RnD) was added immediately before sorting for dead cell exclusion. Cells (T cells or PBMC) were then incubated overnight with 2DG (1 mM) (Merk), Oligomycin (30 µM) (VWR), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) (30 µM) (Merk) or left untreated. The effect of metabolic inhibition was assessed via FLIM (as described herein) 30 min postinitiation of treatment. Remaining cells, following incubation overnight, were subjected to flow cytometric analysis for intracellular cytokine detection.

T cell proliferation

RA patient's PBMC were labelled with Cell Trace Violet (Thermofisher) according to manufacturer's instructions followed by 4-day stimulation with aCD3 (clone: OKT3, 1 µg/mL)

and aCD28 (clone: 28.2, 2 µg/mL) (eBioscience) in cRPMI at 2×10∧6 cells/mL in a 96-well plate (Corning). Cells were then stimulated with PMA/Ionomycin in the presence of BFA and monensin and stained for viability and combinations of extracellular and intracellular antibodies as described herein. Cells were acquired on a four laser LSR Fortessa cytometer (BD). Analysis was performed on FlowJo (V.10) using the Proliferation platform of FlowJo (V.10).

Immunofluorescence

Immunofluorescence was performed as described previously.[13] Briefly, synovial biopsies were fixed in 10% neutral-buffered formalin solution followed by paraffin embedding. Synovial tissue sections, 3 µm thick, were heated for 30 min at 60°C, deparaffinised in xylene and rehydrated in alcohol and deionised water. Antigen retrieval was performed by heating sections in antigen retrieval solution (15 mL of 1 M sodium citrate and 15 mL of 1 M citric acid in deionised water, pH 6.0) in a pressure cooker. Slides were washed in PBS for 5 min. Non-specific binding was blocked using 10% casein in PBS for 30 min. Primary antibodies aCD4 (DAKO, clone: 4B12) and aCD8 (abcam, clone: EP1150Y) were incubated on sections overnight at 4°C, matching sections incubated with secondary antibodies only were used as controls. Slides were washed in PBS/Tween followed by 1-hour incubation at room temperature with secondary Cy2 (catalogue 115-225-146) and Cy3 (catalogue 111-165-144) AffiniPure secondary antibodies (Jackson ImmunoResearch). Slides were washed with PBS/Tween and PBS, before counterstaining of nuclei with DAPI (MilliporeSigma) and cover slide mounting with ProLong Gold Antifade (Thermo Fisher Scientific). Stained cells were visualised with a Leitz DM40 microscope (Leica Microsystems), and images were captured using the AxioCam system and AxioVision V.3.0.6 software (Carl Zeiss).

Statistical analysis

Statistical analysis was performed using Prism V.7 (GraphPad) software. Unpaired two-tailed Mann-Whitney test and unpaired two-tailed standard Student's t-test were used as indicated. One-way and two-way analysis of variance with multiple comparisons were used as indicated when comparing more than two groups. Statistical significance was considered with p values of less than 0.05.

Study approval

Peripheral blood, synovial fluid and synovial tissue samples were collected from patients who were recruited from the Rheumatology Department, St. Vincent's University Hospital, UCD and Tallaght University Hospital, TCD. HC peripheral blood healthy volunteers recruited at Trinity Biomedical Sciences Institute and St. Vincent's University Hospital. All subjects gave fully informed written consent approved by the institutional Ethics Committee and research was performed in accordance with the Declaration of Helsinki.

Patient and public involvement

In this study we analysed peripheral and site specific immune cells obtained from patients with RA and a cohort of 'at-risk individuals'. While no patients were involved in setting the research question, the outcome measures or recruitment plans for the study, the group has hosted a number of patient information evenings where we have described the research, current project and the importance of patient engagement. No patients were asked to advice on interpretation or writing up of results, however, in collaboration with patient partners, we developed a series of patient partnership workshops where lay dissemination of the study research to relevant patient groups was performed, with patient feedback now incorporated in future studies.

RESULTS

Increased T follicular helper (Tfh) cell frequency in IAR and polyfunctional phenotype of RA patient synovial fluid Tfh cells

IAR are anticitrullinated protein antibody positive (ACPA) despite the lack of clinical inflammation. Due to their role in providing B cell help and shaping antibody responses, peripheral blood Tfh cells of IAR and RA patients were examined for evidence of polyfunctionality.[24] High dimensionality flow cytometric analysis was performed utilising the unsupervised algorithm t-SNE for data exploration and the identification of Tfh subpopulations (figure 1A). Peripheral blood Tfh cell ($CD4^+$ $CXCR5^+$) frequency is increased in IAR compared with HC (*p=0.018) and RA (*p=0.02) (figure 1B). Chemokine receptor expression, specifically expression of CCR6 and CXCR3, is a surrogate marker regarding the cytokine profile of Tfh cells. $CCR6^+$ $CXCR3^-$ Tfh share cytokine secretion characteristics with Th17 cells, $CCR6^-CXCR3^-$ with Th2 cells, $CCR6^-CXCR3^+$ with Th1 cells and $CCR6^+CXCR3^+$ Tfh cells demonstrating cytokine expression characteristic of both Th1 and Th17 cells.[25 26] While RA patient synovial fluid is dominated (***p<0.0001) by the recently described peripheral helper T cells (Tph) ($CXCR5^-PD1^+$), Tfh cells are present, although at a reduced frequency compared with peripheral blood (figure 1D, E).[27] Stark differences in the phenotype of RA patient synovial fluid Tfh cells were observed compared with peripheral blood with significantly (*p=0.02) increased frequency of $CXCR3^+$ $CCR6^+$ Tfh cells, indicative of a more plastic synovial Tfh response (figure 1C–F).

Comparable peripheral blood Treg cell frequency of HC, IAR and RA but increased memory to naïve Treg cell distribution in RA patient synovial fluid

Treg cells ($CD4^+CD39^{+/-}CD127^-FOXP3^+CD25^+$) were identified in the peripheral blood of HC, IAR and RA patients with unbiased, unsupervised t-SNE algorithm analysis of flow cytometric data followed by conventional gating (figure 2A, B). The aforementioned approach led to the identification of two Treg cell subpopulations based on the expression pattern of CD39, CCR7, PD-1 and CD45RO, naïve ($CD39^-CCR7^+PD\text{-}1^{-/low}CD45RO^-$) and memory Treg ($CD39^+CCR7^-PD\text{-}1^+CD45RO^+$) (figure 2C). Treg cell frequency as well as their distribution into naïve and memory compartments were comparable between patients with HC, IAR and RA (figure 2D, E). Interestingly, Treg cell frequency is significantly (**p=0.008) increased in the synovial fluid of patients with RA, compared with peripheral blood, however, naïve Treg cells are almost absent from the synovial fluid with a significant (**p<0.0001) increase in the memory compartment (figure 2F). Despite the increased frequency of synovial fluid Treg cells, the overwhelming memory phenotype could be detrimental to the regulation of the immune response due to the previously reported poor stability of FOXP3 expression of memory Treg cells.[28]

T cell activation and differentiation gene pathways are enriched in the synovial tissue prior to disease onset of RA

Due to the profound cellular, metabolic and environmental differences between the peripheral blood and the site of

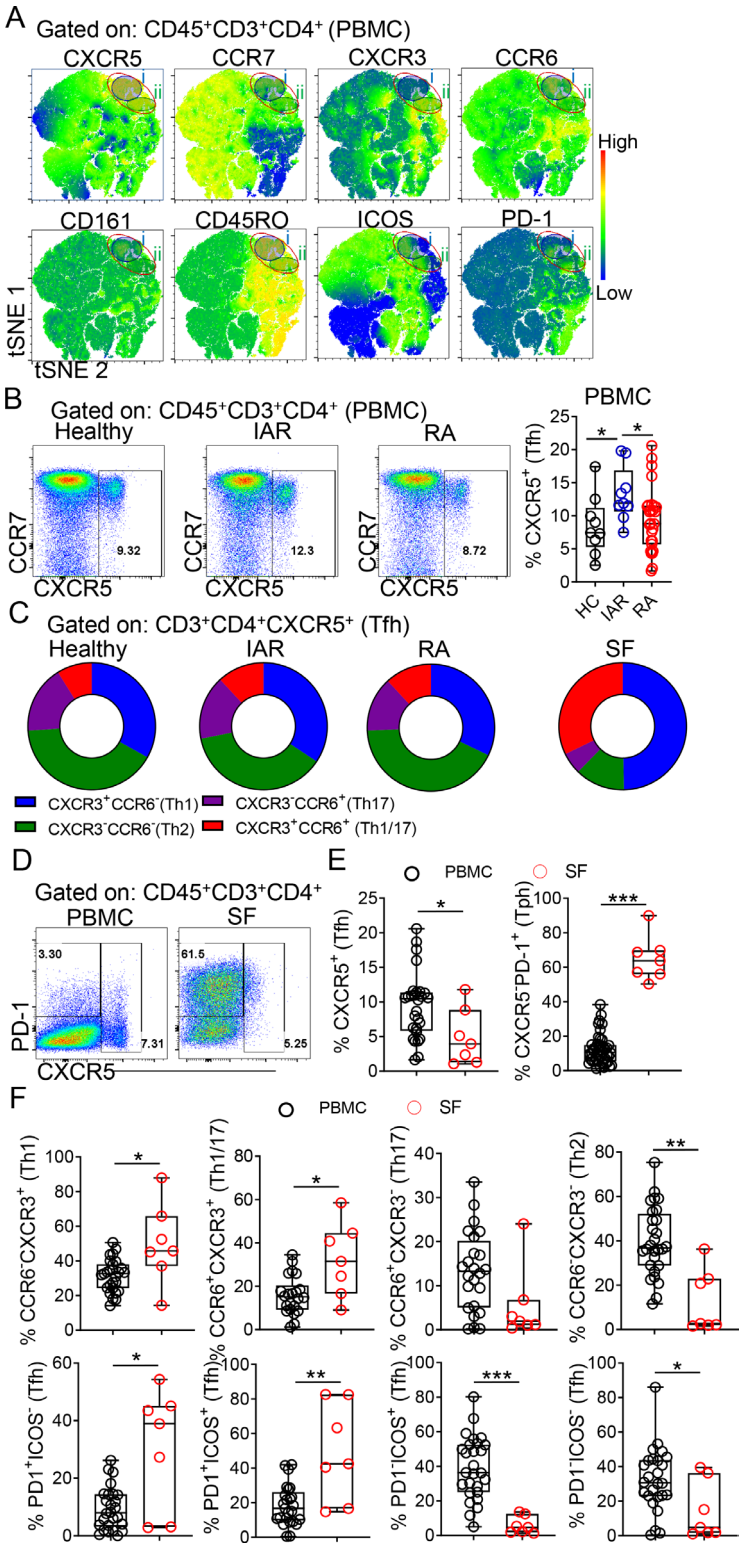

Figure 1 Tfh and Tph cell responses in peripheral blood monomuclear cells (PBMC) of IAR subjects and synovial fluid (SF) of patients with RA. (A) tSNE unsupervised algorithm analysis of RA patient's peripheral blood flow cytometric analysis for the identification of Tfh cells and subpopulations. (B) Frequency of Tfh ($CXCR5^+CD4^+$) cells in the peripheral blood of IAR (n=9), HC (n=9) subjects and RA (n=27) patients. Data are presented as Box and whiskers plots (min to max), symbols represent individual samples. Statistical analysis was performed by using Kruskal-Wallis test, $p<0.05$* was considered significant. (C) Average subpopulation distribution of HC, IAR, RA peripheral blood and RA synovial fluid Tfh cells on the basis of CCR6 and CXCR3 chemokine receptor expression. (D) Representative flow cytometric analysis of patient with RA peripheral blood and synovial fluid Tph ($CXCR5^-PD\text{-}1^+CD4^+$) and Tfh cells. (E) Cumulative flow cytometric analysis data on patient with RA peripheral blood and synovial fluid frequency of Tfh and Tph cells. (F) Patient with RA patient peripheral blood (n=27) and synovial fluid (n=7) subpopulation distribution of Tfh cells on the basis of expression of CXCR3 and CCR6 or PD-1 and ICOS. Data are presented as Box and whiskers plots (min to max), symbols represent individual samples. Statistical analysis was performed by using two-tailed Mann-Whitney test, $p<0.05$* was considered significant. HC, healthy control; IAR, individuals at risk; RA, rheumatoid arthritis.

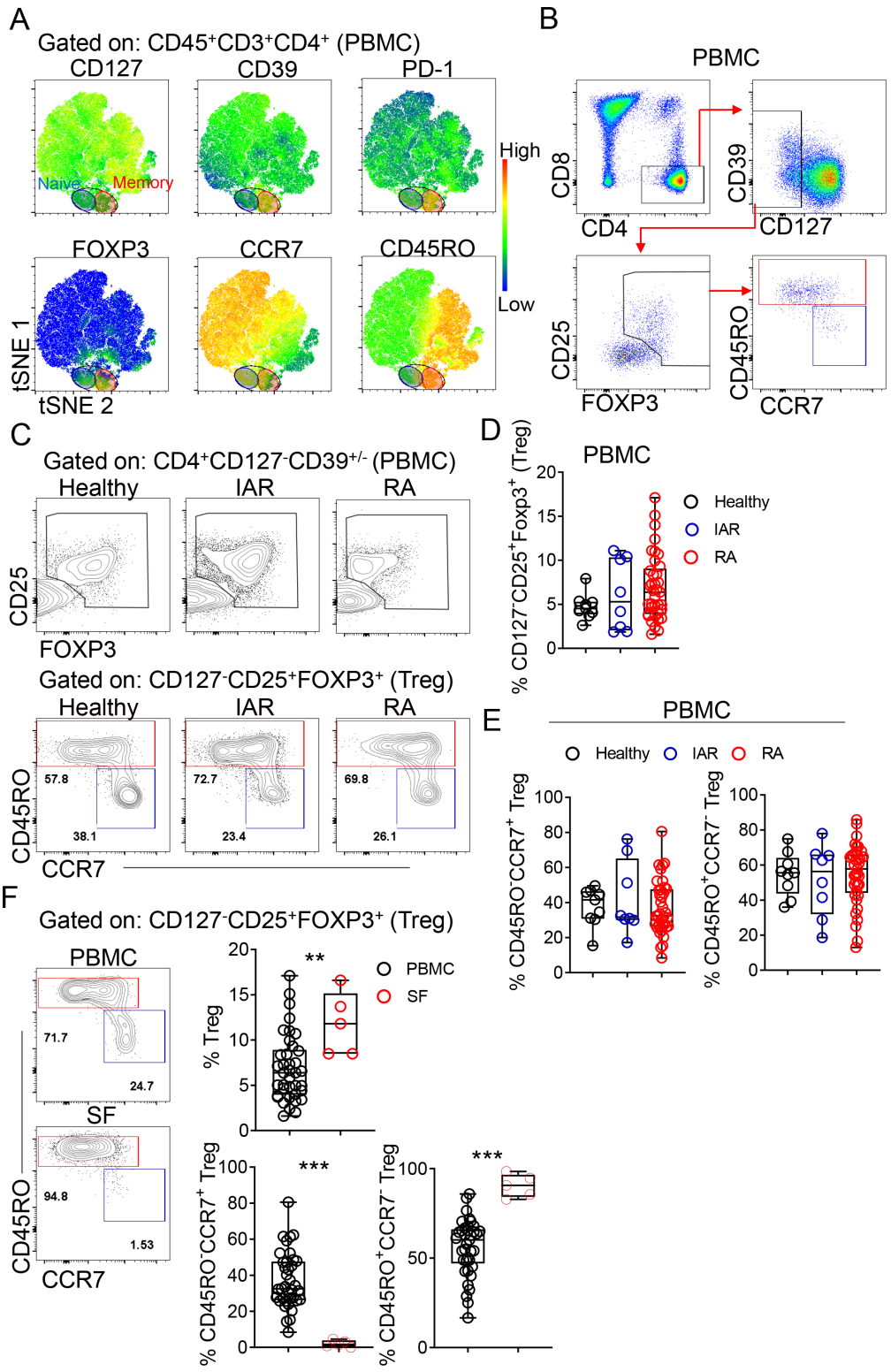

Figure 2 Treg cell frequency in synovial fluid (SF) of patients with RA and peripheral blood mononuclear cells (PBMC) of IAR subjects, RA patients and HC. (A) tSNE unsupervised algorithm analysis of RA patient's peripheral blood flow cytometric analysis for the identification of naïve and memory Treg. (B) Conventional gating followed for the characterisation of naïve and memory Treg following the exploratory identification of relevant markers with tSNE. (C) Representative flow cytometric analysis plots for Treg cell frequency and distribution into naïve (CD45RO-CCR7+) and memory (CD45RO+CCR7-/low) Treg of HC, IAR subjects and RA patients. (D) Peripheral blood Treg cell frequency and (E) their distribution into the naïve and memory Treg cell compartment for HC (n=9), IAR (n=8) and RA (n=38). Data are presented as Box and whiskers plots (min to max), symbols represent individual samples. Statistical analysis was performed by using Kruskal-Wallis test, $p<0.05$* was considered significant. (F) Representative flow cytometric analysis and cumulative data for the synovial frequency and naïve to memory compartment distribution of RA patient peripheral blood (n=38) compared with synovial fluid (n=5) Treg cells. Data are presented as Box and whiskers plots (min to max), symbols represent individual samples. Statistical analysis was performed by using two-tailed Mann-Whitney test, $p<0.05$* was considered significant. HC, healthy control; IAR, individuals at risk; RA, rheumatoid arthritis.

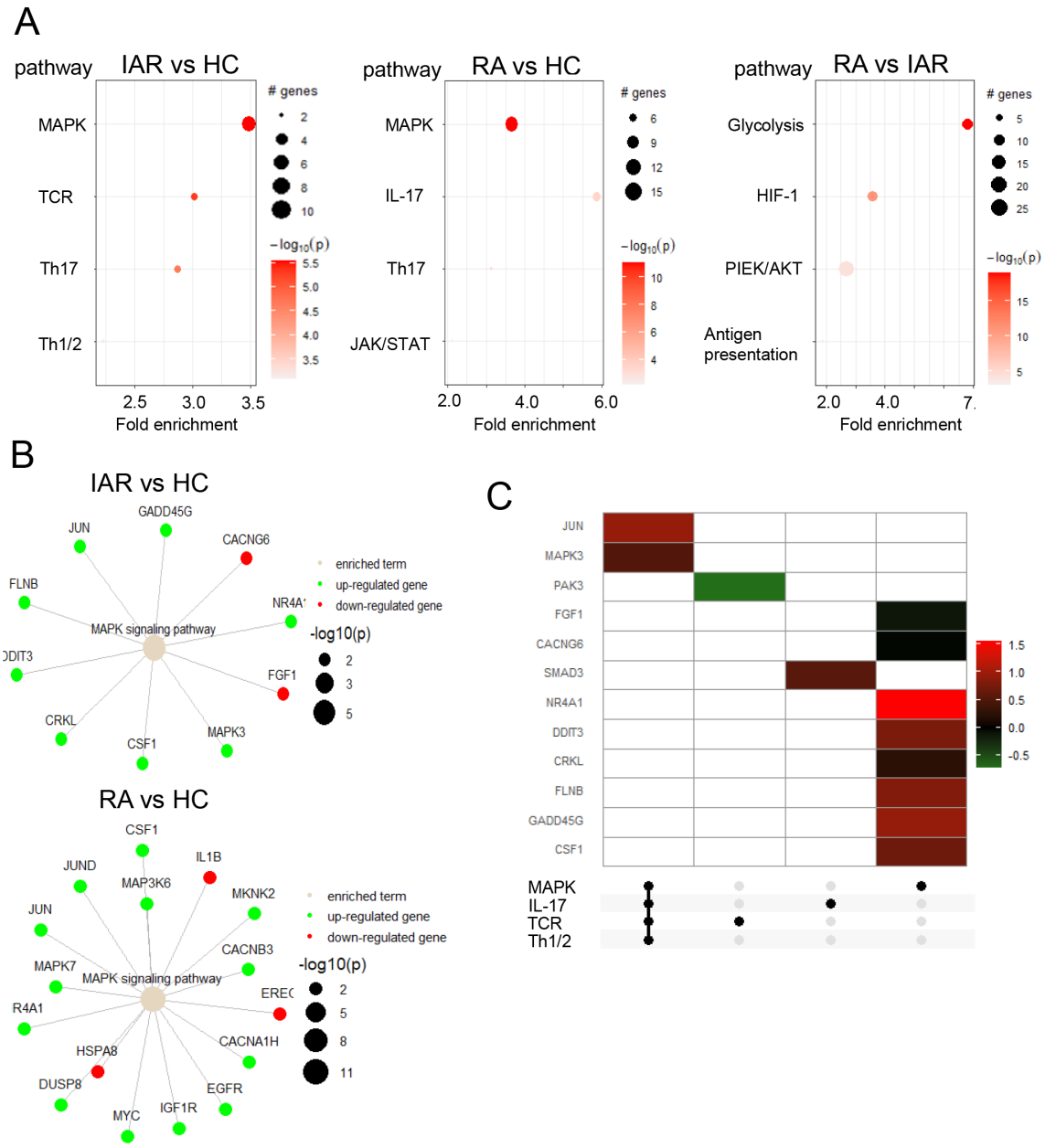

Figure 3 IAR and RA patient synovial tissue samples are enriched for T cell-related gene pathways. (A) Enrichment plots of RNAseq data pathway analysis for significantly upregulated gene pathways for IAR versus HC, RA versus HC and RA versus IAR. Dot size represents number of differentially regulated genes per pathway, colour intensity represents significance and x axis is indicative of the pathways fold enrichment change. (B) Term plot of the MAPK pathway for IAR versus HC and RA versus HC comparison. Only significantly upregulated (green) or downregulated (red) gene members of the pathway are shown. Gene dot size represents significance of change for the corresponding gene. (C) Upset plot highlighting differentially expressed genes of the IAR versus HC comparison and the involvement of those genes in the corresponding pathways. HC, healthy control; IAR, individuals at risk; RA, rheumatoid arthritis.

inflammation in RA, the inflamed joint, examination of synovial tissue T cell responses of IAR could provide a unique insight of T cell involvement at the very early stages of disease pathogenesis. Due to the rarity and low immune cell content of IAR synovial tissue biopsies, analysis of previously obtained single cell RNAseq data from 118 RA patients with active disease, 20 IAR and 44 HC was performed (figure 3). Comparing IAR to HC synovial tissue biopsies, 252 differentially expressed genes were identified. Of the 252 genes, 212 have known interactions belonging in 62 pathways with the KEGG database TCR signalling pathway having the highest p value and an estimated fold enrichment change of 3.1 in IAR compared with HC synovial tissue (figure 3A). Interestingly, pathway enrichment analysis of differentially expressed genes (4802 with 4146 genes with known interactions) in RA compared with HC synovial tissue shows enrichment in T cell activation and differentiation pathways similar to that of IAR (figure 3A, B).

Importantly, the progression from IAR to RA is potentially dependent on 748 differentially expressed genes (615 of known interactions) involved in metabolism (Glycolysis and HIF-1 signalling pathways) as well as key intracellular signalling pathways (PI3K-AKT signalling pathway) (figure 3A).

Highly upregulated genes (JUN, MAPK3) that are key members of several T cell activation-related pathways enriched in IAR and RA synovial tissue biopsies highlight potentially conserved mechanisms driving synovial inflammation from an

early point and should be considered as future targets for therapeutic intervention (figure 3C).

Synovial tissue CD4 T-cell polyfunctionality predates clinical disease onset of RA

RNAseq analysis of HC, RA and IAR synovial tissue biopsies revealed the involvement of T cell activation and polarisation pathways from a preclinical stage of disease. Therefore, we performed extensive flow cytometric analysis of T cell cytokine production for seven key $CD4^+$ T cell-derived cytokines (IL-2, TNF-α, IFN-γ, IL-17A, IL-22, IL-4, granulocyte-macrophage colony-stimulating factor (GMCSF) with potential involvement in RA disease pathogenesis. Characterisation of ex vivo peripheral blood CD4 T cell cytokine production in response to PMA/Ionomycin stimulation revealed comparable cytokine production between HC, IAR and RA patient's CD4 T cells, although with some notable exceptions (online supplemental figure S2). TNF production showed evidence of a stepwise increase with significantly higher expression in IAR compared with HC and RA compared with HC CD4 T cells (*p=0.038 and *p=0.039, respectively) (online supplemental figure S2A). Similarly, IL-2 and IL-22 showed signs of a stepwise increase with significantly higher expression of RA patients-derived CD4 T cells compared with HC (*p=0.024 and **p=0.003, respectively) (online supplemental figure S2A). In order to assess CD4 T cell polyfunctionality, supervised simplified presentation of incredibly complex evaluations (SPICE) algorithm analysis based on Boolean flow cytometric data gating was utilised. SPICE data visualisation revealed similar degree of CD4 T cell polyfunctionality (defined as the simultaneous production of at least three, four or five cytokines) between patients with HC, IAR and RA (online supplemental figure S2B).

The similarities of the peripheral blood CD4 T cell cytokine responses could, however, be misleading regarding the true nature of CD4 T cell involvement at the site of inflammation, the inflamed joint. Flow cytometric analysis of patient with HC, IAR and RA synovial tissue biopsies revealed a stepwise increase in the frequency of synovial $CD3^+$ T cells with a significant (***p=0.0009) enrichment in T cell frequency in patient with RA compared with HC synovial tissue biopsies (figure 4A). Interestingly, there were no observed differences in expression of all seven cytokines tested or polyfunctionality between IAR and RA synovial tissue T cells, indicating that CD4 T cell dysregulation and polyfunctionality pre-date the onset of clinical inflammation of the joint (figure 4B, D). Surprisingly, the small population of CD4 T cells present in the synovial tissue of HC showed remarkably high degree of cytokine production and polyfunctionality (figure 4). Qualitatively, however, HC CD4 T cell responses showed significantly high expression of the immunomodulatory cytokines IL-4 and GMCSF compared with IAR and RA patient synovial CD4 T cells (**p=0.004, **p=0.002 and **p=0.002, ***p<0.001) (figure 4B, D). The aforementioned IL-4 and GMCSF polyfunctionality of HC synovial tissue CD4 T cells raises the potential of immunomodulatory polyfunctionality since IL-4 has previously been shown to suppress neutrophil-induced tissue damage and combination of IL-4 and GMCSF can induce tolerogenic dentritic cells (DC).[29–32]

Synovial tissue frequency of highly polyfunctional $CD4^+$ $CD8^{dim}$ T cells correlates with disease severity in RA

The qualitative T cell polyfunctionality differences between HC and RA patient synovial tissue CD4 T cells described herein highlight the heterogeneity of polyfunctional T cell responses. In order to identify synovial tissue polyfunctional T cell subpopulations with either increased or unique polyfunctionality characteristics, unsupervised clustering of flow cytometric data was performed via the recently described algorithm FlowSOM.[33] Clustering of RA patient's synovial tissue T cells revealed highly polyfunctional T cell clusters with expression of CD4 and CD8 (figure 5). $CD4^+$ $CD8^{dim}$ T cells are present at low frequency in the peripheral blood but accumulate significantly (*p=0.015) at RA patient's synovial fluid (figure 5B, C). While there are no observed differences in the frequency of peripheral blood $CD4^+$ $CD8^{dim}$ T cells in RA and IAR compared with HC, there was a significant (*p=0.019) enrichment of these cells at patient with RA compared with HC synovial tissue biopsies (figure 5D). Flow imaging, a hybrid technology of flow cytometric analysis and immunofluorescence confirmed the expression of CD4 and CD8 by direct ex vivo RA patient peripheral blood and synovial fluid $CD4^+$ $CD8^{dim}$ T cells (figure 5E). $CD4^+$ $CD8^{dim}$ T cells' polyfunctionality was, in addition to FlowSOM, assessed by SPICE (figure 5F). IAR and RA synovial tissue $CD4^+$ $CD8^{dim}$ T cells are highly polyfunctional with significantly increased expression of IFN-γ (***p<0.001 and ***p<0.001, respectively), IL-2 (*p=0.02 and *p=0.01, respectively) but not GMCSF or TNF-α and only RA $CD4^+CD8^{dim}$ T cells showed increased IL-4 expression (**p=0.007) compared with their synovial tissue CD4 T cell counterparts. $CD4^+CD8^{dim}$ T cells from RA patient synovial fluid are not natural killer T cells (NKT) or TCRgd T cells, maintain high levels of CD69 and PD-1 expression and are detectable at ectopic lymphoid structures within the synovial tissue and show evidence of increased proliferative capacity compared with matched $CD4^+$ $CD8^-$ T cells (online supplemental figure S3). Further characterisation of RA patient's peripheral blood revealed that they are not enriched in Treg, Tfh or Tph cells compared with their $CD4^+$ T cell counterparts (online supplemental figure S4B and C). Interestingly, $CD4^+CD8^{dim}$ T cells belong to the T effector (T_{EM}) and differentiated memory (T_{TE}) compartments compared with their CD4 T cell counterparts, which show preference for the central memory (T_{CM}) and naïve (T_N) T cell compartments (*p=0.04 and *p=0.04, respectively) (online supplemental figure S4A). The aforementioned data show heterogeneity within the $CD4^+$ $CD8^{dim}$ T cell compartment and raise the possibility of T_{EM} $CD4^+$ $CD8^{dim}$ T cells with rapid effector function and (T_{TE}) cells with IFN-γ bias.[34]

Recent studies have highlighted the interplay between metabolic programming and T cell responses.[35 36] Therefore, we assessed the metabolic profile of ex vivo flow sorted RA patient peripheral blood $CD4^+$ $CD8^{dim}$ T cells by FLIM. $CD4^+$ $CD8^{dim}$ T cells have significantly (*p=0.01) increased reliance on OXPHOS than glycolysis compared with matched $CD4^+$ $CD8^-$ T cells, as observed by an increase in τ_{avg}, indicative of a memory T cell metabolic profile (figure 6A).[35] In order to assess the degree by which $CD4^+$ $CD8^{dim}$ and $CD4^+$ $CD8^-$ T cell cytokine production rely on a specific metabolic profile, sorted RA patient's $CD4^+$ $CD8^{dim}$ and $CD4^+$ $CD8^-$ T cells were treated with 2DG, oligomycin or FCCP prior flow cytometric analysis. FLIM was utilised to confirm the effectiveness of the metabolic inhibitors used (figure 6B). Interestingly, short-term inhibition of OXPHOS and not glycolysis led to significant reduction in TNF-α, IFN-γ, IL-2 and IL-17A expression by both $CD4^+$ $CD8^{dim}$ and $CD4^+$ $CD8^-$ T cells with FCCP, leading to greater reductions (figure 6C).

Importantly, the synovial tissue frequency of $CD4^+$ $CD8^{dim}$ T cells correlates significantly (**p=0.005) with RA patient's disease severity (figure 6D). It has previously been shown that polyfunctional synovial ex-Th17 cells are resistant to

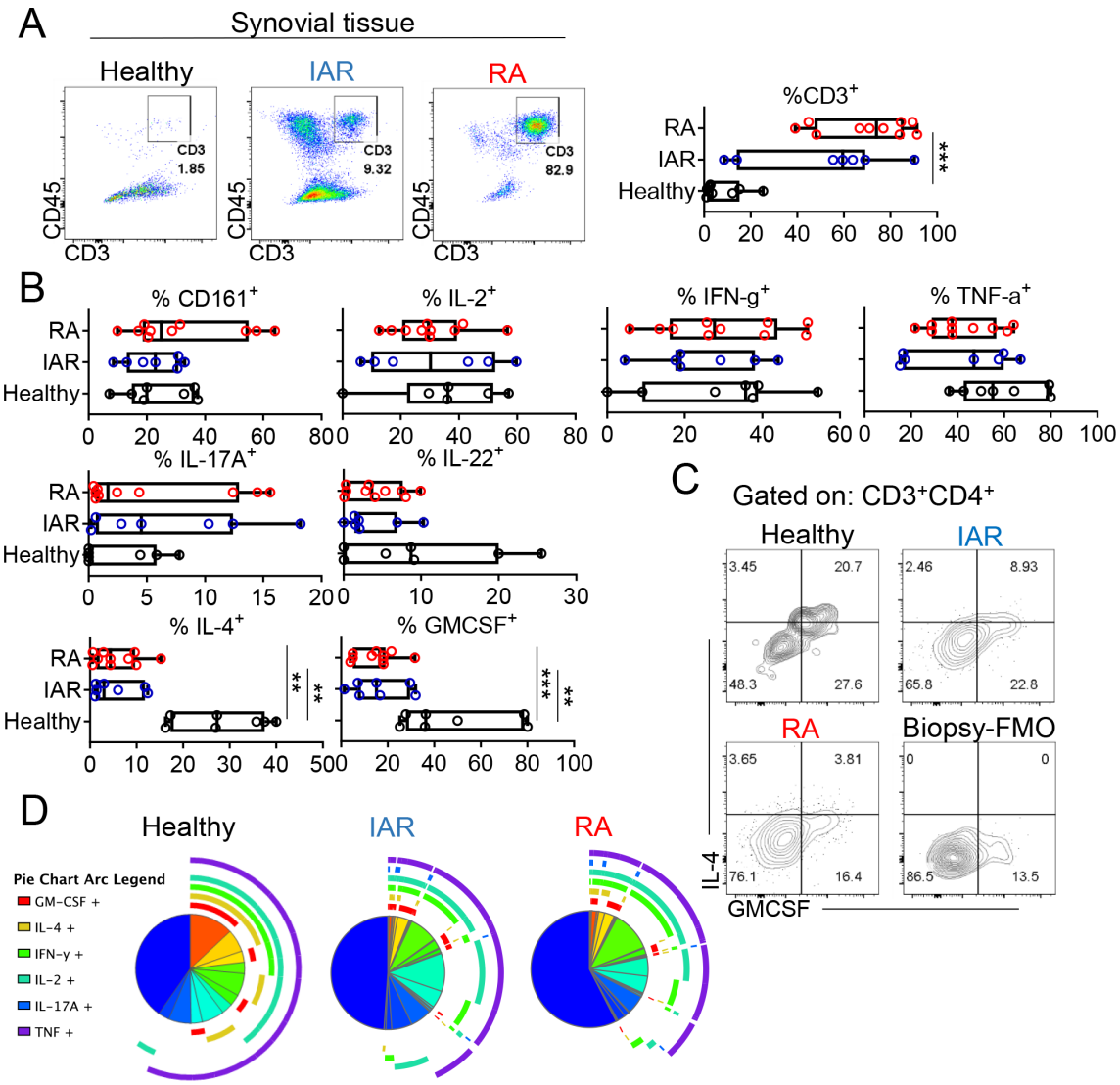

Figure 4 Polyfunctional CD4 T cell responses of HC, IAR and RA patient synovial tissue. (A) Representative flow cytometric analysis of synovial tissue single cell suspension and cumulative data for the frequency of $CD3^+$ cells in HC (n=7), IAR (n=7) and RA (n=10) patient synovial tissue biopsies. (B) Synovial tissue CD4 T cell expression of the indicated markers and cytokines following ex vivo stimulation of HC, IAR and RA patient synovial tissue single cell suspensions. (C) Representative flow cytometric analysis for IL-4 and GM-CSF cytokine expression by synovial tissue CD4 T cells. (D) Spice algorithm visualisation of synovial tissue CD4 T cell cytokine expression and frequency of polyfunctional CD4 T cells. Data are presented as box and whiskers plots (min to max), symbols represent individual samples. Statistical analysis was performed by using Kruskal-Wallis test, $p<0.05$* was considered significant. FMO, fluorescent minus one; HC, healthy control; IAR, individuals at risk; RA, rheumatoid arthritis.

suppression by Treg cells.[4] In order to investigate the susceptibility of $CD4^+CD8^{dim}$ T cells to Treg cell-mediated suppression, we sorted differentially labelled RA patient's $CD4^+$ $CD8^{dim}$ and $CD4^+CD8^-$ T cells and directly assessed their competitive capacity to proliferate in the presence of autologous Treg cells (figure 6F). $CD4^+$ $CD8^{dim}$ T cells showed evidence of limited resistance to Treg cell-mediated suppression compared with their $CD4^+$ $CD8^-$ T cell counterparts as exhibited by the significantly (*p=0.02) increased proliferation index (figure 6F).

The presence of $CD4^+$ $CD8^{dim}$ T cells in both IAR and RA synovial tissue, their absence in HC synovium, in addition to their high polyfunctionality and correlation with disease severity in RA, highlight their involvement in the pathogenesis of RA.

DISCUSSION

Recent advances in flow cytometric analysis and computational tools enabling high dimensionality data exploration, analysis and visualisation have contributed in efforts to identify and characterise polyfunctional T cell responses. Herein, we have presented evidence of T cell dysregulation, based on enrichment in signalling pathways involved in T cell activation and polarisation, at the site of inflammation in RA, prior to onset of clinical disease. The aforementioned T cell dysregulation is characterised by increased synovial tissue CD4 T cell polyfunctionality in RA and importantly, IAR. While, to date, T cell polyfunctionality has been linked with proinflammatory responses, our characterisation of HC synovial tissue CD4 T cells raises the potential for immunomodulatory polyfunctionality and suggests an early tip in the balance between 'protective' and pathogenic polyfunctional T cell responses in RA pathogenesis. Polyfunctional T cell research has been hindered due to lack of cell surface markers of polyfunctionality. Herein, we have identified a population of highly polyfunctional T cells that can be identified based on dual expression of CD4 and CD8. Importantly, this study highlights

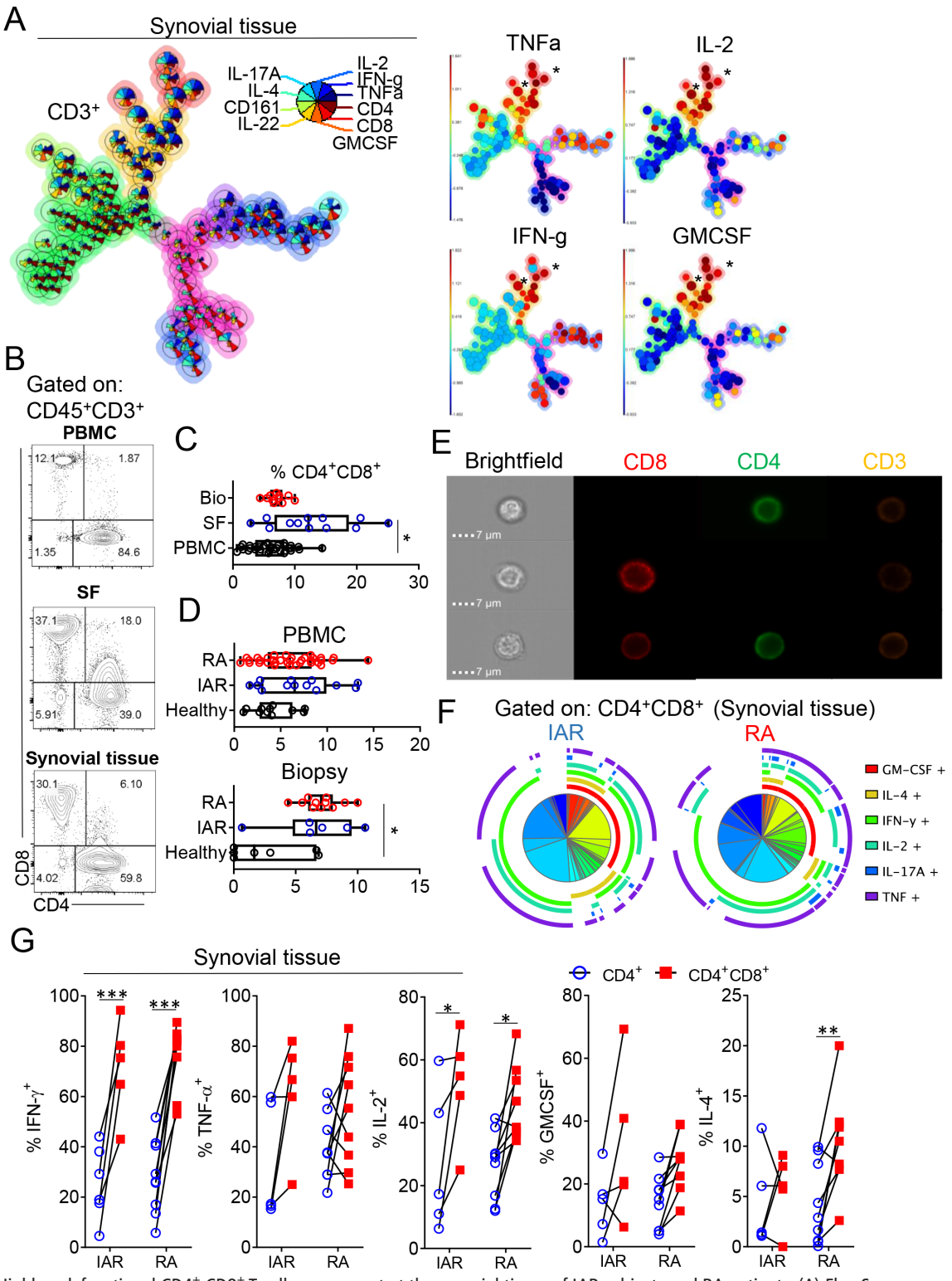

Figure 5 Highly polyfunctional CD4$^+$ CD8$^+$ T cells are present at the synovial tissue of IAR subjects and RA patients. (A) FlowSom unsupervised clustering algorithm analysis of RA patient synovial CD3$^+$ T cells for the identification of phenotypically distinct highly polyfunctional T cell clusters. Symbols indicate polyfunctional CD4$^+$CD8$^+$ DP T cells. (B) Representative flow cytometric analysis of paired RA patient peripheral blood mononuclear cells (PBMC), synovial fluid (SF) and synovial tissue CD3$^+$ T cells. (C) Frequency of CD4$^+$CD8$^+$ DP T cells in the periphery (n=34), synovial fluid (n=13) and synovial tissue (n=11), of RA patients. (D) Frequency of CD4$^+$CD8$^+$ DP T cells in the periphery and synovial tissue of HC (n=11 and n=7 respectively), IAR (n=13 and n=6 respectively), subjects and RA (n=34 and n=11 respectively) patients. (E) Representative imaging flow cytometry of RA patient synovial fluid CD4$^+$, CD8$^+$ and CD4$^+$CD8$^+$ DP T cells. (F) Representative simplified presentation of incredibly complex evaluations (SPICE) algorithm flow cytometric analysis data visualisation for IAR and RA synovial tissue biopsy CD4$^+$CD8$^+$ T cells. (G) Frequency of IAR (n=4–5) and RA (n=9) synovial tissue CD4$^+$ and paired CD4$^+$CD8$^+$ DP T cells expressing the indicated cytokines. Symbols indicate individual samples, two-way ANOVA with Sidak's multiple comparisons test was used (***p<0.001, **p=0.007, *p<0.05). ANOVA, analysis of variance; HC, healthy control; IAR, individuals at risk; RA, rheumatoid arthritis.

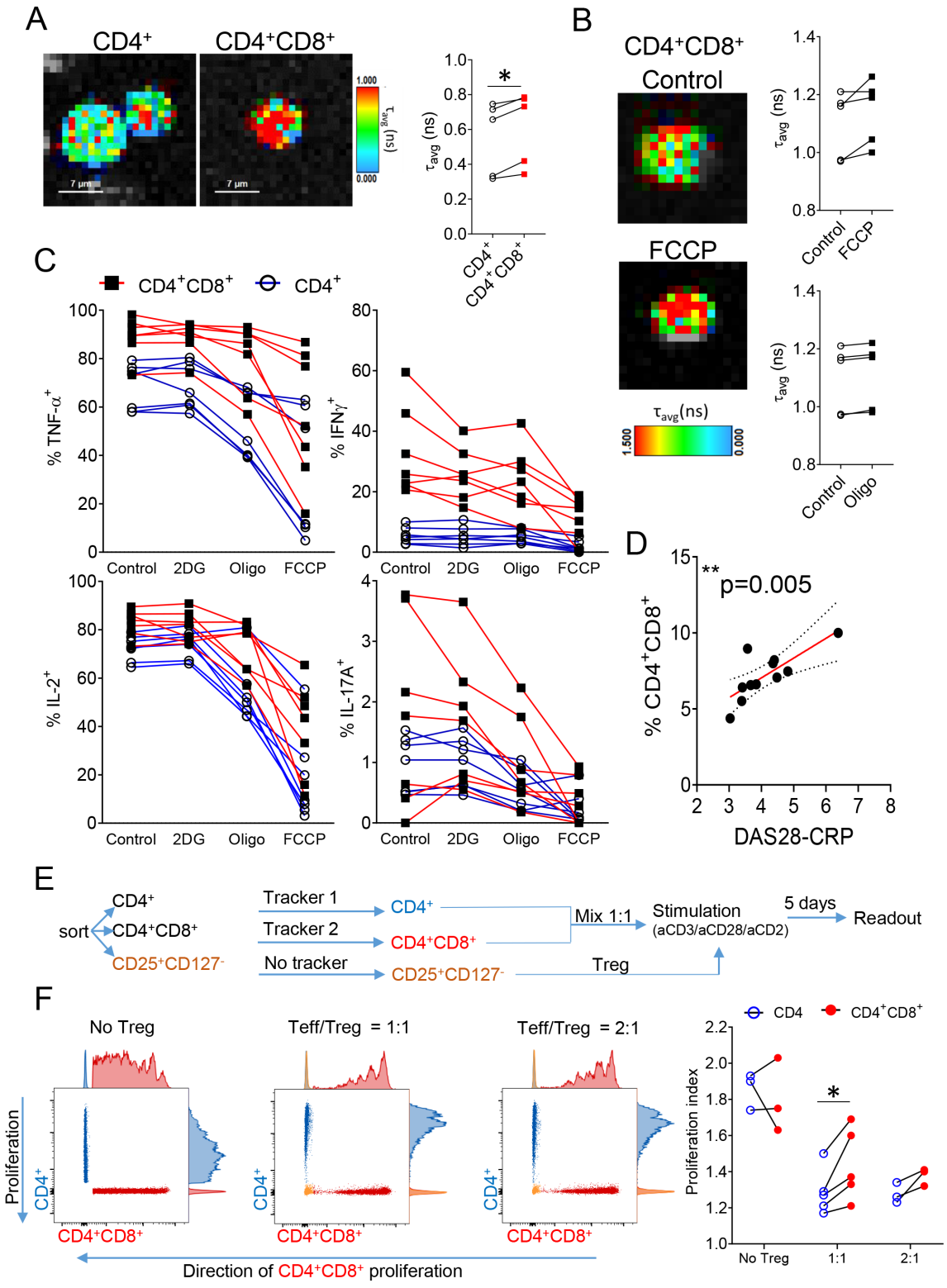

Figure 6 CD4$^+$ CD8$^+$ T cells are metabolically primed and correlate with disease severity in RA. (A) FLIM images and cumulative data of RA patient metabolic assessment of flow sorted peripheral blood CD4$^+$ and CD4$^+$CD8$^+$ DP T cells, (n=5). (B) FLIM images and data on flow sorted CD4$^+$CD8$^+$ DP T cells following treatment with carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) or oligomycin, symbols indicate individual samples (n=4). (C) Paired RA patient CD4$^+$ and CD4$^+$CD8$^+$ DP T cell cytokine responses following treatment with the indicated metabolic inhibitors, (n=7). (D) Linear regression graph for the synovial tissue frequency of RA (n=11) patient CD4$^+$CD8$^+$ DP T cells and disease severity score (DAS28-CRP). (E) Schematic of Treg suppression assay. Briefly, CD4$^+$ and CD4$^+$CD8$^+$ DP T cells were flow sorted differentially labelled and cultured with reducing number of autologous flow sorted Treg cells. Following a 5-day stimulation, T cell proliferation was assessed. (F) Flow cytometric analysis of CD4$^+$ and CD4$^+$CD8$^+$ DP T cells proliferating in the presence of Treg cells, n=3–5 per condition,). Symbols indicate individual samples, two-way ANOVA with Sidak's multiple comparisons test was used (*p<0.05). ANOVA, analysis of variance; FLIM, fluorescent lifetime imaging microscopy; RA, rheumatoid arthritis.

the heterogeneity of polyfunctional T cells and the need for further evaluation of their contribution to autoimmunity.

Understanding polyfunctional T cell responses is instrumental for several facets of the immune response, including response to pathogens, vaccine development and autoimmune disorders. Recent studies have highlighted the role of T cell polyfunctionality in a range of autoimmune disorders, including synovial inflammation of patients with psoriatic arthritis and peripheral blood of children with Down syndrome-associated arthritis.[9 10] Evidence of polyfunctional ex-Th17 cells present in the synovial fluid of patients with RA raised several questions regarding the extend of synovial tissue T cell polyfunctionality and, importantly, the timing of the emergence of polyfunctional T cells during the course of synovial inflammation.[3 4] Arthralgia subjects are ACPA-positive IAR of developing RA.[37] Therefore, the study of T cell responses at the synovial tissue of pre RA, arthralgia subjects offers the opportunity to identify early events of T cell dysregulation in disease pathogenesis. With the aforementioned opportunity, however, several challenges emerge, arthralgia and HC donor synovial tissue biopsies are rare and harbour relatively low numbers of infiltrating immune cells. Although the overall low number of cells recovered from HC synovial tissue biopsies and low frequency of T cells could be a confounding factor in the absence of $CD4^+$ $CD8^{dim}$ T cells from HC synovial biopsies, the unique polyfunctionality characteristics and their accumulation at the synovial tissue of IAR, emphasises their potential as therapeutic targets in RA. Additionally, while the majority of IAR will progress to RA, the timing of that progression can affect the intensity and qualitative characteristics of synovial tissue T cell polyfunctionality and by extension the degree of polyfunctional T cell contribution early in RA disease pathogenesis. In this study, we employed a novel non-invasive 2P-FLIM in order to probe cellular metabolism requiring lower cell numbers compared with commonly used extracellular flux assays[38 39]

T cell polyfunctionality is a by-product of the plasticity of T cell responses, a plasticity that is instrumental for the immune system's ability to respond to a wide array of external threats.[40] Importantly, plasticity is not confounded to a specific T cell population and is rather a crucial characteristic of T cell function including, Tfh cells. RA patient synovial fluid Tfh cells show a mixed phenotype indicative of a plastic response with increased frequency of Th1/Th17-like Tfh cells compared with peripheral blood. In addition, synovial tissue Treg cell responses are dominated by memory Treg cells that could also contribute to the poorly 'checked' synovial immune response due to the previously documented instability of memory Treg FOXP3 expression.[28]

Several aspects regarding the emergence of polyfunctional T cell responses remain elusive and have been hindered by the lack of cell surface markers for the identification of polyfunctional T cells. Previous studies have identified $CD4^+$ $CD8^{dim}$ T cells in the cerebrospinal fluid of multiple sclerosis patients, urological cancer patients, peripheral blood of RA patients and in virus-infected individuals.[41–44] However, little is known regarding the cytokine production, polyfunctionality and phenotype of $CD4^+$ $CD8^{dim}$ T cells. Herein, we have identified $CD4^+$ $CD8^{dim}$ T cells as highly polyfunctional, metabolically primed T cells with their frequency at the site of inflammation in RA correlating with disease severity. Additionally, $CD4^+$ $CD8^{dim}$ T cells demonstrate reliance to OXPHOS, a characteristic of memory T cells capable of rapid recall responses.[35] Although, previous studies have suggested that $CD4^+CD8^{dim}$ T cells are thymic escapees with the potential to give rise to autoreactive T cells several aspects of their ontogeny and function remains poorly understood.[45] Even though $CD4^+CD8^{dim}$ T cells represent a unique population of polyfunctional T cells, the ability to identify them without the need for intracellular cytokine staining offers opportunities for the future characterisation of transcriptional, metabolic and environmental contributors to T cell polyfunctionality.

The effect of treatment on the frequency and characteristics of synovial $CD4^+$ $CD8^{dim}$ T cells could provide further support regarding their contribution to disease pathogenesis and their potential to act as markers of response to treatment. The potential for translatable outcomes from these studies is, however, confounded by the requirement for large numbers of stratified patients, only achievable through a longitudinal multicentre approach. Longitudinal studies could provide further information regarding the contributing role of $CD4^+$ $CD8^{dim}$ T cells in the transition from IAR to RA. These cells, as reported herein, are present in the synovial tissue of IAR subjects and patients with RA with a similar cytokine profile, therefore, it remains to be investigated if confounding factors enhance $CD4^+$ $CD8^{dim}$ T cell pathogenicity and the transition from IAR to RA. Similarly, to other highly polyfunctional T cell populations, $CD4^+CD8^{dim}$ T cells show increased proliferative capacity and, importantly, limited resistance to suppression by autologous Treg cells compared with their CD4 counterparts.[4] Further studies are required in order to investigate the mechanism of DP T cell resistance to suppression by Treg cells and the potential contribution of this phenomenon to $CD4^+CD8^{dim}$ T cells' synovial accumulation.'

While the influencers of synovial tissue polyfunctionality remain elusive, the enrichment of common T cell activation and differentiation pathways in IAR and RA patient's synovial tissue biopsies indicate their potential as candidate regulators of polyfunctional T cell responses. Further characterisation of the synovial pathways identified in this study could lead to the development of methods for the therapeutic manipulation of pathogenic polyfunctional T cell responses.

The data presented, herein, highlight the potential contribution of T cell polyfunctionality early in the pathogenesis of RA and raise the possibility of future therapeutic interventions targeting $CD4^+CD8^{dim}$ T cells and the balance between protective and pathogenic polyfunctional T cells.

Twitter Mary Canavan @mary_canavan and Michael G Monaghan @drmgmonaghan

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ORCID iDs
Achilleas Floudas http://orcid.org/0000-0003-1690-5595
Michael G Monaghan http://orcid.org/0000-0002-5530-4998
Douglas J Veale http://orcid.org/0000-0003-2802-4971
Ursula Fearon http://orcid.org/0000-0001-8084-0429

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CLINICAL SCIENCE

Incidence and risk factors for herpes zoster in patients with rheumatoid arthritis receiving upadacitinib: a pooled analysis of six phase III clinical trials

Kevin L Winthrop ,[1] Peter Nash ,[2] Kunihiro Yamaoka,[3] Eduardo Mysler,[4] Nasser Khan,[5] Heidi S Camp,[5] Yanna Song,[5] Jessica L Suboticki,[5] Jeffrey R Curtis[6]

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[1]School of Public Health, Oregon Health & Science University, Portland, Oregon, USA
[2]Griffith University, School of Medicine, Gold Coast, Queensland, Australia
[3]Rheumatology and Infectious Diseases, University of Occupational and Environmental Health, Sagamihara, Kanagawa, Japan
[4]Rheumatology, Organización Medica de Investigación, Buenos Aires, Argentina
[5]Immunology, AbbVie Inc, North Chicago, Illinois, USA
[6]Rheumatology, University of Alabama at Birmingham, Birmingham, Alabama, USA

Correspondence to
Dr Kevin L Winthrop, School of Public Health, Oregon Health & Science University, Portland, Oregon, USA; winthrop@ohsu.edu

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ABSTRACT

Background Upadacitinib (UPA) is an oral Janus kinase (JAK) inhibitor approved for the treatment of rheumatoid arthritis (RA). JAK inhibitors have been associated with an increased risk of herpes zoster (HZ) in patients with RA.

Objectives To evaluate the incidence and risk factors for HZ in UPA-treated patients with RA from the UPA phase III clinical trial programme.

Methods Exposure-adjusted incidence/event rates for HZ were determined in patients receiving UPA (monotherapy or combination therapy) in six randomised phase III trials (data cut-off on 30 June 2020). HZ incidence and event rates were also determined in patients receiving methotrexate (MTX) monotherapy or adalimumab (ADA) + MTX. Multivariable Cox regression analysis was used to identify HZ risk factors in UPA-treated patients.

Results A total of 5306 patients were included in this analysis. The incidence rate of HZ/100 patient-years (95% CI) was 0.8 (0.3 to 1.9), 1.1 (0.5 to 1.9), 3.0 (2.6 to 3.5) and 5.3 (4.5 to 6.2), in the MTX monotherapy, ADA + MTX, UPA 15 mg and UPA 30 mg groups, respectively. The majority of HZ cases with UPA (71%) involved a single dermatome. Prior history of HZ and Asian region were HZ risk factors in UPA-treated patients.

Conclusion In the UPA phase III RA clinical programme, HZ incidence and event rates were higher with UPA versus ADA + MTX or MTX monotherapy, and higher with the 30 mg versus 15 mg dose. Patients from Asia and those with a history of HZ may be at increased risk of HZ while receiving UPA.

Key messages

What is already known about this subject?

- Upadacitinib (UPA) is an oral Janus kinase (JAK) inhibitor that is approved for the treatment of rheumatoid arthritis (RA) at a dose of 15 mg once daily.
- JAK inhibitors have been linked with an increased risk of herpes zoster (HZ) in patients with RA.

What does this study add?

- In this pooled safety analysis, which included 5306 patients with RA across the UPA phase III clinical trials, including patients who were methotrexate (MTX)-naïve and patients who had an inadequate response to conventional synthetic or biologic disease-modifying antirheumatic drugs, the rate of HZ was higher with UPA compared with MTX monotherapy or adalimumab + MTX, and higher with the 30 mg versus 15 mg dose.
- Risk factors for HZ among UPA-treated patients included Asian region and prior history of HZ (with both UPA doses).

How might this impact on clinical practice or future developments?

- Physicians should be aware of the risk of HZ in patients with RA receiving UPA, particularly those with risk factors.

INTRODUCTION

Herpes zoster (HZ) is a common and debilitating condition caused by reactivation of varicella zoster virus (VZV) and is frequently characterised by a painful vesicular dermatomal rash.[1 2]

The lifetime risk of HZ in the general population is around 30%, with increased risk in the elderly and those who are immunocompromised; in the last decade, incidence has increased globally among adults.[3] A common complication of HZ is postherpetic neuralgia, in which pain persists for months or years following resolution of the rash.[1 2] HZ can also cause complications beyond the skin, including neurological disorders such as meningoencephalitis and HZ oticus (Ramsay Hunt syndrome) and ophthalmic disorders such as uveitis and keratitis. In addition, immunocompromised patients may develop disseminated skin disease, which may also involve non-cutaneous organs such as the lungs or gastrointestinal tract.[1–3]

Patients with rheumatoid arthritis (RA) have approximately a twofold increased risk of HZ compared with the general population.[4 5] This risk can be further increased by immunomodulatory therapies prescribed for the treatment of RA, such as glucocorticoids, and some biologic disease-modifying antirheumatic drugs (bDMARDs).[4] More recently, Janus kinase (JAK) inhibitors, a class of targeted synthetic DMARDs that modulate

signalling downstream of cytokine receptors, have been linked with an increased risk of HZ in patients with RA.[6 7]

Upadacitinib (UPA) is an oral JAK inhibitor engineered to have greater selectivity for JAK1 versus JAK2, JAK3 and TYK2, and is approved for the treatment of RA at a dose of 15 mg once daily.[8 9] The aim of this analysis was to determine the incidence of HZ in the UPA phase III clinical trial programme, and to identify potential risk factors for the development of HZ in UPA-treated patients.

METHODS

Studies design and patients

Data from six randomised phase III trials were included in this analysis: SELECT-EARLY,[10] SELECT-NEXT,[11] SELECT-MONOTHERAPY,[12] SELECT-COMPARE,[13] SELECT-BEYOND[14] and SELECT-CHOICE[15] (online supplemental table S1). Patients were ≥18 years old with active RA and met the 2010 American College of Rheumatology (ACR)/European Alliance of Associations for Rheumatology (EULAR) classification criteria.[16] Active RA was defined as ≥6 swollen joints (of 66) and ≥6 tender joints (of 68), and a high-sensitivity C reactive protein level of ≥3 mg/L (≥5 mg/L for SELECT-EARLY and SELECT-COMPARE, which also required evidence of erosive joint damage and/or autoantibody positivity). The studies enrolled patients with RA who were methotrexate (MTX)-naïve (SELECT-EARLY), or had experienced an inadequate response or intolerance to conventional synthetic DMARDs (csDMARDs) (SELECT-NEXT), MTX (SELECT-MONOTHERAPY and SELECT-COMPARE) or bDMARDs (SELECT-BEYOND and SELECT-CHOICE).

Treatments

In SELECT-MONOTHERAPY, SELECT-EARLY, SELECT-NEXT and SELECT-BEYOND, patients received UPA 15 mg or 30 mg once daily. Patients in SELECT-MONOTHERAPY and SELECT-EARLY received UPA as monotherapy, while those in SELECT-NEXT and SELECT-BEYOND received UPA in combination with csDMARDs. The double-blind, controlled periods of these studies ranged from 12 to 48 weeks. Patients who switched from placebo (PBO) or MTX onto UPA were included in the UPA analysis set from the start of UPA treatment. No switching between different doses of UPA was permitted.

In SELECT-COMPARE, only the 15 mg once daily dose of UPA plus background treatment with MTX was assessed. The double-blind, controlled period was 48 weeks and blinded rescue treatment was permitted at weeks 14, 18, 22 and 26 in patients with an inadequate response ('non-responders'; those who achieved <20% improvement from baseline in swollen joint count and tender joint count); and at week 26 if they also did not achieve Clinical Disease Activity Index ≤10 ('incomplete responders'). Patients who switched from PBO or adalimumab (ADA) onto UPA were included in the UPA analysis set from the start of UPA treatment, and those who switched from UPA to ADA were included in the ADA dataset from the start of ADA treatment. In the UPA 15 mg group, 252/651 (38.7%) patients were incomplete responders or non-responders and switched from UPA to ADA, with 159/327 (48.6%) patients in the ADA group switching from ADA to UPA.[17]

In SELECT-CHOICE, the 15 mg once daily dose of UPA was assessed in patients receiving background csDMARDs. During the 24-week double-blind period, background medication could be added or adjusted in patients with an inadequate response. All patients completing the 24-week double-blind period were eligible to enter an open-label extension in which all patients received UPA 15 mg once daily; patients who switched from abatacept to UPA were included in the UPA dataset from the start of UPA treatment.

Statistical analysis

Treatment-emergent infection was defined as an onset date on or after the first dose of study drug and no more than 30 days (70 days for ADA) after last dose of study drug in cases of premature discontinuation. The duration of the follow-up period differs due to the respective half-lives of UPA (30 days >5 half-lives) and ADA (70 days = 5 half-lives). Non-serious HZ events did not result in mandatory study termination. However, any serious infections (including HZ), if not able to be controlled within 2 weeks, were a reason for discontinuation. HZ recurrence was defined as an event ≥91 days after the first episode.

Rates of treatment-emergent infections for UPA 15 mg once daily (monotherapy or in combination with csDMARDs), UPA 30 mg once daily (monotherapy or in combination with csDMARDs), MTX monotherapy (SELECT-EARLY only) and ADA + MTX (SELECT-COMPARE only) were summarised in terms of exposure-adjusted incidence rate (EAIR), which was calculated as the total number of patients in a particular treatment group who experienced an event adjusted for exposure (with exposure time censored at the first event), and is expressed as n/100 patient-years (PY). Exposure-adjusted event rates (EAERs) were also calculated, defined as the total number of events (including multiple events in the same patient) adjusted for total exposure (number of days on drug), and are expressed as E/100 PY. Events were attributed to the treatment the patient was taking when the event occurred. HZ incidence by baseline concomitant glucocorticoid and/or MTX use and HZ recurrence were also analysed among UPA-treated patients only.

Time to first HZ event with UPA was estimated using Kaplan–Meier analysis. Risk factors for HZ in UPA-treated patients were assessed using multivariable Cox regression models, with baseline MTX use, baseline glucocorticoid dose, prior history of HZ, region, age, body mass index (BMI) and sex as covariates. The covariates were prespecified and no model selection method was applied. HZ onset and change in Disease Activity Score in 28 joints with C reactive protein (DAS28(CRP)) over time was assessed using a multivariable Cox regression model with time to first HZ as response and DAS28(CRP) change from baseline as a time-varying covariate. Prior HZ and HZ vaccination status were captured at baseline as part of the patient's medical history.

Patient and public involvement

This research was done without patient and public involvement.

RESULTS

Patients

A total of 5306 patients were included in this analysis, of whom 314 patients received MTX monotherapy (637.4 PY), 579 patients received ADA + MTX (1051.8 PY), 3209 received UPA 15 mg once daily (7023.8 PY exposure) and 1204 received UPA 30 mg once daily (3091.6 PY) at the time of data cut-off (30 June 2020). The majority of patients were female, and mean age was 53.3–61.1 years across the treatment groups (table 1). Around half of the patients were receiving concomitant glucocorticoids at baseline, with a mean dose of ≥5 mg/day across all groups (table 1). Fewer than 5% of patients reported prior HZ vaccination.

Table 1 Baseline demographics and disease characteristics of patients with and without treatment-emergent HZ event

Characteristic	MTX monotherapy		ADA 40 mg EOW + MTX		Any UPA 15 mg QD		Any UPA 30 mg QD	
	Patients with HZ (n=5)	Patients without HZ (n=309)	Patients with HZ (n=11)	Patients without HZ (n=568)	Patients with HZ (n=204)	Patients without HZ (n=3005)	Patients with HZ (n=150)	Patients without HZ (n=1054)
Female, n (%)	3 (60.0)	237 (76.7)	9 (81.8)	461 (81.2)	174 (85.3)	2407 (80.1)	113 (75.3)	835 (79.2)
Age, years, mean/median	54.8 (8.8)/56.0	53.3 (13.0)/55.0	61.1 (14.3)/62.0	54.0 (11.6)/55.0	57.7 (11.4)/59.0	54.1 (12.0)/55.0	55.9 (10.8)/57.0	55.2 (12.0)/56.0
Time since RA diagnosis, years	3.0 (5.7)	2.6 (5.1)	11.3 (11.9)	8.1 (7.9)	8.0 (8.1)	8.5 (8.4)	7.6 (8.7)	7.0 (8.3)
BMI, kg/m^2, mean/median	27.1 (4.3)/27.7	28.0 (6.4)/27.0	28.1 (4.8)/27.4	29.5 (7.2)/28.0	27.5 (5.6)/26.8	29.2 (6.7)/28.1*	28.3 (7.1)/26.8	29.4 (7.0)/28.4†
Region, n (%)								
North America	1 (20.0)	45 (14.6)	1 (9.1)	121 (21.3)	55 (27.0)	760 (25.3)	46 (30.7)	383 (36.3)
South/Central America	1 (20.0)	89 (28.8)	4 (36.4)	122 (21.5)	39 (19.1)	686 (22.8)	12 (8.0)	141 (13.4)
Europe	2 (40.0)	120 (38.8)	3 (27.3)	275 (48.4)	64 (31.4)	1301 (43.3)	52 (34.7)	428 (40.6)
Asia	0	32 (10.4)	2 (18.2)	16 (2.8)	34 (16.7)	109 (3.6)	30 (20.0)	55 (5.2)
Japan	0	28 (9.1)	0	0	16 (7.8)	46 (1.5)	17 (11.3)	41 (3.9)
Korea	0	0	1 (9.1)	5 (0.9)	10 (4.9)	19 (0.6)	5 (3.3)	7 (0.7)
China/Taiwan/Hong Kong	0	4 (1.3)	1 (9.1)	8 (1.4)	7 (3.4)	37 (1.2)	8 (5.3)	7 (0.7)
Malaysia	0	0	0	3 (0.5)	1 (0.5)	7 (0.2)	0	0
Other	1 (20.0)	23 (7.4)	1 (9.1)	34 (6.0)	12 (5.9)	149 (5.0)	10 (6.7)	47 (4.5)
Prior history of HZ, n (%)	0 (0)	4 (1.3)	1 (9.1)	11 (1.9)	16 (7.8)	50 (1.7)	18 (12.0)	23 (2.2)
History of HZ vaccination, n (%)	0	4 (1.4)‡	1 (10.0)§	14 (2.7)¶	12 (6.3)**	79 (2.8)††	10 (7.1)‡‡	61 (6.1)§§
DAS28(CRP)	6.3 (0.9)	5.9 (1.0)	5.7 (0.7)	5.9 (1.0)¶¶	5.7 (1.0)	5.8 (1.0)***	5.6 (1.0)	5.7 (1.0)†††
Concomitant csDMARDs at baseline, n (%)	N/A	N/A	11 (100.0)	567 (99.8)	143 (70.1)	2405 (80.0)	82 (54.7)	479 (45.4)
Concomitant glucocorticoids at baseline, n (%)	3 (60.0)	161 (52.1)	7 (63.6)	342 (60.2)	105 (51.5)	1656 (55.1)	77 (51.3)	494 (46.9)
Baseline glucocorticoid dose, mg/day‡‡‡	5.0 (0.0)	6.4 (2.4)	6.8 (3.1)	6.4 (2.4)	5.8 (2.3)	6.2 (2.5)	6.0 (2.5)	6.4 (3.4)

Values are mean (SD) unless otherwise stated. Percentages are calculated on non-missing values.
*n=3000.
†n=1048.
‡n=294.
§n=10.
¶n=522.
**n=189.
††n=2773.
‡‡n=141.
§§n=1000.
¶¶n=564.
***n=2990.
†††n=1047.
‡‡‡In patients receiving glucocorticoids at baseline.
ADA, adalimumab; BMI, body mass index; csDMARD, conventional synthetic disease-modifying antirheumatic drug; DAS28(CRP), Disease Activity Score in 28 joints with C reactive protein; EOW, every other week; HZ, herpes zoster; MTX, methotrexate; QD, once daily; RA, rheumatoid arthritis; UPA, upadacitinib.

Overview of infections

The rates of infections and serious infections observed with UPA 15 mg were similar to those observed with MTX monotherapy or ADA + MTX, whereas they were lower than with UPA 30 mg (figure 1). Opportunistic infection (excluding oral candidiasis, tuberculosis and HZ) rates and rates of active tuberculosis were similar across all treatment groups. HZ events occurred with greatest frequency in the UPA 30 mg once daily group (150/1204 (12.5%)), followed by the UPA 15 mg (204/3209 (6.4%)), ADA + MTX (11/579 (1.9%)) and MTX monotherapy (5/314 (1.6%)) groups, with similar comparative results seen using EAIRs (figure 1) and EAERs (online supplemental figure S1).

Overview of HZ in patients receiving UPA

In the UPA 15 and 30 mg once daily groups, 12 (0.3%) and 15 (1.2%) patients had a serious HZ event, respectively. Disseminated HZ (with cutaneous involvement only) occurred in 12 (5.9%) and 11 (7.3%) patients in the UPA 15 mg and 30 mg groups, respectively (table 2). Approximately 75% of HZ cases in patients receiving UPA, and 100% and 82% of cases, in patients receiving MTX monotherapy or ADA + MTX, respectively, involved a single dermatome. Of the total events, ophthalmic involvement occurred in 13 (6.4%) and 3 (2.0%) patients in the UPA 15 and 30 mg once daily groups, respectively, and unilateral involvement with multiple dermatomes was seen in 35 (17.2%) and 27 (18.0%) cases. One case of HZ meningitis was reported in a 64-year-old, male, Japanese patient who had received UPA 30 mg once daily for approximately 18 months and was also receiving loxoprofen 100 mg as required. The patient was withdrawn from the study, and the event resolved after hospital treatment. Of patients who experienced HZ, postherpetic neuralgia was observed in 17/235 events (7.2%) and 13/181 events (7.2%) in the UPA 15 and 30 mg once daily groups, respectively. Hospitalisation due to HZ occurred in 11/235 events (4.7%) in the UPA 15 mg group and in 15/181 events (8.3%) in the UPA 30 mg group.

No clear pattern in the timing of HZ onset with UPA was evident from Kaplan–Meier analysis (figure 2). As expected, the cumulative probability of HZ increased with time, with probabilities of 3.3% (95% CI: 2.7% to 4.0%), 6.7% (95% CI: 5.7% to 7.8%), 8.5% (95% CI: 7.4% to 9.7%) and 10.0% (95% CI: 8.4% to 11.7%) with UPA 15 mg at 1, 2, 3 and 4 years, respectively.

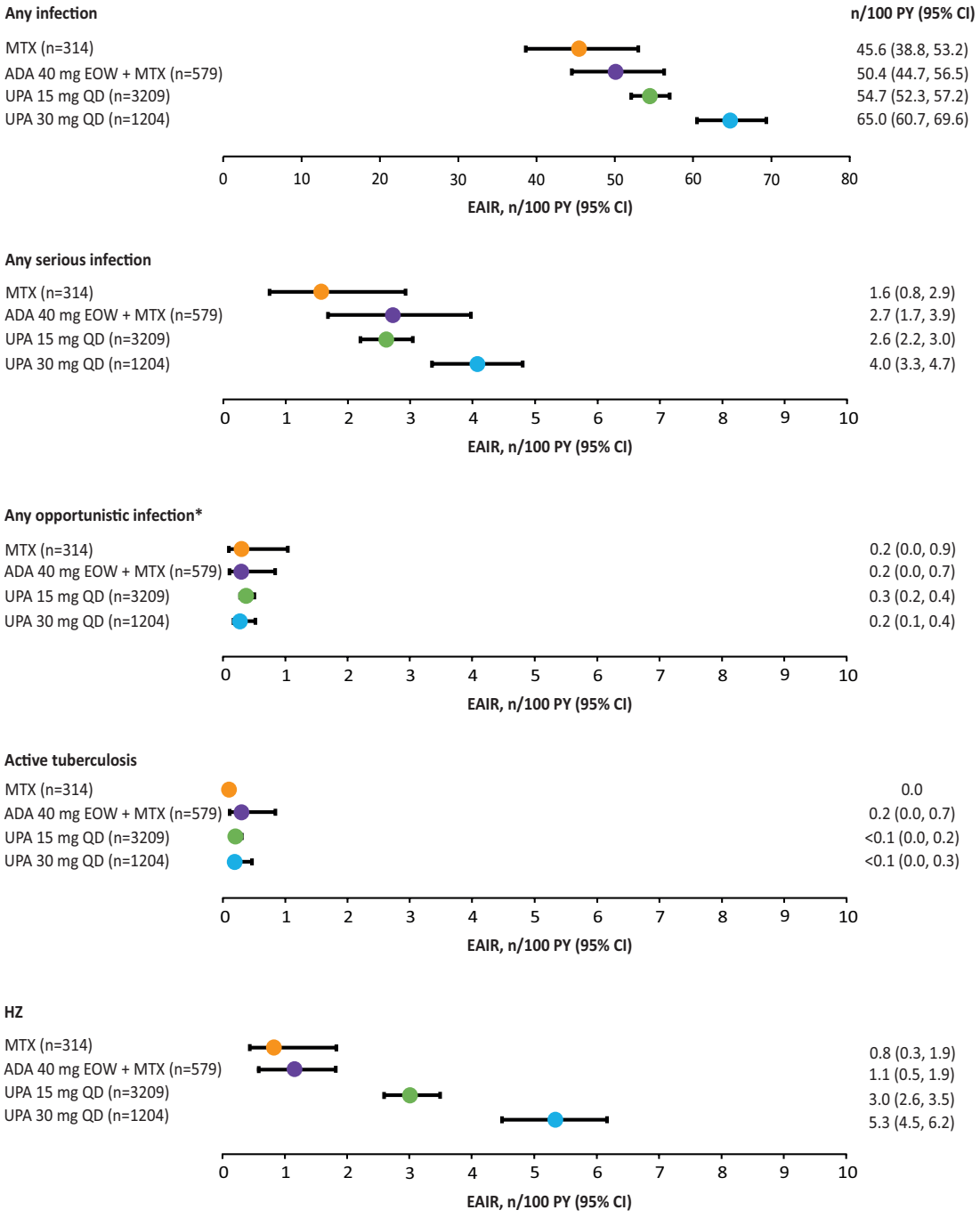

Figure 1 Exposure-adjusted incidence rates of infection. *Excluding oral candidiasis, tuberculosis and HZ. One case of cytomegalovirus infection was observed in the UPA 15 mg arm. ADA, adalimumab; EAIR, exposure-adjusted incidence rate; EOW, every other week; HZ, herpes zoster; MTX, methotrexate; PY, patient years; QD, once daily; UPA, upadacitinib.

In patients who experienced HZ during an UPA trial, 6.4% (13/204) and 9.3% (14/150) had a recurrence of HZ in the UPA 15 and 30 mg once daily groups, respectively, at a median follow-up time after first HZ episode of 476 and 593 days, respectively. Of the 13 recurrences in the UPA 15 mg once daily group, only one was more severe than the initial event; no recurrences in the 30 mg once daily group were more severe than the initial event. The mean age of patients with recurrent HZ was 58.7 years (median 59.0 years), mean BMI was 26.9 kg/m^2 and 23/27 (85.2%) were female. Fifteen (55.6%) were receiving concomitant glucocorticoids at baseline, at a mean dose of 6.3 mg/day.

Risk factors for HZ in patients receiving UPA

Multivariable Cox regression analysis showed that prior history of HZ and Asian region were associated with an increased risk of HZ in both the UPA groups (figure 3). In patients with a prior history of HZ, EAIRs of HZ were 13.9 (95% CI: 8.0 to 22.6) and 22.5 (95% CI: 13.4 to 35.6), respectively, with HRs of 3.3 (1.9–5.6) in the UPA 15 mg group and 3.4 (2.0–5.8) in the UPA 30 mg group compared with patients without a prior history of HZ. In patients from Asia, HZ EAIRs were 10.7 (7.4–14.9) and 17.0 (11.5–24.2), respectively, with Asian patients at a significantly greater HZ risk than patients in other regions (figure 3).

Table 2 Summary of extent of involvement in patients with HZ

Categories, n (%)*	MTX monotherapy (n=314)	ADA 40 mg EOW + MTX (n=579)	Any UPA 15 mg QD (n=3209)	Any UPA 30 mg QD (n=1204)
Total patients with ≥1 HZ event	5 (1.6)	11 (1.9)	204 (6.4)	150 (12.5)
Single dermatome	5 (100)	9 (81.8)	153 (75.0)	110 (73.3)
Ophthalmic involvement	0	2 (18.2)	13 (6.4)	3 (2.0)
Meningoencephalopathic involvement	0	0	0	1 (0.7)
Unilateral involving multiple dermatomes†	0	0	35 (17.2)	27 (18.0)
HZ oticus (Ramsay Hunt syndrome)	0	0	2 (1.0)	1 (0.7)
Disseminated, cutaneous only (no CNS involvement)‡	0	1 (9.1)	12 (5.9)	11 (7.3)
Disseminated with other non-cutaneous organ involvement (no CNS involvement)§	0	0	0	0
Postherpetic neuralgia¶	0	1 (8.3)	17 (7.2)	13 (7.2)
Hospitalisations because of HZ event¶	0	0	11 (4.7)	15 (8.3)
Missing	0	0	5 (2.5)	2 (1.3)

*Patients may fall into >1 category.
†≤2 adjacent dermatomes.
‡≥3 dermatomes, unilateral non-adjacent dermatomes or bilateral dermatomes.
§Involvement of visceral organs (eg, HZ pneumonia or HZ hepatitis).
¶Percentages based on total number of HZ events.
ADA, adalimumab; CNS, central nervous system; EOW, every other week; HZ, herpes zoster; MTX, methotrexate; QD, once daily; UPA, upadacitinib.

Of patients from Asia who developed HZ, the majority were from Japan (although Japanese patients made up the majority of patients from Asia in the study). The EAIRs of HZ in Japanese patients were 10.7 (6.1–17.3) in the UPA 15 mg once daily group and 13.6 (7.9–21.8) in the UPA 30 mg once daily group. Female sex, older age and North American region (vs European region) were also significant risk factors in the UPA 15 mg group only (figure 3).

There was no clear relationship observed between incidence of HZ and concomitant glucocorticoids and/or MTX among UPA-treated patients (figure 3 and online supplemental figure S2). The lack of association between glucocorticoid use and HZ was also observed in univariate analyses (online supplemental figure S3) and in a subanalysis of UPA-treated patients who did not have a prior history of HZ (online supplemental figure S4). In addition, there was no significant association between HZ onset and change in DAS28(CRP) over time (HR 1.0 (0.9–1.2)) in UPA-treated patients.

DISCUSSION

We evaluated the risk of HZ for UPA, a JAK inhibitor, within the phase III clinical trial programme for RA. We identified a dose-dependent risk of HZ with UPA relative to the active comparators MTX monotherapy and ADA in combination with MTX. Similar to HZ reported with other JAK inhibitors, the majority of HZ cases in UPA-treated patients were non-serious and involved a single dermatome. Asian region was a risk factor for HZ among UPA-treated patients (compared with Europe), and a history of HZ before study entry was also a strong risk factor for developing HZ after UPA initiation, a risk factor not previously evaluated in other JAK inhibitor HZ studies. Furthermore, in contrast to previous studies with JAK inhibitors, we did not find an association between HZ and concomitant use of glucocorticoids.

In general, Asian populations appear to have a higher rate of HZ compared with other populations: in a recent systematic review, the highest incidences of HZ were found in the Asia-Pacific region, although there was significant variation within this region and high rates were also observed in Caucasian populations.[18] This apparent increased risk of HZ in Asian populations, particularly Japanese patients, versus other geographical areas has also been previously observed in Japanese and Korean patients receiving baricitinib, peficitinib and tofacitinib,[19–22] as well as with Japanese patients receiving UPA.[23 24] The reasons for this are currently unknown, although it has been suggested that genetic predisposition, regional differences in reporting and

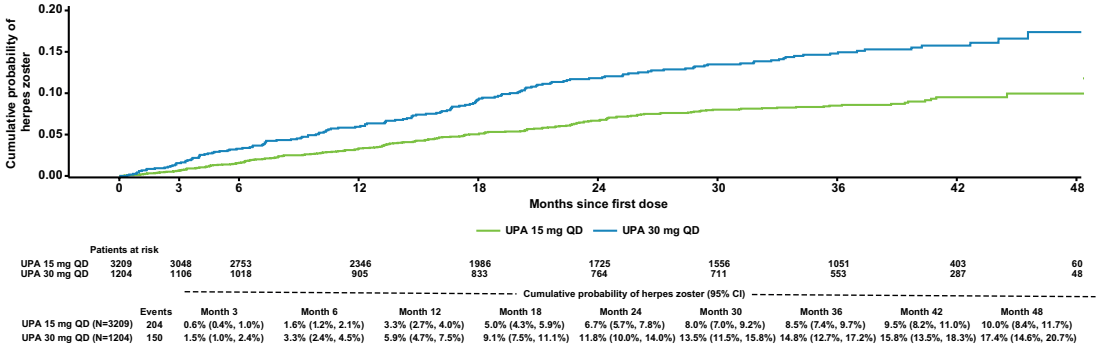

Figure 2 Kaplan–Meier estimate for time to HZ event in patients who received UPA. HZ, herpes zoster; QD, once daily; UPA, upadacitinib.

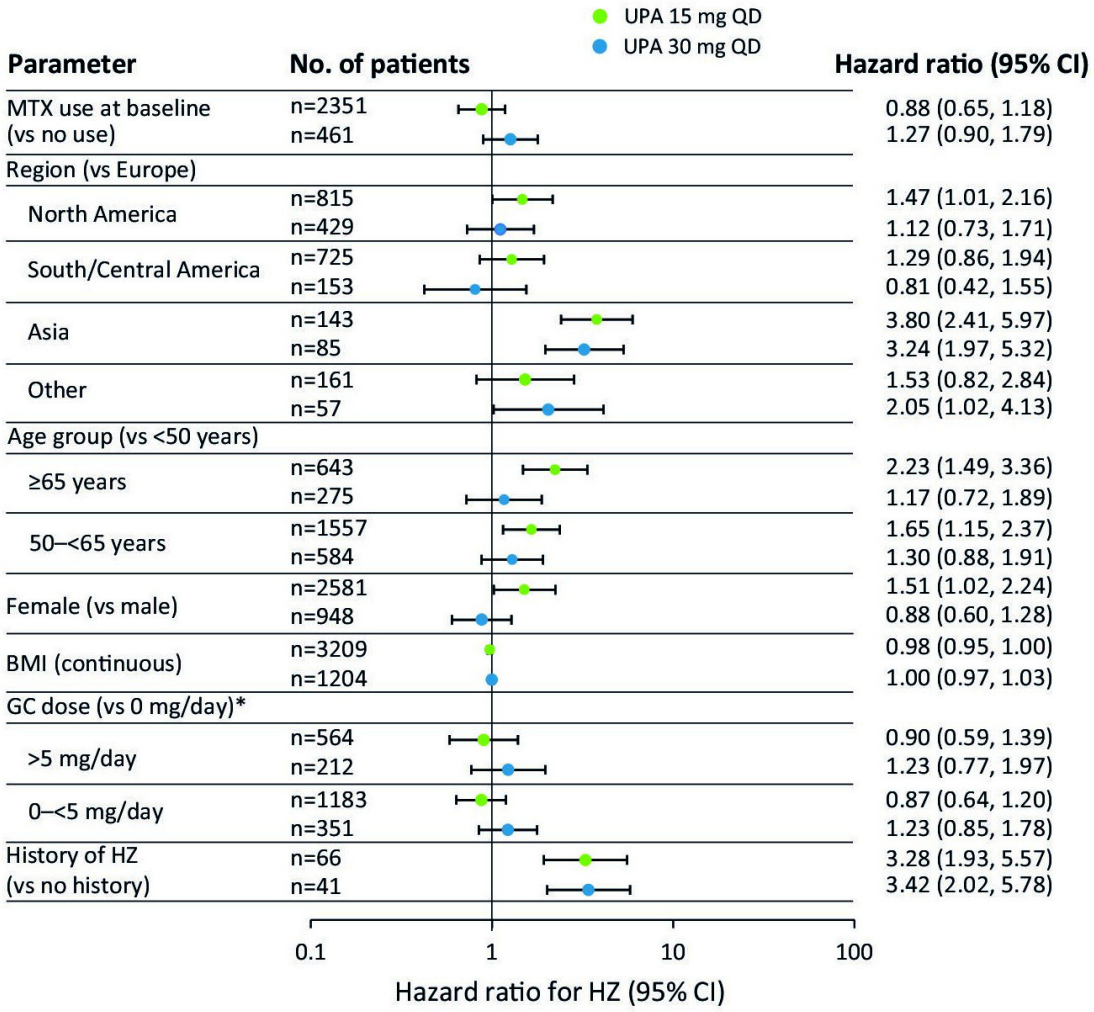

Figure 3 Risk factors for HZ in patients receiving UPA (multivariate analysis). *Prednisone or equivalent dose; doses of ≤10 mg/day were permitted. BMI, body mass index; GC, glucocorticoid; HZ, herpes zoster; MTX, methotrexate; QD, once daily; UPA, upadacitinib.

other cultural or medical factors could be involved.[6 7] Further research is warranted to determine why Asian patients with RA who are treated with JAK inhibitors, particularly those from East Asia, are at higher risk of HZ.

In our study, concomitant glucocorticoids did not significantly increase HZ risk in patients receiving UPA, both in terms of crude incidence rates and in univariate and multivariate analyses. This is in contrast with previous studies, which have shown an increased risk of HZ in patients with RA receiving JAK inhibitors in combination with glucocorticoids.[6 25] For example, Curtis *et al*[25] found a twofold increased risk of HZ in patients with RA receiving tofacitinib together with glucocorticoids versus those receiving tofacitinib alone, with similar results observed by Winthrop *et al*.[6] As glucocorticoids are a well-established risk factor for HZ, it is unclear why we did not observe this in our study, and this potential risk warrants further investigation in real-world data. It may be that the rate of HZ associated with UPA, particularly at the 30 mg dose, dominates other treatment-related risk factors. Our study also found that MTX had no significant effect on HZ incidence, which is consistent with previous results.[6 25]

Current EULAR and ACR guidelines state that live attenuated HZ vaccination may be considered in high-risk patients with autoimmune inflammatory rheumatic diseases, including RA, preferably 2–4 weeks prior to initiation of advanced therapy.[26 27] In this study we were unable to assess the effectiveness of HZ vaccination in patients receiving UPA, as fewer than 5% of patients had received vaccination with the live virus vaccine at baseline, and the study was conducted prior to the introduction of the HZ subunit vaccine (Shingrix).

The strongest risk factor of HZ identified among UPA-treated patients in our study was a prior history of HZ. As individuals typically boost their cell-mediated immunity with an episode of HZ, recurrent HZ is relatively rare, with only 0.4% of individuals in the general population who experience HZ going on to develop another episode later in life.[28] One would speculate that a history of HZ would therefore be protective for most individuals. To our knowledge, a history of HZ was not evaluated as a potential risk factor in the context of other JAK inhibitor studies. However, like other JAK inhibitors, a small percentage of individuals developed recurrent HZ within the phase III programme. In the literature, this has ranged typically between 2.5% and 6% depending on the study.[29 30] This seems consistent with our observation of prior HZ being a strong risk factor (although the rates in our study (6.4% for UPA 15 mg and 9.3% for UPA 30 mg for recurrent HZ) were slightly higher than in these studies), and is suggestive of a pathophysiology of JAK inhibitor-induced HZ beyond that of diminished cell-mediated immunity. It should be noted too, however, that the definition of recurrence can be different across studies.

Other significant risks included female sex, older age and North American region (vs Europe), but these were limited to the UPA 15 mg group only. It is unclear why these factors were not significant in the UPA 30 mg group as well. The increased risk in female patients may reflect the fact that around 80% of patients in this analysis were female, and the risk in North American patients appeared minimal compared with the much larger increased risk in Asian patients with both UPA doses. Conclusions about the risks of HZ in female and North American patients should therefore be made with caution, and additional data may be needed to clarify the risks of HZ in these populations.

In this analysis, HZ with ophthalmic involvement was more frequently reported in the UPA 15 mg group compared with the UPA 30 mg group. It is unclear what the reason may be for this observation. Of the patients who experienced HZ with ophthalmic involvement, 5/13 patients treated with UPA 15 mg and all three patients treated with UPA 30 mg were receiving concomitant glucocorticoids.

Postherpetic neuralgia is a common complication of HZ and is typically defined as pain that persists for ≥90 days after resolution.[3 31] As patients receiving UPA in this study were followed for 30 days after their last dose of study drug, this may mean that some cases of postherpetic neuralgia may not have been recorded. In keeping with this, the rates of postherpetic neuralgia observed in this study (3.8% and 7.3% with UPA 15 mg and 30 mg, respectively) were lower than the rate of 9.1% reported in a large analysis of patients with RA by Forbes *et al*[31]

We would note that the rate of HZ observed with UPA was relatively constant over time, suggesting that there is not a 'high-risk period' shortly after starting therapy, as has been observed for serious bacterial infections in RA.[32] As a corollary, this observation implies that vaccination against HZ remains important over a patient's entire course of UPA treatment, and that it is 'never too late' to vaccinate. However, it should be noted that a recent study suggested that live attenuated HZ vaccination had limited efficacy in preventing HZ in patients with RA receiving tofacitinib,[33] despite patients showing VZV-specific humoral and cell-mediated immune responses.[34] Further research is required to determine the value of HZ vaccination in patients with RA receiving JAK inhibitors, particularly with the recently approved subunit vaccine (Shingrix).

Given the lack of direct comparison in head-to-head studies between JAK inhibitors, we are limited in drawing conclusions regarding the relative risk of HZ with UPA as compared with other JAK inhibitors (tofacitinib, baricitinib and filgotinib).[35] Differences in study design, length of follow-up, inclusion and exclusion criteria, study sites and data analysis limit the ability to compare data across clinical trials. With those limitations in mind, the incidence rates described within the UPA programme are similar to those described in the RA programmes for tofacitinib and baricitinib, with the highest rates in all three programmes observed in the Asian region.[20 22 36] Rates of HZ in patients within the filgotinib clinical programme appear to be lower than those seen in the tofacitinib, baricitinib and UPA clinical programmes across all treatment arms, including both the PBO and active comparator arms, illustrating the challenges in cross-trial comparisons. Taken alone, the rates of HZ appear to be lower for filgotinib compared with other JAK inhibitors, but the relative differences between filgotinib and PBO and other JAK inhibitors and PBO are consistent.[20 22 36–38] Long-term integrated safety data showed EAIRs (95% CI) of 1.1 (0.8 to 1.7) and 1.8 (1.4 to 2.3) for filgotinib 100 mg and 200 mg, respectively, also showing a dose response.[37] In a pooled analysis of phase II, phase III and long-term extension studies, HZ was more common with filgotinib (100 mg and 200 mg) compared with ADA or MTX up to 52 weeks, but comparable versus PBO during the 12-week PBO-controlled period.[37–40] A recent systematic review and network meta-analysis found an increased risk of HZ infection with ADA, etanercept, peficitinib, tofacitinib and UPA compared with filgotinib, although the risk differences became statistically non-significant following a sensitivity analysis.[41]

In conclusion, this analysis provides further support for the need for continued vigilance and monitoring for signs of HZ in patients receiving UPA, particularly in Asian populations and those with a history of HZ. The study also emphasises the importance of future research clarifying whether treatment with UPA or other JAK inhibitor therapies may attenuate the expected benefit and durability of vaccination with the most recent HZ adjuvant vaccine.

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Contributors All authors had access to relevant data and participated in drafting the article and revising it critically for important intellectual content, and all authors approved the final version to be submitted for publication. KLW had full access to all of the data in the study, and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study conception and design: KLW, PN, KY, EM, NK, HSC, YS, JLS and JRC. Acquisition of data: KLW, PN, KY, EM and JRC. Analysis and interpretation of data: KLW, PN, KY, EM, NK, HSC, YS, JLS and JRC.

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Patient consent for publication Not applicable.

Ethics approval Studies were conducted in compliance with the Declaration of Helsinki, International Conference on Harmonization of Technical Regulations for Pharmaceuticals for Human Use guidelines, and applicable local country regulations. All study-related documents were approved by independent ethics committees and institutional review boards of the participating centres. All patients provided written informed consent.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request. AbbVie is committed to responsible data sharing regarding the clinical trials we sponsor. This includes access to anonymised, individual, and trial-level data (analysis datasets), as well as other information (eg, protocols and Clinical Study Reports), provided the trials are not part of an ongoing or planned regulatory submission. This includes requests for clinical trial data for unlicensed products and indications. These clinical trial data can be requested by any qualified researchers who engage in rigorous, independent scientific research, and will be provided following review and approval of a research proposal and statistical analysis plan, and execution of a Data Sharing Agreement.Data requests can be submitted at any time and the data will be accessible for 12 months, with possible extensions considered. For more information on the process, or to submit a request, visit https://www.abbvie.com/our-science/clinical-trials/clinical-trials-data-and-information-sharing/data-and-information-sharing-with-qualified-researchers.html

ORCID iDs
Kevin L Winthrop http://orcid.org/0000-0002-3892-6947
Peter Nash http://orcid.org/0000-0002-2571-788X

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TRANSLATIONAL SCIENCE

Targeting the IL-6–Yap–Snail signalling axis in synovial fibroblasts ameliorates inflammatory arthritis

Rebecca A Symons ,[1] Fabio Colella ,[1] Fraser L Collins ,[1] Alexandra J Rafipay,[1] Karolina Kania,[1] Jessica J McClure,[1] Nathan White,[1] Iain Cunningham,[1] Sadaf Ashraf,[1] Elizabeth Hay,[1] Kevin S Mackenzie,[2] Kenneth A Howard,[3] Anna H K Riemen,[1] Antonio Manzo,[4] Susan M Clark,[1] Anke J Roelofs ,[1] Cosimo De Bari [1]

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[1]Arthritis and Regenerative Medicine Laboratory, Aberdeen Centre for Arthritis and Musculoskeletal Health, Institute of Medical Sciences, University of Aberdeen, Aberdeen, UK
[2]Microscopy and Histology Core Facility, Institute of Medical Sciences, University of Aberdeen, Aberdeen, UK
[3]Interdisciplinary Nanoscience Center (iNANO), Department of Molecular Biology and Genetics, Aarhus University, Aarhus, Denmark
[4]Rheumatology and Translational Immunology Research Laboratories (LaRIT), Division of Rheumatology, IRCCS Policlinico San Matteo Foundation, University of Pavia, Pavia, Italy

Correspondence to
Professor Cosimo De Bari, Institute of Medical Sciences, University of Aberdeen, Aberdeen, UK; c.debari@abdn.ac.uk

RAS and FC contributed equally. AJR and CDB contributed equally.

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ABSTRACT

Objective We aimed to understand the role of the transcriptional co-factor Yes-associated protein (Yap) in the molecular pathway underpinning the pathogenic transformation of synovial fibroblasts (SF) in rheumatoid arthritis (RA) to become invasive and cause joint destruction.

Methods Synovium from patients with RA and mice with antigen-induced arthritis (AIA) was analysed by immunostaining and qRT-PCR. SF were targeted using *Pdgfrα-CreER* and *Gdf5-Cre* mice, crossed with fluorescent reporters for cell tracing and *Yap-flox* mice for conditional *Yap* ablation. Fibroblast phenotypes were analysed by flow cytometry, and arthritis severity was assessed by histology. Yap activation was detected using Yap–Tead reporter cells and Yap–Snail interaction by proximity ligation assay. SF invasiveness was analysed using matrigel-coated transwells.

Results Yap, its binding partner Snail and downstream target connective tissue growth factor were upregulated in hyperplastic human RA and in mouse AIA synovium, with Yap detected in SF but not macrophages. Lineage tracing showed polyclonal expansion of *Pdgfrα*-expressing SF during AIA, with predominant expansion of the *Gdf5*-lineage SF subpopulation descending from the embryonic joint interzone. *Gdf5*-lineage SF showed increased expression of *Yap* and adopted an erosive phenotype (podoplanin+Thy-1 cell surface antigen−), invading cartilage and bone. Conditional ablation of *Yap* in *Gdf5*-lineage cells or *Pdgfrα*-expressing fibroblasts ameliorated AIA. Interleukin (IL)-6, but not tumour necrosis factor alpha (TNF-α) or IL-1β, Jak-dependently activated Yap and induced Yap–Snail interaction. SF invasiveness induced by IL-6 stimulation or Snail overexpression was prevented by Yap knockdown, showing a critical role for Yap in SF transformation in RA.

Conclusions Our findings uncover the IL-6–Yap–Snail signalling axis in pathogenic SF in inflammatory arthritis.

Key messages

What is already known about this subject?

► Synovial fibroblasts (SF) are key drivers of rheumatoid arthritis (RA) pathogenesis; however, the SF lineages involved and the molecular mechanisms underpinning their pathogenic transformation are incompletely understood.

What does this study add?

► This study shows that *Gdf5*-lineage SF, defined by their ontogenic derivation from the joint interzone, are the key pathogenic SF in inflammatory arthritis. Mechanistically, interleukin (IL)-6 signals via Jak to activate Yes-associated protein (Yap), which forms a complex with Snail to drive the invasiveness of SF, and selective targeting of Yap in *Gdf5*-lineage SF ameliorates inflammatory arthritis.

How might this impact on clinical practice or future developments?

► This study supports the targeting of IL-6 and Jak in RA, not only for immunosuppression but also to directly control the Yap–Snail-mediated pathogenic behaviour of SF.

INTRODUCTION

Rheumatoid arthritis (RA) is a common immune-mediated chronic inflammatory disease causing joint damage and deformities. Current treatment consists of synthetic and biological disease-modifying anti-rheumatic drugs aimed at systemic immunosuppression. Nonetheless, some patients fail to respond to treatments, and joint damage progression can still occur despite clinical remission.[1]

A hallmark of RA pathology is synovitis, causing the synovium to thicken and form a pannus that invades cartilage and bone, driven by pathogenic transformation and proliferation of fibroblasts with infiltration of inflammatory/immune cells. A recent study reported two distinct subsets of synovial fibroblasts (SF), immune effector fibroblasts expressing podoplanin (Pdpn) and Thy-1 cell surface antigen (Thy1) found in the sub-lining synovial tissue and promoting inflammation, and destructive Pdpn+Thy1− fibroblasts in the lining layer mediating cartilage and bone damage.[2]

Cell lineage tracing studies have revealed that the adult synovium consists of ontogenetically diverse fibroblast subsets. One subset, the *Gdf5*-lineage cells, descend from the *Gdf5*-expressing joint interzone cells in the embryo.[3–5] Previously, we have shown that these *Gdf5*-lineage cells include the SF in the lining as well as a subset of SF in the sublining.[5 6] The remaining SF are of unknown embryonic origin. It is not known how

the ontogenetically defined SF subsets relate to the subsets identified by Pdpn and Thy1 expression.

The synovial pannus is a tumour-like structure resulting in part from uncontrolled fibroblast expansion. Increased activity of the transcriptional cofactor Yes-associated protein (Yap) is known to cause tissue overgrowth in multiple tissues and organs through stimulating cell proliferation.[7–13] In addition, Yap promotes cell motility and invasion in cancer cells.[14–16] We demonstrated that Yap promotes proliferation of fibroblast-like mesenchymal cells[17] and that increased Yap activity in *Gdf5*-lineage cells underpins synovial lining hyperplasia following acute joint surface injury in mice.[5]

The role of Yap in immune-mediated inflammatory arthritis and the signalling mechanisms that link Yap activity to the pathogenic transformation of SF await clarification. A reduced severity in the K/BxN serum-transfer arthritis model in mice was reported after treatment with the small molecule verteporfin,[18] a non-specific inhibitor of Yap activity. Although the mechanism was not investigated, it was suggested that the beneficial effect was through reduced Yap activity in SF.[18] However, it cannot be excluded that the effects of verteporfin were Yap-independent.[19 20] Furthermore, verteporfin was reported to ameliorate antigen-induced arthritis (AIA) in rabbits by inducing apoptosis of inflammatory cells.[21]

Here, we show that Yap is highly expressed by SF in both human RA and mouse AIA and that conditional genetic ablation of Yap in SF ameliorates AIA. Mechanistically, Yap is Jak-dependently activated by interleukin (IL)-6, a key inflammatory cytokine in RA, and forms a complex with the transcription factor Snail to drive the SF invasive phenotype. Our findings identify the IL-6–Yap–Snail signalling axis as a fibroblast-specific therapeutic target in RA synovitis.

METHODS

Materials and methods are available in the online supplemental information file.

RESULTS

Yap is upregulated in human rheumatoid and mouse immune-mediated synovitis

YAP was upregulated in the hyperplastic compared with quiescent lining of human RA synovium (p=0.0003, figure 1A), and this was accompanied by upregulation of the transcription factor SNAIL (p=0.0003), and the YAP and SNAIL downstream target gene connective tissue growth factor (CTGF) (p=0.0078, figure 1A), a known pathogenic effector in RA.[22] YAP was expressed by CD55+ fibroblasts and not by CD68+ macrophages (figure 1B). *YAP* mRNA expression levels correlated non-significantly with the expression of *SNAIL* (p=0.083) and significantly with the expression of YAP downstream targets *CTGF* (p=0.024) and *GP130* (p=0.001, figure 1C), a transmembrane protein required for IL-6 signalling.[23]

Analysis of mouse AIA synovium confirmed the upregulation of Yap (p<0.0001, figure 2A), Snail (p=0.0022, figure 2B) and Ctgf (p=0.0003, figure 2C) during synovitis. High Yap expression was observed in fibroblast-like cells throughout the synovium and along the periosteal surface, extending into the underlying marrow space at sites of erosive damage (figure 2A and online supplemental figure 1a).

Gdf5-lineage Yap-expressing SF are predominant in arthritis

Next, we used genetic cell-labelling and tracing models to map fibroblast populations in synovitis. To trace individual fibroblasts, we used *Pdgfra-CreER;R26-Confetti* mice (see online supplemental table 1 for transgenic mouse lines used in the study), in which tamoxifen administration activates CreER in cells expressing the pan-fibroblast marker *Pdgfra*, resulting in stochastic expression of one of four fluorescent proteins.[6] Analysis of cyan fluorescent protein (CFP), yellow fluorescent protein (YFP) and red fluorescent protein (RFP) expression (green fluorescent protein (GFP) was rarely detected and was omitted from analysis) 6 days after AIA induction revealed extensive expansion of *Pdgfra*-traced cells in synovium. Multiple small clusters of monochromatic cells were interspersed throughout the synovium, indicating polyclonal cell expansion (figure 3A,B).

We previously showed that a subset of *Pdgfra*-expressing cells in the adult synovium originates from the *Gdf5*-expressing embryonic joint interzone.[5 6] These *Gdf5*-lineage SF are mostly found in the synovial lining but also include a subset of the SF in the sublining.[5] To determine the involvement of the *Gdf5*-lineage SF in synovitis, we induced AIA in *Gdf5-Cre;Tom;Pdgfra-H2BGFP* mice and administered BrdU to label proliferating cells. In these mice, Cre is expressed and permanently switches on Tom expression in cells of the joint interzone and all their progeny, while Cre is not active in the adult knee.[24–26] In addition, *Pdgfra*-expressing fibroblasts are identified by long-lived nuclear GFP. In control knees, *Gdf5*-lineage SF (ie, Tom+GFP+ cells) were mostly quiescent and located predominantly in the synovial lining, while other SF (ie, Tom-GFP+ cells) were found throughout the synovium (figure 3C,D). In contrast, *Gdf5*-lineage SF extensively proliferated and infiltrated the entire synovium in the AIA knee (figure 3C,D), increasing 4.2±0.6-fold (mean±SD, n=5, p<0.001) and constituting the dominant SF lineage in the inflamed synovium (figure 3E). A concomitant increase in the unlabelled cell population (ie, Tom−GFP− cells) reflects the infiltration and expansion of immune cells during synovitis (figure 3E). GFP and Yap co-detection confirmed Yap expression by SF in both the synovial lining and along the periosteal surface (figure 3F), and Yap expression along the bone surface, including at sites of erosion, correlated with the presence of *Gdf5*-lineage cells (online supplemental figure 1).

A recent study reported Pdpn+Thy1+ SF to be immunomodulatory and Pdpn+Thy1− SF to be erosive in inflammatory arthritis.[3] Hence, we investigated the expression of Pdpn and Thy1 in the ontogenetically defined SF subsets. While *Gdf5*-lineage SF included both Pdpn+Thy1+ and Pdpn+Thy1− phenotypic subsets, we observed a striking increase in the percentage of *Gdf5*-lineage SF with a Pdpn+Thy1− (erosive) phenotype during AIA, as compared with control knees (p=0.00005, figure 4A and online supplemental figure 2). In contrast, the other SF adopted primarily a Pdpn+Thy1+ (immunomodulatory) phenotype in response to AIA (p=0.0014, figure 4B and online supplemental figure 2).

To determine whether Yap controls the capacity of SF to remodel and invade extracellular matrix, we used a matrigel-coated Boyden transwell assay.[27] DsiRNA-mediated knockdown of Yap in SF from AIA mice reduced their invasive ability (p=0.006, figure 4C). Notably, knockdown of the Yap paralog, Taz, did not affect mouse SF invasiveness (online supplemental figure 3). In human RA-SF, reduced invasiveness was observed after simultaneous knockdown of YAP and TAZ (p=0.011, figure 4D).

Altogether, these data show extensive proliferation and expansion of Yap-expressing SF throughout the synovium in AIA and reveal the *Gdf5*-lineage SF to be the predominant erosive SF

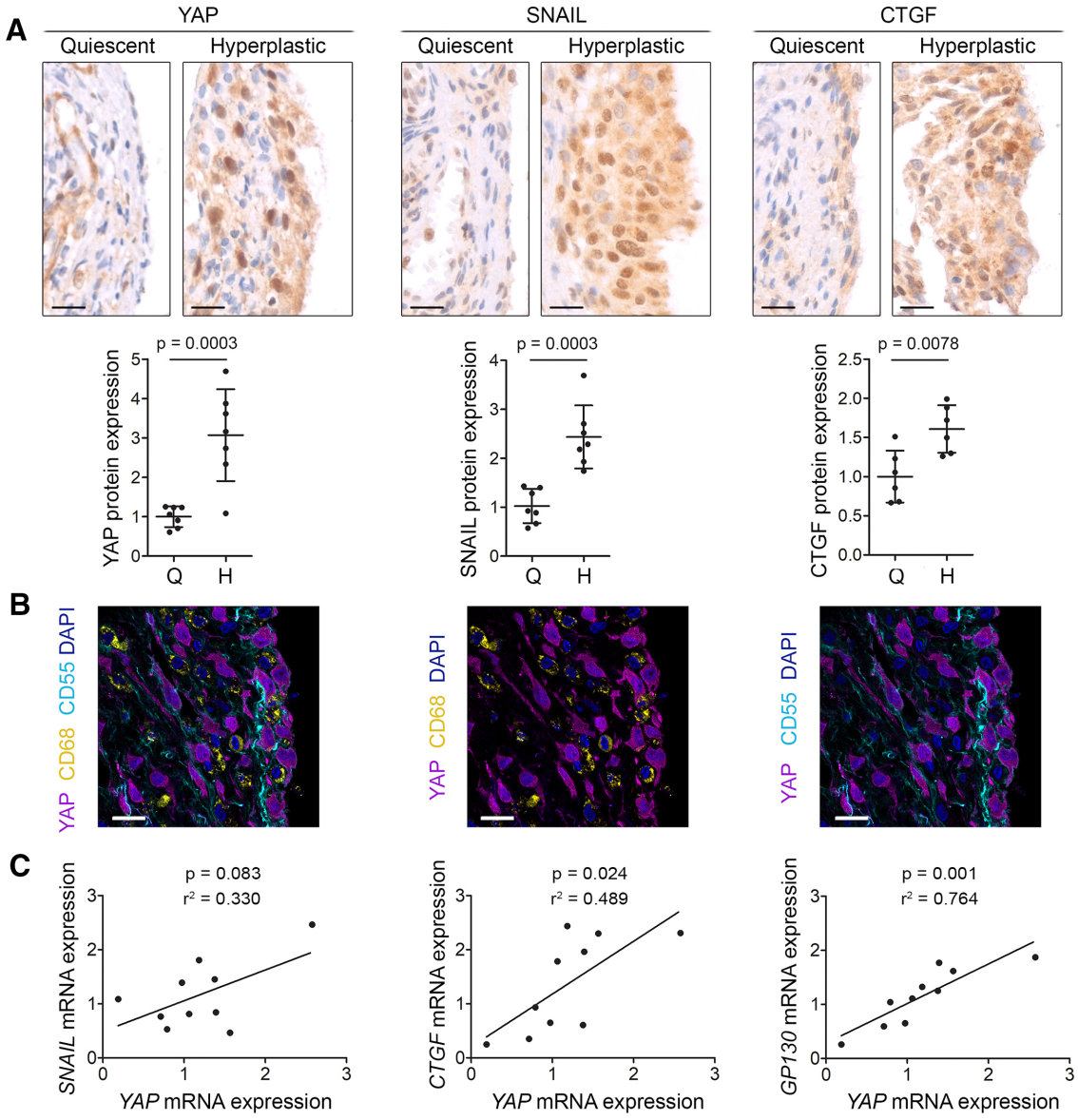

Figure 1 YAP, SNAIL and CTGF are upregulated in hyperplastic rheumatoid arthritis synovium. (A) Immunohistochemical detection of YAP, SNAIL and CTGF in quiescent and hyperplastic areas of human RA synovium (YAP n=7, SNAIL n=7, and CTGF n=6). Haematoxylin counterstain is shown in blue. Scale bars: 20 µm. For isotype negative control stainings, see online supplemental figure 10a–c. Graphs indicate protein expression in quiescent (Q) and hyperplastic (H) synovium based on immunohistochemistry (IHC) staining intensity, with lines and error bars indicating mean±SD (n=6–7). P values indicate statistical significance using an unpaired two-tailed t-test. (B) Expression of YAP (magenta), the SF marker CD55 (cyan) and the macrophage marker CD68 (yellow) in human RA synovium (n=4). DAPI (4′,6-diamidino-2-phenylindole) nuclear counterstain is shown in blue. The same image is shown three times with different channels overlaid, for clarity. Scale bars: 20 µm. For isotype negative control stainings, see online supplemental figure 10d. (C) Correlation between *YAP* gene expression and expression of *SNAIL*, *CTGF* or *GP130* in RA synovial biopsies (n=10) as determined by qRT-PCR. P values indicate results of Pearson's correlation test and R^2 values the square of the correlation coefficient. CTGF, connective tissue growth factor; RA, rheumatoid arthritis; SF, synovial fibroblast; YAP, Yes-associated protein.

subset in immune-mediated synovitis, with Yap being required for SF invasiveness in vitro.

Conditional ablation of *Yap* in SF ameliorates inflammatory arthritis

To determine the role of Yap in *Gdf5*-lineage SF in the pathophysiology of inflammatory arthritis, we conditionally ablated *Yap* in *Gdf5*-lineage cells and induced AIA. A Cre-inducible *Tom* reporter was crossed into the model, allowing detection of *Yap* conditional KO (cKO) target cells. Immunostaining of synovium showed a significant decrease in Yap expression in the cKO mice (p<0.0001, figure 5A). The efficiency of *Yap* cKO was further confirmed at mRNA level by qRT-PCR of sorted cells (p<0.001), which moreover revealed a significantly higher expression of *Yap* in the *Gdf5*-lineage SF compared with other SF after 7 days of AIA (p=0.004; figure 5B and online supplemental figure 4), further supporting a key role for Yap in the *Gdf5*-lineage AIA-SF.

Nine days after arthritis induction, we observed significant decreases in synovial lining hyperplasia (p<0.001), immune infiltrates in synovium (p=0.026) and erosions at the joint margins (p=0.002), and a non-significant trend towards decreased cellular exudate, resulting in an overall arthritis score of 4.9 (95% CI 4.2 to 5.6, n=22) in the cKO mice, compared with 6.5 (95% CI 5.9 to 7.0, n=24) in *Yap*

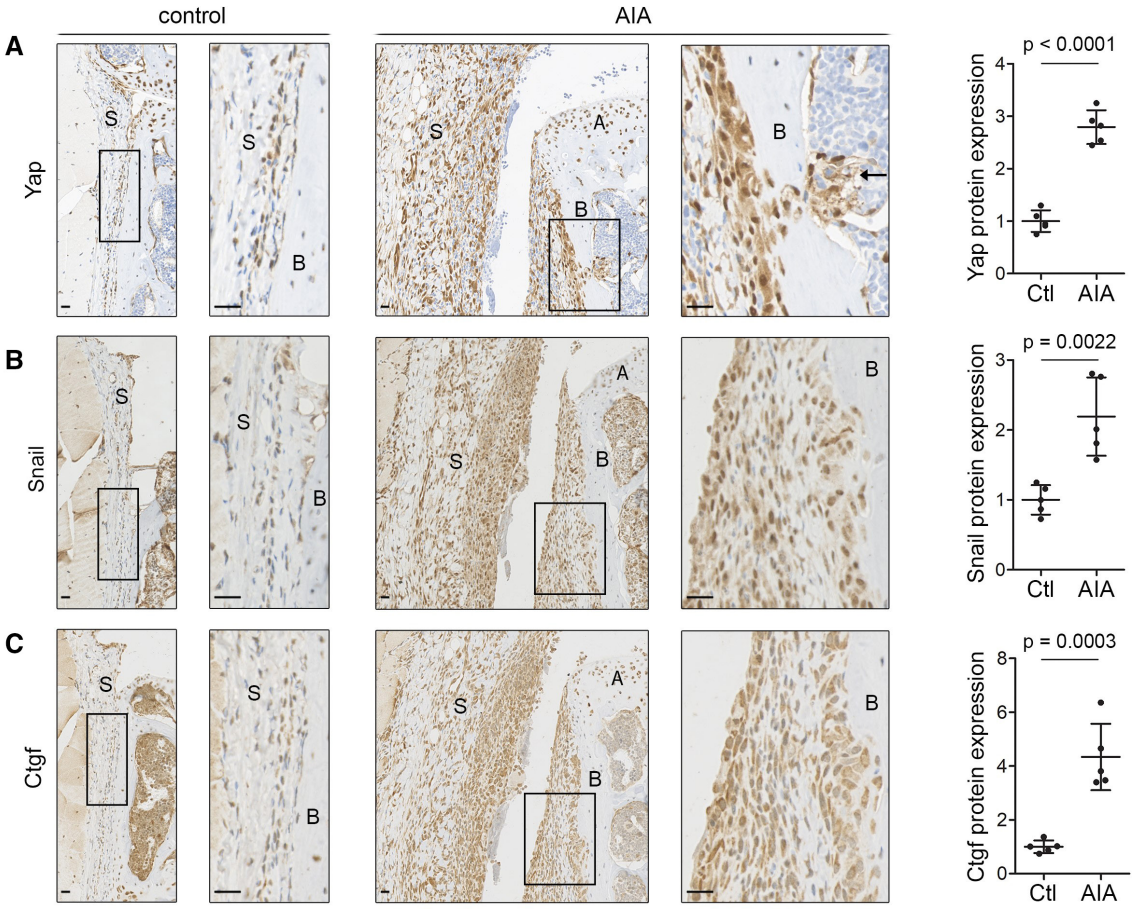

Figure 2 Yap, Snail and Ctgf are upregulated in inflammatory arthritis in mice. (A–C) Immunohistochemical detection of Yap (A), Snail (B) and Ctgf (C) in mouse synovium 6 days after AIA induction (n=5; 2 male mice, 3 female mice, 11–13 weeks). Contralateral knee served as control. Haematoxylin counterstain is shown in blue. Boxed areas on the left are shown at higher magnification on the right. Arrow (A) indicates Yap-expressing cells penetrating through the bone into the underlying marrow space. Scale bars: 20 µm. For isotype negative control stainings, see online supplemental figure 10f–h. Graphs indicate protein expression in synovium based on IHC staining intensity, with lines and error bars indicating mean±SD (n=5). P values indicate statistical significance using an unpaired two-tailed t-test. AIA, antigen-induced arthritis; A, articular cartilage; B, bone; Ctgf, connective tissue growth factor; S, synovium; Yap, Yes-associated protein.

WT mice (p=0.002; figure 5C,D). Despite the decrease in arthritis severity in vivo and diminished cell proliferation after *Yap* KO in vitro (p=0.023) (online supplemental figure 5), extensive expansion of *Gdf5*-lineage cells in synovium during AIA was observed in both *Yap* WT (p<0.001) and *Yap* cKO mice (p<0.001, figure 5E). Accordingly, using BrdU labelling, we detected proliferation of *Gdf5*-lineage cells throughout the synovium in *Yap* cKO mice at 3 and 6 days after AIA induction (figure 5F). These data indicate that Yap is largely dispensable for SF proliferation but required for SF-mediated disease activity in AIA.

Next, we investigated the effects of conditional *Yap* ablation on the expression of key catabolic and inflammatory mediators by qRT-PCR analysis of Tom+ *Gdf5*-lineage SF purified from AIA mice. Compared with *Gdf5*-lineage SF of *Yap* WT mice, the *Gdf5*-lineage SF from *Yap* cKO mice displayed reduced expression levels of critical enzymes mediating SF invasiveness, including Mmp9 (p=0.048), Mmp13 (p=0.044) and Mmp14 (p=0.012),[28–30] as well as the inflammatory cytokine IL-34 (p=0.011) (figure 5G). These data point to a role for Yap in mediating critical arthritogenic effects of SF that contribute to erosive damage and inflammation in AIA.

In RA, erosive damage to bone is caused by increased osteoclastic bone resorption, driven in part by pro-osteoclastogenic factors produced by the SF.[31] Staining for the osteoclast marker TRAP revealed a decreased number of TRAP+ cells along the femoral periosteal surface in female *Yap* cKO mice compared with *Yap* WT controls after AIA (p=0.015; figure 5H and online supplemental figure 6), while no significant difference was observed in the number of TRAP+ cells at endosteal surfaces of the femoral epiphysis (online supplemental figure 6). Accordingly, microCT analysis showed no difference in the trabecular bone loss in the tibial epiphysis between *Yap* WT and *Yap* cKO mice in response to AIA (online supplemental figure 7). Together, these data suggest a role for Yap in SF in stimulating local osteoclast development in female mice.

Next, we sought to validate these data using a conditional tamoxifen-inducible (ci) KO model in which *Yap* is ablated in the SF of the adult knee, using *Pdgfrα-CreER* as driver. Immunodetection of Yap in tissue sections confirmed *Yap* ciKO (figure 6A,B), although with lower efficiency than *Gdf5-Cre*-driven *Yap* cKO (figure 5A). Nonetheless, we again observed a decrease in synovial lining hyperplasia (p=0.013) and arthritis score (p=0.0496) compared with *Yap* WT mice (figure 6C,D).

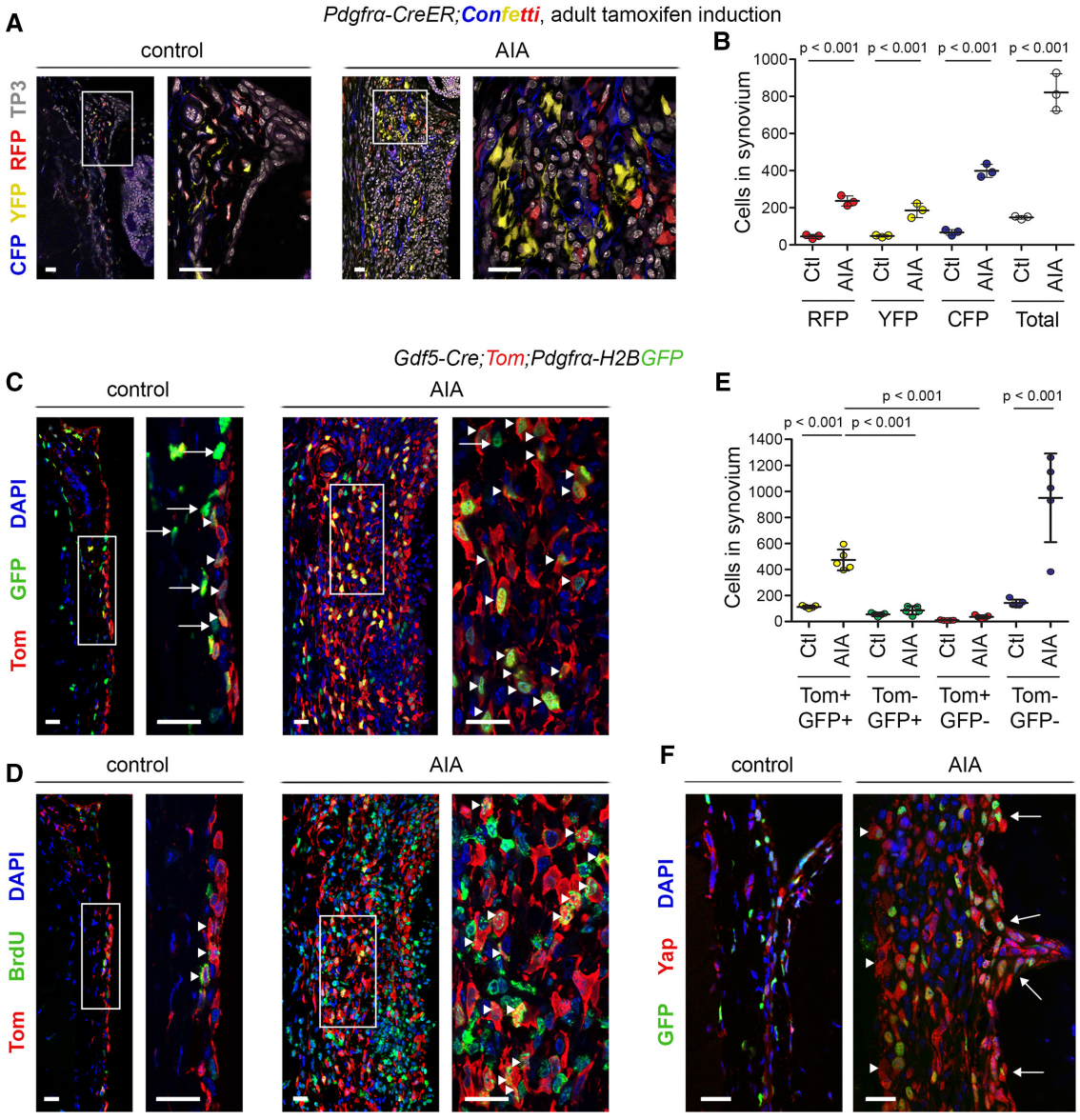

Figure 3 *Pdgfrα*-expressing *Gdf5*-lineage cells expand during inflammatory arthritis. (A,B) Detection of *Pdgfrα*-traced cells, marked by expression of CFP, YFP or RFP, in synovium of *Pdgfrα-CreER;Confetti* mice induced with tamoxifen from 8 weeks of age prior to AIA induction and analysed after 6 days of AIA (n=3; 3 male mice, 14–15 weeks). Contralateral knee served as control. (A) Cells labelled by CFP (blue), YFP (yellow) and RFP (red) in synovium. TO-PRO-3 (TP3) nuclear counterstain is shown in grey. (B) Numbers of CFP, YFP and RFP-labelled cells per mm length of synovium. P values indicate statistical significance based on two-way ANOVA with Tukey's post-test after log transformation (n=3). (C–F) *Gdf5-Cre;Tom;Pdgfrα-H2BGFP* mice 6 days after AIA induction (n=5; 2 male mice, 3 female mice, 11–13 weeks). Mice received bromodeoxyuridine (BrdU) to label proliferating cells from arthritis induction until the end. (C,D) Expansion of Tom-labelled *Gdf5*-lineage cells (red) in synovium during AIA, co-staining for (C) GFP (green), indicative of *Pdgfrα* expression (arrowheads indicate Tom+GFP+ cells; arrows indicate Tom-GFP+ cells), or (D) the BrdU proliferation label (green; arrowheads indicate Tom+BrdU+ cells). Consecutive tissue sections are shown. For isotype negative control stainings, see online supplemental figure 10i,j. (E) Numbers of Tom-labelled and GFP-labelled cells per mm length of synovium. P values indicate statistical significance based on two-way ANOVA with Tukey's post-test after log-transformation (n=5). (F) Detection of Yap (red) in GFP-expressing cells (green) in synovial lining (arrowheads) and along the periosteal surface (arrows). Scale bars: 20 µm. For isotype negative control stainings, see online supplemental figure 10k. Lines and error bars on all graphs indicate mean±SD. AIA, antigen-induced arthritis; ANOVA, analysis of variance.

Together, these findings indicate that Yap has crucial functions in pathogenic SF to promote inflammation and joint destruction, and demonstrate amelioration following its selective targeting in SF in inflammatory arthritis.

IL-6 activates Yap through Jak and induces Yap–Snail interaction in SF to drive their invasiveness

To assess the ability of key inflammatory cytokines to activate Yap, we used a Yap–Tead reporter construct,[32] modified to drive GFP expression following stable lentiviral transduction (online supplemental figure 8). We detected selective Yap activation by IL-6/sIL6R ($p<0.001$), while TNF-α or IL-1β had no effect (figure 7A). Baricitinib, a selective Jak1/2 inhibitor, prevented the IL-6/sIL6R-induced activation of Yap ($p<0.001$, figure 7B), as well as phosphorylation of Stat3 (figure 7C), indicating that IL-6-induced Yap activation requires Jak signalling. IL-6/sIL6R stimulation did not increase *Yap* mRNA expression at least after 24 hours (figure 7D), indicating that its effect may be mediated

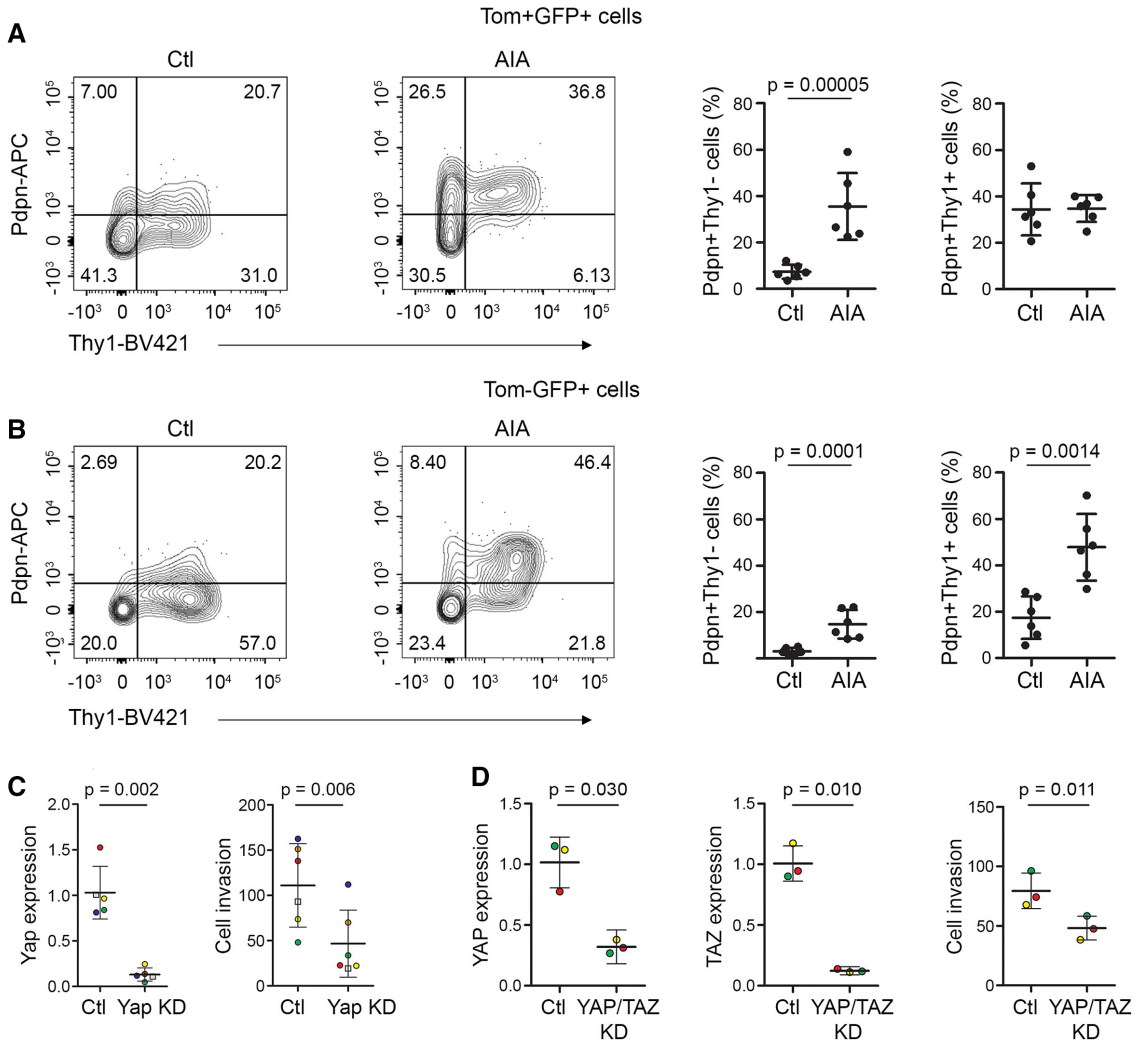

Figure 4 Phenotypic analysis of SF in inflammatory arthritis. (A,B) Freshly isolated cells from knees of adult *Gdf5-Cre;Tom;Pdgfrα-H2BGFP* mice 6 days after AIA induction (n=6; 5 male mice and one female mouse, 11–14 weeks, pooled data from two experiments) were analysed by flow cytometry for the expression of Pdpn and Thy1 within (A) the *Gdf5*-lineage *Pdgfrα*-expressing cells (Tom+GFP+) and (B) the remaining *Pdgfrα*-expressing cells (Tom-GFP+). The contralateral knees served as controls. Graphs show the percentage of cells expressing Pdpn with or without coexpression of Thy1. P values indicate statistical significance based on unpaired two-tailed t-test after log transformation. For gating strategy and staining controls, see online supplemental figure 2. (C) Yap KD decreased AIA-SF invasiveness through matrigel in a transwell assay. Dots are colour-coded to indicate different experiments (n=5 for Yap expression, n=6 for cell invasion) using cells from three different mice for DsiRNA#1 (circles), and from a fourth mouse for DsiRNA#2 (squares). P values indicate results of two-tailed paired Student's t-test. (D) YAP/TAZ KD decreased human RA-SF invasiveness through matrigel in a transwell assay. Dots are colour-coded to indicate independent experiments using cells from different donors (n=3). P values indicate results of two-tailed paired Student's t-test. AIA, antigen-induced arthritis; KD, knockdown; Pdpn, podoplanin; RA, rheumatoid arthritis; SF, synovial fibroblast; Thy1, Thy-1 cell surface antigen.

through post-transcriptional mechanisms. Yap knockdown prevented IL-6/sIL6R-induced invasiveness of normal mouse SF (figure 7E), showing that IL-6 signals via Yap to promote invasion. An IL-6/sIL6R complex was used in these experiments, since SF do not express the IL-6R (online supplemental figure 9) and rely on sIL6R, normally produced by immune cells, to activate IL-6 trans-signalling via gp130.[33]

Since the transcription factor Snail, previously reported to be involved in TNF-α-mediated SF activation in RA,[34] was upregulated alongside Yap during RA and AIA synovitis (figures 1A and 2B), we investigated the functional relationship between Snail and Yap. Snail overexpression increased SF invasiveness (p=0.022), and this was prevented by simultaneous knockdown of Yap (p=0.027, figure 7F), demonstrating that Snail requires Yap to drive SF invasion. Using a proximity ligation assay, we found that treatment of mouse SF or human RA-SF with IL-6/sIL6R induced the formation of Yap–Snail complexes, similar to what is seen after Yap overexpression in mouse SF (figure 7G,H). Taken together, these data indicate that IL-6 trans-signalling in SF activates Yap through Jak and increases Yap–Snail interaction to promote invasiveness.

DISCUSSION

SF are key cells in RA that sustain inflammation and induce tissue damage, but the molecular mechanisms underlying these functional characteristics remain to be fully elucidated. Here, we report that ablation of *Yap* in SF ameliorates immune-mediated inflammatory arthritis in vivo and show that IL-6 increases Yap activity through Jak signalling and promotes interaction of Yap and Snail to drive the pathogenic behaviour of SF.

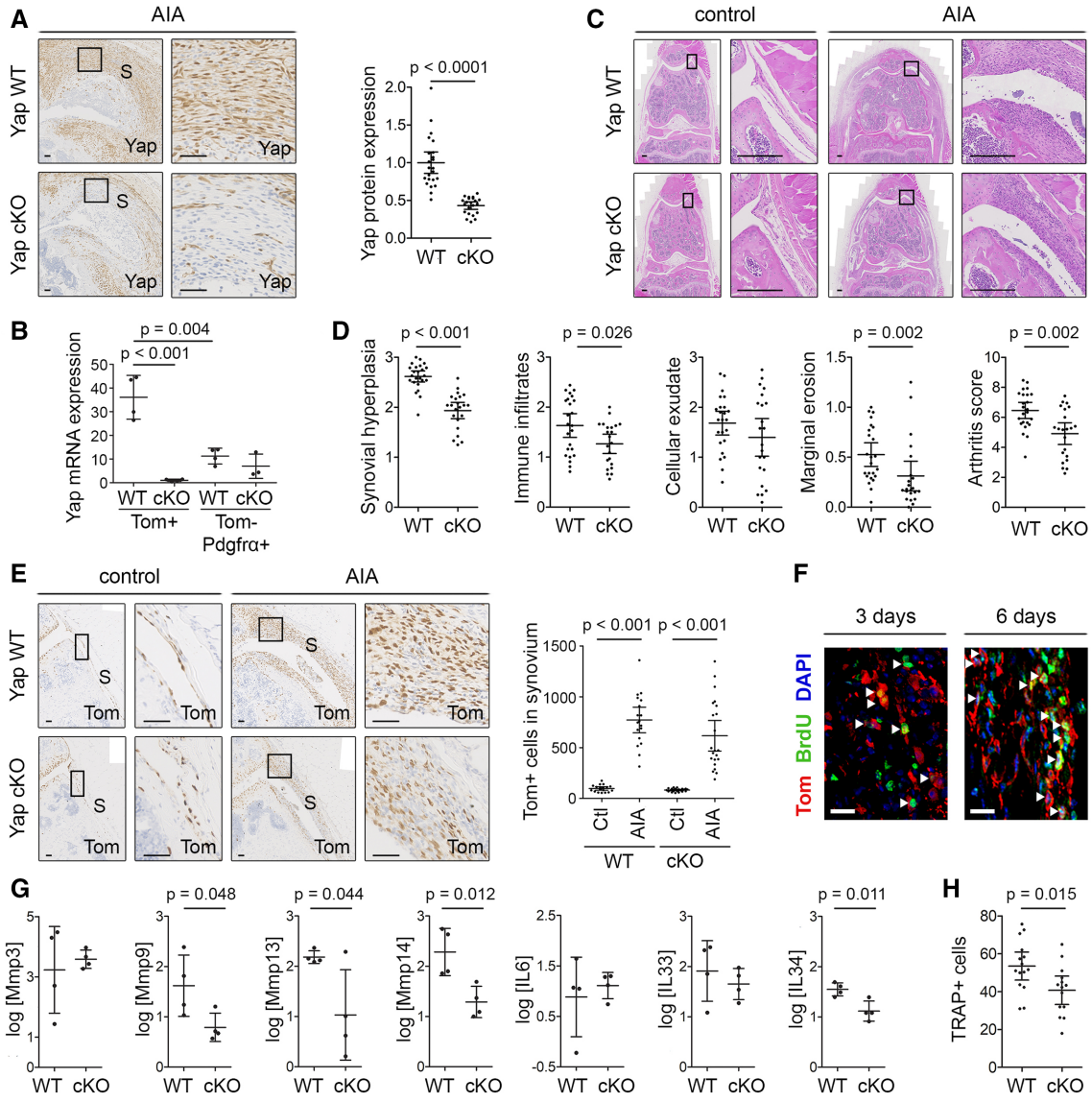

Figure 5 Ablation of *Yap* in *Gdf5*-lineage cells reduces inflammatory arthritis severity. AIA was induced in one knee of *Yap* WT or *Yap* cKO mice, with contralateral knee serving as control, and mice were analysed after 3 or 6 days (F), 7 days (B,G) or 9 days (A,C–E,H). See online supplemental table 2 for mouse genotypes, sex, age and exclusions. (A) Yap expression in inflamed synovium detected by IHC. Images show mice with highest arthritis score in their group. Scale bars: 50 µm. P value: unpaired two-tailed t-test after log transformation (WT: n=24; cKO: n=22; five experiments). (B) *Yap* mRNA expression detected by qRT-PCR in fluorescence-activated cell sorting (FACS)-sorted SF from AIA knees of *Yap* WT (n=4) and *Yap* cKO mice (n=3–4; 1 *Yap* cKO Tom-Pdgfrα+ sorted sample was excluded from analysis due to very low cell yield). P values indicate results of two-way analysis of variance with Tukey's post-test after log transformation. (C,D) H&E staining and histological scoring of arthritis severity. Images show matched control and AIA knees from mice with arthritis scores (WT: 6.33; cKO: 4.66) close to their group average. Scale bars: 200 µm. P values: Mann-Whitney U test (WT: n=24; cKO: n=22; five experiments). (E) Tom+ *Gdf5*-lineage cells in synovium detected by IHC. Images are from the same mice as in (C). Scale bars: 50 µm. Graph shows Tom+ cells per millimetre length of synovium. P values: Kruskal-Wallis test with Dunn's post-test (WT: n=18; cKO: n=22; five experiments). (F) Immunofluorescent detection of BrdU (green) in Tom+ *Gdf5*-lineage cells (red) in the synovium of *Yap* cKO mice at 3 (n=5) or 6 days (n=3) after AIA induction. Scale bars: 20 µm. (G) Expression of Mmps and cytokines detected by qRT-PCR in *Gdf5*-lineage cells FACS-sorted from knees of *Yap* WT (n=4) or *Yap* cKO (n=4) mice. P values: unpaired two-tailed t-test after log transformation. (H) TRAP+ cells along the medial and lateral femoral periosteal surface of female mice. P value: unpaired two-tailed t-test (WT: n=15; cKO: n=14; five experiments). Boxed areas in images on the left are shown at higher magnification on the right. For isotype negative control stainings, see online supplemental figure 10e,f,j. For FACS strategy, see online supplemental figure 4. Data on graphs are shown as mean±95% CI (A, D, E, H) or mean±SD (B, G). AIA, antigen-induced arthritis; S, synovium; SF, synovial fibroblast; WT, wild type; Yap, Yes-associated protein.

In a previous study using the K/BxN serum-transfer mouse model of inflammatory arthritis and mice engrafted with cartilage and RA-SF, treatment with the non-specific Yap inhibitor verteporfin was shown to reduce arthritis severity and cartilage invasion by RA-SF, respectively.[18] However, verteporfin exhibits Yap-independent cytostatic and cytotoxic effects and was previously shown to ameliorate AIA by inducing immune cell apoptosis.[19–21] Additionally, Yap is highly expressed by endothelium and known to regulate endothelial cell proliferation, migration and survival.[35] Indeed, Yap was recently reported to mediate synovial angiogenesis in AIA mice, with verteporfin shown to reduce angiogenesis and synovitis.[35]

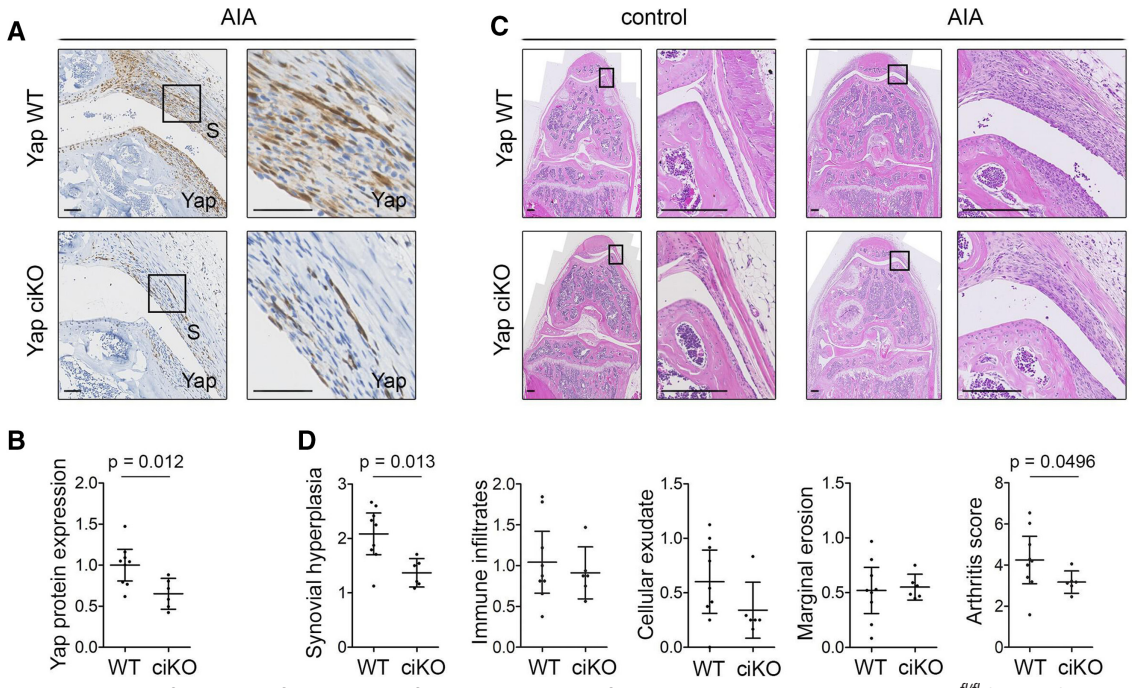

Figure 6 Inducible ablation of *Yap* in *Pdgfrα*-expressing fibroblasts reduces inflammatory arthritis severity. Adult *Yap*$^{fl/fl}$ (Yap WT) or *Pdgfrα-CreER;Yap*$^{fl/fl}$ (Yap ciKO) mice received tamoxifen to activate Cre and KO *Yap*, prior to induction of AIA in one knee, with the contralateral knee serving as control. Histological analysis was performed 9 days after arthritis induction. See online supplemental table 3 for mouse genotypes, sex and age. (A) Yap expression in the inflamed synovium detected by IHC with haematoxylin counterstain. Histological images were selected from the same mice as shown in panel (C). Boxed areas in images on the left are shown at higher magnification on the right. Scale bars: 50 µm. For isotype negative control staining, see online supplemental figure 10f. (B) Yap expression in synovium, based on IHC staining intensity. P value indicates result of two-tailed unpaired t-test (WT: n=9; ciKO n=6; pooled data from two experiments). (C) Synovitis and erosive damage in AIA knees detected by H&E staining. Images show matched control and AIA knees from female mice in the same experiment with arthritis scores (WT: 5.00; ciKO: 2.99) close to the average of their respective groups. Boxed areas in images on the left are shown at higher magnification on the right. Scale bars: 200 µm. (D) Histological assessment of severity of synovial hyperplasia, immune infiltrates, cellular exudate and marginal erosions (all on scale 0–3), and overall arthritis severity (scale 0–12). P values indicate results of Mann-Whitney U test (WT: n=9; ciKO: n=6, pooled data from two experiments). Lines and error bars on all graphs indicate mean±95% CI. AIA, antigen-induced arthritis; S, synovium; WT, wild type; Yap, Yes-associated protein.

Here, we unequivocally show, by conditional ablation of *Yap* in SF using two different mouse models, a specific role for Yap in driving the pathological behaviour of SF in inflammatory arthritis. The recent demonstration of additional roles for Yap in promoting synovial angiogenesis further supports the possible therapeutic benefits of targeting Yap in RA synovitis.[35]

We previously reported that increased Yap activity in *Gdf5*-lineage SF drives synovial lining hyperplasia in a traumatic joint surface injury model in mice.[5] In the present study, we show that ablation of *Yap* in *Gdf5*-lineage SF reduced not only synovial lining hyperplasia but also immune infiltration and erosive damage in inflammatory arthritis. Decreased synovial lining hyperplasia in AIA was also observed when Yap was knocked out in fibroblasts in adult mice, showing this is not a result of developmental defects resulting from Yap KO.

Although the upstream regulators of Yap activity are likely to be context-dependent, we uncover a hitherto unappreciated molecular link between Jak-mediated IL-6 signalling, pivotal in RA pathogenesis, and the Yap-mediated invasive SF phenotype. While IL-6 treatment of SF enhanced invasive behaviour, Yap silencing completely prevented the IL-6-induced SF invasion. JAK inhibition with baricitinib was shown to abrogate cytokine-induced invasive behaviour of SF.[36] Together, this suggests that JAK inhibitors such as baricitinib could ameliorate RA disease in part by modulating YAP activity in SF.

SF, which do not express the IL-6R, undergo pro-inflammatory sIL6R-mediated trans-signalling, facilitated by cell surface expression of GP130.[33] We showed a strong correlation between *YAP* and *GP130* expression in human RA synovium, in accordance with evidence that YAP upregulates *GP130* expression in an autoregulatory loop,[23] indicating that YAP plays a role in amplifying IL-6 signalling and maintaining invasiveness in RA-SF. YAP activation by IL-6 has also been reported in human colorectal cancer cells.[37] It is therefore plausible that the IL-6-GP130-YAP pathway becomes activated in multiple disorders, possibly as a general response to abnormal contexts jeopardising organ/tissue homeostasis.

Snail was previously reported to be highly expressed in RA synovium and SF, and its lentiviral vector-mediated silencing in the joint ameliorated collagen-induced arthritis in rats.[34] We describe the formation of a complex between Yap and Snail in SF from AIA mice and patients with RA. This complex was reported to occur in skeletal stem cells to modulate their physiological functions of self-renewal and lineage commitment,[38] suggesting cell-specific and context-dependent functions of the Yap–Snail complex. Silencing of Yap was sufficient to prevent the invasive phenotype induced by Snail overexpression, demonstrating a requirement for active Yap to mediate SF invasiveness. Intriguingly, while Snail was reported to regulate the TNF-α-mediated activation of SF,[34] in our study activation of Yap occurred downstream of IL-6 only, not TNF-α. Altogether, these findings

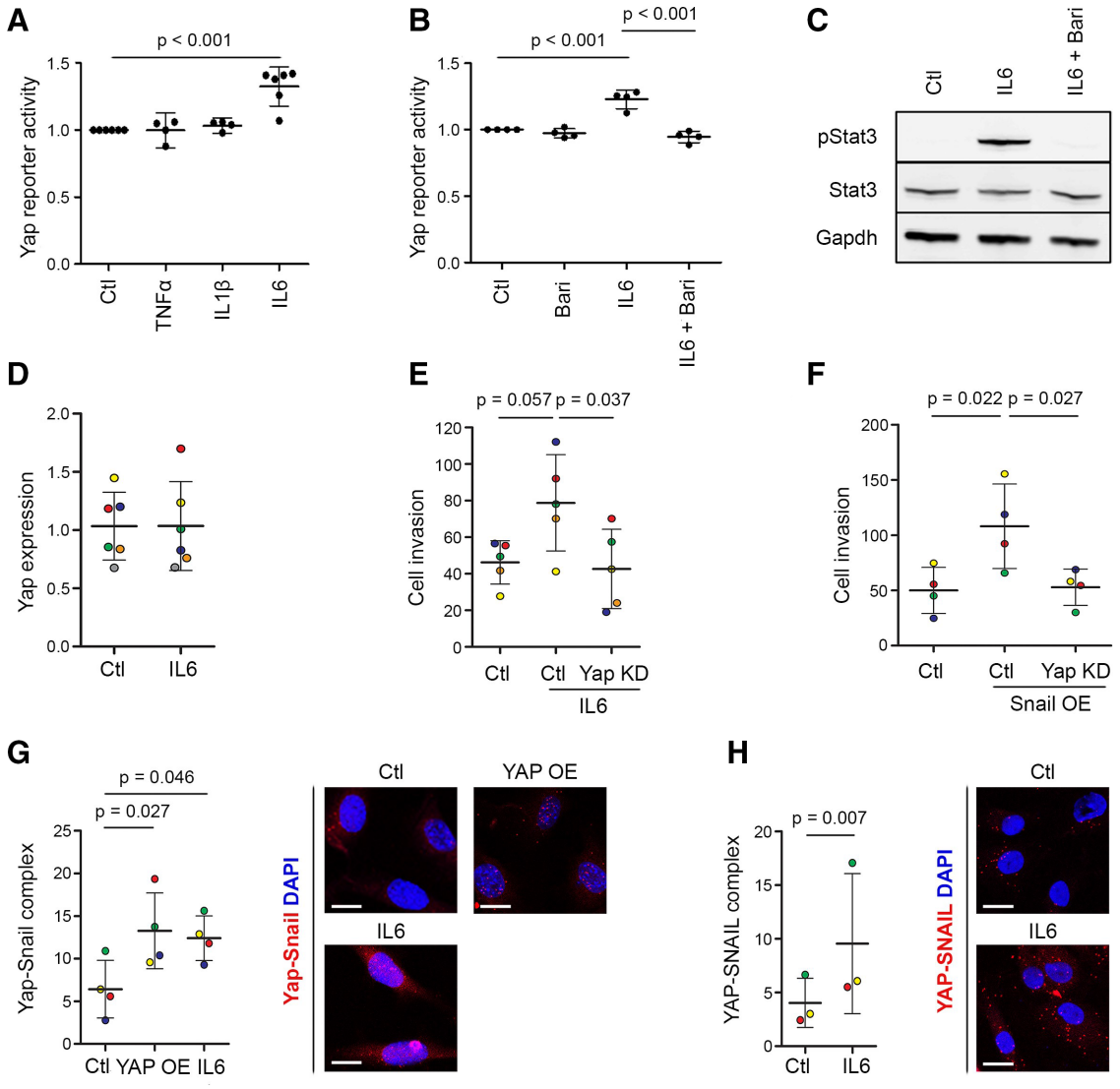

Figure 7 IL-6 activates Yap through Jak and drives SF invasion by stimulating Yap–Snail interaction. (A) IL-6 activates Yap. Yap–Tead GFP reporter cells were stimulated with 10 ng/mL TNF-α, 10 ng/mL IL-1β or 20 ng/mL IL-6/sIL6R, and GFP expression was quantified by flow cytometry (n=4–6 experiments). P value: one-way repeated measures ANOVA with Tukey's post-test, performed on data before normalisation. (B) The Jak inhibitor baricitinib prevents IL-6-induced Yap activation. Yap–Tead GFP reporter cells were treated with IL-6/sIL6R (20 ng/mL) and baricitinib (1 or 2 µM) for 48 hours under vehicle-controlled conditions, and GFP expression was quantified by flow cytometry (n=4 experiments). P values: one-way repeated measures ANOVA with Tukey's post-test, performed on data before normalisation. (C) Baricitinib prevents IL-6/sIL6R-induced Stat3 phosphorylation. Yap–Tead GFP reporter cells were pretreated for 1 hour with baricitinib (10 µM) and then treated with IL-6/sIL6R (140 ng/mL) for 30 min, as indicated, under vehicle-controlled conditions. Data are representative of n=3 experiments. See online supplemental figure 11 for uncropped Western blot images. (D) IL-6/sIL6R treatment (10 ng/mL) does not affect Yap mRNA expression in AIA-SF after 24 hours. (E) Yap KD prevents the increased AIA-SF invasiveness after IL-6/sIL6R treatment (10 ng/mL) for 48 hours. Dots are colour-coded to indicate five independent experiments using cells from four different mice. P values: one-way repeated measures ANOVA with Tukey's post-test. (F) Yap KD prevents the increased invasion of AIA-SF induced by Snail overexpression. Dots are colour-coded to indicate four independent experiments using cells from three different mice. P values: one-way repeated measures ANOVA with Tukey's post-test. (G) Treatment with IL-6/sIL6R (5 ng/mL) for 4 hours increased Yap–Snail complex formation in mouse SF, detected using proximity ligation assay. Transfection with constitutively active YAP-S127A was used as a positive control. Dots are colour-coded to indicate independent experiments using cells from different mice (n=4). P values: repeated measures one-way ANOVA with Dunnet's post-test. Scale bars: 20 µm. (H) Treatment with IL-6/sIL6R (5 ng/mL) for 4 hours increased YAP–SNAIL complex formation in human SF, detected using proximity ligation assay. Dots are colour-coded to indicate independent experiments using cells from different donors (n=3). P value: two-tailed paired Student's t-test. Scale bars: 20 µm. Lines and error bars on all graphs indicate mean±SD. ANOVA, analysis of variance; IL, interleukin; KD, knockdown; OE, overexpression; SF, synovial fibroblast; Yap, Yes-associated protein.

configure a synergistic cooperation of the inflammatory cytokine network in RA to activate SF and transform them into destructive cells via inducing Yap–Snail interaction.

A recent study reported functionally distinct fibroblast subsets in RA synovium, with destructive fibroblasts restricted to the synovial lining layer and immune effector fibroblasts located in the synovial sub-lining.[2] In our study, we show that while in normal knee joint synovium the *Gdf5*-lineage SF were largely confined to the synovial lining, as previously reported,[5] in AIA synovitis the *Gdf5*-lineage SF underwent extensive proliferation

and expansion throughout the synovium, indicating a derangement of the anatomical segregation of fibroblast lineages during synovitis. Our findings show the *Gdf5*-lineage SF to become erosive and support a role for Yap in the destructive SF, possibly through modulation of MMP expression.[28–30] The reduced immune cell infiltrates in the synovium of *Yap* cKO mice with AIA suggests that the *Gdf5*-lineage also includes immune effector SF and that Yap may be needed in these cells to sustain inflammation. Indeed, *Gdf5*-lineage SF from *Yap* cKO AIA mice displayed reduced expression levels of IL-34, an inflammatory cytokine produced by RA-SF able to support osteoclastogenesis[39] and reported to be associated with synovitis severity in patients with RA.[40]

In summary, we report a novel IL-6-Jak-Yap–Snail signalling axis linking immune-mediated inflammation with pathogenic SF in RA. Our findings position Yap in RA-SF at the crossroads between the activated immune system and their destructive phenotype, and suggest Yap for SF-targeted therapy in RA.

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Contributors RAS and FC: experimental design, data acquisition, analysis and interpretation, and writing of the manuscript. FLC and AJRa: experimental design, data acquisition and analysis. KK, JJM, NW and IC: data acquisition and analysis. SA and EH: data acquisition. KSM: data analysis. KAH: experimental design. AHKR and AM: provided human tissue samples. SMC: experimental design, data acquisition and analysis. AJR: conceptualisation, experimental design, data acquisition, analysis and interpretation, and writing of the manuscript. CDB: conceptualisation, experimental design, data analysis and interpretation, and writing of the manuscript. All authors edited and approved the manuscript. CDB is acting as guarantor.

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Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval Human synovial tissue samples were obtained from patients with a clinical diagnosis of rheumatoid arthritis after informed consent, under the auspices of the NHS Grampian Biorepository and IRCCS Policlinico San Matteo Foundation. Animal experimental protocols were approved by the UK Home Office and the Animal Welfare and Ethical Review Committee of the University of Aberdeen.

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Data availability statement Data are available upon reasonable request. All data relevant to the study are included in the article or uploaded as supplemental information.

ORCID iDs
Rebecca A Symons http://orcid.org/0000-0002-2811-7919
Fabio Colella http://orcid.org/0000-0002-3929-7273
Fraser L Collins http://orcid.org/0000-0002-1156-6873
Anke J Roelofs http://orcid.org/0000-0001-8993-1984
Cosimo De Bari http://orcid.org/0000-0002-5113-862X

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CLINICAL SCIENCE

Efficacy and safety of risankizumab for active psoriatic arthritis: 24-week results from the randomised, double-blind, phase 3 KEEPsAKE 1 trial

Lars Erik Kristensen,[1] Mauro Keiserman,[2] Kim Papp,[3] Leslie McCasland,[4] Douglas White,[5] Wenjing Lu,[6] Zailong Wang,[6] Ahmed M Soliman,[6] Ann Eldred,[6] Lisa Barcomb,[6] Frank Behrens[7]

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For numbered affiliations see end of article.

Correspondence to
Dr Lars Erik Kristensen, The Parker Institute, Bispebjerg and Frederiksberg Hospital, Copenhagen University Hospital, Frederiksberg, Denmark; lars.erik.kristensen@regionh.dk

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ABSTRACT

Objective To evaluate risankizumab, a biological therapy that inhibits interleukin 23, in patients with active psoriatic arthritis (PsA) who have responded inadequately or are intolerant to ≥1 conventional synthetic disease-modifying antirheumatic drug (csDMARD).

Methods In the randomised, placebo-controlled, double-blind KEEPsAKE 1 trial, 964 patients with active PsA were randomised (1:1) to receive risankizumab 150 mg or placebo at weeks 0, 4 and 16. The primary endpoint was the proportion of patients achieving ≥20% improvement in American College of Rheumatology criteria (ACR20) at week 24. Here, we report the results from the 24-week double-blind period; the open-label period with all patients receiving risankizumab is ongoing.

Results At week 24, a significantly greater proportion of patients receiving risankizumab achieved the primary endpoint of ACR20 (57.3% vs placebo, 33.5%; p<0.001). Significant differences were also observed for risankizumab versus placebo for the first eight ranked secondary endpoints, including skin and nail psoriasis endpoints, minimal disease activity and resolution of enthesitis and dactylitis (p<0.001). Adverse events and serious adverse events were reported at similar rates in the risankizumab and placebo groups. Serious infections were reported for 1.0% and 1.2% of patients receiving risankizumab and placebo, respectively. There was one death in the risankizumab group (urosepsis deemed unrelated to the study drug).

Conclusions Risankizumab treatment results in significantly greater improvement of signs and symptoms of PsA compared with placebo and is well tolerated in patients with active PsA who have responded inadequately or are intolerant to ≥1 csDMARD.

Trial registration number NCT03675308.

Key messages

What is already known about this subject?

- Despite the range of available therapies for psoriatic arthritis, efficacious, well-tolerated therapeutic options are needed to treat the diverse disease manifestations in patients who have not responded adequately to standard treatment.

What does this study add?

- Risankizumab 150 mg at weeks 0, 4 and 16 significantly improved the signs and symptoms of psoriatic arthritis, including joint symptoms, enthesitis and dactylitis, and skin and nail manifestations of psoriasis, in patients with inadequate response or intolerance to ≥1 conventional synthetic disease-modifying antirheumatic drug.
- Risankizumab was well tolerated, with a safety profile similar to that observed in patients with psoriasis, and no new safety signals were identified.

How might this impact on clinical practice or future developments?

- The results from the phase 3 KEEPsAKE 1 trial demonstrate the efficacy of risankizumab to treat the diverse clinical manifestations of psoriatic arthritis.
- Risankizumab may provide an additional therapeutic option for patients in whom standard therapies are inadequate.

INTRODUCTION

Psoriatic arthritis (PsA) is a chronic, systemic, inflammatory disease characterised by co-occurring musculoskeletal inflammation and psoriasis. The diverse clinical manifestations of PsA include arthritis, enthesitis, dactylitis, axial involvement, and skin and/or nail psoriasis. The impact of PsA on patients' function; pain; fatigue; emotional well-being and ability to participate in work, social and leisure activities reduces patients' quality of life[1] and contributes to the individual and societal burden of the disease.[2]

Treating all facets of PsA is important for meaningfully improving patients' quality of life. First-line PsA treatment includes non-steroidal anti-inflammatory drugs, local corticosteroid injections for musculoskeletal symptoms and topical therapies for psoriasis. For patients with poor prognostic factors or who do not respond adequately to first-line treatments, systemic therapy with conventional synthetic disease-modifying antirheumatic drugs (csDMARDs), antitumour necrosis factor therapy and other biological therapies are recommended.[3] Despite the range of available PsA therapies,

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efficacious, well-tolerated therapeutic options are needed for patients who have experienced inadequate responses or intolerances to available therapies.

Risankizumab is a humanised IgG1 monoclonal antibody that specifically inhibits interleukin 23 (IL-23) by binding to its p19 subunit. Risankizumab is approved in multiple countries to treat moderate-to-severe plaque psoriasis.[4] The KEEPsAKE 1 trial is evaluating the efficacy and safety of risankizumab to treat active PsA in patients who had responded inadequately or were intolerant to ≥1 csDMARD. The companion KEEPsAKE 2 trial (NCT03671148) is evaluating similar endpoints in a patient population that includes patients with a history of inadequate response or intolerance to biological agents.[5] The results of the initial 24-week double-blind period of the ongoing KEEPsAKE 1 study are reported herein.

METHODS

Study design and treatment

This phase 3, global, multicentre study included a screening period; a 24-week double-blind, placebo-controlled, parallel-group period; and a 204-week open-label period. Patients were randomised (1:1, stratified by baseline psoriasis (≥3%/<3% body surface area), presence of dactylitis (yes/no), presence of enthesitis (yes/no) and current csDMARD use (0/≥1)) by interactive response technology to receive subcutaneously administered risankizumab 150 mg or matching placebo in a blinded fashion at weeks 0, 4 and 16 during the double-blind period. Study visits occurred at weeks 0, 4, 8, 12, 16 and 24. Patients who had not achieved ≥20% improvement in swollen and/or tender joint count at both weeks 12 and 16 could add or modify concomitant therapies. Except for the baseline and primary endpoint visits, study visits could be modified to accommodate COVID-19-related restrictions; these included out-of-window study visits, phone calls and/or at-home visits for patients unable to attend onsite visits. The study drug was not administered to patients with suspected or confirmed COVID-19 infection; study drug administration and study visits could be resumed after patients recovered from infection.

This study was conducted in accord with the protocol, operations manual, International Council for Harmonisation guidelines, applicable regulations and guidelines governing clinical study conduct and the ethical principles that have their origin in the Declaration of Helsinki. The study protocol, informed consent document and all study materials were reviewed and approved by the independent ethics committee or institutional review board. All patients provided written informed consent to participate in the study.

Patient and public involvement

Patients and/or the public were not involved in the design, conduct, reporting or dissemination plans of this research.

Patients

Eligible patients were adults (aged ≥18 years) with active PsA (symptom onset ≥6 months, meeting the Classification Criteria for Psoriatic Arthritis, ≥5 of 68 tender and ≥5 of 66 swollen joints, ≥1 erosion based on centrally read radiograph (hands and/or feet) or high-sensitivity C reactive protein (hsCRP) ≥3.0 mg/L and active plaque psoriasis (≥1 psoriatic plaque(s) of ≥2 cm in diameter or nail psoriasis)). All patients had experienced an inadequate response, intolerance or contraindication to ≥1 csDMARD (csDMARD-IR). Continuation of concomitant therapy with ≤2 csDMARDs at protocol-approved doses was allowed. No prior exposure to biologics was permitted; however, prior exposure to targeted synthetic disease-modifying antirheumatic drug was allowed.

Assessments

Efficacy assessments

The primary endpoint was the proportion of patients who achieved ≥20% improvement in American College of Rheumatology criteria (ACR20) at week 24. Multiplicity-controlled ranked secondary endpoints included (1) change from baseline in Health Assessment Questionnaire-Disability Index (HAQ-DI); (2) proportion of patients who achieved ≥90% reduction in Psoriasis Area and Severity Index 90 (PASI 90); (3) proportion of patients who achieved ACR20 at week 16; (4) proportion of patients who achieved minimal disease activity (MDA); (5) change from baseline in modified Nail Psoriasis Severity Index (mNAPSI), a composite score incorporating grading (0–3) of pitting, onycholysis and oil-drop dyschromia and crumbling and absence/presence (0/1) of leukonychia, splinter haemorrhages, hyperkeratosis and red spots in the lunula[6]; (6) change from baseline in Physician's Global Assessment of Fingernail Psoriasis Score (PGA-F), based on the worse of nail bed or nail matrix signs of disease severity (0 (clear) to 4 (severe)[7 8]), (7) proportion of patients who achieved resolution of enthesitis (Leeds Enthesitis Index=0; prespecified analysis of pooled data from KEEPsAKE 1 and KEEPsAKE 2); (8) proportion of patients who achieved resolution of dactylitis (Leeds Dactylitis Index=0; prespecified analysis of pooled data KEEPsAKE 1 and KEEPsAKE 2); (9) change from baseline in PsA-modified Total Sharp Score (PsA-mTSS)[9]; (10) change from baseline in 36-Item Short-Form Health Survey Physical Component Summary (SF-36 PCS) score; and (11) change from baseline in Functional Assessment of Chronic Illness Therapy-Fatigue Questionnaire (FACIT-Fatigue) score. Except for ACR20 at week 16, all ranked secondary endpoints were evaluated at week 24. Non-ranked secondary endpoints included the proportions of patients who achieved ≥50% and ≥70% improvement in ACR criteria (ACR50/70) at week 24. Post hoc analyses included the proportions of patients who achieved Disease Activity in Psoriatic Arthritis (DAPSA) remission (REM; DAPSA score ≤4), low disease activity (LDA) +REM (DAPSA score ≤14), ≥50% and ≥85% reduction in DAPSA.

Safety assessments

Safety was evaluated throughout the study and included adverse event (AE) monitoring, physical examinations, vital sign measurements and clinical laboratory testing for haematology and chemistry. An independent data monitoring committee periodically reviewed unblinded safety data until the week 24 interim analysis.

Statistical analysis

A sample size of 440 patients per treatment group was estimated to provide ≥90% power to detect a ≥25% difference in ACR20 response rates, assuming a placebo response rate of 35%. This sample size was estimated to provide approximately 80% power to detect a standardised effect size of 0.20 in change from baseline in PsA-mTSS.

Efficacy analyses were conducted on the full analysis set, which included all randomised patients who received one or more doses of the study drug. For categorical efficacy endpoints, missing data unrelated to COVID-19 were handled by non-responder imputation, and missing data due to COVID-19 (infection or

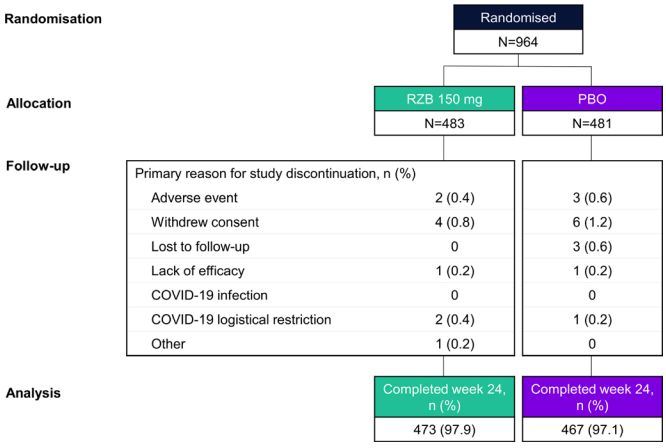

Figure 1 Patient disposition. PBO, placebo; RZB, risankizumab.

logistical restrictions) were handled by multiple imputation. Observations after patients initiated rescue therapy or concomitant medications for PsA that could have meaningfully impacted efficacy assessments were imputed as non-responders (categorical endpoints) or considered as missing and excluded from the model (continuous endpoints). Categorical efficacy endpoints were compared using the Cochran-Mantel-Haenszel test with adjustment for stratification factors. Continuous efficacy endpoints were analysed using mixed-effect model for repeated measures incorporating factors of treatment, visit, stratification factors and baseline values. Radiographic endpoints were analysed using an analysis of covariance model incorporating linear extrapolation to impute missing data or data after discontinuation of study drug or initiation of rescue medication. To increase sample size due to the smaller number of patients with enthesitis and dactylitis at baseline, data for the resolution of enthesitis and dactylitis were pooled from KEEPsAKE 1 and KEEPsAKE 2 (prespecified); these analyses were adjusted for common stratification factors and study. All primary and ranked secondary efficacy endpoints were tested with multiplicity adjustment via a fixed sequence testing procedure to control the family-wise type I error rate at $\alpha=0.05$ (two-sided). Safety analyses were conducted on the safety analysis set, which included all patients who received one or more doses of study drug.

RESULTS

Patients

A total of 964 patients were enrolled at 186 sites in 38 countries, and 97.5% completed the double-blind period between 25 March 2019 and 8 October 2020 (figure 1). No patients withdrew due to COVID-19 infection, and three patients (<0.3%) withdrew due to COVID-19 logistical restrictions. Less than 3% of patients in either group had missing data due to COVID-19 or the primary and all secondary endpoints (online supplemental table S1).

Demographical and baseline characteristics were generally balanced between groups (table 1). Patients were considered csDMARD-IR based on inadequate response (85.2%), intolerance (14.4%) or contraindication (0.4%) to prior therapy with ≥1 csDMARD. csDMARDs used previously by >10% of patients included methotrexate (89.9%), sulfasalazine (21.5%) and leflunomide (12.8%). The proportion of patients using concomitant csDMARDs was similar between the risankizumab and placebo groups (76.0% vs 76.7%); of concomitant csDMARDs, only methotrexate was reported for >10% of patients (61.6%).

Table 1 Demographics and characteristics at baseline

Characteristic	RZB 150 mg N=483	Placebo N=481
Women, n (%)	231 (47.8)	247 (51.4)
Age (years), median (range)	52 (20–85)	52 (22–79)
Race, n (%)		
White	454 (94.0)	451 (93.8)
Black/African American	4 (0.8)	2 (0.4)
Asian	13 (2.7)	22 (4.6)
Native Hawaiian/Pacific Islander	3 (0.6)	1 (0.2)
American Indian/Alaskan Native	1 (0.2)	0
Multiple	8 (1.7)	5 (1.0)
Not Hispanic/Latino, n (%)	390 (80.7)	389 (80.9)
BMI, kg/m^2, mean (SD)	30.7 (6.4)	30.3 (6.2)
PsA duration, years, mean (SD)	7.1 (7.0)	7.1 (7.7)
Swollen joint count,* mean (SD)	12.1 (7.8)	12.2 (8.0)
Tender joint count,† mean (SD)	20.8 (14.1)	20.5 (12.8)
Patient's assessment of pain,‡ mean (SD)	57.1 (22.6)	57.1 (22.6)
PtGA of disease activity,‡ mean (SD)	57.9 (21.8)	57.4 (22.1)
PGA of disease activity,‡ mean (SD)	61.3 (17.6)	62.4 (17.0)
HAQ-DI, mean (SD)	1.15 (0.66)	1.17 (0.65)
hsCRP, mg/L, mean (SD)	11.9 (15.9)	11.3 (14.1)
PsA-mTSS, mean (SD)	13.0 (29.9)	13.5 (29.0)
Presence of psoriasis affecting ≥3% BSA, n (%)	273 (56.5)	272 (56.5)
BSA,§ %, mean (SD)	16.8 (19.7)	16.5 (20.8)
PASI,§ mean (SD)	10.9 (10.1)	10.0 (10.4)
Presence of nail psoriasis, n (%)	309 (64.0)	338 (70.6)
mNAPSI,¶ mean (SD)	18.1 (16.4)	16.6 (16.0)
PGA-F,¶ mean (SD)	2.1 (1.0)	2.0 (1.0)
MDA, n (%)	2 (0.4)	6 (1.2)
Presence of enthesitis,** n (%)	297 (61.5)	290 (60.3)
LEI,†† mean (SD)	2.7 (1.5)	2.6 (1.5)
Presence of dactylitis,‡‡ n (%)	148 (30.6)	147 (30.6)
LDI,§§ mean (SD)	98.6 (120.4)	92.5 (125.5)
SF-36 PCS, mean (SD)	35.2 (8.1)	35.2 (7.7)
FACIT-Fatigue, mean (SD)	29.4 (11.3)	29.3 (11.2)
Prior csDMARDs,¶¶ n (%)		
0	2 (0.4)	2 (0.4)
1	338 (70.0)	311 (64.7)
2	105 (21.7)	136 (28.3)
≥3	38 (7.9)	32 (6.7)
Concomitant medication use, n (%)		
MTX***	314 (65.0)	315 (65.5)
csDMARD other than MTX†††	52 (10.8)	49 (10.2)
MTX and another csDMARD	20 (4.1)	29 (6.0)
Oral corticosteroids	101 (20.9)	87 (18.1)
NSAID	296 (61.3)	314 (65.3)

*Based on 66 joints.
†Based on 68 joints.
‡Scored as millimetres on a 100 mm horizontal Visual Analogue Scale.
§Among patients with ≥3% BSA affected by psoriasis (RZB, n=273; PBO, n=271).
¶Among patients with nail psoriasis (RZB, n=309; PBO, n=338).
**LEI >0.
††Among patients with LEI >0.
‡‡LDI >0.
§§Among patients with LDI >0.
¶¶Includes 32 patients who reported prior treatment with apremilast (RZB, n=11 (2.3%); PBO, n=21 (4.4%)) and five patients who reported prior treatment with tofacitinib (RZB, n=2 (0.4%); PBO, n=3 (0.6%)).
***As monotherapy or in combination with another csDMARD.
†††Sulfasalazine, leflunomide or apremilast without MTX.
BMI, body mass index; BSA, body surface area; csDMARD, conventional synthetic disease-modifying antirheumatic drug; FACIT-Fatigue, Functional Assessment of Chronic Illness Therapy-Fatigue; HAQ-DI, Health Assessment Questionnaire-Disability Index; hsCRP, high-sensitivity C reactive protein; LDI, Leeds Dactylitis Index; LEI, Leeds Enthesitis Index; MDA, minimal disease activity for PsA; mNAPSI, modified Nail Psoriasis Severity Index; MTX, methotrexate; NSAID, non-steroidal anti-inflammatory drug; PASI, Psoriasis Area and Severity Index; PBO, placebo; PGA, physician global assessment; PGA-F, Physician's Global Assessment of Fingernail Psoriasis; PsA, psoriatic arthritis; PsA-mTSS, PsA-modified Total Sharp Score; PtGA, patient's global assessment; RZB, risankizumab; SF-36 PCS, 36-Item Short-Form Health Survey Physical Component Summary.

Table 2 Primary and secondary efficacy endpoints

Parameter	RZB 150 mg N=483	Placebo N=481	Difference (95% CI)	P value
Primary endpoint				
ACR20 at week 24, n (%)	277 (57.3)	161 (33.5)	24.0 (18.0 to 30.0)	<0.001*
Ranked secondary endpoints				
Change in HAQ-DI at week 24, mean (95% CI)	−0.31 (−0.36,−0.27)	−0.11 (−0.16,−0.06)	−0.20 (−0.26 to 0.14)	<0.001*
PASI 90 at week 24,† n (%)	143 (52.3)	27 (9.9)	42.5 (35.6 to 49.3)	<0.001*
ACR20 at week 16, n (%)	272 (56.3)	161 (33.4)	23.1 (16.8 to 29.4)	<0.001*
MDA at week 24, n (%)	121 (25.0)	49 (10.2)	14.8 (10.2 to 19.4)	<0.001*
Change in mNAPSI at week 24,‡ mean (95% CI)	−9.8 (−11.0, −8.6)	−5.6 (−6.7, −4.4)	−4.2 (−5.7 to −2.7)	<0.001*
Change in PGA-F at week 24,‡ mean (95% CI)	−0.8 (−1.0, −0.7)	−0.4 (−0.5, −0.3)	−0.4 (−0.6 to −0.3)	<0.001*
Resolution of enthesitis at week 24,§ n (%)	215 (48.4)	156 (34.8)	13.9 (7.6 to 20.2)	<0.001*
Resolution of dactylitis at week 24,¶ n (%)	128 (68.1)	104 (51.0)	16.9 (7.5 to 26.4)	<0.001*
Change in PsA-mTSS at week 24, mean (95% CI)	0.23 (0.02, 0.44)	0.32 (0.11, 0.53)	−0.09 (−0.4 to 0.2)	0.50
Change in SF-36 PCS at week 24, mean (95% CI)	6.5 (5.8, 7.2)	3.2 (2.5, 3.9)	3.3 (2.4 to 4.2)	<0.001
Change in FACIT-Fatigue, at week 24, mean (95% CI)	6.5 (5.6, 7.3)	3.9 (3.1, 4.7)	2.6 (1.5 to 3.7)	<0.001
Non-ranked secondary endpoints				
ACR50 at week 24, n (%)	162 (33.4)	54 (11.3)	22.2 (17.3 to 27.2)	<0.001
ACR70 at week 24, n (%)	74 (15.3)	23 (4.7)	10.5 (6.9 to 14.2)	<0.001

All changes are from baseline. Results for binary endpoints are based on non-responder imputation incorporating multiple imputation if there were missing data due to COVID-19 or non-responder imputation if there were no missing data due to COVID-19. Results for continuous endpoints are based on mixed models for repeated measures, except for PsA-mTSS, which was based on the analysis of covariance model.
*Statistically significant under overall type I error control.
†Among patients with ≥3% body surface area affected by psoriasis at baseline (RZB, n=273; PBO, n=272).
‡Among patients with nail psoriasis at baseline (RZB, n=309; PBO, n=338).
§Defined as LEI=0 among patients with LEI >0 at baseline. Prespecified analysis of pooled data from the KEEPsAKE 1 and KEEPsAKE 2 trials (RZB, n=444; PBO, n=448).
¶Defined as LDI=0 among patients with LDI>0 at baseline. Prespecified analysis of pooled data from the KEEPsAKE 1 and KEEPsAKE 2 trials (RZB, n=188; PBO, n=204).
ACR 20/50/70, ≥20/50/70% improvement in American College of Rheumatology score; FACIT-Fatigue, Functional Assessment of Chronic Illness Therapy-Fatigue Questionnaire; HAQ-DI, Health Assessment Questionnaire-Disability Index; LDI, Leeds Dactylitis Index; LEI, Leeds Enthesitis Index; MDA, minimal disease activity; mNAPSI, modified Nail Psoriasis Severity Index; PASI 90, ≥90% reduction in Psoriasis Area and Severity Index; PBO, placebo; PGA-F, Physician's Global Assessment of Fingernail Psoriasis; PsA-mTSS, psoriatic arthritis-modified Total Sharp Score; RZB, risankizumab; SF-36 PCS, 36-Item Short-Form Health Survey Physical Component Summary.

Efficacy assessments

A significantly greater proportion of patients treated with risankizumab versus placebo achieved the primary endpoint of ACR20 at week 24 (57.3% vs 33.5%; p<0.001; table 2) and the secondary endpoint of ACR20 at week 16 (56.3% vs 33.4%; p<0.001; table 2). ACR component results at week 24 are shown in online supplemental table S2. Higher ACR20 response rates were observed at week 24 in patients treated with risankizumab versus placebo in all prespecified subgroups defined by demographics (eg, age, sex, race, body mass index), baseline disease characteristics (eg, duration of PsA, presence of enthesitis, presence of dactylitis) and use of prior or concomitant therapy as analysed using the Cochran-Mantel-Haenszel test. Specifically, higher ACR20 response rates were observed in patients treated with risankizumab versus placebo, regardless of whether patients received concomitant csDMARDs (57.9% vs 35.9%) or risankizumab as monotherapy (55.5% vs 26.2%; online supplemental table S3).

Rapid improvements in PsA signs and symptoms were observed in patients treated with risankizumab. After a single dose, a greater proportion of patients in the risankizumab group achieved ACR20 at week 4 than did patients in the placebo group; this result persisted through week 24 (figure 2A). Similar outcomes were observed for ACR50 and ACR70, as greater proportions of patients treated with risankizumab versus placebo achieved these endpoints at week 24 (nominal p value <0.001 for both; table 2); greater improvement was observed for patients receiving risankizumab compared with placebo by week 4 for ACR50 (figure 2B) and week 8 for ACR70 (figure 2C).

Among patients with enthesitis and/or dactylitis at baseline in the KEEPsAKE 1 and KEEPsAKE 2 studies (prespecified pooled analyses), greater proportions of patients treated with risankizumab versus placebo achieved resolution of their enthesitis or dactylitis (p<0.001 for both). Unpooled results from KEEPsAKE 1 for these endpoints were consistent with the pooled results, demonstrating greater improvement with risankizumab versus placebo (resolution of enthesitis, 51.2% vs 37.2%; nominal p<0.001; resolution of dactylitis, 66.9% vs 54.4%; nominal p=0.034). Changes from baseline in PsA-mTSS were not different between patients treated with risankizumab versus placebo (table 2). The proportion of patients demonstrating no radiographic progression (change from baseline of PsA-mTSS <0 or PsA-mTSS <0.5) is provided in online supplemental table S4.

Among patients with ≥3% body surface area affected by psoriasis at baseline, a significantly greater proportion of patients treated with risankizumab versus placebo achieved PASI 90 (52.3% vs 9.9%; p<0.001; table 2); differences were observed starting at week 8 and persisted through week 24 (figure 3). Significantly greater improvements in nail outcomes (mNAPSI and PGA-F) were observed for patients treated with risankizumab versus placebo among patients with psoriatic nail disease at baseline (p<0.001 for both; table 2).

Patients treated with risankizumab demonstrated improved physical function as evidenced by a significantly greater decrease from baseline in HAQ-DI (p<0.001; table 2). In a prespecified analysis of patients with HAQ-DI ≥0.35 at baseline, a greater percentage of patients achieved the minimal clinically important difference in HAQ-DI (improvement ≥0.35 from baseline)[10] at week 24 in the risankizumab group (50.3%) compared with the placebo group (27.9%; nominal p≤0.001). In addition, greater improvements from baseline were observed for both SF-36 PCS and FACIT-Fatigue

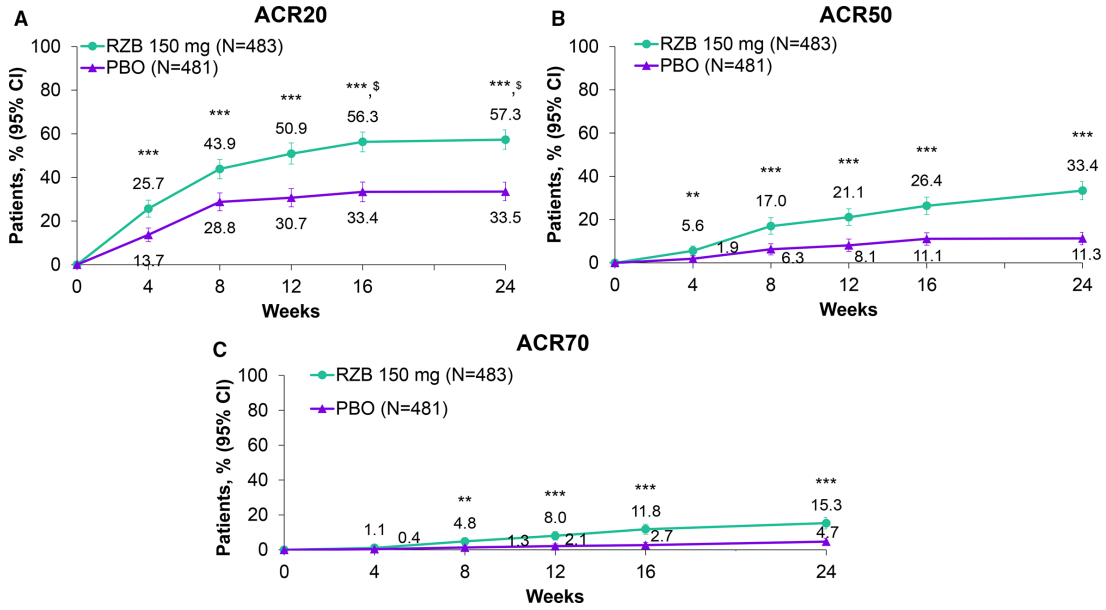

Figure 2 ACR responses over time. (A) ACR20, (B) ACR50 and (C) ACR70 response rates for risankizumab 150 mg and placebo over the 24-week, double-blind treatment period. ACR20/50/70, ≥20%/50%/70% improvement in American College of Rheumatology score; PBO, placebo; RZB, risankizumab. ***P≤0.001 versus PBO. $Statistically significant under overall type I error control. **P≤0.01.

in the risankizumab group compared with the placebo group (nominal p<0.001 for both).

Significantly greater proportions of patients treated with risankizumab versus placebo achieved MDA, a comprehensive measure of disease activity, at week 24 (25.0% vs 10.2%; p<0.001; table 2). Post hoc analyses of DAPSA outcomes (REM and LDA+REM, ≥50% and ≥85% score reductions) are reported in online supplemental table S5.

Safety

Treatment-emergent adverse events (TEAEs) were reported at similar frequencies in the risankizumab and placebo groups (40.4% and 38.7%, respectively; table 3). Most TEAEs were mild or moderate. Serious AE rates were comparable between groups. One death was reported for an 81-year-old male patient with dementia in the risankizumab group; the patient was hospitalised for pneumonia (week 8), subsequently developed urosepsis and died during week 13. One patient in the risankizumab group and two in the placebo group experienced COVID-19-related TEAEs. TEAEs leading to study drug discontinuation were rare (0.8% of patients in either group). TEAEs reported for ≥2% of patients in either group included nasopharyngitis, upper respiratory infection, increased alanine transaminase, increased aspartate transaminase and headache; all were reported at similar frequencies for patients in both groups (table 4).

Rates of AEs of safety interest were low and generally comparable between groups (table 3). However, injection site reactions were more frequently reported for patients in the risankizumab group; none of the reactions were serious, and no anaphylactic reactions were reported. Serious infections were reported for five patients in the risankizumab group and six patients in the placebo group.

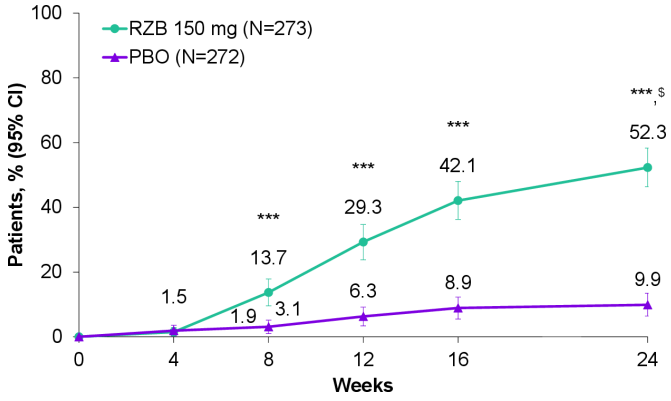

Figure 3 PASI 90 response over time. Among patients with ≥3% body surface area affected by psoriasis at baseline. PASI 90, ≥90% reduction in Psoriasis Area and Severity Index; PBO, placebo; RZB, risankizumab. ***P≤0.001 versus PBO. $Statistically significant under overall type I error control.

Table 3 Safety summary

Patients, n (%)	RZB 150 mg N=483	Placebo N=481
TEAE	195 (40.4)	186 (38.7)
COVID-19-related TEAE	1 (0.2)	2 (0.4)
Serious AE*	12 (2.5)	18 (3.7)
Severe TEAE*	10 (2.1)	9 (1.9)
TEAE leading to discontinuation of study drug	4 (0.8)	4 (0.8)
Death	1 (0.2)†	0
Serious infections‡	5 (1.0)	6 (1.2)
Active tuberculosis	0	0
Herpes zoster§	2 (0.4)	1 (0.2)
Any other opportunistic infections	0	0
Malignancy	0	2 (0.4)
Anaphylactic reactions	0	0
Injection site reactions¶	3 (0.6)	0
MACE	0	0

*Except for pneumonia, which was reported for two patients (0.4%) in the placebo group, no serious AE or severe TEAE was reported for >1 patient in either group.
†One death (urosepsis) in an 81-year-old male patient.
‡RZB: urosepsis (one patient, resulting in death), cellulitis (one patient), gastroenteritis (one patient), COVID-19 pneumonia (one patient) and viral upper respiratory tract infection leading to pneumonia (one patient); placebo: pneumonia (two patients), oral bacterial infection (one patient), dysentery (one patient), appendicitis (one patient) and cellulitis (one patient).
§All non-serious, resolved with oral antiviral agents and did not result in discontinuation of the study drug.
¶All non-serious and did not result in discontinuation of the study drug.
AE, adverse event; MACE, major adverse cardiovascular event; RZB, risankizumab; TEAE, treatment-emergent AE.

Table 4 Frequently reported TEAEs

Patients, n (%)	RZB 150 mg N=483	Placebo N=481
TEAEs reported for ≥2% of patients in either group		
Nasopharyngitis	16 (3.3)	14 (2.9)
Upper respiratory tract infection	12 (2.5)	20 (4.2)
Increased ALT	13 (2.7)	10 (2.1)
Increased AST	10 (2.1)	7 (1.5)
Headache	10 (2.1)	8 (1.7)

ALT, alanine aminotransferase; AST, aspartate aminotransferase; RZB, risankizumab; TEAE, treatment-emergent adverse events.

Herpes zoster was reported for two patients receiving risankizumab and one patient receiving placebo; all were non-serious, resolved with oral antiviral treatment and did not result in treatment discontinuation. No active tuberculosis or other opportunistic infections were reported. No malignancies were reported for patients receiving risankizumab; one event each of breast cancer and non-small-cell lung cancer was observed in the placebo group.

Mean changes in haematology and clinical chemistry (except liver function tests) were small, not clinically meaningful and comparable between the risankizumab and placebo groups. Grade 3 transaminase elevations (based on Common Terminology Criteria for Adverse Events version 4.03) were reported for <2% of patients in either group (nine patients receiving risankizumab and four patients receiving placebo). Grade 3 transaminase elevations in the nine patients receiving risankizumab were transient and were not accompanied by elevations in bilirubin. Grade 3 transaminase elevations in eight of the nine patients either (1) coincided with initiation of isoniazid or fenofibrate or (2) occurred in patients with underlying medical conditions of hepatic steatosis or hepatic cytolysis syndrome. The remaining patient had grade 1 and grade 2 transaminase levels at screening and baseline and experienced a single grade 3 elevation on study day 57. Subsequent transaminase levels for this patient were at or below baseline levels while the patient continued to receive risankizumab.

DISCUSSION

Currently available csDMARDs demonstrate variable efficacy in treating the diverse clinical manifestations of PsA, and additional therapeutic agents are needed to address the range of rheumatological and dermatological signs and symptoms of disease. At week 24 of the phase 3 KEEPsAKE 1 study, risankizumab 150 mg significantly improved clinical manifestations of PsA in patients who had an inadequate response or were intolerant to one or more csDMARDs, as evidenced by the achievement of the primary efficacy endpoint (ACR20) and secondary endpoints evaluating physical function, skin and nail psoriasis and resolution of enthesitis and dactylitis.

Evidence of improved joint symptoms (ACR20/50/70) was observed at early time points and increased over time through week 24. Risankizumab was effective, regardless of concomitant csDMARD therapy, as similar efficacy rates were observed in patients treated with risankizumab as monotherapy or in combination with one or more csDMARDs. Risankizumab treatment also markedly reduced hsCRP levels. Across KEEPsAKE 1 and KEEPsAKE 2, significantly greater proportions of patients treated with risankizumab versus placebo achieved resolution of dactylitis and enthesitis. There was no difference in change from baseline in PsA-mTSS between groups at week 24.

Risankizumab treatment led to the achievement of PASI 90 in over 50% of patients with ≥3% of body surface area affected by psoriasis at baseline. Many patients with PsA have psoriatic nail disease, which is associated with substantial disease burden and negatively impacts quality of life.[11–13] Risankizumab treatment resulted in significant improvements from baseline in nail psoriasis (mNAPSI and PGA-F) among patients with psoriatic nail disease at baseline.

Significantly greater improvements in HAQ-DI and greater improvements in SF-36 PCS and FACIT-Fatigue scores demonstrate the benefits of risankizumab treatment on physical function. Together, these findings support the potential for risankizumab treatment to reduce the substantial patient burden of PsA.

By week 24, 25% of patients treated with risankizumab versus 10% in the placebo group had achieved MDA, a comprehensive measure of PsA activity and a recommended target for PsA treatment when using a treat-to-target approach,[14] further demonstrating the efficacy of risankizumab to treat the varied manifestations of PsA.

Risankizumab was generally well tolerated over 24 weeks of treatment. Notably, rates of opportunistic infection (ie, herpes zoster) were low with no reported cases of candidiasis or active tuberculosis. This safety profile is consistent with safety findings in previous studies of risankizumab in patients with psoriasis,[15 16] and no new safety concerns were identified.

Several therapeutics targeting the IL-23/IL-17 pathway are approved to treat PsA.[17] Risankizumab's mechanism of action, specifically targeting the p19 subunit of IL-23, has been previously established,[18 19] and the KEEPsAKE 1 study results further support this mechanism of action for the treatment of PsA. The demonstrated efficacy and consistent safety profile of risankizumab, along with a 3-month dosing interval, further support the value of risankizumab as a treatment option for patients with PsA.

This study is being conducted during the COVID-19 pandemic. COVID-19-related logistical restrictions have been well managed, and completion of the double-blind period was not affected. Few patients had missing data due to COVID-19, and missing data did not impact efficacy conclusions. Further, there were no serious COVID-19-related safety issues. This study is currently limited by the availability of short-term data; the ongoing extension study will evaluate the maintenance of efficacy and long-term safety. The generalisability of these results may be limited by enrichment of the study population by requiring ≥5 tender and ≥5 swollen joints and at least one erosion based on centrally read radiograph or hsCRP ≥3.0 mg/L.

In summary, results from the 24-week double-blind portion of the KEEPsAKE 1 trial demonstrate that risankizumab is well tolerated and effective for treating diverse clinical manifestations of PsA in patients who have had an inadequate response or intolerance to csDMARD therapy. Risankizumab may provide an additional therapeutic option for patients in whom standard therapies are inadequate.

Author affiliations
[1]The Parker Institute, Copenhagen University Hospital, Bispebjerg and Frederiksberg, Copenhagen, Denmark
[2]Rheumatology Section, Pontifical Catholic University, School of Medicine, Porto Alegre, Brazil
[3]Probity Medical Research–K Papp Clinical Research, Waterloo, Ontario, Canada
[4]Department of Rheumatology, Loyola University Medical Center, Maywood, Illinois, USA, and Department of Veterans Affairs, Hines VA Hospital, Hines, Illinois, USA
[5]Rheumatology Department, Waikato Hospital, Hamilton, New Zealand, and Waikato Clinical School, University of Auckland, Auckland, New Zealand
[6]AbbVie Inc, North Chicago, Illinois, USA
[7]CIRI/Rheumatology & Fraunhofer Institute for Translational Medicine and Pharmacology (ITMP), Fraunhofer Cluster of Excellence for Immune-Mediated Disease (CIMD), Goethe University, Frankfurt, Germany

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CLINICAL SCIENCE

Very low prevalence of ultrasound-detected tenosynovial abnormalities in healthy subjects throughout the age range: OMERACT ultrasound minimal disease study

Jeanette Trickey ,[1,2] Ilfita Sahbudin,[1,2] Mads Ammitzbøll-Danielsen ,[3] Irene Azzolin,[4] Carina Borst ,[5] Alessandra Bortoluzzi,[6] George AW Bruyn ,[7,8] Philippe Carron ,[9,10] Coziana Ciurtin ,[11] Georgios Filippou ,[12] Jacek Fliciński,[13] Daniela Fodor,[14] Hélène Gouze ,[15] Marwin Gutierrez,[16,17] Hilde Berner Hammer ,[18,19] Ellen-Margrethe Hauge,[20,21] Annamaria Iagnocco,[22] Kei Ikeda ,[23] Rositsa Karalilova ,[24] Helen Isobel Keen,[25] Marion Kortekaas,[26,27] Giuliana La Paglia,[28] Gustavo Leon,[29,30] Peter Mandl,[31] Mihaela Maruseac,[32] Marcin Milchert ,[13] Mohamed Atia Mortada ,[33] Esperanza Naredo,[34] Sarah Ohrndorf ,[35] Carlos Pineda ,[36] Mads Nyhuus Bendix Rasch,[20] Cristina Reátegui-Sokolova ,[36,37] Garifallia Sakellariou ,[38] Teodora Serban,[39] Cesar A Sifuentes-Cantú,[40,41] Maria S Stoenoiu,[42] Takeshi Suzuki,[43] Lene Terslev,[3] Ilaria Tinazzi ,[44] Florentin Ananu Vreju,[45] Ruth Wittoek ,[10,46] Maria-Antonietta D'Agostino ,[47] Andrew Filer [1,2]

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For numbered affiliations see end of article.

Correspondence to
Dr Andrew Filer, Institute of Inflammation and Ageing, University of Birmingham, Birmingham, UK;
a.filer@bham.ac.uk

JT and IS contributed equally. M-AD and AF contributed equally.

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ABSTRACT

Objectives This study aimed to determine the prevalence of ultrasound-detected tendon abnormalities in healthy subjects (HS) across the age range.

Methods Adult HS (age 18–80 years) were recruited in 23 international Outcome Measures in Rheumatology ultrasound centres and were clinically assessed to exclude inflammatory diseases or overt osteoarthritis before undergoing a bilateral ultrasound examination of digit flexors (DFs) 1–5 and extensor carpi ulnaris (ECU) tendons to detect the presence of tenosynovial hypertrophy (TSH), tenosynovial power Doppler (TPD) and tenosynovial effusion (TEF), usually considered ultrasound signs of inflammatory diseases. A comparison cohort of patients with rheumatoid arthritis (RA) was taken from the Birmingham Early Arthritis early arthritis inception cohort.

Results 939 HS and 144 patients with RA were included. The majority of HS (85%) had grade 0 for TSH, TPD and TEF in all DF and ECU tendons examined. There was a statistically significant difference in the proportion of TSH and TPD involvement between HS and subjects with RA (HS vs RA p<0.001). In HS, there was no difference in the presence of ultrasound abnormalities between age groups.

Conclusions Ultrasound-detected TSH and TPD abnormalities are rare in HS and can be regarded as markers of active inflammatory disease, especially in newly presenting RA.

Key messages

What is already known about this subject?
- Little is known about the prevalence of sonographic tenosynovial abnormalities in healthy subjects (HS) across the age range.

What does this study add?
- This is the largest cohort of healthy subjects with tendons scanned by ultrasound.
- There is very low prevalence of tendon synovial hypertrophy or power Doppler abnormalities in tendons of HS even in old age.
- Ultrasound-detected inflammation in digit flexor and extensor carpi ulnaris tendons in patients suspected to be in the early stages of rheumatoid arthritis (RA) should not be discounted as physiological, even in older age.

How might this impact on clinical practice or future developments?
- Ultrasound-detected tenosynovial abnormalities can be regarded as robust findings in the clinical management of early RA.

INTRODUCTION

Tenosynovitis (TS) of hand and wrist tendons is common in early untreated inflammatory polyarthritis.[1] However, clinical examination alone may not detect this pathology,[2] especially as conventional rheumatoid arthritis (RA) disease activity scoring systems focus on joints, not tendons. The use of MRI and ultrasound examination is more sensitive and has shown that the prevalence of detecting TS in patients with early RA is higher than by physical examination alone.[3]

There has been extensive focus on the sensitivity and role of ultrasound in detecting subclinical synovial inflammation.[4 5] Ultrasound has been shown to be highly sensitive in the detection of tenosynovial inflammation, with recent studies demonstrating that ultrasound-detected hand and wrist TS has a role in predicting outcome in early RA and flare in clinical remission.[6 7]

Although recent studies using MRI have focused on the prevalence of tendon abnormalities in healthy subjects (HS),[8] there are limited data on the prevalence of ultrasound-detected 'TS' abnormalities in HS, with data arising from small comparison cohorts (ie, case–control studies focused on patients with rheumatic diseases). Furthermore, current studies were not focused on the prevalence of sonographic tendon abnormalities in HS within the age range of 40–70 years when RA commonly presents.[9] The prevalence of such abnormalities therefore remains unknown in this group.

The objective of this Outcome Measures in Rheumatology (OMERACT) ultrasound study was therefore to determine the prevalence of ultrasound-detected tendon abnormalities characterising the presence of TS in HS according to the age range.

METHODS

Adult HS (18–80 years) were recruited between August 2017 and December 2018 in 23 ultrasound centres in 14 countries with experience of participating in OMERACT ultrasound studies. To ensure a wide range of age coverage, recruitment was obtained from a large range of populations: university or hospital research staff, health service workers, students, volunteers from local advertising or national cohorts such as the Birmingham 1000 Elders group[10] in the UK. Exclusion criteria were current or previous history of any form of inflammatory arthritis, joint trauma of hands or wrist in the previous month; hand or wrist pain of o≥10/100 on the Visual Analogue Scale; hand osteoarthritis according to American College of Rheumatology (ACR) criteria[11]; history of infection; and recent or current use of medications that may affect ultrasound assessment (see online supplemental table 1). An additional 12 HS were excluded after data collection but before ultrasound analysis due to autoimmune, infectious or musculoskeletal conditions identified from medical history that could confound the results. Demographic data including body mass index (BMI) were collected. Metacarpophalangeal, proximal interphalangeal, metatarsophalangeal and wrist joints were clinically examined by an independent assessor in each centre, and subjects were excluded if synovitis was found.

Ultrasound assessment of bilateral digit flexors (DFs) 1–5 and extensor carpi ulnaris (ECU) tendons was performed using a multiplanar approach. The presence of hypoechoic tenosynovial hypertrophy (TSH) and power Doppler signal within tenosynovial power Doppler (TPD) was defined and graded using the OMERACT ultrasound scoring system for TS in RA.[12] The ungraded presence of tenosynovial effusion (TEF) was recorded. Adequate gel was used to avoid compression. Views were recorded according to European League Against Rheumatism (EULAR) standard reference scan guidelines.[13] Musculoskeletal specific preset parameters were used to optimise imaging for greyscale and power Doppler and reduce variability. Details of probes, machines and experience of sonographers in all centres can be found in online supplemental table 2. Quality and grading of recorded images were confirmed by a review of all images for the first HS recruited in each centre by an experienced blinded independent assessor (IS) in the hub centre. Any disagreement was then fed back to the centre and consensus was achieved to ensure reliability in subsequent scans.

Data for a comparison cohort of DMARD-naive patients presenting as patients with new early arthritis with RA fulfilling ACR-EULAR 2010[14] and/or 1987 criteria[15] at presentation were extracted from the Birmingham Early Arthritis (BEACON) inception cohort.[6] The following data were collected: 68 tender and 66 swollen clinical counts, age, sex, symptom duration, early morning stiffness duration, medication, erythrocyte sedimentation rate (ESR), C reactive protein (CRP), rheumatoid factor and anti-citrullinated protein antibody status. This cohort underwent identical baseline tendon ultrasound assessment except for the presence of TEF.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics V.26. Significance for the binary variable gender was assessed using Fisher's exact test. The continuous variables age and BMI (for all subjects) and early morning stiffness, CRP and joint counts (for patients with RA) were not normally distributed; significance was therefore assessed using the Kruskal-Wallis test. The tendon gradings were dichotomised into either present (grades 1–3) or absent (grade 0). Fisher's exact test was used to compare the proportions of grade 1–3 TSH, TPD or TEF between age groups in HS, and between HS and patients with RA.

RESULTS

One thousand and forty-nine HS were recruited and 939 HS were included after exclusions of subjects with protocol deviations (see flowchart in online supplemental figure 1). Baseline data for 144 patients with RA were randomly extracted from the BEACON database and matched with a cohort of 144 HS by age, sex and smoking status where possible. Table 1 shows the demographic and ultrasound characteristics of the two populations. Full ultrasound grading results are available in online supplemental table 2 and example of grading in online supplemental figure 2.

Healthy subjects

The median age of HS was 43 years (30–57). HS were grouped into three age groups: HS Y (young, 18–39 years) HS M (middle, 40–59 years) and HS O (old, 60–80 years) for analysis. The majority of volunteer HS were healthcare professionals (423, 45.0%). Other occupational groups included clerical staff (156, 16.6%), students (95, 10.1%), manual workers (68, 7.2%) and teachers (34, 3.6%).

A total of 11 237 tendons were scanned; 98% of these tendons were grade 0 for TSH, TPD and TEF (online supplemental table 3). The distribution of tendon abnormalities, when found, was symmetrical with no significant difference between right and left hands (online supplemental table 4). TEF was more frequently detected than TSH or TPD ($p<0.001$) (online supplemental table 5).

The majority (791/939, 84.2%) of HS presented grade 0 overall for all ultrasound lesions examined (TSH, TPD and TEF) in all DF 1–5 and ECU tendons. In particular 99% (931/939) of HS had grade 0 for TPD in all tendons scanned. There were no statistically significant differences between age groups (table 1 and figure 1).

Abnormalities were detected in 148 individuals across 939 HS and were of grade 1 severity, with the exception of one grade 2 for TSH in an ECU tendon. The ECU tendons had significantly more grade ≥1 for TSH than the DF 1–5 tendons ($p<0.05$) (online supplemental table 6).

Table 1 Demographics and tendon changes (grade 1–3 TSH and power Doppler) for HS and patients with RA

	HS Y 18–39 year	HS M 40–59 year	HS O ≥60 years	HS Y/M/O P value	RA	RA versus age-matched and sex-matched HS* P value
n	405	350	184		144	
Age (years), median (IQR)	29 (25–33)	49 (44–54)	68 (62–72)	<0.001	54 (45–67)	1.000
Female, n (%)	268 (66.2)	285 (81.4)	117 (63.6)	<0.001	106 (73.6)	0.924
BMI, median (IQR)	23 (22–24)	25 (21–28)	26 (23–28)	<0.001	27 (24–32)	<0.001
Smoking						
Never (%)	316 (78)	241 (68)	115 (63)		68 (47)	0.021
Ever (%)	88 (22)	109 (31)	66 (36)		75 (52)	
Current (%)	47 (12)	56 (16)	12 (7)		28 (19)	
EMS (min), median (IQR)	n/a	n/a	n/a	n/a	60 (15–120)	n/a
Symptom duration (weeks), median (IQR)	n/a	n/a	n/a	n/a	26 (13–52)	n/a
CRP (mg/L), median (IQR)	n/a	n/a	n/a	n/a	7 (3–20)	n/a
DAS28 CRP, median (IQR)	n/a	n/a	n/a	n/a	5.1 (4.1–5.8)	n/a
Tender joint,† median (IQR)	0 (0–0)	0 (0–0)	0 (0–0)	n/a	17 (11–27)	<0.001
Swollen joint,† median (IQR)	0 (0–0)	0 (0–0)	0 (0–0)	n/a	6 (3–11)	<0.001
DF 1 TSH grade ≥1, n (%)	1 (0.1)	0 (0)	1 (0.3)	0.490	15 (5.2)	<0.001
DF 2 TSH grade ≥1, n (%)	1 (0.1)	2 (0.3)	0 (0)	0602	50 (17.3)	<0.001
DF 3 TSH grade ≥1, n (%)	2 (0.2)	1 (0.1)	2 (0.6)	0.432	50 (17.3)	<0.001
DF 4 TSH grade ≥1, n (%)	2 (0.2)	1 (0.1)	1 (0.3)	1.000	28 (9.8)	<0.001
DF 5 TSH grade ≥1, n (%)	1 (0.1)	4 (0.6)	0 (0)	0.220	36 (12.5)	<0.001
ECU TSH grade ≥1, n (%)	7 (0.9)	9 (1.3)	1 (0.3)	0.293	65 (22.6)	<0.001
DF 1 TPD grade ≥1, n (%)	1 (0.1)	0 (0)	1 (0.3)	0.490	10 (3.5)	0.002
DF 2 TPD grade ≥1, n (%)	0 (0)	1 (0.1)	0 (0)	0.568	36 (12.6)	<0.001
DF 3 TPD grade ≥1, n (%)	1 (0.1)	0 (0)	0 (0)	1.000	40 (13.9)	<0.001
DF 4 TPD grade ≥1, n (%)	0 (0)	0 (0)	1 (0.3)	0.194	20 (7)	<0.001
DF 5 TPD grade ≥1, n (%)	0 (0)	0 (0)	0 (0)	n/a	23 (8.1)	<0.001
ECU TPD grade≥1, n (%)	0 (0)	0 (0)	0 (0)	n/a	62 (21.7)	<0.001
Total grade tendon score,‡ mean (range)	0.04 (0-2)	0.05 (0-4)	0.04 (0-2)		3.02 (0-21)	
Total count of tendons grade ≥1,§ mean (range)	0.03 (0-2)	0.05 (0-4)	0.03 (0-2)		1.69 (0-11)	
Individuals with grade ≥1 TSH, n (%)	12 (3.0)	10 (2.8)	4 (2.1)		76 (52.8)	
Individuals with grade ≥1 TPD, n (%)	2 (0.5)	1 (0.3)	2 (1.1)		63 (43.7)	
Individuals with grade ≥1 TEF, n (%)	50 (12.2)	46 (13.2)	29 (15.8)		n/a	

*RA and HS age matched and sex matched to compare ultrasound graded tendon findings.
†Patients with RA had 66/68 joint counts; HS had joint counts of MCPs, PIPs, wrists and MTPs.
‡Total grade tendon score is the per patient sum of all grades of TSH and TPD tendon abnormalities.
§ Total count of tendons grade ≥1 includes TSH and TPD.
BMI, body mass index; CRP, C reactive protein; DAS28, Disease Activity Score in 28 joints; DF, digit flexor; ECU, extensor carpi ulnaris; EMS, early morning stiffness; HS, healthy subjects; M, middle; MCP, metacarpophalangeal; MTP, metatarsophalangeal; O, old; PIP, proximal interphalangeal; RA, rheumatoid arthritis; TEF, tenosynovial effusion; TPD, tendon power Doppler; TSH, tenosynovial hypertrophy; Y, young.

There was no statistically significant difference in the proportion of TSH or TPD ≥1 in HS with manual professions, or in those who practice sports or hobbies which may have high impact on the upper limbs (online supplemental tables 7 and 8).

Patients with RA

Patients with RA were matched with 144 HS by age (within 2 years) and sex, and with smoking status in 116/144 HS. TS as defined by TSH and power Doppler grade ≥1 in DF and ECU tendons was more prevalent in patients with RA (52.8%) compared with HS (0.9%). There were significantly more TSH and TPD grade ≥1 detected in patients with RA compared with age-matched and sex-matched HS (p=0.002 to <0.001) (online supplemental table 9).

DISCUSSION

Our study is the first to assess tendon involvement in large numbers of HS, encompassing the age incidence of RA with 367 HS over 50 years, and showing a very low prevalence of abnormal findings. The few abnormalities observed were almost exclusively grade 1 in severity. Due to the large population assessed, we provide conclusive data validating and expanding on the findings of existing studies with few HS.[16–18]

TEF was more prevalent than TSH or TPD in HS. Although MRI studies have suggested TEF to be almost ubiquitous in DF tendons in HS,[19] we have shown that ultrasound detects smaller numbers: less than 2% of DF tendons even in the older age group. Visualisation of tendons in two dimensions is the most likely cause of this difference. Tenosynovial abnormalities on ultrasound were significantly more prevalent in early RA compared with matched HS.

By explicitly selecting only subjects with minimal joint pain and without overt osteoarthritis, and by using a non-random recruitment strategy to ensure inclusion of an older cohort, HS in this study may have fewer tendon changes than an unselected general population of 60–80 year olds. However, it was not our purpose to document the presence of tendon abnormalities in

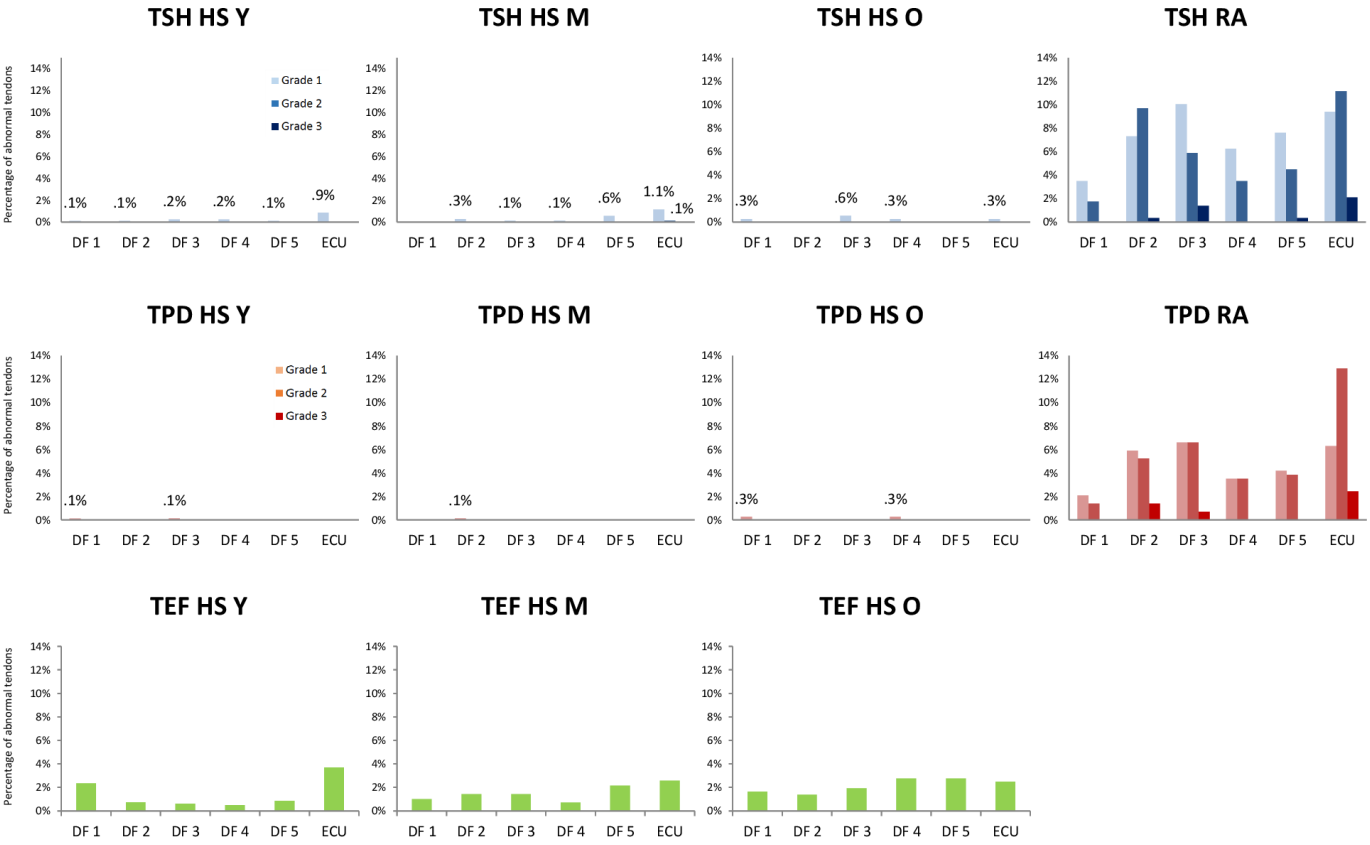

Figure 1 Percentage of tendons with grade 1–3 TSH and TPD, and presence of TEF in DF tendons 1–5 and ECU for HS according to age groups, compared with patients with RA. TEF measured only in HS. HS Y, 18–39 years; HS M, 40–59 years; HS O, 60–80 years. DF, digit flexor; ECU, extensor carpi ulnaris tendon; HS, healthy subjects; M, middle; O, old; RA, rheumatoid arthritis; TEF, tenosynovial effusion; TPD, tenosynovial power Doppler; TSH, tenosynovial hypertrophy; Y, young.

unselected primary or secondary care early arthritis clinics or in osteoarthritis, but to assess if HS with no symptoms may have ultrasound inflammatory abnormalities. The lack of a formal reliability study which would have been logistically difficult in such a large study, and the consecutive, not blinded recruitment may be seen as potential limitations. We mitigated these by designing a blinded central regrading strategy of the first HS scan performed by each centre.[20]

The very low prevalence of TSH and TPD across a large age range in HS suggests that these findings can be seen as potentially pathological, and not simply the consequence of ageing, by health professionals performing ultrasound in early arthritis or disease management clinics. The interpretation of such findings should depend on the clinical context. In addition, DF and ECU tendons can be easily examined during routine ultrasound examination and so could be included in abbreviated scanning protocols.

Author affiliations
[1]Institute of Inflammation and Ageing, University of Birmingham, Birmingham, UK
[2]NIHR Birmingham Biomedical Reserarch Centre, University Hospitals Birmingham NHS Foundation Trust, Birmingham, UK
[3]Center for Rheumatology and Spine Diseases, Rigshospitalet, Copenhagen, Denmark
[4]Academic Rheumatology Center, MFRU, Department of Clinical and Biological Science, University of Turin, Turin, Italy
[5]Department of Dermatology, Medical University of Vienna, Vienna, Austria
[6]Section of Rheumatology, Department of Medical Sciences, University of Ferrara and Azienda Ospedaliera-Universitaria di Ferrara, Cona, Italy
[7]MC Hospital Group, Lelystad, Netherlands
[8]Reumakliniek Flevoland, Lelystad, Netherlands
[9]Department of Internal Medicine and Paediatrics, University Hospital Ghent, Ghent, Belgium
[10]VIB Center for Inflammation Research, Ghent University, Ghent, Belgium
[11]Centre for Adolescent Rheumatology, Division of Medicine, University College London, London, UK
[12]University Hospital, Rheumatology Unit, ASST Fatebenefratelli Sacco, Milan, Italy
[13]Department of Internal Medicine, Rheumatology, Diabetes, Geriatrics and Clinical Immunology, Pomeranian Medical University, Szczecin, Poland
[14]2nd Internal Medicine, UMF Iuliu Haţieganu Cluj-Napoca, Cluj-Napoca, Romania
[15]Hopital Ambroise-Pare, Boulogne-Billancourt, France
[16]Clinica Reumatologica, Università Politecnica delle Marche, Ancona, Italy
[17]Instituto Nacional de Rehabilitacion Luis Guillermo Ibarra Ibarra, Mexico, Mexico
[18]Rheumatology, Diakonhjemmet Sykehus, Oslo, Norway
[19]Faculty of Medicine, University of Oslo, Oslo, Norway
[20]Department of Rheumatology, Aarhus University Hospital, Aarhus, Denmark
[21]Department of Clinical Medicine, Aarhus University, Aarhus, Denmark
[22]Academic Rheumatology Centre, Università degli Studi di Torino, Turin, Italy
[23]Department of Allergy and Clinical Immunology, Chiba University Hospital, Chiba, Japan
[24]University Hospital Kaspela, Medical University of Plovdiv Hospital, Plovdiv, Bulgaria
[25]Medicine and Pharmacology, UWA, Perth, WA, Australia
[26]Leiden University Medical Center, Leiden, Netherlands
[27]Flevoziekenhuis, Almere, Netherlands
[28]Rheumatology Unit, Asst-Fbf-Sacco, Luigi Sacco Hospital, Milan, Italy
[29]Instituto Nacional de Rehabilitacion, Mexico, Mexico
[30]Hospital Nacional Edgardo Rebagliati Martins, Lima, Peru
[31]Department of Rheumatology, Medical University of Vienna, Vienna, Austria
[32]Department of Rheumatology, Cliniques universitaires Saint-Luc, Brussels, Belgium
[33]Department of Rheumatology Rehabilitation and Physical Medicine, Zagazig University, Zagazig, Egypt
[34]Rheumatology, Hospital Universitario Fundación Jiménez Díaz, Madrid, Spain
[35]Department of Rheumatology and Clinical Immunology, Charité–Universitätsmedizin Berlin, Campus Mitte, Humboldt–Universität zu Berlin, Freie Universität Berlin, Berlin, Germany

[36]Rheumatology, Instituto Nacional de Rehabilitación Luis Guillermo Ibarra Ibarra, Mexico, Mexico
[37]Universidad San Ignacio de Loyola, Lima, Peru
[38]Istituti Clinici Scientifici Maugeri SpA SB IRCCS, Pavia, Italy
[39]S.C. Reumatologia, ASL3 Genovese, Ospedale La Colletta, Genoa, Italy
[40]Hospital Universitario Fundación Jiménez Díaz, Madrid, Spain
[41]Department of Immunology and Rheumatology, Instituto Nacional de Ciencias Medicas y Nutricion Salvador Zubiran, Tlalpan, Mexico
[42]Institut de Recherche Expérimentale et Clinique, Université catholique de Louvain, Rheumatology Department, Cliniques universitaires Saint-Luc, Brussels, Belgium
[43]Division of Allergy and Rheumatology, Japanese Red Cross Medical Center, Shibuya, Japan
[44]Unit of Rheumatology, IRCCS Ospedale Sacro Cuore Don Calabria, Negrar, Italy
[45]Department of Rheumatology, University of Medicine and Pharmacy of Craiova, Craiova, Romania
[46]Department of Internal Medicine and Paediatrics, Ghent University, Ghent, Belgium
[47]Università Cattolica del Sacro Cuore, Fondazione Policlinico Universitario Agostino Gemelli IRCCS, Roma, Italy

Twitter Philippe Carron @PhilippeCarron and Andrew Filer @A_Filer

Contributors JT and IS were equal contributors. MA-D and AF were equal contributors.

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ORCID iDs
Jeanette Trickey http://orcid.org/0000-0001-9275-5627
Mads Ammitzbøll-Danielsen http://orcid.org/0000-0003-4878-0432
Carina Borst http://orcid.org/0000-0002-3451-7820
George AW Bruyn http://orcid.org/0000-0001-7020-5798
Philippe Carron http://orcid.org/0000-0001-9254-6171
Coziana Ciurtin http://orcid.org/0000-0002-8911-4113
Georgios Filippou http://orcid.org/0000-0002-1647-2083
Hélène Gouze http://orcid.org/0000-0002-5333-0316
Hilde Berner Hammer http://orcid.org/0000-0001-7317-8991
Kei Ikeda http://orcid.org/0000-0003-0574-9611
Rositsa Karalilova http://orcid.org/0000-0001-8030-0529
Marcin Milchert http://orcid.org/0000-0002-0943-8768
Mohamed Atia Mortada http://orcid.org/0000-0002-7198-9878
Sarah Ohrndorf http://orcid.org/0000-0001-5943-4688
Carlos Pineda http://orcid.org/0000-0003-0544-7461
Cristina Reátegui-Sokolova http://orcid.org/0000-0003-3421-2717
Garifallia Sakellariou http://orcid.org/0000-0002-1849-5123
Ilaria Tinazzi http://orcid.org/0000-0002-5231-4250
Ruth Wittoek http://orcid.org/0000-0002-6367-9466
Maria-Antonietta D'Agostino http://orcid.org/0000-0002-5347-0060
Andrew Filer http://orcid.org/0000-0001-8164-6249

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CLINICAL SCIENCE

Choose wisely: imaging for diagnosis of axial spondyloarthritis

Torsten Diekhoff ,[1] Iris Eshed ,[2] Felix Radny,[1] Katharina Ziegeler ,[1] Fabian Proft ,[3] Juliane Greese,[1] Dominik Deppe,[1] Robert Biesen ,[4] Kay Geert Hermann,[1] Denis Poddubnyy [5]

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[1]Department of Radiology, Charité Universitätsmedizin Berlin, Berlin, Germany
[2]Radiology, Sheba Medical Center, Tel Hashomer, Israel
[3]Department of Gastroenterology, Infectiology and Rheumatology, Charite Universitatsmedizin Berlin Campus Benjamin Franklin, Berlin, Germany
[4]Department of Rheumatology and Clinical Immunology, Charité Universitätsmedizin Berlin, Berlin, Germany
[5]Division of Gastroenterology, Infectious Diseases and Rheumatology, Charité Universitätsmedizin Berlin, Berlin, Germany

Correspondence to
Torsten Diekhoff, Department of Radiology, Charité Universitätsmedizin Berlin, Berlin, Berlin, Germany; torsten.diekhoff@charite.de

TD and IE contributed equally.

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ABSTRACT

Objective To assess the diagnostic accuracy of radiography (X-ray, XR), CT and MRI of the sacroiliac joints for diagnosis of axial spondyloarthritis (axSpA).

Methods 163 patients (89 with axSpA; 74 with degenerative conditions) underwent XR, CT and MR. Three blinded experts categorised the imaging findings into axSpA, other diseases or normal in five separate reading rounds (XR, CT, MR, XR +MR, CT +MR). The clinical diagnosis served as reference standard. Sensitivity and specificity for axSpA and inter-rater reliability were compared.

Results XR showed lower sensitivity (66.3%) than MR (82.0%) and CT (76.4%) and also an inferior specificity of 67.6% vs 86.5% (MR) and 97.3% (CT). XR +MR was similar to MR alone (sensitivity 77.5 %/specificity 87.8%) while CT+MR was superior (75.3 %/97.3%). CT had the best inter-rater reliability (kappa=0.875), followed by MR (0.665) and XR (0.517). XR +MR was similar (0.662) and CT+MR (0.732) superior to MR alone.

Conclusions XR had inferior diagnostic accuracy and inter-rater reliability compared with cross-sectional imaging. MR alone was similar in diagnostic performance to XR+MR. CT had the best accuracy, strengthening the importance of structural lesions for the differential diagnosis in axSpA.

Key messages

What is already known about this subject?

⇒ Current imaging guidelines recommend radiography of the sacroiliac joints as first-line modality, followed by MRI when axial spondyloarthritis is suspected. Recently, low-dose CT was introduced for detection of structural lesions in the sacroiliac joints; however, its impact for diagnostic workup is still unclear.

What does this study add?

⇒ Radiography is inferior to MRI and CT of the sacroiliac joints in establishing the diagnosis when axial spondyloarthritis is suspected.

⇒ Combined radiography and MRI had no added value on readers' performance or inter-rater reliability compared with MRI alone and diagnostic scenarios with radiography as first imaging showed low specificity.

⇒ CT shows superior specificity and positive likelihood ratio and only a small shortfall in sensitivity compared with MRI, underlining the importance of structural lesions for the differential diagnosis.

How might this impact on clinical practice or future developments?

⇒ Radiography should be avoided whenever MRI is readily available, and current guidelines must be re-evaluated. CT is a highly specific alternative whenever MRI is inconclusive, unfeasible or not available.

INTRODUCTION

The European Alliance of Associations for Rheumatology guidelines still recommend X-ray (XR) as first-line imaging in axial spondyloarthritis (axSpA) and MRI if the diagnosis cannot be established by XR and clinical features.[1 2] While XR may miss early changes and has low inter-rater reliability, MR has proven to be superior in detecting erosions[3] and depicts periarticular and intra-articular fatty metaplasia and active inflammation of bone marrow and soft tissues.[4] Therefore, the question arises whether XR should always be used as a first-line imaging test or could be replaced by cross-sectional techniques.

A third modality that is gaining increasing attention as the gold standard for detecting structural lesions is CT.[5] While conventional CT is also unable to assess bone marrow changes and active inflammation of the sacroiliac joint (SIJ), it provides higher spatial resolution, thinner slices and direct depiction of the cortical bone compared with standard MR. This is one of the reasons why structural lesions in MR are not included in the Assessment of Spondyloarthritis international Society (ASAS) definition of positive imaging.[6] However, much knowledge has been gained since, and there is increasing evidence suggesting that MR can be used for structural assessment as well[7 8]—but it is still open how this impacts the diagnosis. While structural lesions might be less important in terms of classifying patients for study purposes, they are decisive for the differential diagnosis.[9 10] For example, a common condition in women, osteitis condensans, shows bone marrow oedema, sclerosis and fat metaplasia in the SIJ and might be, therefore, difficult to distinguish from axSpA.[11] The presence or absence of structural lesions such as erosions can have a decisive role in differentiating axSpA from non-inflammatory mechanical conditions.

In light of this complex diagnostic situation, the aim of the present study was to compare the three major modalities, XR, MR and CT of SIJ, regarding their capabilities in the diagnosis and differential diagnosis of axSpA in patients with low back pain using the final judgement of the rheumatologist as standard of reference.

METHODS

Subjects

The SacroIliac joint MAgnetic resonance imaging and Computed Tomography (SIMACT) study, in which patients underwent XR, MR and CT, is already well described in the literature.[3 7] For the second study, patients also underwent MR and dual-energy CT of the SIJ, from which conventional CT images were reconstructed. All patients had chronic back pain and were referred for imaging with suspected or known axSpA. Patients were excluded if one of the modalities was not available.

Anonymisation

Images were anonymised separately and read independently in the following five sessions: XR, MR and CT alone, XR plus MR and CT plus MR. Thus, every patient was presented five times to each reader either with just one modality (XR, MR and CT) or with two modalities (XR plus MR and CT plus MR). Oblique-coronal T1-weighted and short-tau inversion recovery sequences were provided in the MR datasets, and CT volumes were reconstructed in 4 and 1 mm slices in oblique-coronal orientation.

Image assessment

Three expert musculoskeletal radiologists, completely blinded to identifying information, clinical data including the clinical diagnosis and findings of the other modalities and previous imaging as well as the prevalence of axSpA within the study population, used an online electronic case report form to answer the following questions for each image dataset:

1. Grading using the modified New York Criteria (mNYC): grade 0–4 for each SIJ.
2. Are unequivocal structural lesions compatible with axSpA present: yes or no?
3. Does MR fulfil the ASAS criteria for MR-positivity (MR only): yes or no?
4. Overall impression: normal or pathologic?
5. If pathologic: axSpA or other?

Readers were advised to give their personal expert opinion.

Statistical analysis

Descriptive statistics was computed for clinical parameters and scoring results, where agreement of at least two readers was used to report results by modality. Sensitivity and specificity and positive and negative likelihood ratios (LRs) were calculated using the expert rheumatologists' assessment as standard of reference. Fleiss' kappa was used for assessing the inter-rater reliability of the imaging diagnosis. Percent of patients with MR and diagnostic accuracy values were calculated for four different clinical scenarios:

A. : Clinical standard with XR followed by MR if mNYC negative.
B. : Radiography followed by MR if no SIJ shows sacroiliitis grade 3 or 4.
C. : MR only.
D. : CT followed by MR if negative.

Calculations were performed using Microsoft Excel, Graphpad Prism (version 9) and SPSS (version 27).

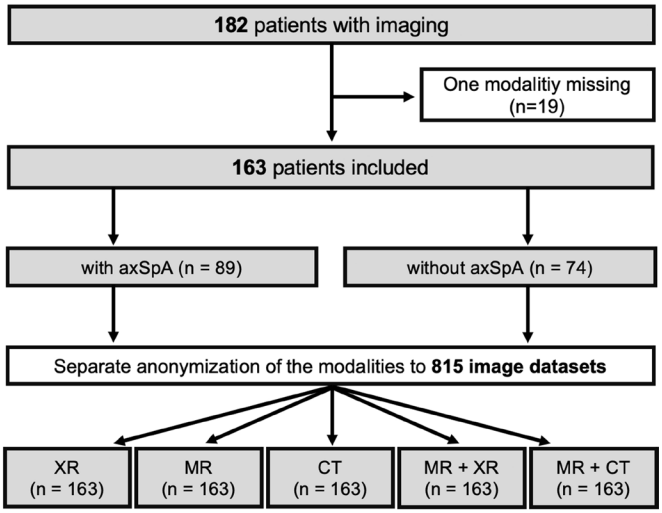

Figure 1 Flow chart of patient inclusion. After excluding 19 patients with missing imaging data, a total of 163 patients were included, 89 with the final diagnosis of axSpA. The image datasets were anonymised into five different chunks: radiography, MR and CT alone and MR combined with XR, and MR combined with CT. The datasets were separately presented to the readers. axSpA, axial spondyloarthritis; XR, X-ray.

RESULTS

Subjects

We analysed a total of 163 patients (see figure 1)—89 (54.6%) with axSpA, 56 (34.4%) with degenerative or mechanical SIJ disease such as osteoarthritis or osteitis condensans ilii, and 18 (11%) with non-specific back pain unrelated to SIJ. Mean age was 38.2±10.6 years, and symptom duration 79.3±89.5 months; 82.3% had inflammatory back pain, 50% were female and 60.2% HLA-B27-positive. Patient characteristics are summarised in online supplemental 1.

Imaging findings

Grading results based on the mNYC are provided as means of all readers in online supplemental 2. Stages of axSpA in the study population ranged from early disease to established axSpA with advanced structural damage. 83 XRs, 70 CTs and 75 MRs were scored positive for structural damage. Interestingly, the number of patients positive for structural lesions increased when scoring XR and MR (81) compared with MR alone but decreased for CT and MR (71).

Diagnostic modalities

Sensitivity, specificity and the corresponding LRs for each modality alone and their combinations are shown in figure 2 and in more detail in online supplemental 3. Symptom duration had no effect on diagnostic accuracy (see online supplemental 4).

XR showed lower sensitivity than MR and CT and also an inferior specificity compared with MR and CT. XR +MR was similar to MR alone in terms of sensitivity and specificity, while CT +MR was superior to MR alone. Imaging examples are shown in figure 3.

Inter-rater reliability

Inter-rater agreement was substantial for XR with a Fleiss' kappa of 0.517 (95% CI 0.428 to 0.605) and MR (0.665, 95% CI 0.576 to 0.753) but almost perfect for CT (0.875, 95% CI 0.786 to 0.964). CR+MR had similar inter-rater agreement (0.662,

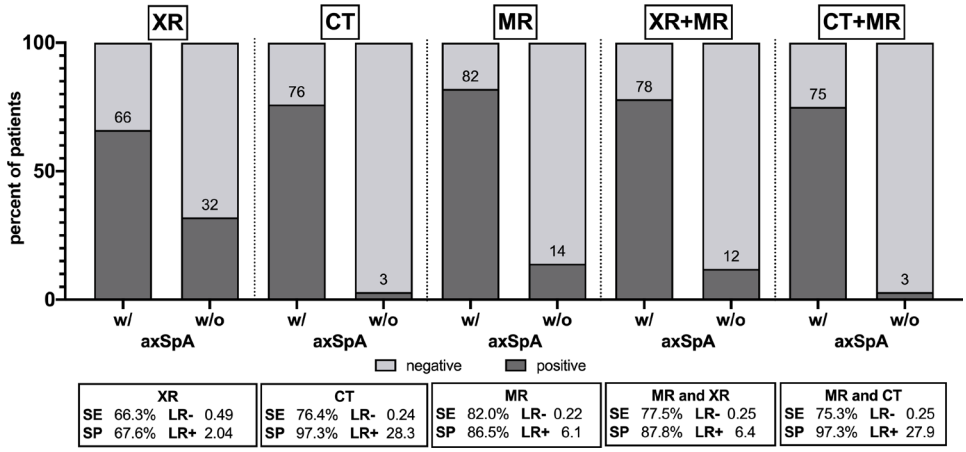

Figure 2 Frequency of positive and negative findings in radiography (XR), CT, MRI and combinations with resulting diagnostic accuracy values. Numbers are percentages of positive imaging results in patients with and without axSpA. axSpA, axial spondyloarthritis; LR−/+, negative/positive likelihood ratio; SE, sensitivity; SP, specificity.

95% CI 0.573 to 0.751) compared with MR alone, while CT +MR showed higher reliability (0.732, 95% CI 0.643 to 0.821).

Clinical scenarios

The evaluation of the different scenarios is presented in figure 4. An increase of the threshold of radiographic positivity improves the specificity of diagnostic imaging but also increases the number of MRs needed for diagnosis (from 49% to 75%) and still performs inferiorly compared with MR alone. CT before MR shows (similar to CT alone) a high specificity and might be an alternative whenever MR is unavailable.

DISCUSSION

In this study, we designed a unique reading exercise asking three expert radiologists to separately review a total of 815 image datasets acquired in a mixed cohort of 163 patients with early to established axSpA, non-specific low back pain, and SIJ degeneration. Our study has two key results: first, radiography is neither

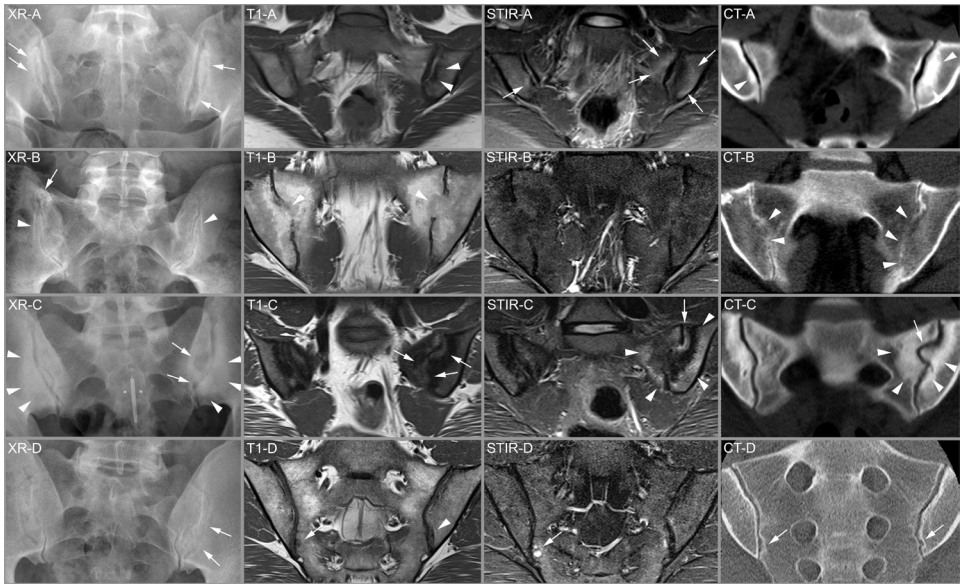

Figure 3 Imaging examples. (A) Female patient with osteitis condensans (23 years old, HLA-B27 negative, normal CRP). Radiography suggests bilateral erosions and joint space blurring (arrows) with mild sclerosis. However, cross-sectional imaging shows no erosions but some bone marrow oedema (arrows) and sclerosis (arrowheads) consistent with the final diagnosis. (B) Male patient with axSpA (53 years old, HLA-B27 positive, long history of back pain). Radiography shows only mild blurring of the joint space (arrowheads) and capsular calcification (arrow) and was deemed negative by all readers. However, MR and CT show extensive ankylosis (arrowheads) with preservation of only a small portion of the joint space, suggesting advanced axSpA. (C) Female patient with mechanical joint disease (34 years old, HLA-B27 negative, normal CRP). Radiography and T1W MR show extensive sclerosis (arrowheads) and irregularities (arrows) on the left side, MR-STIR extensive bone marrow oedema (arrowheads) and joint fluid (arrow). Both were misclassified by the readers as positive for axSpA. In this patient, only CT ruled out erosions (arrow) and confirmed the diagnosis of osteitis condensans and iliosacral complex as an anatomical variant. (D) Male patient with axSpA (40 years old, HLA-B27 positive, normal CRP). Radiography shows only minor irregularities (arrows) and was deemed negative. MR shows small cysts (arrows) and minor irregularities (arrowhead) as well as some bone marrow oedema on STIR but was judged negative by two of the three readers. Only CT shows very tiny erosions, confirming the diagnosis of axSpA (arrows). axSpA, axial spondyloarthritis; CRP, C reactive protein; STIR, short-tau inversion recovery; XR, X-ray.

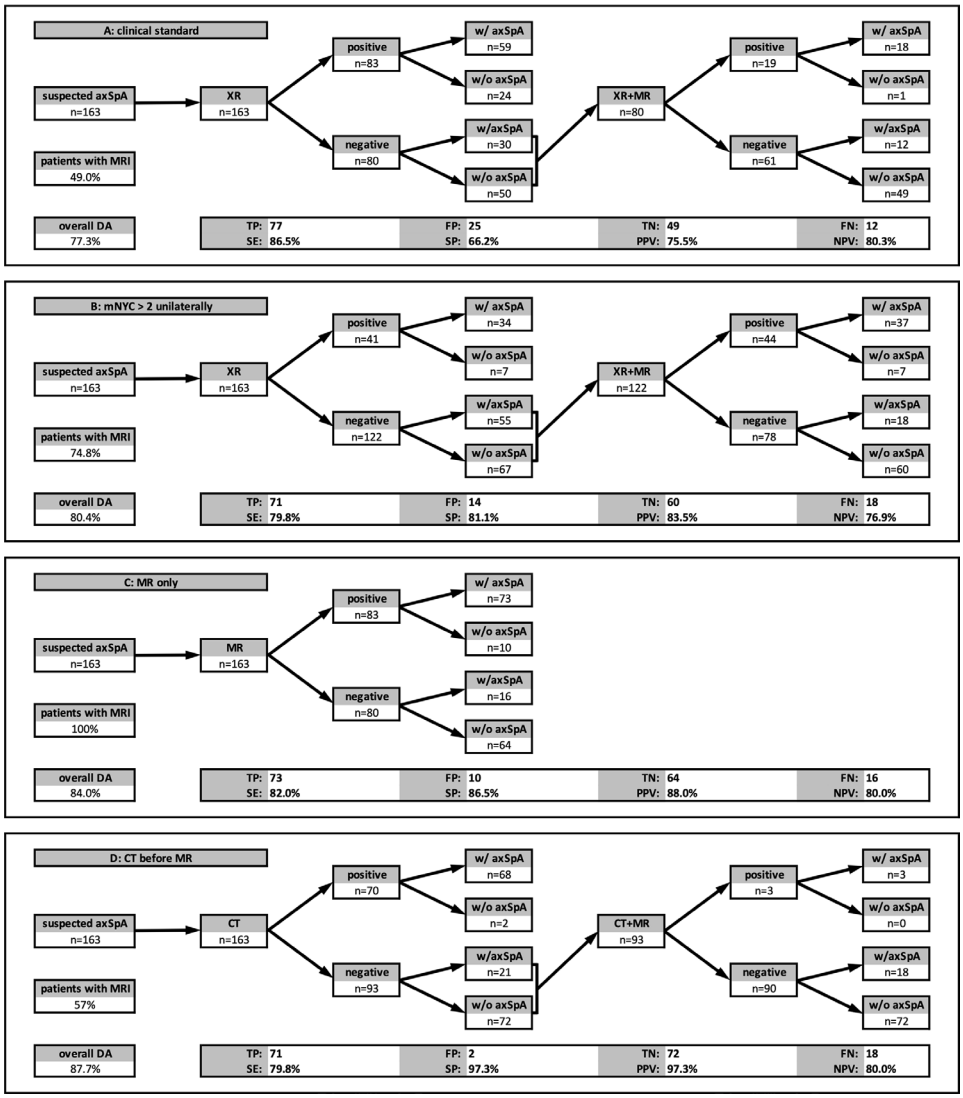

Figure 4 Clinical scenarios. (A) the current clinical standard (MR in patients with mNYC negative XR) shows the highest sensitivity but only poor specificity. (B) XR considered positive if sacroiliitis grade three or four unilaterally is present. This increases the specificity, but MR still must be performed in nearly 75% of patients. (C) MR alone outperforms the scenarios with XR as imaging of first choice showing better overall diagnostic accuracy. (D) CT as first-line imaging showed the best diagnostic accuracy and specificity. However, only 3% of CT-negative patients are positive when adding MR, calling into question, whether the additional MRI is beneficial, whatsoever. axSpA, axial spondyloarthritis; DA, diagnostic accuracy; FN, false-negative; FP, false-positive; mNYC, modified New York Criteria; NPV: negative predictive value; PPV: positive predictive value; TN, true-negative; TP, true positive; SE: sensitivity; SP: specificity; XR, X-ray.

sensitive (66%), nor specific (67%) or reliable (kappa=0.52) in diagnosing axSpA and contributes little when added to MR. Second, CT is similarly sensitive (76% vs 82%) but far more specific (97% vs 87%) than standard MR and the most reliable imaging method in our analysis. When added to MR, CT improves specificity far more markedly than it reduces sensitivity. The current clinical standard can be improved by increasing the threshold for XR positivity (eg, grade 3 or 4 unilaterally) or by omitting XR completely.

Our results underline the importance of structural lesions for the differential diagnosis when axSpA is suspected. CT is certainly the gold standard for structural lesions, displaying the cortical and trabecular bone directly and benefitting from superior resolution and thinner slices compared with MR. The inferior inter-rater reliability of MR alone or in combination with CT might be attributable to the variety of findings that can be detected (eg, fatty metaplasia or bone marrow oedema) and need to be taken into account by readers. Their combinations might be non-specific or complex to interpret. Furthermore, MR is prone to artefacts in bone marrow that might mimic erosions and, thus, lead to false-positive interpretation[12] while other changes such as sclerosis can mask other important lesions. Therefore, specific definitions must be established and followed when reading MR.[13]

Previous studies have already shown that MR is superior to radiography in detecting structural changes[3] and can be improved further by using more sophisticated pulse sequences that generate images with greater tissue contrast[7 8] or CT-like images.[12 14] Other investigators have reported the potential of (low-dose) CT for detecting structural lesions of the SIJ[15] or spine.[5] Our analysis provides more data in terms of differential diagnosis and diagnostic pathways when SpA is suspected, suggesting that XR adds little once MR has been performed or is easily available, although XR might provide some additional information relevant for the differential diagnosis of back pain, for example, on a hip joint disease. Furthermore, bone marrow

changes in MR seem to be less specific for axSpA and interpretation complicated for the differential diagnosis,[4 16 17] providing evidence that CT can be a reasonable sensitive and highly specific alternative to MR. Also, CT might be a useful addition, if MR images are ambiguous. When only CT (with or without XR) is readily available, we would recommend adding MR only if the changes seen on the CT scan are inconclusive (ie, very early disease without clear structural changes). However, in view of its radiation exposure, we explicitly do not recommend CT rather than MR as the first-line imaging method. As low-dose CT is comparable to XR in terms of radiation exposure,[3] we prefer CT over XR.

Our results contribute to the current discussion within ASAS and other groups, as to whether XR should be a first-line imaging test in suspected axSpA or be replaced by cross-sectional techniques. The authors conclude that it is advisable to avoid XR whenever MR is readily available while clinicians may fall back on XR if MR is not available. However, costs of misdiagnosis and of undertreatment or overtreatment must be included in this calculation. Further studies might address the cost-effectiveness of XR compared with cross-sectional imaging.

Limitations of our study include the use of a rough scoring system not providing details on detected lesions. Our focus here was on global scoring relevant for diagnostic decision-making. About 35% of our axSpA patients did not show characteristic or sufficient inflammatory SIJ changes to meet the ASAS definition of an active MR. Although available—yet not for all patients—we did not include dual-energy CT data, which might have added information undepictable by conventional CT[18] because we deliberately focused on conventional techniques widely used in routine clinical practice. There were small differences between the two CT protocols, but they were comparable in terms of radiation exposure (low-dose protocols).

Furthermore, we only assessed imaging findings. Access to clinical information might have improved readers' diagnostic accuracy. Thus, our approach does not fully capture clinical reality as our aim was to provide an unbiased assessment of imaging findings only. Although no information on sex was provided, complete blinding to sex is usually not possible in pelvic imaging (radiography, MR). Also, we present reading data from three expert radiologist—the performance of imaging outside specialised centres might be considerably worse. While we did not assess intrareader variability in this study, we expect variability to be low because images were assessed by radiologists with expertise in SpA. Also, we did not analyse other aspects of imaging such as radiation exposure, costs or time effectiveness. Imaging is an important part in establishing the diagnosis in suspected axSpA, and imaging findings will always have an impact on the final diagnosis made by the rheumatologist. However, the current reading exercise was unrelated to the clinical diagnostic strategy in order to rule out in the approach. Further, the 'yes' or 'no' response system used in this study is closer to the classification than to a diagnostic approach in patients. The readers in our study were experienced radiologists with expertise in SpA. Therefore, results might be different when images are assessed by non-expert readers. Continuous training of radiologists and rheumatologists in the interpretation of imaging findings is the only way to improve diagnostic confidence in routine clinical practice. Finally, no follow-up data were available.

In conclusion, XR is inferior to cross-sectional imaging and should be replaced by MR or CT for differential diagnosis. While MR is the most sensitive imaging technique, it lacks specificity compared with CT. CT alone has high diagnostic accuracy despite its insensitivity to bone marrow lesions such as fatty metaplasia or osteitis. Adding CT to MR improves specificity at a minor expense of sensitivity.

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ORCID iDs
Torsten Diekhoff http://orcid.org/0000-0003-3593-1449
Iris Eshed http://orcid.org/0000-0002-4655-9606
Katharina Ziegeler http://orcid.org/0000-0002-9763-2420
Fabian Proft http://orcid.org/0000-0003-4306-033X
Robert Biesen http://orcid.org/0000-0002-0434-7832
Denis Poddubnyy http://orcid.org/0000-0002-4537-6015

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TRANSLATIONAL SCIENCE

Genomic sequencing and functional analyses identify MAP4K3/GLK germline and somatic variants associated with systemic lupus erythematosus

Huai-Chia Chuang,[1] Wei-Ting Hung,[2] Yi-Ming Chen ,[2] Pu-Ming Hsu,[1] Jeng-Hsien Yen ,[3] Joung-Liang Lan,[2,4,5] Tse-Hua Tan [1,6]

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For numbered affiliations see end of article.

Correspondence to
Professor Tse-Hua Tan, Immunology Research Center, National Health Research Institutes, Zhunan, Miaoli Count 350, Taiwan; ttan@nhri.edu.tw
Dr Joung-Liang Lan; d8838@mail.cmuh.org.tw
Dr Jeng-Hsien Yen; jehsye@kmu.edu.tw
Dr Huai-Chia Chuang; cinth@nhri.org.tw

H-CC and W-TH contributed equally.

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ABSTRACT

Objectives MAP4K3 (GLK) overexpression in T cells induces interleukin (IL)-17A production and autoimmune responses. GLK overexpressing T-cell population is correlated with severity of human systemic lupus erythematosus (SLE); however, it is unclear how GLK is upregulated in patients with SLE.

Methods We enrolled 181 patients with SLE and 250 individuals without SLE (93 healthy controls and 157 family members of patients with SLE) in two independent cohorts from different hospitals/cities. Genomic DNAs of peripheral blood mononuclear cells were subjected to next-generation sequencing to identify GLK gene variants. The functional consequences of the identified GLK germline or somatic variants were investigated using site-directed mutagenesis and cell transfection, followed by reporter assays, mass spectrometry, immunoblotting, coimmunoprecipitation, and in situ proximity ligation assays.

Results We identified 58 patients with SLE from Cohort #1 and #2 with higher frequencies of a somatic variant (chr2:39 477 124 A>G) in GLK 3′-untranslated region (UTR); these patients with SLE showed increased serum anti-double-stranded DNA levels and decreased serum C3/C4 levels. This somatic variant in 3′-UTR enhanced GLK mRNA levels in T cells. In addition, we identified five patients with SLE with GLK (A410T) germline variant in Cohort #1 and #2, as well as two other patients with SLE with GLK (K650R) germline variant in Cohort #1. Another GLK germline variant, A579T, was also detected in one patient with SLE from Cohort #2. Both GLK (A410T) and GLK (K650R) mutants inhibited GLK ubiquitination induced by the novel E3 ligase makorin ring-finger protein 4 (MKRN4), leading to GLK protein stabilisation.

Conclusions Multiple GLK germline and somatic variants cause GLK induction by increasing mRNA or protein stability in patients with SLE.

Key messages

What is already known about this subject?

► Both heritable and environmental factors are linked to systemic lupus erythematosus (SLE) pathogenesis. MAP4K3 (GLK) overexpression in T cells induces interleukin (IL)-17A production and autoimmune responses. The frequency of GLK overexpressing T cells is correlated with severity of human SLE.

What does this study add?

► GLK 3′-untranslated region (UTR) (T635C), GLK 3′-UTR (A644C), GLK (A410T) or GLK (K650R) variant-induced GLK overexpression through the stabilisation of GLK mRNAs or proteins may be involved in SLE pathogenesis. To our knowledge, this is the first identification of the novel E3 ligase MKRN4 that induces GLK protein degradation. GLK (A410T) and GLK (K650R) variants block MKRN4-induced Lys48-linked ubiquitination of GLK.

How might this impact on clinical practice or future development?

► SLE is difficult to be diagnosed at the early stage. Our findings suggest that individuals harbouring GLK variants or MKRN4 dysregulation/mutation accompanied by other risk factors could be at high risk for SLE.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic, complex and systemic autoimmune disease with multiorgan damages.[1] Both heritable and environmental factors are linked to SLE pathogenesis.[2–4] About 95% of patients with SLE display an induction of antinuclear autoantibodies.[5] Increased serum anti-double stranded DNA (dsDNA) autoantibody levels are correlated with enhanced SLE disease activity, whereas serum complement C3/C4 levels are inversely correlated with SLE disease activity.[6] Autoantibodies trigger complement responses and amplify inflammation, leading to multiorgan damages in patients with SLE.[7] Moreover, Th17 ($CD4^+$ IL-17A-producing T) cells contribute to autoimmune responses by recruiting macrophages and facilitating B-cell activation.[8]

Makorin ring-finger protein 4 (MKRN4, also named MKRN4P, RNF64, ZNF127L1) is a putative ubiquitin-protein E3 ligase, which was thought to be a pseudogene prior to 2010 due to the deficiency of MKRN motif, the lack of introns and the presence of a poly-A region.[9 10] In 2011, the deposited full-length MKRN4 mRNA sequence was deposited (NCBI accession number: NG_004713.4).[11] MKRN4 protein in fact, similar to other MKRN family members, contains four conserved C3H domains, one conserved MKRN Cys-His motif, and one conserved C3HC4 ring-finger domain. The MKRN family of ubiquitin E3 ligases includes MKRN1, MKRN2, MKRN3 and MKRN4.[9 12]

MKRN4 shares 81% amino acid identity with MKRN1, 46% amino acid identity with MKRN2 and 52% amino acid identity with MKRN3.[9 12] To date, the functions and targets of MKRN4 remain completely unknown.

The serine/threonine kinase MAP4K3 (also named GLK) directly interacts with and phosphorylates PKCθ in T cells, resulting in T-cell activation.[13] GLK overexpression in murine T cells induces IL-17A production and T-cell hyperactivation, leading to autoimmune inflammatory diseases.[14] Moreover, GLK is overexpressed in T cells in patients with autoimmune diseases including SLE[13 15–18]; GLK-overexpressing T-cell population is correlated with the disease severity of patients with SLE.[13 17] To date, the mechanism of GLK overexpression in patients with SLE remains unclear. Here we explored whether GLK genetic variants occur in patients with SLE by next-generation sequencing using two independent cohorts of patients with SLE from different cities.

RESULTS

Both GLK somatic and germline variants occur in patients with SLE

To identify GLK gene variants in patients with SLE, we isolated genomic DNAs of peripheral blood mononuclear cells (PBMCs) from 101 patients with SLE and 163 individuals without SLE (6 healthy controls (HCs) and 157 family members of patients with SLE) (Cohort #1, Taichung Veterans General Hospital, located in Taichung City in central Taiwan; online supplemental table S1). The genomic DNAs were subjected to next-generation sequencing for GLK exons and the 3′-untranslated region (UTR) with the sequencing depth of around 100 000 reads. One GLK somatic variant 3′-UTR (T635C), hg19 human reference genome chr2:39 477 124 A>G, was identified in patients with SLE and individuals without SLE (HCs and family members without SLE) with variant frequencies of 0%–5.3% and 0%–2.3%, respectively (figure 1A and B, left panels). The means of variant frequencies of SLE versus groups without SLE were not significantly different due to high SD (1.47%) of the variant frequency in patients with SLE. Interestingly, several patients with SLE showed higher frequency of this GLK somatic variant 3′-UTR (T635C) compared with the group without SLE (figure 1B, left panel and online supplemental figure S1). To investigate the potential significance of these frequency values, we determined the cut-off value of the somatic mutation frequencies between patients with SLE and individuals without SLE by using the values of mean plus 3SD of individuals without SLE (2.7%, 99.7% of normal distribution) according to Westgard rules. Seventeen (16.83%) of 101 patients with SLE (or 10 (12.99%) of 77 family members without SLE), but no HCs nor family members without SLE, showed a variant frequency of 2.7% or higher (figure 1B, left panel; online supplemental figure S1 and table S2). Next, we studied whether there is a potential association between the high frequency of the GLK somatic variant 3′-UTR (T635C) and SLE. We found that higher frequencies (>2.7%) of GLK somatic variant 3′-UTR (T635C) were associated with SLE in Cohort #1 ($p<0.0001$; table 1). Additional four GLK somatic missense variants were also identified in other Cohort #1 patients with SLE but not in individuals without SLE (table 1). Moreover, a GLK germline variant (50.6% read frequency) 3′-UTR (A644C) (chr2:39,477,115 T>G) was also identified in another female patient with SLE of Cohort #1 (0.581% allele frequency; table 2); this variant is a previously annotated single nucleotide polymorphism (SNP), rs191224999. It is noted that one male family member without SLE of this patients with SLE also harboured this germline variant (table 2). In addition to GLK 3′-UTR, three (all females) of 101 Cohort #1 patients with SLE showed a GLK germline variant at the codon p.Ala410 to Thr (GCA to ACA) with 1.163% allele frequency (figure 1A and table 2); this variant is a previously annotated SNP, rs148167737. There are no Cohort #1 individuals without SLE harbouring this GLK (A410T) variant/SNP (table 2). Interestingly, two of the three Cohort #1 patients with SLE with GLK p.Ala410Thr variant belong to the same family F7 (table 2). In addition, a second GLK germline variant, p.Lys650Arg, was identified in other two female patients with SLE from Cohort #1 (0.158% allele frequency; figure 1A and table 2); this germline variant is also the same as another previously annotated SNP, rs200566214. The two patients with SLE with GLK p.Lys650Arg germline variant belong to the same family F26 (figure 1A and table 2), whereas their healthy brother did not have GLK p.Lys650Arg variant. There are no Cohort #1 individuals without SLE harbouring the GLK (K650R) variant. The three abovementioned GLK germline variants were further confirmed by Sanger sequencing (figure 1C).

To validate the abovementioned GLK gene variants in patients with SLE, we further recruited the second cohort containing 80 patients with sporadic SLE and 87 non-familial HCs from a different hospital (Kaohsiung Medical University Hospital; figure 1A, right panel and online supplemental table S3) located in another city, Kaohsiung City, in southern Taiwan. The genomic DNAs of PBMCs from Cohort #2 were subjected to next-generation sequencing with the sequencing depth of 100 000 to 3 00 000 reads. Consistently, the most prevalent GLK somatic variant in Cohort #1, GLK 3′-UTR (T635C), was also identified in Cohort #2 patients with SLE (table 1). The frequencies of this GLK somatic variant were also significantly increased in patients with SLE compared with those of HCs in Cohort #2 (figure 1B, right panel); 37 of 80 patients with SLE showed the variant frequency higher than 2.7%, whereas only 14 of 87 HCs did (figure 1B, right panel). Consistent with Cohort #1, GLK somatic variant 3′-UTR (T635C) with higher frequency (>2.7%) also showed a significant association with SLE in Cohort #2 ($p<0.0001$; table 1). Moreover, GLK 3′-UTR (A644C) germline variant, identified in one patient with SLE in Cohort #1, was also detected in two female patients with SLE in Cohort #2 (table 2). In addition, GLK p.Ala410Thr germline variant, identified in three patients with SLE in Cohort #1, was also detected in two female patients with SLE in Cohort #2 (table 2). It is noted that one male non-familial HC from Cohort #2 had GLK p.Ala410Thr germline variant (table 2). No additional patients with SLE from Cohort #2 showed any GLK p.Lys650Arg germline variant detected in two patients with SLE from Cohort #1 (table 2). Another GLK germline variant, p.Ala579Thr, was also detected in one female patient with SLE in Cohort #2 but not in Cohort #1 (table 2); this germline variant is not an annotated SNP. Furthermore, other two Cohort #2 patients with SLE each has four or five GLK somatic missense variants (table 1); these somatic variants were not detected in any Cohort #1 patients. Next, we studied whether the abovementioned GLK gene variants are involved in GLK dysregulation.

Somatic and germline variants in the GLK 3′-UTR cause induction of GLK mRNA levels

AU-rich elements (AREs) in the 3′ UTR induce mRNA destabilisation.[19 20] GLK 3′-UTR (T635C) somatic variant and (A644C) germline variant were in a putative ARE, which contained 62 nucleotides with 69.4% AU nucleotides (figure 2A); therefore,

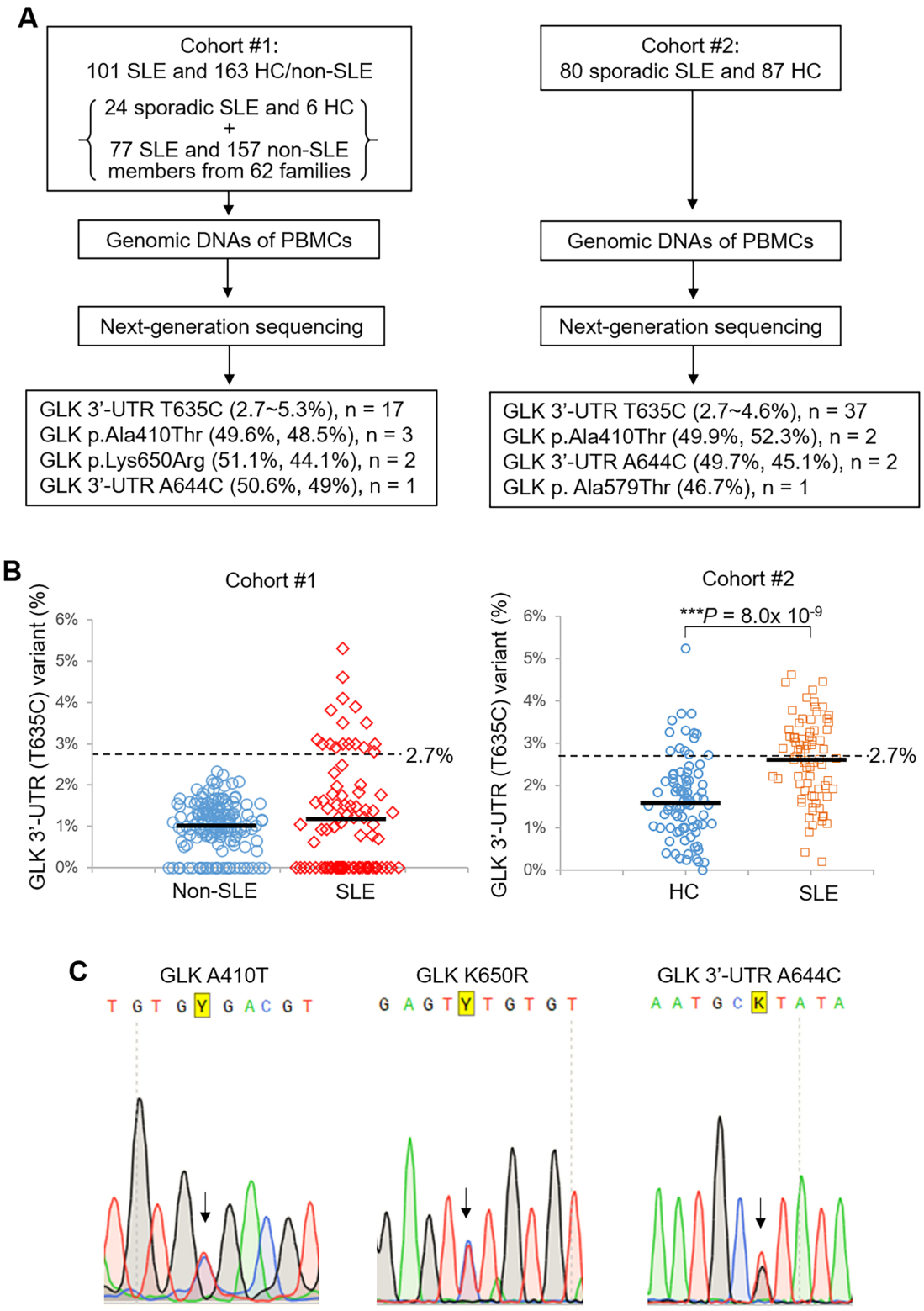

Figure 1 GLK somatic and germline variants occur in PBMCs of SLE patients. (A) Schematic diagram of the screening design to identify GLK gene variants in patients with SLE by next-generation sequencing. (B) The variant frequency of GLK 3′-UTR (T635C) variant in 163 individuals without SLE (6 HCs and 157 members without SLE from individual families) and 101 patients with SLE (sporadic and familial) from Cohort #1 (left panel). The value 2.7% was mean plus 3SD (1.0% + 3 × 0.58%=2.74%) of 3′-UTR (T635C) variant frequencies in the group without SLE. The frequency of GLK 3′-UTR (T635C) variant in 87 HCs and 80 patients with SLE (all sporadic) from Cohort #2 (right panel). Bars denote means of variant frequency. ***P<0.0001 (two-tailed Student's t-test). (C) Sanger-sequencing chromatograms for heterozygous variants at GLK (A410T), (K650R) and 3′-UTR (A644C). Arrows indicate the bases with distinct nucleotides. Y denotes mixed bases of C and T nucleotides; K denotes mixed bases of G and T nucleotides. HCs, healthy controls; PBMCs, peripheral blood mononuclear cells; SLE, systemic lupus erythematosus; UTR, untranslated region.

Table 1 GLK gene somatic variants resulting in codon or 3′-UTR changes in patients with SLE of Cohort #1 and Cohort #2

Cohort	Locus	Type	Ref	GLK coding	Codon/3′-UTR change	Patient number	Mutation frequency among reads	SLE ID#	Control number	Association with SLE
#1 (SLE, n=101) (control, n=163)	chr2:39 477 820	SNV	G	c.2624C>G	p.Thr875Ser	1	0.064	S5	0	p=0.383
	chr2:39 494 337	SNV	A	c.2025T>G	p.Cys675Trp	1	0.070		0	p=0.383
	chr2:39 507 491	SNV	C	c.1635G>A	p.Ala546Thr	2	0.044	S1	0	p=0.146
							0.076	S4		
	chr2:39 552 878	SNV	A	c.800T>A	p.Leu267Ter	1	0.033	S7	0	p=0.383
	chr2:39 477 124	SNV	A	c.3320T>C	3′-UTR U635C*	17	>0.027	S2, S7, S8, S16, S17, S29, S30, F10-4, F13-1, F14-1, F14-2, F15-1, F15-2, F18-1, F18-2, F19-3, F53-3	0	p<0.0001
#2 (SLE, n=80) (control, n=87)	chr2:39 499 454	INDEL	G	c.1942_1943 insert A	p.Ala648fs	1	0.016	B52	0	p=0.479
	chr2:39 553 291	SNV	T	c.658A>G	p.Met220Val	1	0.028		0	p=0.479
	chr2:39 553 305	SNV	A	c.644T>C	p.Phe215Ser	1	0.028		0	p=0.479
	chr2:39 553 354	SNV	C	c.595G>A	p.Ala199Thr	1	0.029		0	p=0.479
	chr2:39 492 369	SNV	G	c.2111C>A	p.Pro704Gln	1	0.026	B53	0	p=0.479
	chr2:39 499 497	SNV	C	c.1900G>T	p.Asp634Tyr	1	0.070		0	p=0.479
	chr2:39 517 440	SNV	G	c.1307C>T	p.Pro436Leu	1	0.031		0	p=0.479
	chr2:39 570 569	SNV	C	c.270G>A	p.Met90Ile	1	0.039		0	p=0.479
	chr2:39 583 402	SNV	C	c.233G>T	p.Gly78Val	1	0.026		0	p=0.479
	chr2:39 477 124	SNV	A	c.3320T>C	3′-UTR U635C*	37	>0.027	B14, B15, B16, B21, B22, B23, B24, B26, B29, B30, B31, B32, B37, B38, B39, B40, B41, B42, B47, B48, B53, B54, B55, B58, B61, B63, B64, B65, B67, B69, B70, B71, B73, B75, B76, B77, B79	14	p<0.0001

GLK coding, GLK variant coding that is a reverse sequence on chromosome 2.
Association of GLK somatic variants with SLE was determined by Fisher's exact test (two-tailed).
'F' denotes family member in Cohort #1; 'S' denotes patient with sporadic SLE in Cohort #1; 'B' indicates patient with sporadic SLE in Cohort #2.
Ref, DNA coding from the human genome hg19 reference.
*Variant occurs in both Cohort #1 and Cohort #2.
.fs, frameship; INDEL, insertion/deletion; SLE, systemic lupus erythematosus; SNV, single nucleotide variant; UTR, untranslated region.

we studied whether GLK 3′-UTR (T635C) somatic variant or (A644C) germline variant affects GLK mRNA levels using luciferase (Luc) reporter assays. The reporter activity of GLK 3′-UTR (T635C)-Luc was significantly increased (2.35 times) compared with that of wild-type GLK 3′-UTR-Luc (figure 2B, left panel). Besides GLK 3′-UTR (T635C) mutation, GLK 3′-UTR (A644C) mutation also drastically enhanced the reporter activity (figure 2B, right panel). These results suggest that the GLK 3′-UTR (T635C) or (A644C) variant increases GLK mRNA levels. Consistently, analysis of T cells from a cohort reported previously[13] also showed that GLK mRNA levels were increased in 84.6% (11 of 13) of patients with SLE compared with those of HCs (online supplemental figure S2).

GLK 3′-UTR (T635C) somatic variant is associated with increased anti-dsDNA and decreased serum C3/C4 levels

To study the clinical consequences of the GLK 3′-UTR (T635C) somatic variant, we analysed clinical parameters of patients with SLE from Cohort #1. Patients with SLE with higher variant frequency (>2.7%) of GLK 3′-UTR (T635C) showed an induction of anti-dsDNA autoantibody levels compared with those of patients with SLE with lower variant frequency (<2.7%) (figure 2C). Consistently, the patients with SLE with higher variant frequency also showed decreased serum complement C3 and C4 levels during the follow-up period (figure 2D and E); these patients also showed decreased cell counts of white blood cells (WBCs) and lymphocytes in the peripheral bloods (figure 2F and G). Interestingly, the patients with SLE with higher variant frequency (>2.7%) of GLK 3′-UTR (T635C) somatic variant showed a higher mean value of SLE disease activity index (SLEDAI), although statistically insignificant, compared with that of patients with SLE with lower variant frequency (<2.7%) (figure 3A). Furthermore, higher frequencies (>2.7%) of GLK 3′-UTR (T635C) somatic variant in Cohort #1 were associated (p=0.057) with higher scores of SLEDAI (online supplemental table S4). It is plausible that the association between GLK 3′-UTR (T635C) somatic variant and SLEDAI may achieve statistical significance after enrolling more patient samples. In addition, higher frequencies (>2.7%) of GLK 3′-UTR (T635C) somatic variant in Cohort #1 were not associated with the development of rashes, oral ulcer, arthritis, serositis, neuropsychiatric, nephritis, as well as the treatment with cyclophosphamide (Endoxan), mycophenolate mofetil, cyclosporine or azathioprine (online supplemental table S4).

Table 2 GLK gene germline variants* resulting in codon or 3′-UTR change in patients with SLE of Cohort #1 and Cohort #2

Locus	Ref	GLK coding	Codon/3′-UTR change	Annotated SNP	Cohort	Patient number	SLE ID#	Allele frequency in SLE	Allele frequency in control	Allele frequency in world
chr2:39 477 115	T	c.3329A>C	3′-UTR (A644C)	rs191224999	#1	1	F52-01	1/172 (0.005814)	1†/136 (0.007353)	0.000050
					#2	2	B24 B71	2/160 (0.012500)	0	
chr2:39 519 957	C	c.1228G>A	p.Ala410Thr	rs148167737	#1	3	S10, F7-01 F7-04	2/172 (0.01163)	0	0.000601
					#2	2	B33 B45	2/160 (0.012500)	1‡/174 (0.005747)	
chr2:39 499 448	T	c.1949A>G	p.Lys650Arg	rs200566214	#1	2	F26-01 F26-02	1/172 (0.001581)	0	0.000231
					#2	0	none	0	0	
chr2:39 505 607	C	c.1735G>A	p.Ala579Thr	ND	#2	1	B19	1/160 (0.006250)	0	ND

GLK coding, GLK variant coding that is a reverse sequence on chromosome 2.
Cohort #1, SLE, n=101 (24 patients with sporadic SLE and 77 patients with SLE from 62 families); non-SLE, n=163 (6 healthy controls and 157 family members without SLE from 62 families).
Cohort #2, SLE, n=80 (patients with sporadic SLE); healthy control, n=87 (non-familial healthy controls).
Allele frequencies were calculated using unrelated patients and controls; if from individual families, only one patient with SLE and one member without SLE from each family are included.
'F' denotes family member in Cohort #1; 'S' denotes patient with sporadic SLE in Cohort #1; 'B' indicates patient with sporadic SLE in Cohort #2.
*Single nucleotide variants.
†One male family member without SLE control (F52-02) from Cohort #1 harboured this variant.
‡One male non-familial healthy control from Cohort #2 harboured this variant.
Ref, DNA coding from the human genome hg19 reference; SLE, systemic lupus erythematosus; UTR, untranslated region.

Consistent with the data derived from Cohort #1, Cohort #2 patients with SLE with a higher variant frequency (>2.7%) of GLK 3′-UTR (T635C) also showed significantly decreased serum C3/C4 levels, WBC counts and platelet counts (figure 3A–D). These patients in Cohort #2 showed an increased mean value of anti-dsDNA levels but without statistical significance (figure 3E); this may be due to a smaller patient number of Cohort #2 than that of Cohort #1. Similar to the results of Cohort #1, the GLK 3′-UTR (T635C) somatic variant in Cohort #2 was also not associated with any organ damage or therapeutic treatment (online supplemental figure S3B and table S5). The data suggest that patients with SLE with the GLK 3′-UTR (T635C) somatic variant could develop severe SLE symptoms such as inflammation and lymphocytopaenia.

GLK germline variants A410T and K650R elicit GLK protein stabilisation

Five patients with SLE (three in Cohort #1 and two in Cohort #2) harboured GLK (A410T) germline variant (table 2). Two other patients with SLE from one family in Cohort #1 harboured GLK (K650R) germline variant (table 2). One patient with SLE (#S5) in Cohort #1 harboured two GLK (T875S and C675W) somatic variants (table 1). In Cohort #2, GLK (A579T) germline variant was identified from one patient with SLE (table 2). Two non-familial patients with SLE (#B52 and #B53) in Cohort #2 harboured multiple somatic variants (table 1). To study the functional consequence of these GLK variants that altered GLK codons, we performed mutagenesis and immunoblotting analyses. The protein levels of GLK (A410T) and GLK (K650R) mutants were increased compared with those of wild-type GLK in transfected Jurkat T cells (figure 4A) and HEK293T cells (figure 4B), whereas protein levels of GLK (C675W) and GLK (T875S) mutants were modestly increased (online supplemental figure S4A). Moreover, GLK levels were also increased by GLK (A579T) germline variant identified from one Cohort #2 patient, as well as GLK (A199T), GLK (A648fs), GLK (G78V), GLK (M90I), GLK (P436L) and GLK (D634Y) somatic variants identified from two Cohort #2 non-familial patients (#B52 and #B53) (online supplemental figure S4B).

GLK (A410T) and GLK (K650R) variants were the two most prevalent germline variants in both Cohort #1 and #2; thus, we further investigated the mechanism of GLK protein induction by GLK (A410T) and GLK (K650R) variants. To study whether GLK (A410T) or GLK (K650R) variant enhances its protein stability, the protein half-life of GLK was determined by cycloheximide pulse-chase experiments. GLK (A410T) and GLK (K650R) mutants showed longer GLK protein half-life in HEK293T cells (figure 4C), suggesting that GLK (A410T) and GLK (K650R) mutants are resistant to protein degradation. To identify the protease that targets and degrades GLK proteins, individual immunocomplexes of wild-type GLK, GLK (A410T) mutant and GLK (K650R) mutant were subjected to mass spectrometry-based proteomics analyses. The mass data revealed a novel E3 ligase, MKRN4, as an interacting protein of wild-type GLK or GLK (K650R) mutant but not GLK (A410T) mutant (figure 4D–F). To date, there are no known functions of MKRN4, a putative ubiquitin E3 ligase of MKRN family. After close examination of MKRN4 protein sequence, we found that MKRN4 did not show any deficiency in MKRN family conserved domains (figure 4G). Thus, we tested whether MKRN4 induces GLK protein degradation. Remarkably, MKRN4 overexpression induced GLK protein degradation in HEK293T cells and Jurkat T cells (figure 5A and B). MKRN4-induced GLK degradation was blocked by the proteasome inhibitor MG132 (figure 5C). In addition, the protein–protein interaction between GLK and MKRN4 was confirmed by in situ proximity ligation assays (figure 5D).

GLK (A410T) and GLK (K650R) variants block MKRN4-induced Lys48-linked ubiquitination of GLK

To investigate the molecular mechanism of GLK protein stabilisation by A410T or K650R mutation, we first tested whether MKRN4 induces the ubiquitination and subsequent proteasomal degradation of GLK. Immunoprecipitation and immunoblotting analyses showed that MKRN4 overexpression induced Lys48-linked ubiquitination of GLK (figure 6A), and the MKRN4-induced GLK ubiquitination was further enhanced by MG-132 treatment (figure 6A). Conversely, Lys48-linked ubiquitination of GLK was blocked by A410T or K650R mutation of GLK (figure 6B). To identify MKRN4-induced ubiquitination residues on GLK, MKRN4 immunocomplex was subjected to mass spectrometry-based analyses (online supplemental figure S5A).

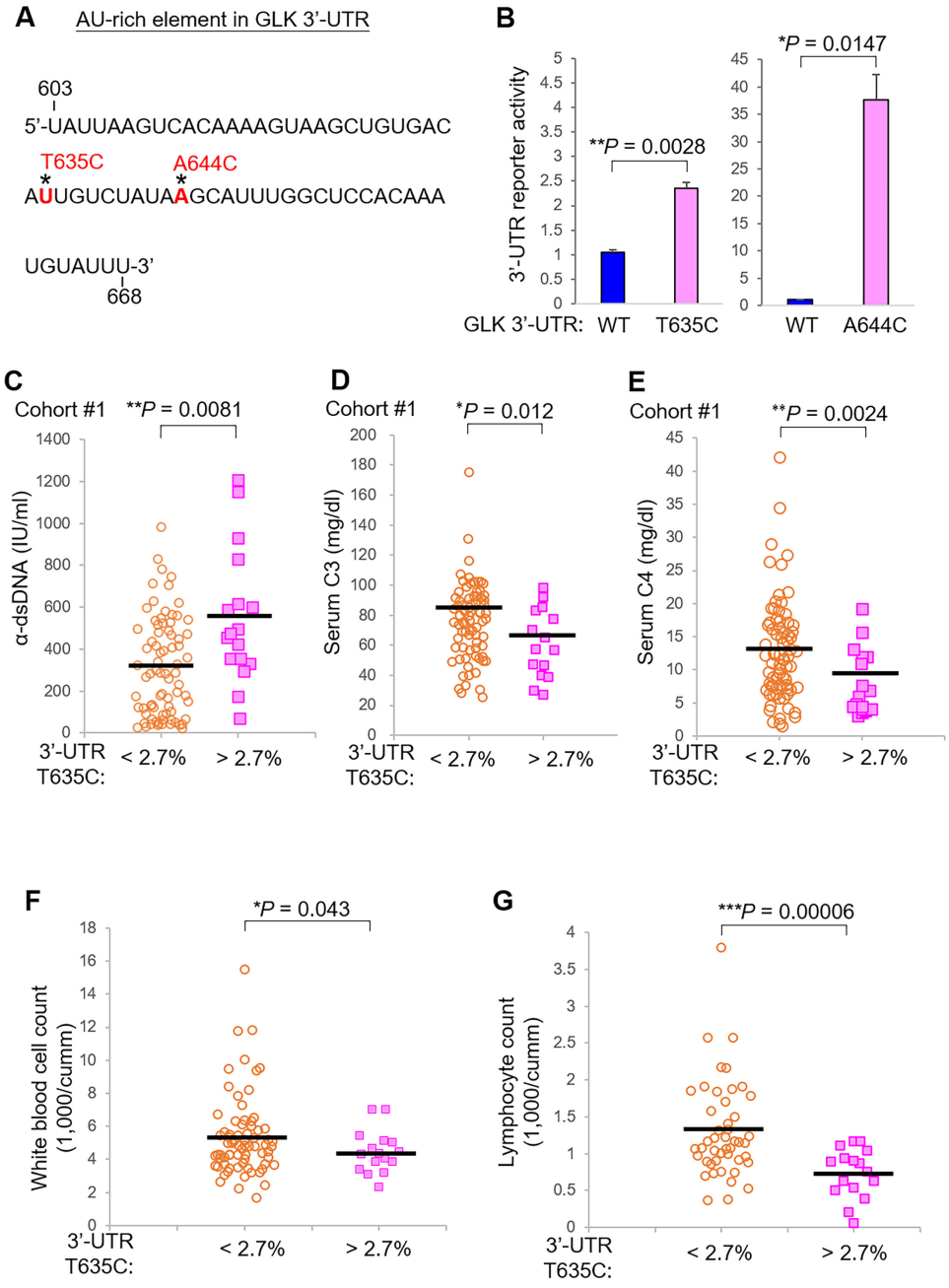

Figure 2 GLK 3′-UTR (T635C) variant results in GLK overexpression and is associated with severe symptoms from Cohort #1. (A) The AU-rich element (UTR nucleotide number: 603 to 668) in the GLK 3′-UTR. Asterisks indicate the location of GLK 3′-UTR (T635C) or (A644C) variant. (B) Bar charts of GLK 3′-UTR reporter activity in 3′-UTR wild-type, T635C or A644C mutant-expressing Jurkat T cells. The reporter activity of GLK 3′-UTR-gaussia luciferase (Luc) was normalised to the secreted alkaline phosphatase. Means±SEM are shown. (C) Anti-double-stranded DNA antibody (α-dsDNA) levels in the sera of Cohort #1 patients with SLE with a lower (<2.7%, n=77) or higher (>2.7%, n=17) variant frequency. (D) Serum C3 levels of Cohort #1 patients with SLE with a lower (<2.7%, n=77) or higher (>2.7%, n=17) variant frequency. (E) Serum C4 levels of Cohort #1 patients with SLE with a lower (<2.7%, n=76) or higher (>2.7%, n=17) variant frequency. (F) White blood cell count in the peripheral blood of Cohort #1 patients with SLE with a lower (<2.7%, n=74) or higher (>2.7%, n=16) variant frequency. (G) Lymphocyte count in the peripheral blood of Cohort #1 patients with SLE with a lower (<2.7%, n=58) or higher (>2.7%, n=16) variant frequency. Bars denote means of levels. *P <0.05; **p<0.01; ***p<0.001 (two-tailed Student's t-test). SLE, systemic lupus erythematosus; UTR, untranslated region.

Interestingly, Lys650 residue of GLK proteins in the MKRN4 immunocomplex was identified as a MKRN4-targeted GLK ubiquitination site (figure 6C). Besides Lys650 residue, three additional lysine residues (Lys526, Lys550 and Lys620) were also identified as MKRN4-induced GLK ubiquitination sites (online supplemental figure S5A). Individual mutations of these three lysine residues did not block the MKRN4-induced K48-linked ubiquitination of GLK (online supplemental figure S5B). Conceivably, GLK (K650R) mutation would block MKRN4-induced ubiquitination of Lys650 residue on GLK, leading to GLK protein stabilisation. In addition, GLK (A410T) mutation is in the GLK proline-rich domain (figure 6D), which mediates protein–protein interaction.[21 22] We next studied whether the interaction between MKRN4 and GLK is attenuated by GLK (A410T) mutation. To avoid the false-positive result due to the kinase-domain-mediated dimerisation between GLK

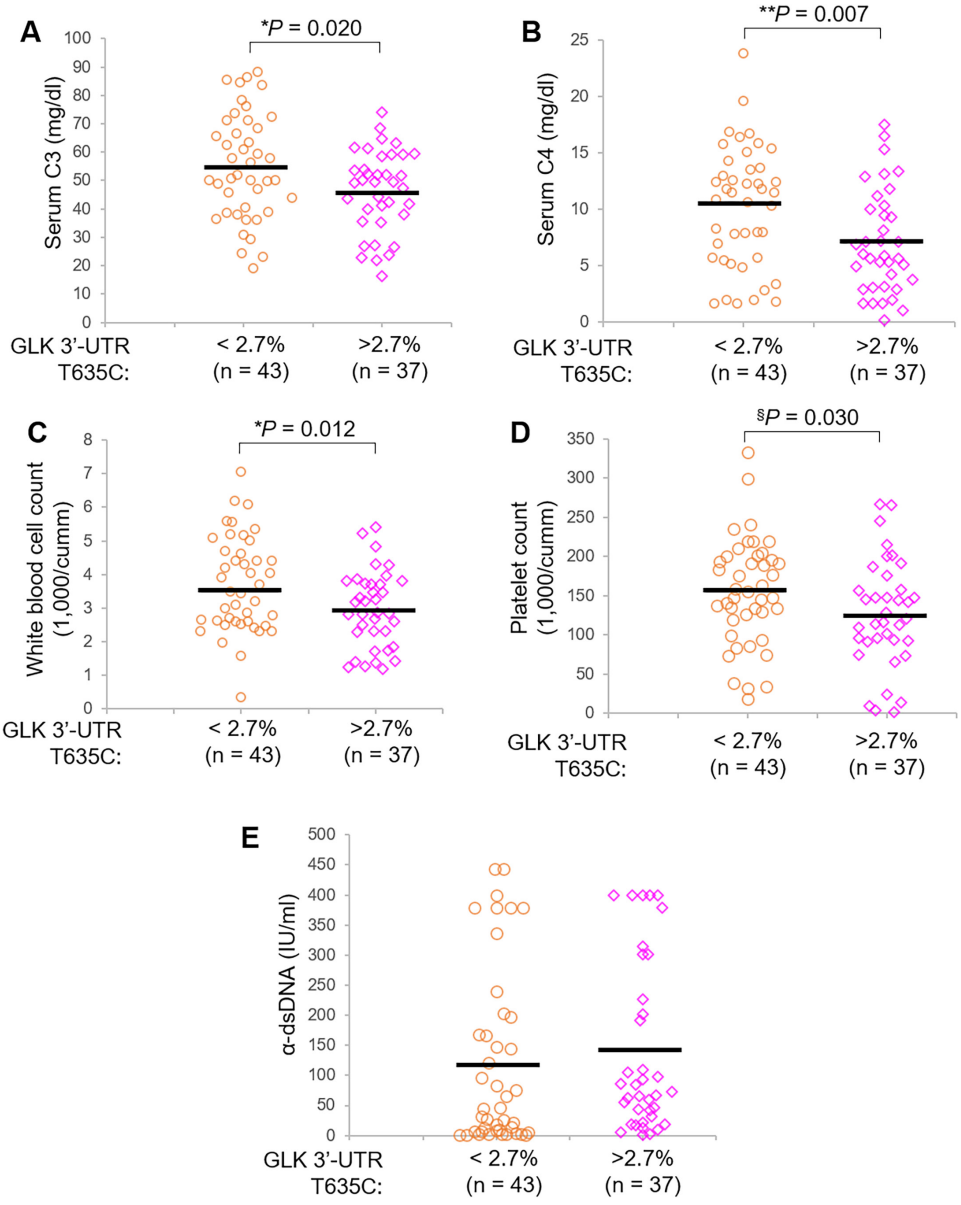

Figure 3 GLK 3′-UTR (T635C) variant is also associated with severe symptoms of patients with SLE from Cohort #2. Complement C3 levels (A) and complement C4 levels (B) in the sera of Cohort #2 patients with SLE with a lower (<2.7%, n=43) or higher (>2.7%, n=37) mutation frequency. White blood cell count (C) and platelet count (D) in the peripheral blood of Cohort #2 patients with SLE with a lower (<2.7%, n=43) or higher (>2.7%, n=37) mutation frequency. (E) Anti-double stranded DNA antibody (α-dsDNA) levels in the sera of Cohort #2 patients with SLE with a lower (<2.7%, n=43) or higher (>2.7%, n=37) mutation frequency. Bars denote means of levels. *P<0.05; **p<0.01 (two-tailed student's t-test); §p<0.05 (one-tailed Student's t-test). SLE, systemic lupus erythematosus; UTR, untranslated region.

(A410T) mutant and the endogenous wild-type GLK, GLK proteins without the GLK kinase domain (GLKΔN) were used. Coimmunoprecipitation analysis showed that the interaction between MKRN4 and wild-type GLKΔN was abolished by GLK (A410T) mutation (figure 6E). This result was consistent with our proteomics data that no MKRN4 peptides were detected in GLK (A410T) immunocomplex (figure 4F). Interestingly, GLK (K650R) mutation did not attenuate the GLK–MKRN4 interaction (figure 6F). This result suggests that GLK (A410T) mutation blocks its interaction with MKRN4, leading to GLK protein stabilisation. Collectively, either A410T or K650R mutation stabilises GLK proteins by preventing MKRN4-mediated ubiquitination and subsequent proteasomal degradation.

Our data showed that both GLK somatic and germline variants in patients with SLE lead to increased GLK levels. Induction of GLK in T cells contributes to IL-17A production and subsequent autoimmune responses.[14 17] Thus, we studied whether the identified GLK variants are correlated with IL-17A induction in patients with SLE. The serum IL-17A levels were significantly increased in patients with SLE who harboured GLK germline or somatic variants compared with those of HCs in Cohort #1 (online supplemental figure S6), while IL-17A levels were modestly increased in patients with SLE without GLK variants (online supplemental figure S6). It is interesting that two patients with SLE (#S9 and #S12) without GLK variants showed high levels of serum IL-17A, which could be due to dysregulation or mutation of MKRN4. Collectively, these results suggest that GLK variants contribute to induction of GLK levels and overproduction of IL-17A, leading to autoimmune responses.

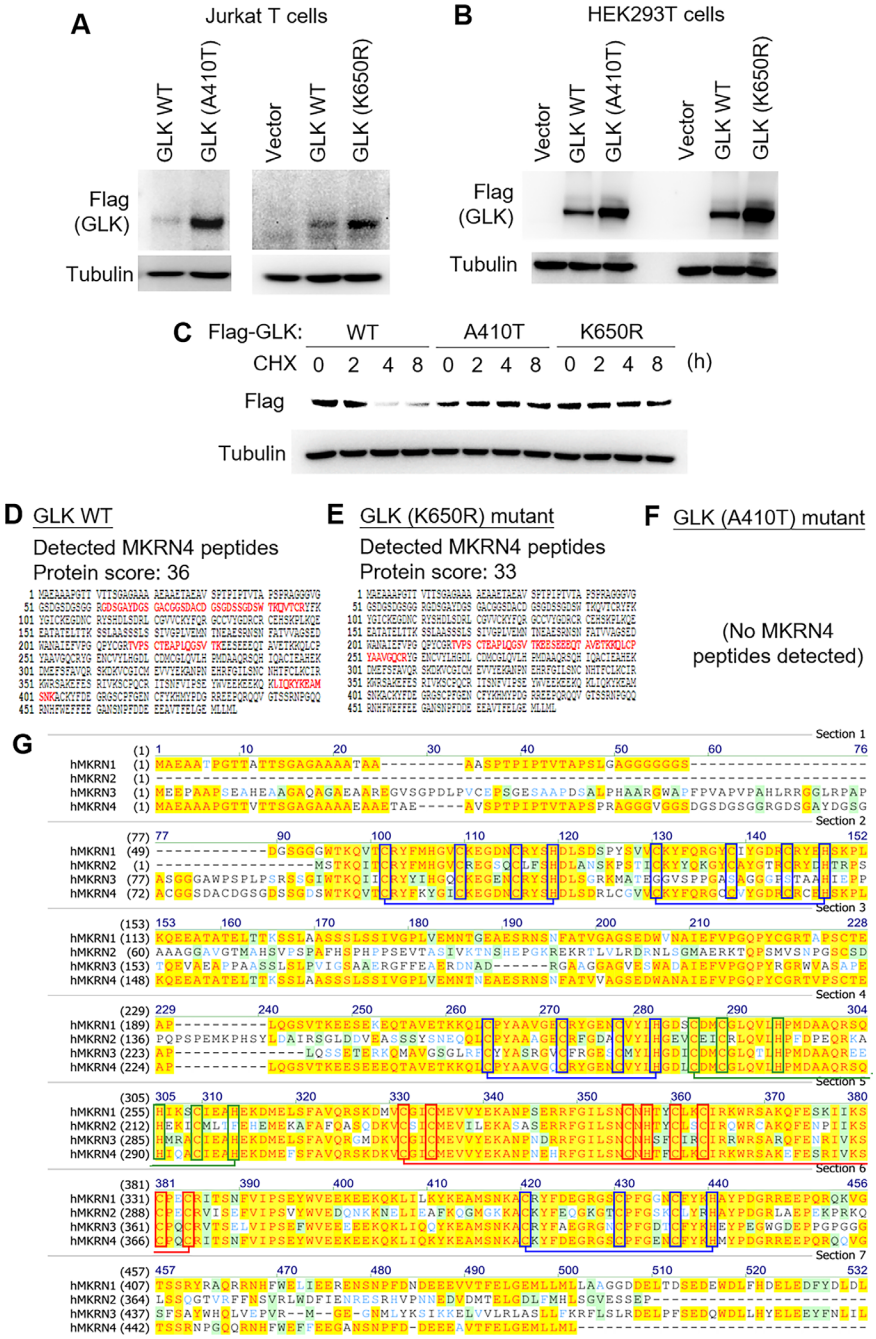

Figure 4 GLK (A410T) or GLK (K650R) variant enhances GLK protein stability. (A) Immunoblotting of Flag-tagged GLK and tubulin proteins from Jurkat T cells transfected with Flag-GLK WT), A410T mutant, or K650R mutant plasmid. Transfected Jurkat T cells were harvested at 48 hours post-transfection, followed by immunoblotting analyses. (B) Immunoblotting of Flag-tagged GLK and tubulin proteins from HEK293T cells transfected with Flag-GLK WT, A410T mutant or K650R mutant plasmid. Transfected HEK293T cells were harvested at 12 hours post-transfection, followed by immunoblotting analyses. (C) Cycloheximide pulse-chase experiments using HEK293T cells. immunoblotting of Flag-tagged GLK (anti-FLAG) and tubulin proteins from HEK293T cells transfected with Flag-GLK WT), A410T mutant or K650R mutant plasmid. Transfected cells were treated with 100 µg/mL CHX for up to 24 hours. (D, E) The detected peptide sequences (red colour) of the endogenous MKRN4 proteins by mass spectrometry analyses using the Flag-tagged GLK immunocomplex isolated from the Jurkat T cells that were transfected with either Flag-GLK WT or Flag-GLK (K650R) mutant plasmid. (F) No MKRN4 peptides detected using the Flag-tagged GLK (A410T)-immunocomplex. The immunocomplex was isolated from the Jurkat T cells that were transfected with Flag-GLK (A410T) plasmid. (G) Protein sequence alignment of human MKRN1, MKRN2, MKRN3 and MKRN4. Amino acids highlighted in yellow and dark green represent the conserved and the similar amino acids, respectively. Amino acids shown in light green represent the weakly similar amino acids. Red, blue and green boxes denote C3HC4 ring domain, C3H domain and MKRN motif, respectively. CHX, cycloheximide; WT, wild-type.

DISCUSSION

A key finding of this study was the identification of one recurrent somatic variant (3′-UTR (T635C)) and four germline variants (3′-UTR (A644C), A410T, A579T or K650R) of GLK in a subgroup of patients with SLE from two independent cohorts. These variants cause GLK overexpression. GLK overexpressing

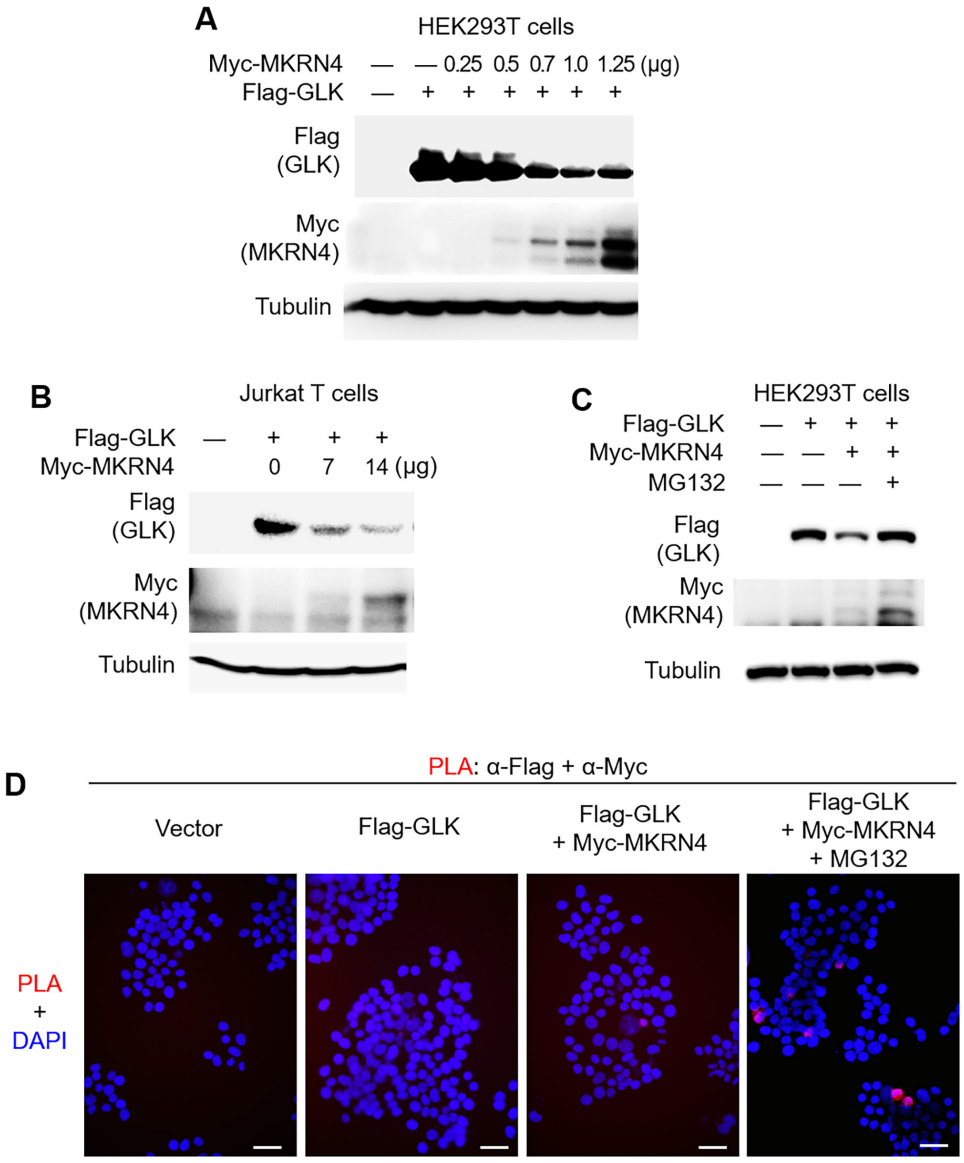

Figure 5 The novel E3 ligase MKRN4 induces proteasomal degradation of GLK. (A) Immunoblotting of Flag-tagged GLK (anti-FLAG), Myc-tagged MKRN4 (anti-MYC) and tubulin proteins from Jurkat T cells cotransfected with Flag-GLK plus increasing amounts of Myc-MKRN4 plasmids. (B) Immunoblotting of Flag-tagged GLK (anti-FLAG), Myc-tagged MKRN4 (anti-MYC) and tubulin proteins from HEK293T cells cotransfected with Flag-GLK plus increasing amounts of Myc-MKRN4 plasmids. (C) Immunoblotting of Flag-tagged GLK (anti-FLAG), Myc-tagged MKRN4 (anti-MYC) and tubulin proteins from HEK293T cells cotransfected with Flag-GLK plus Myc-MKRN4 plasmids. Cells were treated with 25 μM MG132 for 2 hours before being harvested. (D) In situ PLA assays of the interaction between Myc-tagged MKRN4 and Flag-tagged GLK proteins in HEK293T cells. Cells were treated with 25 μM MG132 for 2 hours before being harvested. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Imaging was detected by Leica DM2500 upright fluorescence microscope. Original magnification, ×200. scale bars, 50 μm. PLA, proximity ligation assay.

and GLK$^+$ IL-17A$^+$ T-cell subpopulations are correlated with SLE disease activity of human patients with SLE.[17] Previous reports demonstrate that GLK overexpression in T cells induces IL-17A overproduction, leading to autoimmune responses[14]; conversely, GLK inhibitor blocks IL-17A production from human SLE T cells and attenuates disease severity of autoimmune disease mice.[17] The findings reported here suggest that the 3′-UTR (T635C), 3′-UTR (A644C), A410T, A579T or K650R variant-induced GLK overexpression through the stabilisation of GLK mRNAs or proteins may contribute to SLE pathogenesis.

One of the exciting findings in this report is the identification of the novel E3 ligase MKRN4 that induces GLK protein degradation. This is the first report revealing that MKRN4 is an E3 ubiquitin ligase instead of a pseudogene. MKRN4 ubiquitinated GLK at Lys650 residue. Consistently, GLK (K650R) mutation blocked MKRN4-induced Lys48-linked ubiquitination of GLK; GLK (A410T) mutation attenuated its association with MKRN4. Interestingly, GLK (A648fs) somatic frameshift variant results in the lack of Lys650 residue—the MKRN4-targetted GLK ubiquitination site; therefore, GLK (A648fs) mutation causes GLK protein induction by preventing MKRN4-induced protein degradation. Thus, A410T, K650R or A648fs variant of GLK causes GLK protein stabilisation by blocking MKRN4-mediated GLK ubiquitination.

SLE is a multigenic disease associated with genetic, environmental and gender factors.[23 24] Patients with SLE with GLK 3′-UTR (T635C) somatic variant showed more severe inflammation (increased anti-dsDNA antibody and decreased C3/C4 levels) and lymphocytopaenia than those of patients with SLE without this somatic variant. T-cell-specific GLK transgenic mice

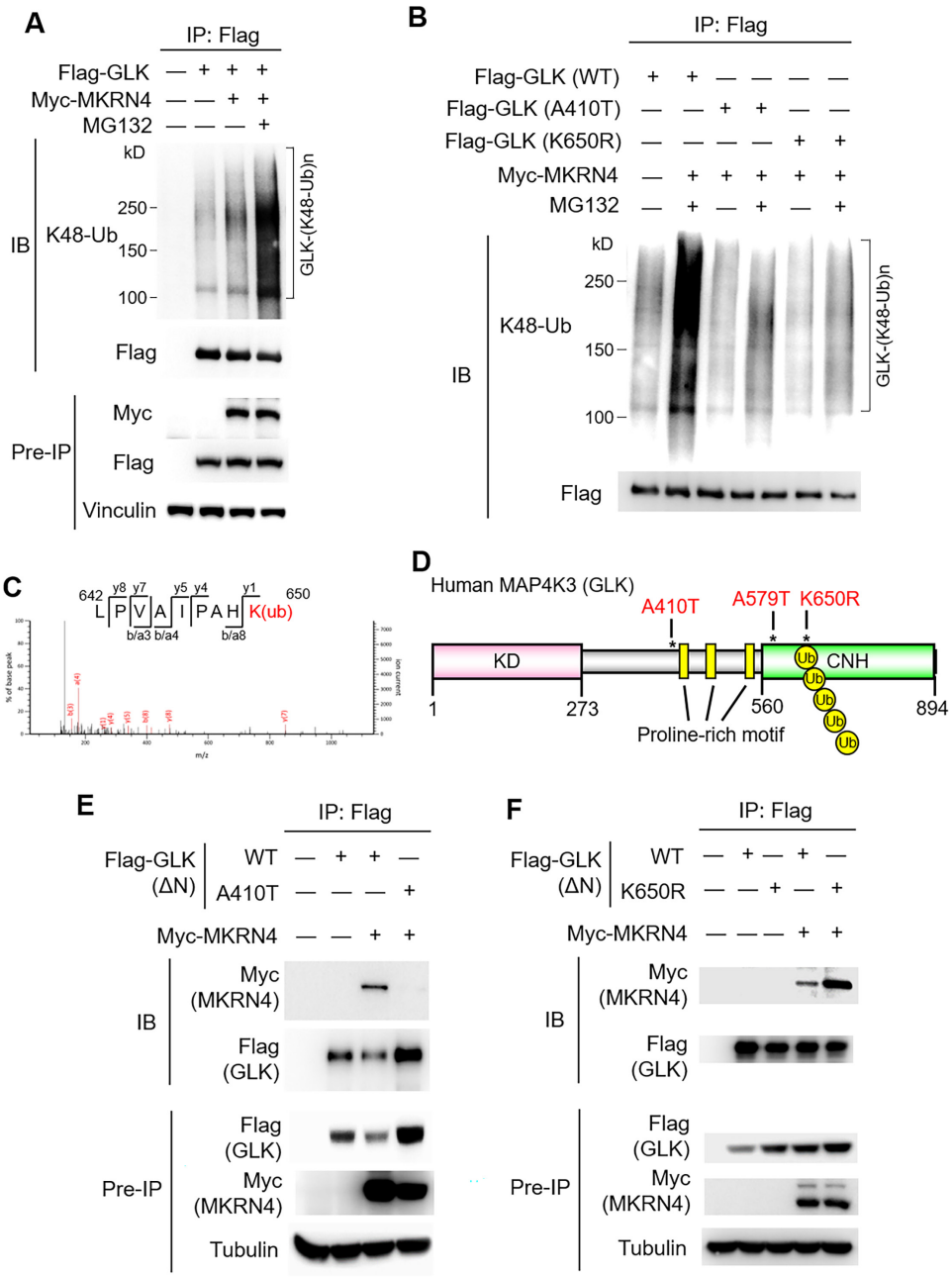

Figure 6 GLK (A410T) or GLK (K650R) mutant is resistant to MKRN4-induced GLK ubiquitination. (A) MKRN4-induced GLK ubiquitination. Flag-tagged GLK proteins were immunoprecipitated from lysates of HEK293T cells cotransfected with Flag-GLK plus Myc-MKRN4 plasmids, followed by immunoblotting with anti-Lys48-linked ubiquitination or anti-FLAG antibody. Cells were treated with 25 µM MG132 for 2 hours before being harvested. (B) Reduced GLK ubiquitination by GLK (A410T) or GLK (K650R) variant. Flag-tagged GLK proteins were immunoprecipitated from lysates of HEK293T cells cotransfected with Myc-MKRN4 plus Flag-GLK WT), A410T mutant, or K650R mutant, followed by immunoblotting with anti-Lys48-linked ubiquitination or anti-FLAG antibody. Cells were treated with 25 µM MG132 for 2 hours before being harvested. (C) Mass spectrometry analysis of the GLK peptides from the MKRN4 immunocomplex. The GLK peptide sequences containing Ub-Lys650 residue of GLK proteins that were detected in the MKRN4 immunocomplex are shown. (D) The structural domains of human MAP4K3 (GLK). Asterisks indicate the locations of A410T, K650R and A579T variants on GLK. (E) Coimmunoprecipitation of Flag-tagged GLKΔN with Myc-tagged MKRN4 proteins from lysates of HEK293T cells cotransfected with Myc-MKRN4 plus either Flag-GLKΔN (deletion of amino acids 1–272) wild-type or Flag-GLKΔN (A410T) mutant plasmids. (F) Coimmunoprecipitation of Flag-tagged GLK with Myc-tagged MKRN4 proteins from lysates of HEK293T cells transfected with Myc-MKRN4 plus either Flag-GLKΔN wild-type or Flag-GLKΔN (K650R) mutant plasmids. CNH, citron-homology domain; KD, kinase domain; WT, wild-type.

display high levels of autoantibodies and severe inflammation.[14] Thus, the severe SLE symptoms may be due to GLK overexpression induced by T635C variant, as well as other somatic or germline variants of GLK. GLK 3′-UTR (T635C) somatic variant (>2.7% frequency) occurs in 17 (16.8%) of 101 patients with SLE from Cohort #1. Notably, none of any HCs nor family members without SLE from all 62 families in Cohort #1 showed high frequency of this somatic variant. These results suggest that the GLK 3′-UTR (T635C) somatic variant in Cohort #1 is not inherited and is independent of their family environment. In Cohort #2, 37 (46.3%) of 80 patients with SLE and 14 (16.1%) of 87 HCs harboured GLK 3′-UTR (T635C) somatic variant

(>2.7% frequency); the numbers of patients with SLE and HCs who harboured GLK 3′-UTR (T635C) somatic variant in Cohort #2 were higher than those of Cohort #1. Interestingly, the age of SLE in Cohort #2 (median: 43.5 years old) was older than that of Cohort #1 (median: 31 years old). Somatic mutation accumulation is associated with ageing due to the increase of clonal haematopoiesis[25 26]; therefore, the increased frequencies of GLK 3′-UTR (T635C) in Cohort #2 patients with SLE (with older age than Cohort #1) may be due to clonal haematopoiesis increasing with age. However, median age of the control group was not significantly different between Cohort #1 (median: 40 years old) and Cohort #2 (median: 42 years old); the Cohort #2 HC group showed a slightly higher mean value of GLK 3′-UTR (T635C) somatic variant frequency than that of Cohort #1. Notably, Cohort #2 individuals were enrolled from the heavy industrial city Kaohsiung in southern Taiwan.[27] Besides age, it is also possible that environmental pollutants may induce somatic variants on GLK, which could be one of the risk factors for SLE. Consistently, two patients with SLE (#B52 and #B53) from Cohort #2 had multiple somatic variants resulting in GLK codon changes and GLK protein induction. The findings suggest that individuals harbouring GLK variants accompanied by other risk factors could be at high risk for SLE.

GLK (A410T), (K650R) and 3′-UTR (A644C) variants are previously annotated, germline-transmitted SNPs. Besides these three SNPs, GLK (A579T) variant is a newly identified germline variant. Due to germline transmission, family members without SLE of the patients with SLE with these four GLK germline variants may also have these variants. Consistently, we noted that one male family member without SLE of a female patient with SLE also harboured GLK 3′-UTR (A644C) variant (table 1). The data suggest that complex risk factors, such as gender factors, in combination with GLK variant contribute to SLE pathogenesis. Two patients with SLE with GLK (K650R) variant were female siblings, whereas their healthy brother did not harbour GLK (K650R) variant. Due to the lack of DNA samples from other family members of patients, it is unclear whether GLK (A410T) variant, (K650R) variant or (A579T) variant occurs in other family members. Nevertheless, our findings suggest that individuals or family members with these four identified GLK germline variants may need to be vigilant for SLE or other autoimmune diseases. In addition, GLK (A410T) and (K650R) variants are the previously annotated SNP rs148167737 and SNP rs200566214, respectively; both SNPs are prevalent in Asia. The prevalence of GLK (A410T) variant/SNP in the world population is 0.000601, whereas it is 0.0017 in Asia, 0.0013 in East Asia and 0.0027 in other Asian (Asian individuals excluding South or East Asian) regions.[28] The prevalence of GLK (K650R) variant/SNP in the world population is 0.000231, while it is 0.0036 in Asia, 0.0042 in East Asia and 0.0022 in other Asian regions.[28] Notably, GLK 3′-UTR (A644C) variant, the SNP rs191224999, is barely identified in the world population (0.00005),[28] but is frequently identified in Vietnamese population (0.014; NCBI BioProject number: 515199) and Korean population (0.0015).[29] These three Asia-prevalent SNPs may be associated with the higher prevalence of SLE in Asia[30] than that worldwide.

Multiple GLK somatic variants on the coding region (C675W, T875S, A579T, A199T, A648fs, G78V, M90I, P436L, D634Y and P704Q) also caused GLK protein stabilisation; therefore, many GLK codons could be somatically mutated, leading to GLK protein induction and subsequent autoimmune responses. At least one third of patients with SLE show a high frequency of GLK overexpressing T cells,[13] while 39% (71 of 181) of patients with SLE have (A410T) germline variant/SNP, (K650R) germline variant/SNP, (A579T) germline variant, 3′-UTR (A644C) germline variant or 3′-UTR (T635C) somatic variant of GLK. Besides the aforementioned GLK variants in coding region and 3′-UTR, it is likely that, mutations or epigenetic changes on the GLK promoter region, as well as downregulation/mutation of MKRN4 may also lead to GLK overexpression and subsequent induction of autoimmune responses. Moreover, genomic analyses of GLK using other SLE cohorts in Western countries may provide additional insights about GLK overexpression-mediated SLE pathogenesis. Taken together, individuals who harbour the aforementioned GLK germline or somatic variants may be at high risk for SLE.

Author affiliations
[1]Immunology Research Center, National Health Research Institutes, Zhunan, Taiwan
[2]Division of Allergy, Immunology and Rheumatology, Taichung Veterans General Hospital, Taichung, Taiwan
[3]Department of Internal Medicine, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung City, Taiwan
[4]Rheumatology and Immunology Center, China Medical University Hospital, Taichung, Taiwan
[5]College of Medicine, China Medical University, Taichung, Taiwan
[6]Department of Pathology and Immunology, Baylor College of Medicine, Houston, Texas, USA

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Contributors H-CC performed experiments, literature search, data analysis, data interpretation, study design, statistical analyses and manuscript writing. W-TH collected family data, provided patient samples, analysed clinical data and performed statistical analyses. Y-MC collected family data and provided patient samples. P-MH performed biochemistry experiments. J-HY provided patient samples and analysed clinical data. J-LL conceived the study, provided patient samples and analysed clinical data. T-HT conceived the study, supervised experiments, interpreted data and wrote the manuscript.

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ORCID iDs
Yi-Ming Chen http://orcid.org/0000-0001-7593-3065
Jeng-Hsien Yen http://orcid.org/0000-0002-1291-1739
Tse-Hua Tan http://orcid.org/0000-0003-4969-3170

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TRANSLATIONAL SCIENCE

Human SLE variant *NCF1*-R90H promotes kidney damage and murine lupus through enhanced Tfh2 responses induced by defective efferocytosis of macrophages

Linyu Geng ,[1,2] Jian Zhao,[1] Yun Deng,[1] Ivan Molano,[1] Xue Xu,[1] Lingxiao Xu ,[1] Phillip Ruiz,[3] Quanzhen Li,[4] Xuebing Feng,[2] Miaojia Zhang,[5] Wenfeng Tan ,[5] Diane L Kamen,[1] Sang-Cheol Bae ,[6] Gary S Gilkeson,[1,7] Lingyun Sun ,[2] Betty P Tsao[1]

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For numbered affiliations see end of article.

Correspondence to
Dr. Betty P Tsao, Division of Rheumatology & Immunology, Medical University of South Carolina, Charleston, SC 29425, USA; tsaob@musc.edu and Dr. Lingyun Sun, The Affiliated Drum Tower Hospital of Nanjing University Medical School, Nanjing, People's Republic of China; lingyunsun@nju.edu.cn

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ABSTRACT

Objectives We previously identified a hypomorphic variant, p.Arg90His (p.R90H) of neutrophil cytosolic factor 1 (*NCF1*, a regulatory subunit of phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2 complex), as an putative causal variant for systemic lupus erythematosus (SLE), and established a knock-in (KI) H90 variant in the C57BL/6 background to study how this variant promotes lupus development.

Methods Wild type (WT) and KI littermates were assessed for immune profiles and lupus-like features. Disease activity and renal damage of patients with SLE were assessed by systemic lupus erythematosus disease activity index (SLEDAI) and renal items of systemic lupus international collaborating clinics (SLICC), respectively.

Results Compared with WT littermates, 5-week-old homozygous KI mice had reduced oxidative burst, splenomegaly, elevated type I interferon (IFN-I) scores, increased ratios of splenic follicular T helper 2 (Tfh2) to either T follicular regulatory (Tfr) or Tfh1 cells, increased ANA^+ follicular, germinal centre and plasma cells without spontaneous kidney disease up to 1 year of age. Pristane treatment exacerbated the immune dysregulation and induced IFN-I-dependent kidney disease in 36-week-old H90 KI female mice. Decreased efferocytosis of macrophages derived from KI mice and patients with homozygous H90 SLE promoted elevated ratios of Tfh2/Tfr and Tfh2/Tfh1 as well as dysregulated humoral responses due to reduced voltage-gated proton channel 1 (Hv1)-dependent acidification of phagosome pH to neutralise the decreased electrogenic effect of the H90 variant, resulting in impaired maturation and phagosome proteolysis, and increased autoantibody production and kidney damage in mice and patients with SLE of multiple ancestries.

Conclusions A lupus causal variant, NCF1-H90, reduces macrophage efferocytosis, enhances Tfh2 responses and promotes autoantibody production and kidney damage in both mice and patients with SLE.

INTRODUCTION

Systemic lupus erythematosus (SLE), a multisystemic chronic autoimmune disease with a strong genetic predisposition, is characterised by increased type I interferon (IFN-I) production, disturbed T cell

Key messages

What is already known about this subject?

⇒ A p.Arg90His (p.R90H) variant of the neutrophil cytosolic factor 1 (*NCF1*) gene that reduces reactive oxygen responses is one of the strongest common variants associated with systemic lupus erythematosus (SLE) in multiple ancestries, and is associated with altered formation of neutrophil extracellular traps, high serum interferon activity and antiphospholipid syndrome in SLE.

What does this study add?

⇒ Young naïve C57BL6 (B6) mice expressing this human H90 risk variant (knock-in (KI)) developed spontaneous autoimmunity, including, splenomegaly, elevated type I interferon scores, increased ratios of splenic follicular T helper 2 (Tfh2) to either T follicular regulatory (Tfr) or Tfh1, increased ANA^+ follicular, germinal centre B cells and plasma cells, but did not progress into full blown lupus-like kidney disease as they aged.

⇒ Pristane treatment induced proliferative glomerulonephritis and proteinuria in 36-week-old H90 KI B6 female mice, exhibiting increased Tfh2 coupled with decreased Tfr and Tfh1 proportions, robust germinal centre formation and IgG autoantibody production.

⇒ The *NCF1*-H90 variant, that impaired apoptotic cell clearance by macrophages in B6 mice and patients with SLE, expanded Tfh2-cells in a CD40-dependent manner and increased risk for kidney damage in patients with SLE from multiple ethnic populations, is the causal variant within the SLE-associated NCF1 locus.

How might this impact on clinical practice or future developments?

⇒ Patients with SLE carrying the *NCF1*-H90 genotype have increased risk for active disease course and kidney damage, and targeted interventions of key pathogenic mechanisms could be considered for therapy development.

homoeostasis, autoreactive B-cells and deposition of autoantibodies and immune complexes causing tissue injury.[1] Genome-wide association studies have identified over 100 SLE-linked risk loci.[2] Recently, we identified a p.Arg90His (p.R90H, rs201802880) substitution encoded in neutrophil cytosolic factor (NCF1), an essential component of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex 2 (NOX2), as a novel and robust genetic risk variant for autoimmune diseases including SLE.[3] Subsequently, the association between the hypofunctional H90 variant and SLE was independently validated.[4 5]

The phagocyte NOX2 complex, containing two catalytic transmembrane subunits and three regulatory cytosolic subunits (including the NCF1 protein, also known as $p47^{phox}$),[6] can be activated by engulfment of apoptotic cells,[7 8] termed efferocytosis. This engulfment triggers phosphorylation and subsequent binding of NCF1 to the membrane NOX2 component, initiates electron transfer from NADPH across the membrane to molecular oxygen forming superoxide anion—a precursor to reactive oxygen species.[3] The consequences of electron efflux during oxidative burst is counteracted by proton release via voltage-gated proton channel 1 (Hv1),[9 10] leading to endolysosomal acidification by the vacuolar-type H^+ ATPase (V-ATPase),[11] and proteolytic degradation of the cellular corpse by activated lysosomal proteases.[12 13] While NOX2 is known to inhibit murine lupus-like responses to dying cells via the light chain 3 (LC3)-associated phagocytosis,[14] much less is known about how p.R90H NCF1 affects specific stages of efferocytosis by macrophages, and its downstream effect leading to lupus manifestations.

Pathogenic autoantibodies in SLE, long-lived with a high load of somatic mutation, have characteristic features of T-cell-dependent germinal centre (GC) reactions with the participation of follicular helper T (Tfh) cells.[15] Tfh cells, including Tfh1, Tfh2 and Tfh17 subsets, have distinct functions to impact on autoimmune diseases.[16 17] Additionally, follicular regulatory T (Tfr) cells inhibit Tfh cell-mediated antibody responses.[18] The imbalanced Tfh subsets and Tfh/Tfr ratios could contribute to the onset or progression of SLE.[19 20] However, the mechanisms by which phagocytes affect the adaptive immune system and the ensuing helper T cell responses during efferocytosis are not entirely clear.

Considering the high homology between mouse and human *NCF1* and the lack of *NCF1B*/*1C* pseudogenes in mice, we established a C57BL/6 (B6) mouse model with a knock-in (KI) H90 hypomorphic variant in the Ncf1 locus by CRISPR/Cas9 genome editing (online supplemental figure 1). We present novel data showing the same link between the H90 hypomorphic genotype to lupus-like phenotype in a mouse model and patients with SLE (online supplemental methods), demonstrating this variant is the causal variant in the *NCF1* locus.

RESULTS

Ncf1-H90 KI mice demonstrate lupus-like features induced by pristane

We confirmed the effect of H90 allele on reactive oxygen species (ROS) production by phorbol myristate acetate (PMA) stimulated polymorphonuclear leucocytes from patients with SLE[3 4] by showing reduced extracellular ROS in KI (figure 1A) and similar intracellular ROS levels (figure 1B) in PMA-stimulated splenocytes from *Ncf1*-R90H wild type (WT) and KI 5-week-old littermates. The hypofunctional *Ncf1*-H90 variant predisposed to early-onset autoimmunity, as shown by splenomegaly (figure 1C) and increased IFN-I scores (figure 1D,E) in 5-week-old H90 KI mice compared with R90 WT littermates. These KI mice showed no evidence of glomerulonephritis or glomerular IgG deposition (online supplemental figure 2A) at either 5 weeks or 52 weeks in a specific pathogen-free environment (online supplemental figure S2B–F).

We next tested the genetic effect in the pristane-induced lupus model. Compared with pristane-treated female WT mice (PRI-WT), 36-week-old female KI littermates (PRI-KI) developed robust lupus-like manifestations including splenomegaly (figure 1F) with increased spleen indices (figure 1G and online supplemental figure S2G), IFN-simulated gene expression levels and IFN-I scores (figure 1H and online supplemental figure S2H,I), enlarged PNA^+ GC within splenic follicles (figure 1I) and elevated IgG antibodies to anti-Smith (Sm), double-stranded DNA (dsDNA) and ribonucleoprotein (RNP) antibody using autoantigen arrays (figure 1J). Serology findings in female PRI-KI were confirmed by ELISA, including elevated levels of IgG anti-RNP (online supplemental figure S2K), ANA (online supplemental figure S2L), total IgG (online supplemental figure S2M) and a trend towards increased anti-dsDNA levels (online supplemental figure S2J). Furthermore, 36-week-old female PRI-KI littermates developed elevated blood urea nitrogen (figure 1K) and serum creatinine levels (figure 1L), aggravated glomerulonephritis with increased glomerular scores (figure 1M), and increased glomerular deposition of IgG and complement C3 (figure 1N) that positively correlated with increased IFN-I scores (online supplemental figure S2N). These data showed the R to H substitution of *Ncf1* on the B6 background enhanced pristane-induced lupus-like glomerulonephritis in females, and the extent of kidney disease correlated with upregulated IFN-I scores.

Induced humoral response of SLE-associated variant *Ncf1*-H90 in KI mice

To dissect the cellular basis for splenomegaly in 5-week-old naïve H90 KI mice, immunophenotyping revealed significantly higher absolute numbers of $CD3^+$ T cells and $CD19^+$ B cells (online supplemental figure S3A), in comparison to WT littermates, but no significant difference in myeloid-derived cells (online supplemental figure S3B–G). Further analysis of B cell subsets of splenocytes showed that H90 KI mice had significantly greater frequencies of follicular B cells ($CD19^+CD23^{high}CD21^{int}$, figure 2A, left), plasma cells ($CD138^+B220^-$, figure 2A, right), GC B cells ($CD19^+GL7^+CD95^+$, figure 2B) and ANA^+ autoreactive follicular B cells (figure 2C) in H90 KI mice compared with their WT littermates. No significant differences were noted between WT and KI littermates in the absolute number of marginal zone B cells ($CD19^+CD23^{low}CD21^{high}$, figure 2A, left) or B1a B cells (online supplemental figure S3F,H). Compared with Tfh cells observed in WT spleens, the spleens of H90 KI littermates exhibited elevated frequencies of Tfh cells ($CD4^+Foxp3^-CXCR5^{++}PD\text{-}1^{++}$, figure 2D,E, online supplemental figure S3I) with increased frequencies of Tfh2 cells ($CD4^+Foxp3^-CXCR5^{++}PD\text{-}1^{++}CCR6^-CXCR3^-$, figure 2G), and decreased frequencies of Tfh1 cells ($CD4^+Foxp3^-CXCR5^{++}PD\text{-}1^{++}CCR6^-CXCR3^+$, figure 2G), and Tfr cells ($CD4^+Foxp3^+CXCR5^{++}PD\text{-}1^{++}$, figure 2D,E), resulting in increased Tfh/Tfr (figure 2F), Tfh2/Tfr and Tfh2/Tfh1 ratios (figure 2H). We observed no significant difference in either the frequency or the number of T regulatory cells (Treg) ($CD4^+CD25^+Foxp3^+$, online supplemental figure S3J,K), Th1 ($CD4^+IFN\text{-}\gamma^+$, online supplemental figure S3K, upper), Th2 ($CD4^+IL\text{-}4^+$, figure. S3K, middle), Th17 ($CD4^+IL\text{-}17^+$, online supplemental figure S3K, lower) or Tfh17 (figure 2G) cells in 5-week-old KI mice compared with their WT littermates.

Consistent with 5-week-old KI mice (figure 2I and online supplemental figure S4B–E, G–K), spleen cells of PRI-KI female mice

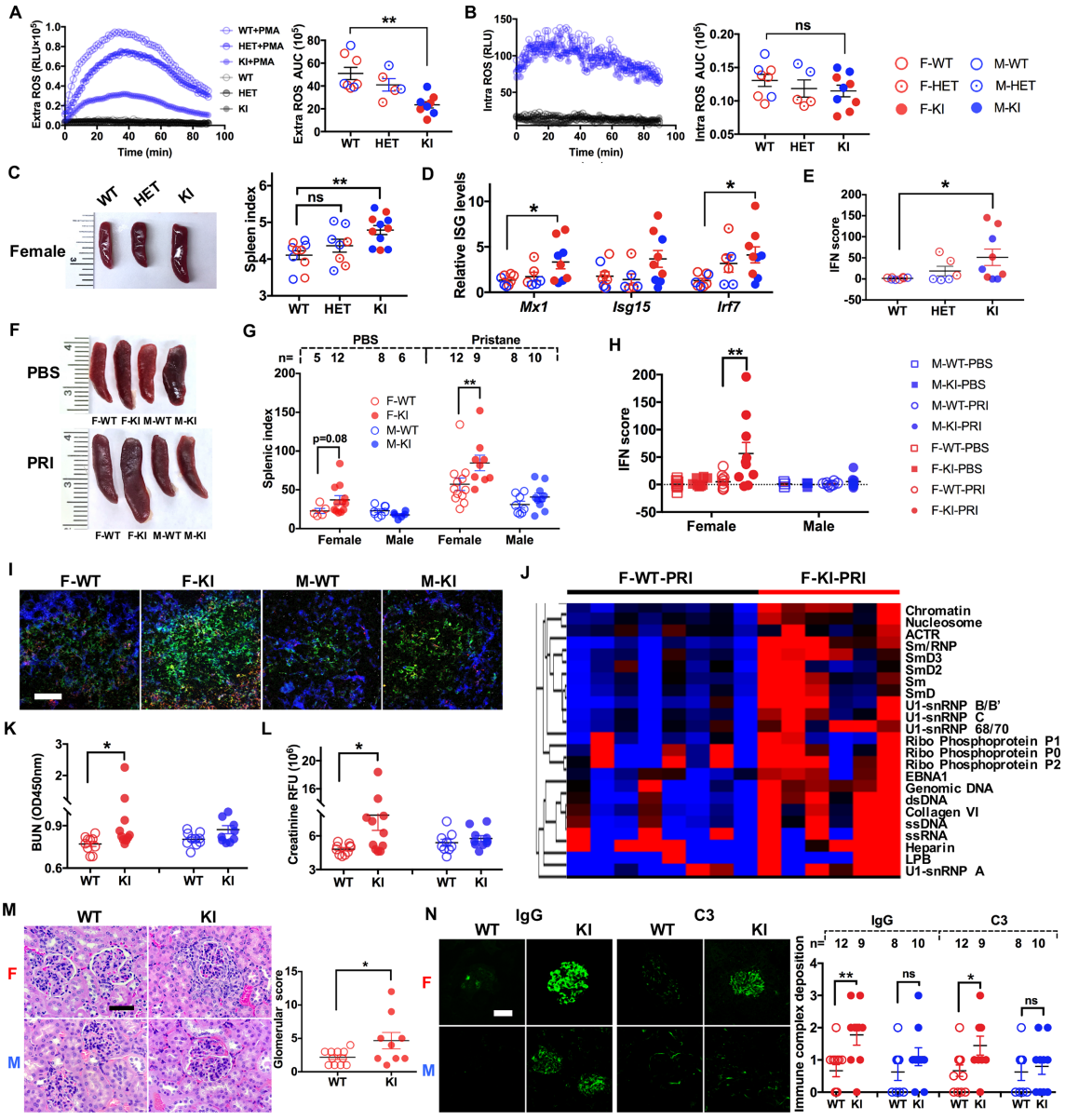

Figure 1 Young *Ncf1*-H90 KI mice, exhibiting reduced extracellular oxidative burst, splenomegaly and increased IFN scores, developed autoantibodies and lupus-like kidney disease after pristane injection. (A, B) Extracellular (left) and intracellular (right) ROS production over time measured by isoluminol-enhanced chemiluminescence in PMA stimulated splenocytes from 5-week-old *Ncf1*-H90 WT and KI littermates, AUC was calculated from RLU values. (C–E) Representative image of spleen (C), relative expression levels of *ISGs* (D) and calculated IFN-I scores (E) in 5-week-old littermates. (F–H) Representative image of spleen (F), splenic index (G) and calculated IFN-I scores (H) in 36-week-old pristane-injected littermates. (I) PNA^+ GC formation in pristane-induced 36-week-old mice. Bar: 50 µm, magnification: ×400; red, $CD3^+$ T cells; blue, B220 +B cells; green, PNA^+ GC cells. (J–L). Heat map of IgG autoantibody profiles by autoantigen array (J), Blood urea nitrogen (BUN; K) and serum creatinine (L) levels in sera of 36-week-old mice. (M, N) Representative kidney sections and their calculated scores from 36-week-old mice, stained with H&E (M), IgG (N, left) and C3 (N, right). Bar: 50 µm, magnification: ×400. AUC, area under the curve; C3, complement 3; GC, germinal centre; F, female, red dot; HET, heterozygous; IFN-I, type I interferon; IgG, immunoglobin G; *ISGs*, IFN-simulated genes; KI, knock-in; M, male, blue dot; PMA, phorbol myristate acetate; PBS, phosphate buffered saline; PRI, pristane; RNP, ribonucleoprotein antibody; RLU, relative luminescence units; ROS, reactive oxygen species; RFU, relative fluorescence units; WT, wild type.

exhibited increased frequencies of Tfh and Tfh2 subsets (figure 2I and online supplemental figure S4A,F,G) accompanied with decreased frequencies of Tfh1 (figure 2I and online supplemental figure S4H) and Tfr cells (figure 2I). Compared with Tfh cells, Tfh2 subset numbers correlated strongly with the number of plasma cells, ANA^+ autoreactive B cells, GC B cells ($CD19^+GL7^+CD95^+$) and activated B cells ($IgG2c^+B220^-$, figure 2K,L). The positive correlations of Tfh2/Tfh1 and Tfh2/Tfr with expansion of autoreactive B cells and plasma cells (figure 2L) suggest that the *Ncf1*-H90 variant caused increased follicular T subset ratios, facilitating GC formation and autoantibody production.

H90 variant drives Tfh2/Tfr and Tfh2/Tfh1 responses in *Ncf1*-H90 KI mice through enhancing CD40 expression levels on BMDM during the efferocytosis process

We next asked how the adaptive immune system could be affected by the expression of the H90 variant in innate immune cells.

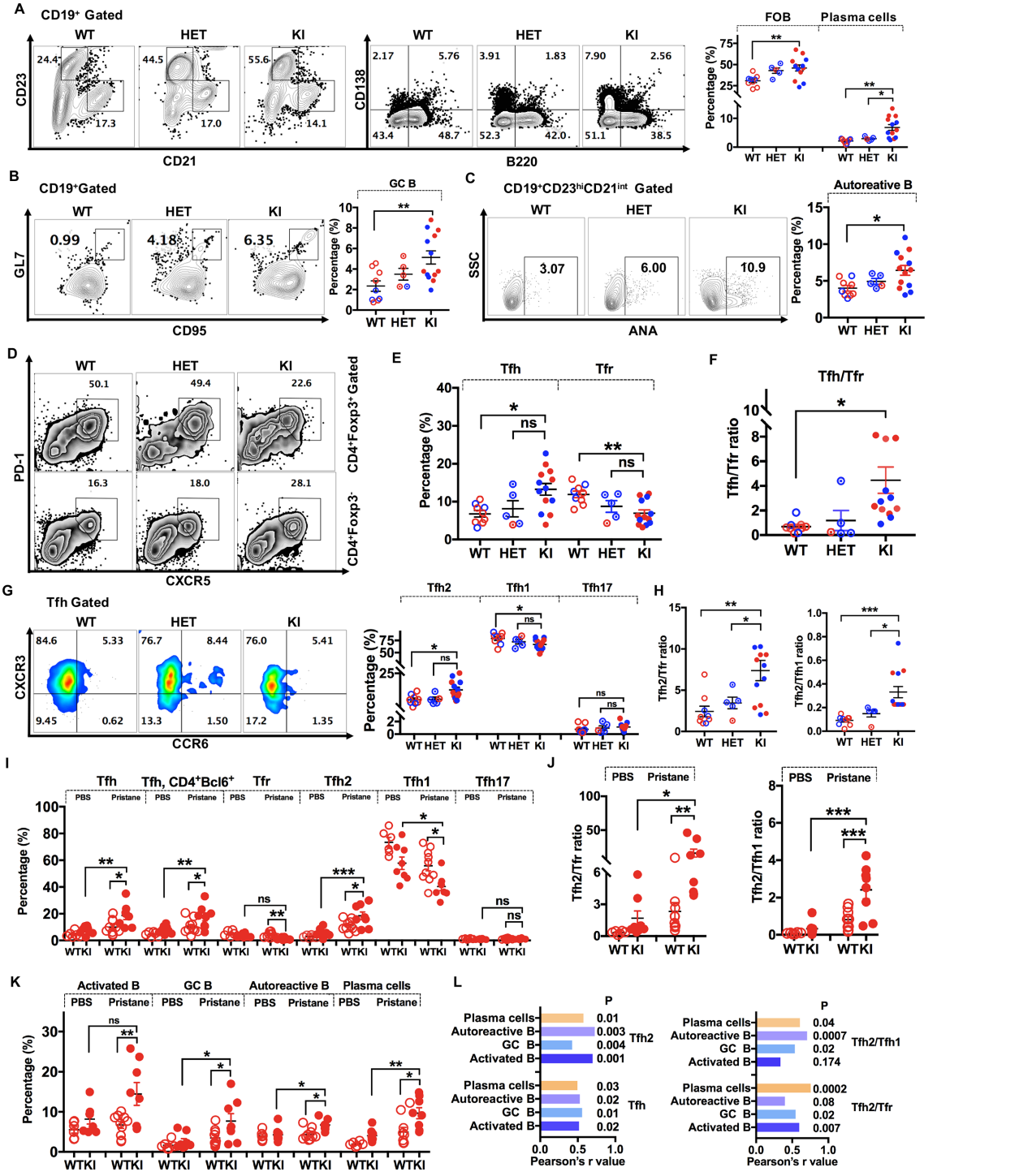

Figure 2 Both 5-week-old naïve KI and 36-week-old pristane-injected female KI mice had increased splenic frequencies of GC B cells, plasma cells, autoreactive follicular B cells and ratios of Tfh2/Tfr and Tfh2/Tfh1. (A–H) Gating strategy or frequencies of CD19$^+$CD23^{hi}CD21^{int} follicular B cells (FOB) and CD138$^+$B220$^-$ plasma cells (A), CD19$^+$CD95$^+$GL7$^+$ GC B cells (B), ANA$^+$ autoreactive B cells from FOB (C), CXCR5^{++}PD-1^{++} cells from CD4$^+$Foxp3$^-$ or CD4$^+$Foxp3$^+$ cells (D, lower; E, left; Tfh, T follicular helper cells; D, upper; E, right; Tfr, T follicular regulatory cells), CCR6$^-$CXCR3$^-$ cells from Tfh cells (G, Tfh2), CD4$^+$Foxp3$^-$CXCR5^{++}PD-1^{++}CCR6$^-$CXCR3$^+$ Tfh1 cells (G) and CD4$^+$Foxp3$^-$CXCR5^{++}PD-1^{++}CCR6$^+$CXCR3$^-$ Tfh17 cells (G), Tfh/Tfr ratios (F) and Tfh2/Tfr ratios (H) in 5-week-old murine spleen cells. (I–K) Flow-cytometric plots or frequencies of CD4$^+$Foxp3$^-$CXCR5^{++}PD-1^{++} Tfh cells (I), CD4$^+$Bcl6$^+$ Tfh cells (I), CD4$^+$Foxp3$^+$CXCR5^{++}PD-1^{++} Tfr cells (I), CD4$^+$Foxp3$^-$CXCR5^{++}PD-1^{++}CCR6$^-$CXCR3$^-$ Tfh2 cells (I), Tfh1 (I), Tfh17 (I), Tfh2/Tfr ratio (J, left), Tfh2/Tfh1 ratio (J, right), IgG2c$^+$B220$^-$ activated B cells (K, left), CD19$^+$CD95$^+$GL7$^+$ GC B cells (K), CD19$^+$ANA$^+$ autoreactive B cells and plasma cells (K) in splenocytes of 36-week-old mice. (L) Correlation analysis of Tfh, Tfh2, Tfh2/Tfr and Tfh2/Tfh1 with B cell subsets in splenocytes of 36-week-old mice. GC, germinal centre; HET, heterozygous; KI, knock-in; PBS, phosphate buffer saline; PRI, pristane; WT, wild type; red dot, F, female; blue dot, M, male.

Given that lupus is well known for defective efferocytosis, and *NCF1* is a regulatory subunit of phagocyte NADPH oxidase, we assessed H90 genotypic effects on efferocytosis by bone marrow derived macrophages (BMDM). F4/80$^+$CD11b$^+$ BMDM (online supplemental figure S5A,B) from WT and H90 KI mice were incubated in the presence or absence of apoptotic cells (AC)

(irradiated WT thymocytes; online supplemental figure S5C) and then co-cultured with WT splenocytes or naïve CD4$^+$ T cells for 72 hours. Interestingly, efferocytosis of H90 BMDM significantly increased the frequency of Tfh cells (figure 3A,B and online supplemental figure S5H), Tfh2 cells (figure 3C,D and online supplemental figure S5H) with increased proliferative capacity (figure 3E and online supplemental figure S5F), suppressed the frequency of Tfr (figure 3F and online supplemental figure S5H) and Tfh1 cells (figure 3C and online supplemental figure S5H), leading to increased Tfh2/Tfr (figure 3G) and Tfh2/Tfh1 ratios (figure 3H) in co-cultures. Efferocytosis of H90 BMDM had no significant effect on the rate of proliferation or apoptosis of BMDM (online supplemental figure S5D,E), on splenocytes (online supplemental figure S5G) or proportions of Th1, Th2, Th17, Treg and Tfh17 subsets (figure 3C and online supplemental figure S5I) in co-culture system, indicating that H90 variant of BMDM could promote WT CD4$^+$ T cell differentiation into Tfh2 during efferocytosis.

We further assessed the H90 allele effect on costimulatory factors expressed on BMDM during efferocytosis. Compared with that of WT littermates, the AC-BMDM from H90 KI mice had increased CD40 (M1 marker) but reduced CD163 and CD206 (M2 markers) expression levels (figure 3I). Further, Tfh (figure 3J), Tfh2 (figure 3K) and Tfr (figure 3L) polarisation, Tfh2/Tfr (figure 3M) and Tfh2/Tfh1 ratios (figure 3N) by AC-H90 BMDM could be abrogated by anti-CD40 antibodies (Ab) but not by antibodies to CD80 or ICOSL, supporting that the H90 variant drove CD4$^+$ T cell polarisation in *Ncf1*-H90 KI mice through promoting an inflammatory phenotype of BMDM via enhanced CD40 levels during efferocytosis. Importantly, only direct contact, but not trans-well assay, of AC-H90 BMDM with naïve WT CD4$^+$ T cells induced the Tfh and Tfr polarisation (online supplemental figure S5J,K), supporting that R90H variant acted through BMDM surface levels of the co-stimulatory factors rather than releasing immunomodulatory molecules.

Efferocytosis required intact activity of NOX2 and Hv1 and defective efferocytosis process led to increased CD40 levels of BMDM from *Ncf1*-H90 KI mice

NOX activation promotes acidification and proteolytic activity within inflammatory macrophage efferosomes.[21] To determine the mechanisms of efferocytosis-induced polarisation of BMDM from H90 KI mice, we tracked the fate of ingested AC in WT and H90 KI BMDM. Compared with that of WT littermates, BMDM from KI mice showed decreased phagocytic ability both at 15 and 30 min by two independent assays of immunofluorescence (figure 4A and online supplemental figure S6C,D) and flow cytometry (figure 4B), which was abrogated by cytochalasin D (figure 4A,B), a classic phagocytosis inhibitor.[22]

To investigate the digestive process of ingested AC in BMDM, we compared kinetics of different maturation events between WT and H90 KI littermates. Lysotracker fluorescence intensity was significantly lower in H90 KI efferosomes compared with that of WT littermates at 3 and 6 hours (figure 4C and online supplemental figure S6F), and no size difference between the two groups (online supplemental figure S6E), suggesting reduced acidification of efferosomes induced by H90 variant. Dequenched-bovine serum albumin (DQ-BSA) is a fluorogenic substrate for proteases we used to coat AC that produces a bright fluorescence on AC degradation. The fraction of efferosomes with cleaved DQ-BSA was significantly lower in H90 BMDM at 3 and 6 hours compared with that of WT BMDM (figure 4D), indicating impaired proteolysis of efferosomal cargo in H90 BMDM. The early endosome maker early endosome antigen 1 (EEA-1) and late endosome/lysosome marker lysosomal-associated membrane protein 1 (LAMP1) encircled intact and colocalised with digested AC within WT and KI BMDM at 3 or 6 hours after ingestion, and colocalization of LC3 (figure 4E,F). Both EEA-1 and LAMP1 coalescing with apoptotic material on efferosomes was significantly reduced in H90 KI BMDM compared with WT BMDM (figure 4E,F), which positively correlated with their phagocytic rate (figure 4G), suggesting that H90 variant caused defective maturation of apoptotic cell-containing phagosome. Taken together, these results demonstrated that efferosomes in BMDM from H90 KI mice had reduced acidification, delayed maturation and defected proteolysis following ingestion of AC.

The reduced NOX2-derived ROS production (online supplemental figure S6A,B) by the hypomorphic H90 *Ncf1* variant might affect phagocyte functions by modulating phagosome (efferosome) pH via proton release by V-ATPase and Hv1 during efferocytosis process. To explore the relative contribution of V-ATPase and/or Hv1 in pumping protons to neutralise the ROS produced by activated NOX2, we assessed effects of the ROS stimulator t-butyl hydroperoxide (TBHP), the ROS inhibitor diphenyleneiodonium chloride (DPI), Hv1 inhibitor 2-guanidino-benzimidazole (2GBI) or V-ATPase inhibitor bafilomycin A1 (BafA1), respectively, in our co-culture systems. We observed that the phagocytic rate (figure 4H and online supplemental figure S6G), the acidification of efferosomes in AC-treated BMDM (figure 4I and online supplemental figure S6H) and proteolytic degradation of AC in BMDM (figure 4J and online supplemental figure S6J) were inhibited by 2GBI and DPI, but increased by TBHP, as measured by cell tracker, lysotracker and DQ-BSA cleavage, respectively. Further, in contrast to TBHP, the oxidant scavenger DPI and pH manipulator 2GBI, which ablated NOX2-induced ROS by WT BMDM, reduced recruitment of LC3 and LAMP1 in WT and KI efferosomes (figure 4L and online supplemental figure S6I), but not LC3 and EEA-1 recruitment (figure 4K). Nevertheless, treatment of AC-activated BMDM from 10-week-old female WT mice with 2GBI or DPI during efferocytosis induction, which ablated Hv1 induced acidification and NOX2-induced ROS by BMDM, respectively, promoted the differentiation of Tfh (online supplemental figure S6K) and Tfh2 cells (figure 4M) but prevented the differentiation of Tfh1 cells (figure 4N) and Tfr cells (figure 4O and online supplemental figure S6L) from naïve CD4$^+$ T cells in AC-activated macrophage co-cultures. These effects could be abolished by treatment with TBHP or anti-CD40 Ab. No difference was found for BafA1 treatment. Collectively, our findings support that the H90 variant lowered NOX2-ROS, together with lower Hv1-dependent, but not V-ATPase dependent acidification to modulate phagosome pH, results in a defective efferocytosis process of BMDM and increased ratios of Tfh2/Tfr polarisation.

R90H variant drives humoral response in patients with lupus nephritis during the efferocytosis process

To determine if our findings in H90 KI mice could be confirmed in patients with SLE, we assessed the efferocytosis process of peripheral blood monocyte derived macrophages from patients with SLE carrying the H90 variant (online supplemental table S1 and S2, figure S7A–D), which showed decreased phagocytic ability at 30 min (figure 5A,I). Compared with those from R90-encoding patients with SLE (R90), AC-macrophages from H90 patients co-cultured with CD4$^+$ T cells from healthy controls showed enhanced Tfh (figure 5B and online supplemental figure S7E,F) and Tfh2 frequencies (figure 5C and online supplemental

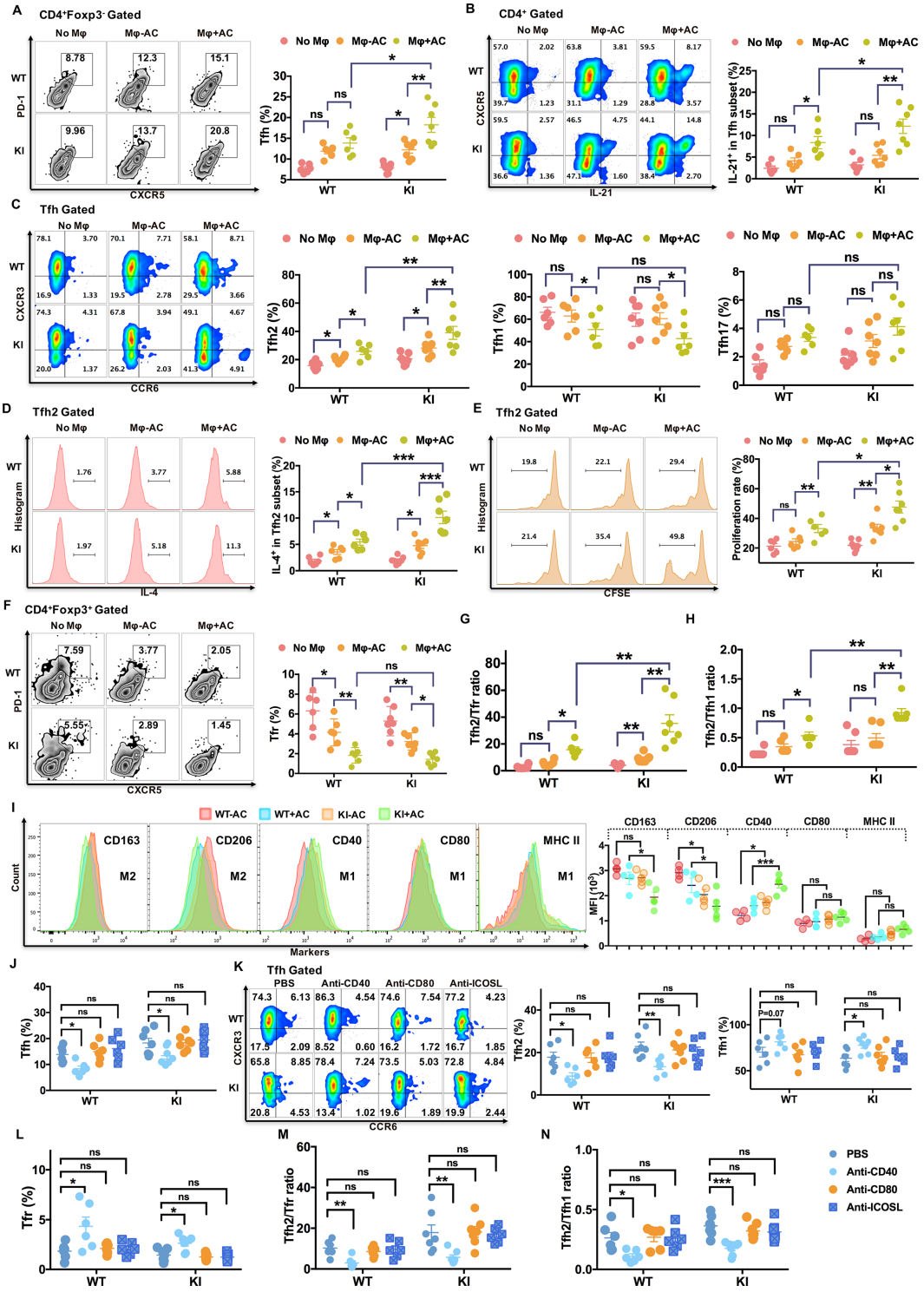

Figure 3 The *Ncf1*-R90H variant promoted Tfh2 proliferation and increased ratios of Tfh2/Tfr and Tfh2/Tfh1 in WT CD4$^+$ T cell co-cultures through elevated CD40 levels on BMDM from KI mice during efferocytosis processes. (A, C, F, G–H) Flow-cytometric plot (left) and summary graph (right) of Tfh (A), Tfh2 (C), Tfh1 (C), Tfh17 (C), Tfr (F), Tfh/Tfr ratios (G) and Tfh2/Tfr ratios (H) differentiated from WT naïve CD4$^+$ T cells co-cultured with AC treated BMDM from 10-week-old female *Ncf1*-H90 WT and KI mice. (B, D). Flow-cytometric plot (left) and summary graph (right) of IL-21 (B) levels in Tfh cells, IL-4 (D) levels in Tfh2 cells differentiated from naïve WT CD4$^+$ T cells co-cultured with AC treated BMDM from 10-week-old female *Ncf1*-H90 WT and KI mice. (E) Increased proliferative rate of WT Tfh2 cells co-cultured with AC treated BMDM from 10-week-old female *Ncf1* KI mice. I. Expression levels of CD163, CD206, CD40, CD80 and MHC-II levels in AC treated BMDM from 10-week-old female *Ncf1* KI mice. (J–L) Differentiation of Tfh (J), Tfr (L), Tfh2 (K) instead of Tfh1 (K) co-cultured with BMDM was suppressed by anti-CD40 Ab, but not anti-CD80 and anti-ICOSL Ab. (M–N) Ratio of Tfh2/Tfr (M) and Tfh2/Tfh1 (N) differentiated from naïve WT CD4$^+$ T cells co-cultured with AC treated BMDM from 10-week-old female *Ncf1*-H90 WT and KI mice treated with or without anti-CD40 Ab. Ab, antibodies; AC, apoptotic cells; BMDM, bone marrow derived macrophages; IL, interleukin; KI, knock-in; Mϕ, macrophage; Mϕ-AC, bone marrow derived macrophages without AC treatment; Mϕ+AC, bone marrow derived macrophages with AC treatment; MHC-II, major histocompatibility complex-II; *NCF1*, neutrophil cytosolic factor 1; No Mϕ, naïve CD4$^+$ T cells or splenocytes without co-culture with macrophages; Tfh, T follicular helper cells; Tfr, T follicular regulatory cells; WT, wild type.

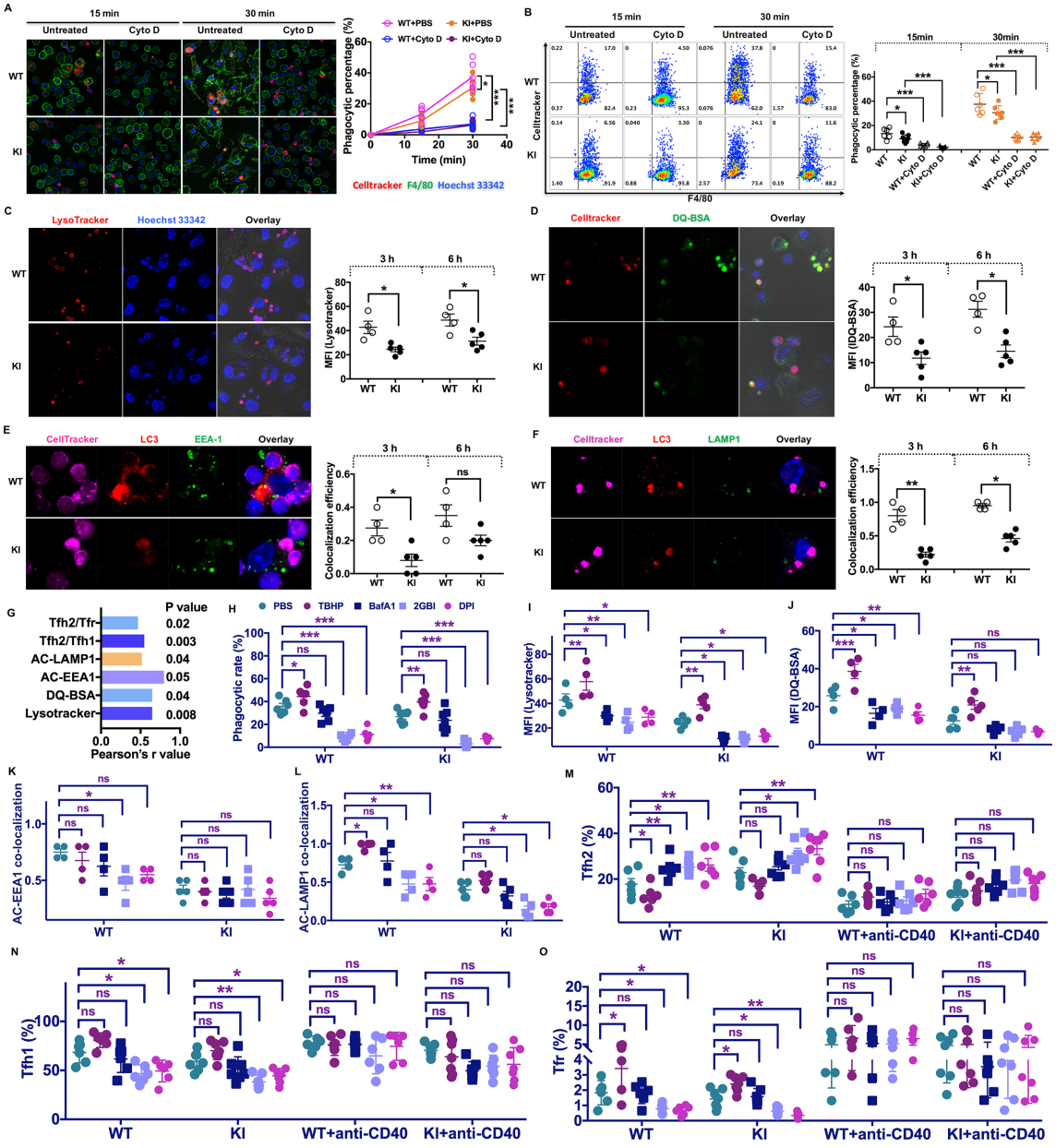

Figure 4 Efferocytosis required intact activity of NOX2 and Hv1 and defective efferocytosis processes led to increased CD40 levels on BMDM from *Ncf1*-H90 KI mice. (A–B) Defective apoptotic cell uptake by BMDM from 10-week-old female *Ncf1*-H90 KI mice assessed by confocal microscope (A) and flow cytometry (B), BMDM: AC=1:5. Cyto D, 10 µg/mL. Original magnification, ×400. Red: Celltracker; Green: F4/80; Blue: Hoechst 33342. (C) Reduced acidification of efferosome in AC-treated BMDM (3 hours) from female H90 KI mice. Original magnification, ×600. Zoom in, 1.0. (D) Defected proteolytic degradation of AC labelled with self-quenched immune complexes (DQ-BSA conjugate with an anti-bovine serum albumin antibody) in BMDM from H90 KI mice. Original magnification, ×600. Zoom in, 2.0. (E–F) Phagosomes containing apoptotic cell fragments failed to mature in BMDM from *Ncf1* KI mice after co-culturing for 3 hours. Celltracker-labelled apoptotic thymocytes were incubated with BMDM for 3 hours (left) or 6 hours. The colocalization of AC and EEA-1 (E) or LAMP1 (F) plus late endosomes were visualised by confocal microscopy. Original magnification, ×600. For E, zoom in, 3.0; For F, zoom in, 5.0. (G) Correlation of phagocytic rate of WT and KI BMDM with lysotracker MFI, DQ-BSA MFI, AC-EEA1 and AC-LAMP1 colocalization rate and the frequencies of Tfh2/Tfr and Tfh2/Tfh1 differentiated from naïve $CD4^+$ T cells co-cultured with AC treated BMDM. (H–J) Phagocytic rate of AC (H), acidification of efferosome (I) and proteolytic degradation (J) in AC-treated BMDM was inhibited by BafA1, 2GBI and DPI, but increased by TBHP. (K, L) The colocalization of AC and LAMP1 (L) plus late endosomes in AC-treated BMDM was inhibited by BafA1, 2GBI and DPI, increased by TBHP, but not EEA-1 (K). (M–O) Differentiation of Tfh2 (M), Tfh1 (N) and Tfr (O) regulated by BMDM was promoted by 2GBI and DPI, suppressed by TBHP, but converted by anti-CD40 Ab. Ab, antibodies; AC, apoptotic cells; BafA1, bafilomycin A1, V-ATPase inhibitor, 10 nM; BMDM, bone marrow derived macrophages; Cyto D, cytochalasin D; DPI, diphenyleneiodonium chloride; DQ-BSA, dequenched-bovine serum albumin; EEA-1, early endosome antigen 1; KI, knock-in; LAMP1, lysosomal-associated membrane protein 1; LC3, microtubule-associated protein 1A/1B-light chain 3; *NCF1*, neutrophil cytosolic factor 1; MFI, mean fluorescence intensity; NOX2, NADPH oxidase derived ROS inhibitor, 25 µM; ROS, reactive oxygen species; ROS inducer, 50µM; TBHP, t-butyl hydroperoxide, Tfh, T follicular helper cells; Tfr, T follicular regulatory cells; 2GBI, 2-guanidino-benzimidazole, Hv1 voltage-gated proton channel inhibitor, 20 µM.

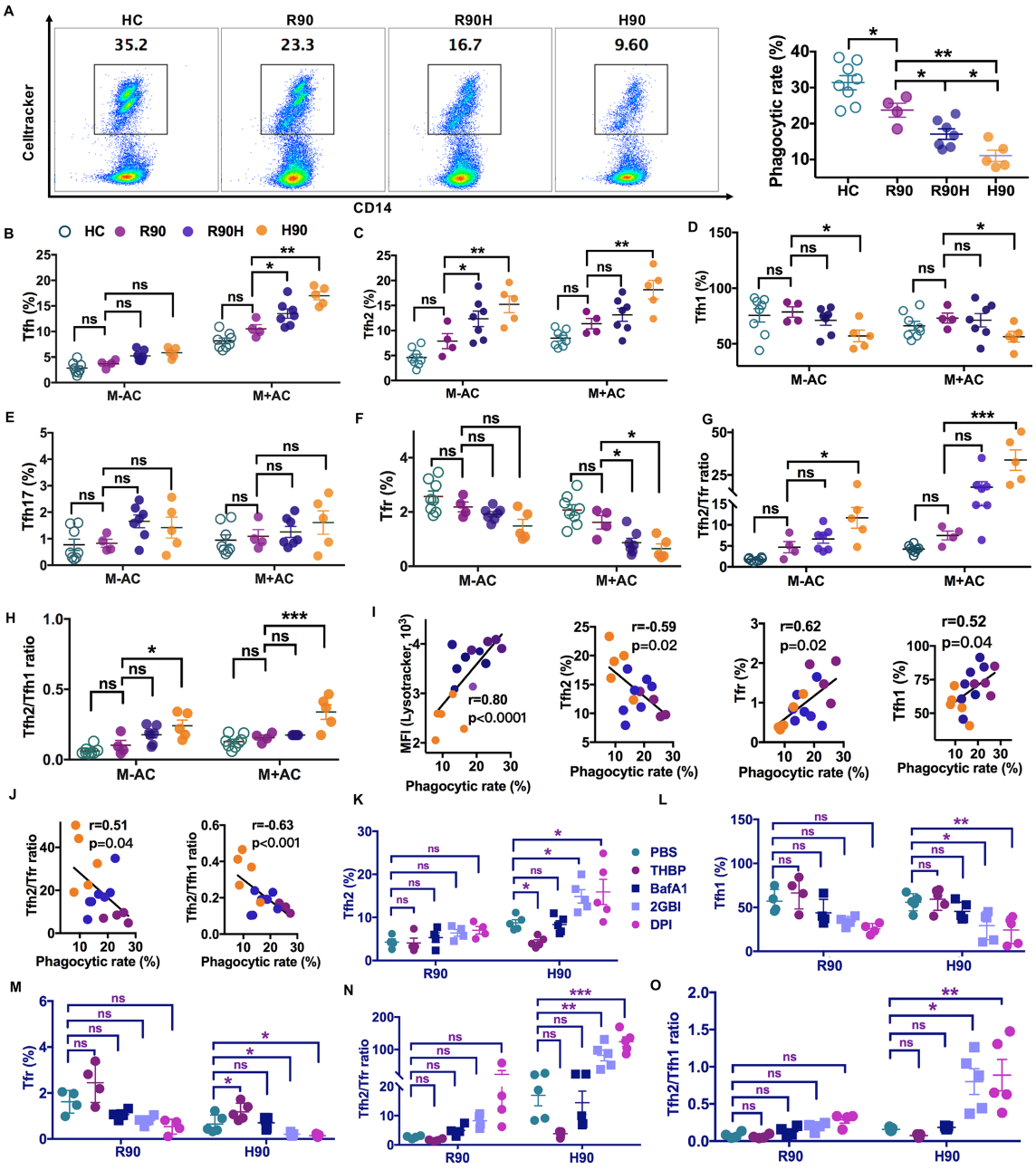

Figure 5 Reduced rate of efferocytosis in monocyte-derived macrophages from patients with SLE exhibited a dose-effect of *NCF1*-H90 variant, correlated negatively with increased Tfh2 response, Tfh2/Tfr and Tfh2/Tfh1 ratios that required intact NOX2 and Hv1 activity. (A) Reduced phagocytic rate (A) in AC-treated macrophages (3 hours) from patients with lupus with H90 genotype. R90, patients with SLE with R90 genotype; H90, patients with SLE with H90 genotype; R90H, patients with SLE with heterozygous genotype. (B–H) Summary graph of Tfh (B), Tfh2 (C), Tfh1 (D), Tfh17 (E), Tfr (f), the ratios of Tfh2/Tfr (G) and Tfr2/Tfh1 (H) differentiated from naïve CD4$^+$ T cells co-cultured with AC treated macrophages from patients with SLE. (I–J) Correlation of phagocytic rate of macrophages from patients with SLE with lysotracker MFI (I), the frequencies of Tfh2 (I), Tfr (I), Tfh1 (I), Tfh2/Tfr (J) and Tfh2/Tfh1 (J) differentiated from naïve CD4$^+$ T cells co-cultured with AC treated macrophages. (K–O) Differentiation of Tfh2 (K), Tfh1 (L), Tfr (M), Tfh2/Tfr (N) and Tfh2/Tfh1 (O) from naïve CD4$^+$ T cells co-cultured with AC treated macrophages from R90 and H90 patients with SLE was promoted by 2GBI and DPI, suppressed by TBHP. AC, apoptotic cells; BafA1, bafilomycin A1, V-ATPase inhibitor, 10nM; DPI, diphenyleneiodonium chloride, HC, healthy control; Hv1, voltage-gated proton channel inhibitor, 20 µM; M+AC, macrophages with apoptotic cell treatment; M-AC, macrophages without apoptotic cell treatment; macrophage: AC=1:5; MFI, meanfluorescenceintensity; NADPH oxidase 2 derived ROS inhibitor, 25µM; *NCF1*, neutrophil cytosolic factor 1; NOX2, NADPH oxidase 2 complex; PBMCs, peripheral blood mononuclear cells; ROS, reactive oxygen species; SLE, systemic lupus erythematosus; TBHP, t-butyl hydroperoxide, ROS inducer, 50 µM.

figure S7E,G) correlated with the phagocytic capacity (figure 5I, for Tfh, r=0.62, p=0.03), but decreased Tfh1 (figure 5D,I and online supplemental figure S7E,G) and Tfr frequency (figure 5F,I and online supplemental figure S7E,H), resulting in increased ratios of Tfh2/Tfr (figure 5G,J) and Tfh2/Tfh1 ratios (figure 5H,J). Other T cell subsets including Th1, Th2, Th17, Treg and Tfh 17 (figure 5E and online supplemental figure S7E,G) were unaffected. The phagocytic rate (online supplemental figure S7J,K), acidification status (online supplemental figure S7I), the polarisation of Tfh2, Tfh1 and Tfr in

macrophage co-cultures were inhibited by 2GBI and DPI, but increased by TBHP (figure 5K–O), confirming the role of H90 variant in decreased production of NOX2-ROS and Hv1-dependent acidification in Tfh2/Tfr and Tfh2/Tfh1 polarisation during efferocytosis.

To test whether H90 variant exacerbates Tfh2 responses in circulating SLE peripheral blood mononuclear cells (PBMCs), we compared immune cell subsets and disease activities among patients with SLE. Compared with that of R90 patients with SLE, H90 patients with SLE demonstrated increased frequencies of circulating Tfh (figure 6A), Tfh2 cells (figure 6B,C) and plasmablast ($CD19^{+}CD27^{high}$; figure 6D), but decreased proportions of Tfh1 (figure 6B) and Tfr (figure 6E). Patients with SLE carrying two copies of H90 had significantly higher ratios of circulating Tfh2/Tfr (figure 6F) and Tfh2/Tfh1 (figure 6G) in circulating PBMCs than patients with SLE of other genotypes. Further, kidney sections from H90 patients with lupus nephritis exhibited aggravated glomerulonephritis (figure 6H, upper and middle) and increased deposition of IgG and C3 (figure 6H, lower). Additionally, patients with SLE carrying one or two copies of H90 variant had increased risk for developing renal damage than those carrying 90R allele in both Asian (Chinese and Korean) and African American populations (figure 6I and online supplemental figure S7L-N). Together, our data indicate the H90 allele is the causal variant of *NCF1* and predisposes to development of lupus-like kidney disease through enhanced Tfh2 responses induced by defective macrophage efferocytosis (figure 7).

DISCUSSION

Here, we showed that the hypofunctional allele encoding H90 *Ncf1* is a SLE causal variant by using a new H90.B6 KI mouse model for both in vivo and in vitro studies and by confirming mechanistic findings from mice in patients with SLE. Compared with WT B6 littermates, 5-week-old H90.B6 KI mice exhibited splenomegaly containing elevated numbers of follicular and GC B cells, plasma cells, Tfh2 cells and elevated IFN-I levels. While this single, common risk allele was insufficient for spontaneous lupus development, female but not male KI mice developed enhanced lupus-like autoantibodies and kidney disease in a pristane-induced lupus model, presumably via TLR7/MyD88 signalling and elevated IFN-I production.[23] We observed positive correlations of IFN-I scores of spleen cells with kidney disease features (online supplemental figure S2N) and with increased splenic ratios of Tfh2/Tfh1 and Tfh2/Tfr (figure 2L). The aggravated lupus-like disease in H90 KI female mice, compared with their WT female littermates, was attributed to, at least partially, decreased expression of IFN-negative regulator genes, *Usp18* and *Socs1* (online supplemental figure S2H).[24] While association between H90 *NCF1* genotype and elevated expression of IFN-I-regulated genes was reported in whole blood from patients with rheumatoid arthritis,[4] this genetic effect on expanded Tfh2 subset and decreased Tfh1 and Tfr subsets was novel as shown in splenocytes from 5-week-old naïve and 36-week-old pristane-treated mice, and in PBMCs from patients with SLE. The genetic effect on exacerbated lupus kidney disease in pristane-injected KI mice was confirmed in patients with SLE, as shown by elevated cellular infiltration in glomeruli and deposition of IgG and complement C3 in kidney biopsies of lupus nephritis patients carrying two copies of H90 variant, and by association between the H90 variant carriage and the presence of renal damage (renal SDI>0, defined by levels of glomerular filtration rate and proteinuria, and end stage kidney disease) in East Asian and African American patients with SLE.

The p.R90H *NCF1* variant is one of the strongest common risk variant for SLE with an OR >3 in Asian and European populations.[3 4] We expanded the previous association and showed a causal relationship between H90 variant and reduced oxidative burst in PMA-stimulated macrophages and spleen cells from KI mice (figure 1A; Online supplemental figure 6A). Another NOX2 regulatory subunit encoding gene, *NCF2*, has hypofunctional missense variants predisposing to SLE,[7 25] and haploinsufficiency of *Ncf2*-accelerated murine lupus.[26] Comparing to previous null mutations or knockout mouse models, our study directly tested functional consequences of a human p.R90H *NCF1* variant, in the absence of copy number variations, on a normal mouse background, which were subsequently confirmed in patients with SLE. This polymorphism associates with impaired formation of neutrophil extracellular traps, high serum IFN-I activity and antiphospholipid syndrome (APS) in patients with SLE,[27] we now extended its association with kidney damage measured by renal items of the SDI. Renal manifestations of APS could contribute to kidney injury.

How does this hypofunctional NCF1, a subunit of phagocyte NOX2, affect adaptive autoimmune responses? A well-established phagocyte defect in SLE is defective disposal of ACs by macrophages.[28] Perturbed clearance disrupts homoeostasis and disease pathologies arise due to loss or altered function of molecules involved in different phases of efferocytosis.[8] Defects in the AC clearance by macrophages have provided mechanistic insights in studying human lupus.[29–31] Null alleles of subunits of NOX2 complex cause chronic granulomatous disease (CGD) characterised by infections and autoimmunity, and mouse models of CGD exhibit delayed deposal of AC by BMDM and blood monocytes and enhanced cross-presentation of AC-associated antigens accompanied by production of inflammatory mediators.[21 32 33] Corroborating with these previous reports, our co-cultures of AC and BMDM from naïve H90 KI mice showed delayed AC engulfment via LC3-associated phagocytosis, reduced phagosome acidification and proteolysis of ingested apoptotic cargo compared with AC co-cultured with BMDM from WT littermates. We showed that efficient disposal of AC required functional Ncf1 and Hv1, the NOX2-ROS stimulator TBHP treatment of KI BMDM increased AC phagocytic rate, acidification and promoted proteolysis to levels observed in untreated WT BMDM. The treatment with either ROS or Hv1 inhibitors significantly suppressed all phases of efferocytosis we measured (figure 4H–L). Our data were as anticipated, given that activities of NOX2 and Hv1 are tightly coupled in regulating ROS generation, which in turns regulates acidification and proteolysis in efferosomes.[21 34] We also confirmed that phagosomal acidification during efferocytosis of AC required activity of V-ATPase that its inhibitor BafA1 significantly diminished acidification in both WT and KI BMDM.[21]

To the best of our knowledge, we showed for the first time a mechanistic connection between defective efferocytosis due to the hypoactive H90 variant and imbalanced Tfh2, Tfh1 and Tfr subsets caused by elevated surface expression of CD40 on BMDM from naïve H90 KI mice pretreated with AC (figures 3I and 7). This difference could interact with WT naïve $CD4^{+}$ T cells to induce Tfh2 expansion through proliferation accompanied with reduced Tfh1 and Tfr subsets, resulting in elevated ratios of Th2/Tfh1 and Tfh2/Tfr (figures 3 and 7). This defective efferocytosis and reduced phagosome acidification in monocyte-derived macrophages from H90 patients with SLE also promoted elevated ratios of Tfh2/Tfh1 and Tfh2/Tfr similarly (figures 5J and 7). The phagocytic rate of AC by monocyte-derived macrophages positively correlated with the extent of phagosome

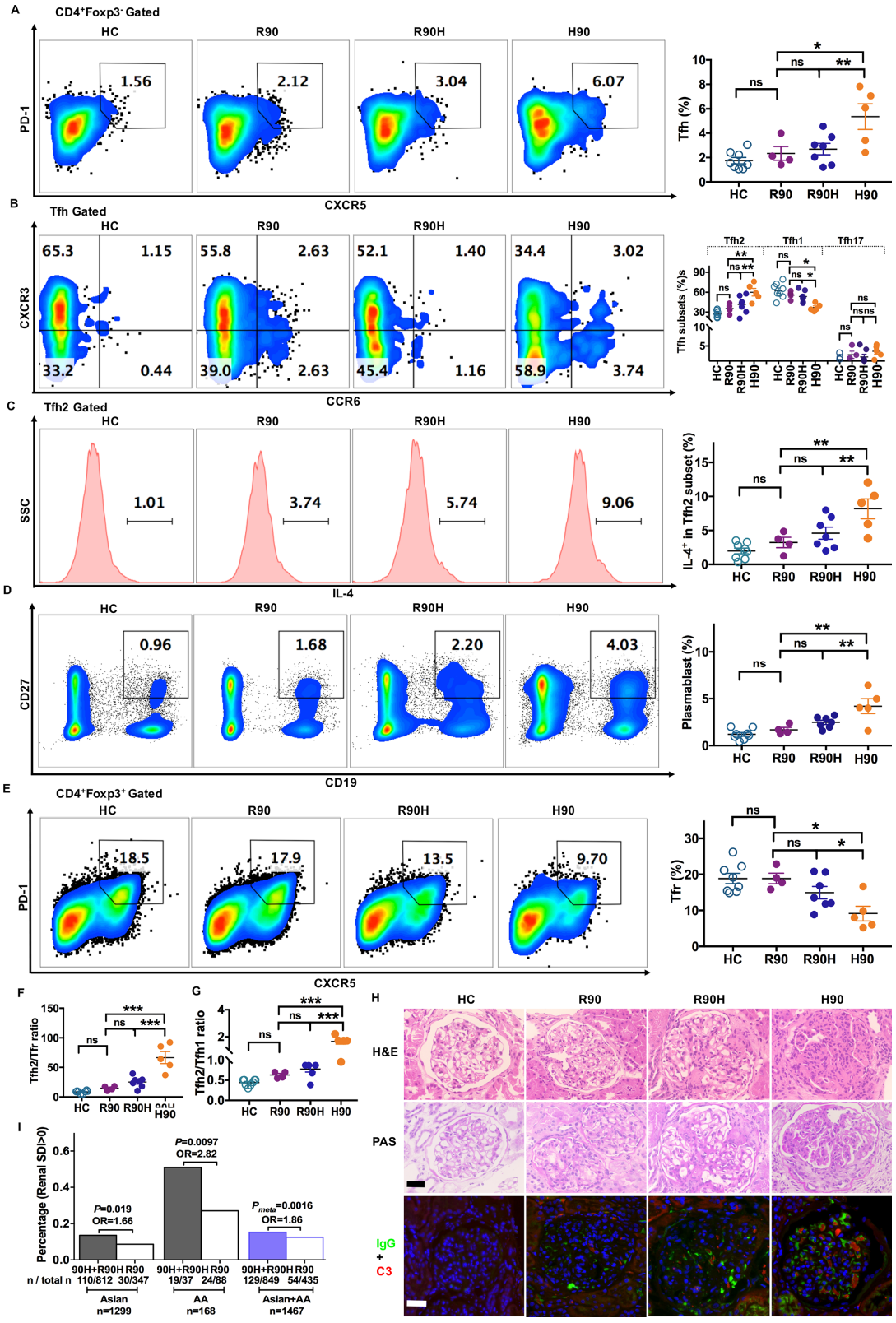

Figure 6 Association of *NCF1*-H90 variant with elevated levels of plasmablasts, Tfh2, deposition of immune complexes and renal damage accrual in patients with SLE. (A–E) Flow-cytometric plot (left) and summary graph (right) of circulating Tfh (A), Tfh2 (B), Tfh1 (B), Tfh17 (B), IL-4$^+$ levels in Tfh2 (C), plasma blasts (D) and Tfr (E) in circulating PBMCs from patients with SLE and HC. (F–G) The ratio of circulating Tfh2/Tfr (F) and Tfh2/Tfh1 (G) in circulating PBMCs from patients with SLE and HC. (H) Representative kidney sections in patients with SLE and HC, stained with H&E (upper), PAS (middle) and IgG and C3 (lower). Bar: 50 µm, magnification: ×400. (I) Significant association of the H90 risk genotypes with renal SDI score in Asian (Korean, n=867 and Chinese, n=432) and African American patients with SLE (n=168) through meta-analysis. AC, apoptotic cells; HC, healthy control; IL, interleukin; *NCF1*, neutrophil cytosolic factor 1; PBMCs, peripheral blood mononuclear cells; PAS, periodic Acid-Schiff staining; SLE, systemic lupus erythematosus; Tfh, T follicular helper cells; Tfr, T follicular regulatory cells; R90, patients with SLE with R90 genotype; H90, patients with SLE with H90 genotype; R90H, patients with SLE with heterozygous genotype; SDI, SLE damage Index.

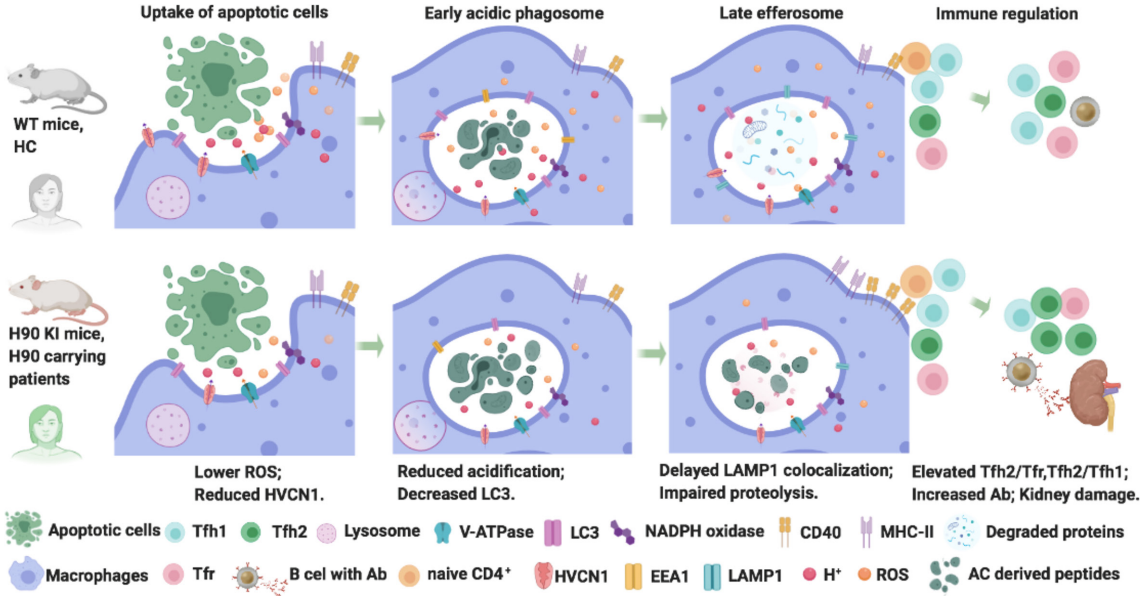

Figure 7 Proposed model for H90 variant-mediated phagocytosis of AC by macrophages. In WT mice or HC (top panel), during the encounter of a phagocyte with an AC, once internalised, the phagosome is acidified by V-ATPase, proton channels like Hv1 proton channel (HVCN1) and NOX2 complex and the membrane of the phagosome begins to fuse with lysosomes. AC proteins within phagosomes will be ingested by accelerating acidification, maturation and proteolysis of phagosomes. In H90 KI mice or patients with SLE carrying H90 variant (lower panel), H90 variant induced lower NOX2-ROS and Hv1-dependent acidification led to defective efferocytosis of macrophages from female H90 KI mice, as shown by reduced acidification, impaired maturation and proteolysis of phagosome, which led to slower digestion of AC, persistence of AC proteins within phagosomes and enhanced presentation of an AC-associated protein antigen with increased CD40 expression. Created with BioRender.com. AC, apoptotic cells; HC, healthy control; KI, knock-in; NOX2, NADPH oxidase 2 complex; ROS, reactive oxygen species; SLE, systemic lupus erythematosus; V-ATPase, vacuolar-type H^+ ATPase; WT, wild type.

acidification, and either Tfr or Tfh1 frequency; while negatively correlated with Tfh2 frequency (figure 5I). Figure 7 depicts the graphic summary of our mechanistic findings that link defective efferocytosis of AC caused by a hypomorphic H90 variant to Tfh subset imbalance and autoantibody production.

Tfh cells are essential for GC reaction in humoral immunity, which provide B-cell help for proliferation, isotype switching and somatic hypermutation mainly through CD40L–CD40 expression and cytokines.[35] While increased circulating Tfh cells in patients with SLE are established, the underlying Tfh subsets are inconsistently reported. A key role of Tfh subsets in SLE is supported by correlation of disease activity with circulating Tfh2 frequency positively and Tfh1 frequency negatively,[36] by association of altered frequencies of Tfh2 and Tfh1 subsets with the presence of high IgG, IgE and autoantibodies in patients with SLE[36] and by positive correlation between ratios of circulating Tfh/Tfr and disease activity.[37] Consistent with these findings, we observed expanded Tfh2 cell proliferation and decreased numbers of Tfh1 and Tfr cells in spleens from as early as 5-week-old naïve *Ncf1*-H90 KI mice (figure 3E), showing the genetic basis of elevated ratios of Tfh2/Tfh1 and Tfh2/Tfr. These same genetic effects were evident in spleens from pristane-treated female KI mice, exhibiting increased ratios of Tfh2/Tfr and Tfh2/Tfh1 accompanied with elevated numbers of GC B cells, autoreactive B cells and plasma cells, which suggested the contribution of imbalanced Tfh subsets in abnormal GC responses in murine lupus. Similarly, SLE PBMCs from those carrying the homozygous H90 variant genotype, compared with those carrying other genotypes, exhibited decreased Tfh1 frequency, and elevated circulating Tfh and plasmablast (figure 6A,D). While focusing on H90 genetic effects, we observed no evidence for expansion of either circulating Tfh1 or Tfh17 frequency previously reported in patients with SLE.[36 38]

The pattern of reduced frequency of circulating Tfh1 and/or Tfr, but increased Tfh2 has been reported in multiple diseases affected with kidney injury, which associates with kidney injury[39 40] and could be attenuated by immunosuppressive treatment in disease remission.[41] Similarly, we observed the increased Tfh2 responses accompanied with decreased Tfh1 and Tfr compartment in H90 patients with SLE, compared with patients carrying other genotypes, independent of their medication and disease duration (online supplemental figure S7M,N). This imbalanced follicular T cell pattern due to defective efferocytosis drives exuberant GC responses and autoantibody production, which could promote kidney damage over time.[3 27]

Both follicular and extra follicular humoral responses to contribute to lupus development.[42] Important players of extrafollicular responses include age-associated B cells (ABCs, $CD11c^+$T-bet^+ B cells) and T peripheral helper cells (Tph, PD-$1^{hi}CXCR5^-$), development of ABCs require help from Tfh1.[43] We found no difference for $CD19^+CD11C^+$ B cells enriched with ABCs ($p=0.32$) and Tph cells ($p=0.18$, online supplemental figure S4I) between the 5-week-old female WT and KI group, limiting the role of extrafollicular immune responses in our mouse model.

Limitations of this study include the following: In addition to macrophages, other Ncf1 expressing cells, including neutrophils, DC and B cells, that could impact on lupus development were not studied here.[4 27] Defects other than abnormal apoptotic cell clearance might be involved in Ncf1-mediated pathway for lupus development, which awaits future experiments. Other stimuli of phagocytosis, such as immune complex coated beads that are relevant for lupus pathogenesis,[1] were not included in our studies. As the first study of this new mouse model of H90 KI mice, we chose to study efferocytosis because clearance of apoptotic cells has a crucial role in lupus development.[21] The number

of patients with SLE used in the in vitro experiments and kidney biopsy study was small, which increased the probability of biased representation. However, we observed consistent effects of *NCF1*-p.R90H in these two non-overlapping small sampling of patients with SLE, which led us to test and to identify genetic association between the H90 allele with kidney damage in 1467 patients with SLE (figure 6I).

In summary, we developed H90 KI on the normal mouse background that exhibit spontaneously developed elevated IFN-I scores, skewed Tfh subsets accompanied with robust GC responses and plasma cell development at the young age of 5 weeks. Defective efferocytosis by macrophages derived from H90 bearing individuals upregulated circulating Tfh2 expansion together with reduced Tfh1 and Tfr cells and increased plasmablasts, promoting antibody production. Patients with SLE carrying the H90 variant had elevated circulating Tfh2 correlating with plasmablast numbers and disease activity, exacerbated glomerulonephritis and increased risk of kidney damage. Our data highlight the common p.R90H *NCF1* variant as a causal risk variant with robust impact on SLE pathogenesis, and provide the rationale to target its underpinning mechanisms for future treatment development.

Author affiliations
[1]Division of Rheumatology and Immunology, Medical University of South Carolina, Charleston, South Carolina, USA
[2]Department of Rheumatology and Immunology, The Affiliated Drum Tower Hospital of Nanjing University Medical School, Nanjing, Jiangsu, China
[3]Department of Surgery, University of Miami School of Medicine, Miami, Florida, USA
[4]Department of Immunology and Internal Medicine, The University of Texas Southwestern Medical Center, Dallas, Texas, USA
[5]Department of Rheumatology, The First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu, China
[6]Department of Rheumatology, Hanyang University Hospital for Rheumatic Diseases and Hanyang University Institute for Rheumatology, Seoul, The Republic of Korea
[7]Ralph H Johnson VA Medical Center, Medical University of South Carolina, Charleston, South Carolina, USA

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Contributors BPT and GG designed the study; LS provided the kidney biopsy section of patients with lupus. XF, MZ, WT and LX provided the DNA samples of patients with lupus. DLK, GG and S-CB provided renal damage scoring and DNA samples of patients with lupus; IM, LG, JZ, YD and XX conducted experiments and association studies of patients with lupus; LG and JZ analysed data and performed statistical analyses; PR performed renal score assessment of mice; Q-ZL conducted the autoantigen array; BPT and LG drafted the manuscript; all authors edited and reviewed the manuscript.

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Competing interests None declared.

Patient consent for publication Not required.

Ethics approval Humans participants were recruited with informed consents approved by the Ethics Committee of the Affiliated Drum Tower Hospital of Nanjing University Medical School (2016-027), the First Affiliated Hospital of Nanjing Medical University (2020-SR-044), Hanyang University Hospital for Rheumatic Diseases (HYUH 2001-06-001-024) and Medical University of South Carolina Institution Review Boards (HR 7148), respectively. Animal subjects were approved by the Medical University of South Carolina Institutional Animal Care (IACUC-2019-00711 and IACUC-2018-00619).

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ORCID iDs
Linyu Geng http://orcid.org/0000-0002-8983-2326
Lingxiao Xu http://orcid.org/0000-0003-3418-0727
Wenfeng Tan http://orcid.org/0000-0002-5160-2023
Sang-Cheol Bae http://orcid.org/0000-0003-4658-1093
Lingyun Sun http://orcid.org/0000-0002-8563-2036

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TRANSLATIONAL SCIENCE

Antifibrotic factor KLF4 is repressed by the miR-10/TFAP2A/TBX5 axis in dermal fibroblasts: insights from twins discordant for systemic sclerosis

Maya Malaab,[1] Ludivine Renaud ,[1] Naoko Takamura,[1] Kip D Zimmerman,[2] Willian A da Silveira,[3] Paula S Ramos ,[1,4] Sandra Haddad,[5] Marc Peters-Golden,[6] Loka R Penke,[6] Bethany Wolf,[4] Gary Hardiman,[3] Carl D Langefeld,[2] Thomas A Medsger,[7] Carol A Feghali-Bostwick [1]

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For numbered affiliations see end of article.

Correspondence to
Dr Carol A Feghali-Bostwick, Medicine, Medical University of South Carolina, Charleston, South Carolina, USA; feghalib@musc.edu

MM, LR and NT contributed equally.

MM, LR and NT are joint first authors.

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ABSTRACT

Objectives Systemic sclerosis (SSc) is a complex disease of unknown aetiology in which inflammation and fibrosis lead to multiple organ damage. There is currently no effective therapy that can halt the progression of fibrosis or reverse it, thus studies that provide novel insights into disease pathogenesis and identify novel potential therapeutic targets are critically needed.

Methods We used global gene expression and genome-wide DNA methylation analyses of dermal fibroblasts (dFBs) from a unique cohort of twins discordant for SSc to identify molecular features of this pathology. We validated the findings using in vitro, ex vivo and in vivo models.

Results Our results revealed distinct differentially expressed and methylated genes, including several transcription factors involved in stem cell differentiation and developmental programmes (*KLF4*, *TBX5*, *TFAP2A* and *homeobox* genes) and the microRNAs *miR-10a* and *miR-10b* which target several of these deregulated genes. We show that *KLF4* expression is reduced in SSc dFBs and its expression is repressed by *TBX5* and *TFAP2A*. We also show that KLF4 is antifibrotic, and its conditional knockout in fibroblasts promotes a fibrotic phenotype.

Conclusions Our data support a role for epigenetic dysregulation in mediating SSc susceptibility in dFBs, illustrating the intricate interplay between CpG methylation, miRNAs and transcription factors in SSc pathogenesis, and highlighting the potential for future use of epigenetic modifiers as therapies.

Key messages

What is already known about this subject?

- Systemic sclerosis (SSc) is a complex connective tissue disease of unknown aetiology.
- Disease concordance in twins with SSc is low, implicating a potential role for epigenetics in disease manifestation.

What does this study add?

- Using global DNA methylation and gene expression, we identified novel programmes deregulated in dermal fibroblasts of a unique cohort of twins discordant for SSc.
- Using gain and loss of function studies, we confirmed the functional impact of differentially expressed and methylated transcription factors and miRNAs in mediating dermal fibrosis in SSc.

How might this impact on clinical practice or future developments?

- Since no effective therapy that can halt the progression of fibrosis exists, novel insights into disease pathogenesis and identification of novel potential therapeutic targets are critically needed.

INTRODUCTION

Systemic sclerosis (SSc) is a complex connective tissue disease whose hallmarks include autoimmunity, inflammation, fibrosis and vasculopathy. SSc predominantly affects women and is the connective tissue disease with the worst survival.[1] Diffuse cutaneous SSc (dcSSc) is characterised by rapid development of skin, lung and other organ fibrosis within 3–5 years, after which skin fibrosis regresses but damages to internal organs persist. Limited cutaneous SSc (lcSSc) shows gradual skin progression with delayed internal organ involvement. Most research studies and clinical trials have focused on dcSSc,[2] although dermal fibrosis is a hallmark of both dcSSc and lcSSc. The aetiology and molecular mechanisms underlying SSc remain elusive and no effective therapeutic treatment exists.

In monozygotic (MZ) twins, the rate of disease concordance is low (4.2%),[3] suggesting an important role for epigenetic and environmental factors in SSc susceptibility in addition to a genetic predisposition.[4] Environmental factors can influence a trait through epigenetic regulation, and epigenetic marks can impact gene expression, thus governing cell function and response to environmental stimuli.[5] Additionally, early mutations that occurred before the primordial germ cell specification have recently been shown to contribute to phenotypic discordance observed in MZ twins,[6] a contribution previously underestimated. Due to replication errors, mutations that are specific to one twin happened in 15% of MZ twin pairs, revealing the importance of early cell lineages and mutations that are dependent on DNA methylation. Epigenetic mechanisms play a role in the pathogenesis of

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autoimmune diseases, and epigenome-wide association studies revealed the existence of differentially methylated (DM) regions associated with systemic lupus erythematosus[7] and psoriasis,[8] two other autoimmune rheumatic diseases. Inhibition of DNA methyltransferases has shown promising antifibrotic properties in SSc fibroblasts.[9–11] Epigenetic changes have been reported in SSc fibroblasts.[12 13] Epigenetic changes in SSc are emerging as important mediators of disease processes including fibrosis and angiogenesis.[11] As a result, epigenetic modifying drugs may provide viable therapies in SSc.[9]

To our knowledge, our study is the first to combine RNA sequencing (RNAseq) and genome-wide DNA methylation analyses in dermal fibroblasts (dFBs), the effector cells in fibrosis,[14] of twins discordant for SSc. This unique cohort is the ideal design to assess the role of epigenetic variations in disease aetiology.[15]

Our study identified several transcription factors (TFs) involved in stem cell differentiation that were DM and differentially expressed (DE) in SSc, including homeobox (*HOX*) genes, *TBX5* and *TFAP2A*, suggesting that SSc is mediated by an aberrant resumption of developmental pathways. MicroRNAs (miRNAs) of the *miR-10* family encoded within *HOX* gene clusters have a direct regulatory role on these TFs, and are also DE, highlighting the deregulation of complex mRNA–miRNA regulatory networks in SSc. We also identified *KLF4* as a major regulator of fibrosis, cell differentiation and extracellular matrix (ECM) accumulation, and determined that *TBX5* and *TFAP2A* regulate *KLF4*, which in turn regulates *WNT* and *HOX* genes. Mice with conditional fibroblast-targeted loss of KLF4 showed increased dermal hydroxyproline levels, and dFBs from these mice had increased expression levels of fibrotic genes. Overexpression of *KLF4* in dFBs and human skin tissues in organ culture prevented the TGFβ-induced fibrotic response. Our data suggest that epigenetic events shape the fibrosis in dFBs that leads to SSc.

METHODS

See online supplemental file 1 for details of the methods.

RESULTS

Gene expression profiling of twins discordant for SSc

The DE analysis 'lcSSc twins versus healthy twins' (n=8) returned 897 DE genes in dFBs of lcSSc twins (online supplemental figure 1A and table 1), 511 upregulated (q<0.1, log2FC>0.6) and 386 downregulated (q<0.1, log2FC<−0.6) genes. The heatmap showed two clear clusters for healthy and lcSSc samples (online supplemental figure 1B). To validate these results, another DE analysis was computed for 'lcSSc twins versus unrelated controls' (n=8) and revealed 2164 DE genes in lcSSc twins (online supplemental figure 2A,B and table 2) out of which 308 DE genes are in common with DE genes from the 'lcSSc twins versus healthy twins' comparison (online supplemental figure 2C and table 3). One striking feature of the lcSSc twins' dataset is that 12 homeobox genes are present, 5 of which are significantly upregulated (*HOXB3*, *HOXB7*, *HOXB8*, *HOXC10*, *PITX1*) and 7 are significantly downregulated (*HOXB13*, *HOXD3*, *HOXD9*, *HOXD10*, *HOXD11*, *HOXD13*, *HOXD-AS2*) (figure 1A; online supplemental figure 2D). Of note, *WNT5A* was significantly upregulated in dFBs of lcSSc twins (online supplemental figure 2E).

The systems level analysis showed enrichment of pathways and ontologies pertaining to ECM components, organ morphogenesis, regulatory region involved in DNA binding and integral component of the plasma membrane (table 1; online supplemental table 4). Note that the *HOX* genes are hit in the query list for 'animal organ morphogenesis' and 'transcription regulatory region sequence-specific DNA binding' along with several T-box genes (*TBX2*, *TBX3*, *TBX15*) and TFs that bind the consensus sequence 5'-GCCNNNGGC-3' (*TFAP2A*, *TFAP2B*) (online supplemental figure 2E). We used qPCR to validate several genes of interest (figure 1B). The expression levels of *TFAP2A* were increased in lcSSc samples compared with unrelated controls while *HOXD10* and *HOXD11* levels were reduced, in agreement with the RNAseq data. A significant decrease in expression levels of *KLF4* was observed, consistent with DE analysis 'lcSSc twins versus unrelated controls' (online supplemental table 2). We also detected an increase in *HOXA13* levels and a decrease in *HOXC5* levels in lcSSc samples (figure 1B).

The comparison of 'dcSSc twins versus healthy twins' (n=7) returned 76 DE genes (online supplemental figure 3A and table 5). The heatmap revealed that the transcriptomic signatures of dcSSc and healthy samples were not markedly different, therefore no clear clustering of the samples was observed; dcSSc and healthy samples appear to be randomly intertwined (online supplemental figure 3B), suggesting that the transcriptomic signature of dFBs in patients with dcSSc is not as deep and well-defined as in their healthy twins. Three HOX genes are present in this dataset (figure 1C: *HOXB-AS3* is downregulated and *HOXD10*/*HOXD11* are upregulated in dFBs of dcSSc twins) as well as the T-box gene *TBX5* (upregulated; online supplemental table 5). The systems level analysis did not identify any pathways, likely due to a limited number of DE genes (table 2; online supplemental table 6).

By comparing the DE genes in lcSSc and dcSSc twins, we identified nine genes in the intersect (figure 1D): *CDK18*, *IL-6*, *KLF5*, *LIF*, *MST1R* and *RGCC* were commonly upregulated in both disease subtypes while *HOXD10*, *HOXD11* and *PRDM6* were upregulated in dcSSc twins but downregulated in lcSSc twins.

Methylation profiling of twins discordant for SSc

Methylation profiling of 10 twin pairs discordant for SSc identified 174 DM CpG sites ($p<10^{-04}$) between healthy and SSc twins, 67% of which mapped to gene bodies (116 genes), while the remainder mapped to intergenic regions, and these 116 DM CpG sites in dFBs mapped to 83 distinct genes (online supplemental table 7). A total of 55 CpG sites showed a large reduction (W Beta <−0.20) and 16 showed a large increase (W Beta >0.20) in DNA methylation status in the SSc twins. At the gene level, 35 DM genes were identified ($p<10^{-04}$) in dFBs of SSc twins, out of which 13 had increased methylation and 22 had decreased methylation (figure 1E).

Next, we sought to identify genes that are both DM and DE (figure 1F). In lcSSc twins, *HOXB3* and *TFAP2A* showed an increase in both gene expression and DNA methylation while *HOXB8* and *HOXC10* had an increase in gene expression but a reduction in DNA methylation status as compared with healthy twins (online supplemental figure 4A). In dcSSc twins, *TBX5* and *UNC5B* were upregulated and less methylated compared with their healthy twins (online supplemental figure 4B).

miR-10a and *miR-10b* mediate *HOXD10*, *TFAP2A*, *TBX5* and *COL1A1* dysregulation

The *HOX* gene clusters have coevolved with and contain many miRNAs,[16] including *miR-10a* and *miR-10b* (figure 2A). *miR-10a* is located upstream of *HOXB4* on chromosome 17, while *miR-10b* is located upstream of *HOXD4* on chromosome 2.

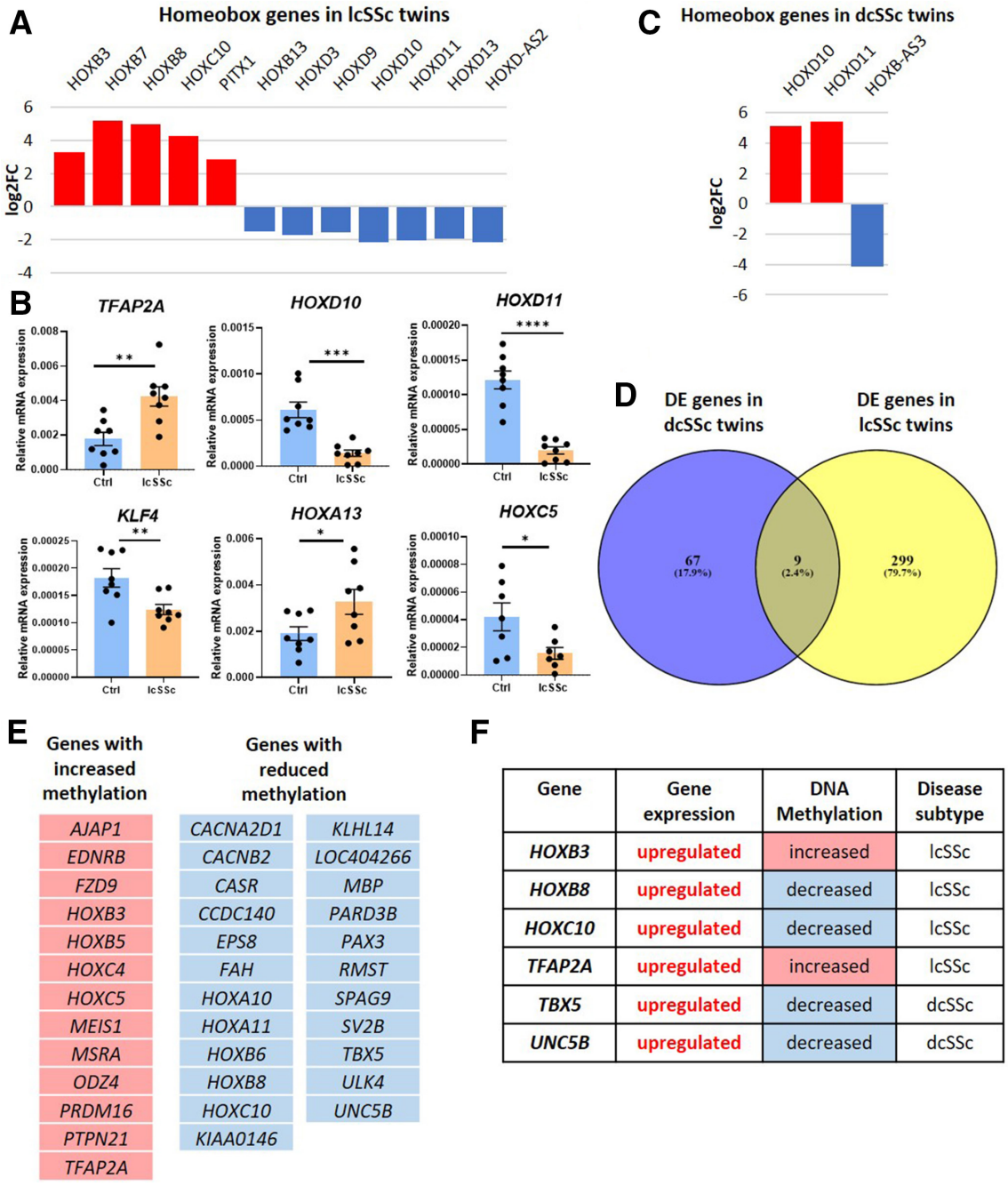

Figure 1 RNAseq and DNA methylation results. (A) RNAseq revealed that several homeobox genes are DE in dFBs of lcSSc twins compared with healthy twins and validated with unrelated controls. Significance criteria in RNAseq analysis: q-value <0.1, log2FC>0.6 for upregulation, log2FC<−0.6 for downregulation. (B) Validation of several genes of interest by qPCR in lcSSc twins (lcSSc) and unrelated controls (Ctrl). (C) Homeobox genes DE in dcSSc twins compared with healthy twins. Significance criteria in RNAseq analysis: q-value <0.1, log2FC>0.6 for upregulation, log2FC<−0.6 for downregulation. (D) Venn diagram for the comparison of DE genes in dcSSc and lcSSc twins. (E) Genes with increased and reduced methylation status identified in the DNA methylation analysis of dFBs. Significance criteria in DNA methylation analysis: p value $<10^{-04}$, W Beta >0.20 for increased methylation, W Beta <−0.20 for reduced methylation. (F) Genes that are simultaneously DE and differentially methylated in dFBs of lcSSc (validated dataset) and dcSSc twins. dcSSc, diffuse cutaneous SSc; DE, differentially expressed; dFBs, dermal fibroblasts; lcSSc, limited cutaneous SSc; SSc, systemic sclerosis.

Both *miR-10a-5p* and *miR-10b-5p* expression levels were significantly reduced in lcSSc dFBs compared with healthy twins (figure 2B–C). In the dcSSc subtype, both miRNAs were also decreased, although not significantly for *miR-10b-5p*. Since *miR-10a-5p* and *miR-10b-5p* are downregulated, we expect a negative correlation with their target genes. Accordingly, *TFAP2A* and *TBX5* levels were upregulated in lcSSc and dcSSc dFBs, respectively (figure 1B; online supplemental table 5). This negative correlation was also observed with HOXD10 in dcSSc dFBs (figure 1C) but not in the lcSSc dFBs in which HOXD10 is downregulated (figure 1A,B), suggesting that other regulatory mechanisms govern HOXD10 in lcSSc dFBs.

These two miRNAs share many target genes because they have seed sequences that only differ by one base. By comparing the list of predicted gene targets obtained from TargetScan for *miR-10a-5p* and *miR-10b-5p* to the DE genes lists in lcSSc (validated)

Table 1 Functional enrichment in lcSSc twins

Enrichment	Hit in query list
PW: ensemble of genes encoding ECM and ECM-associated proteins (7.04E−11)	ADAMTSL1, ADAMTS5, ANGPTL1, BMP6, CHRDL1, CILP2, CLEC14A, COL7A1, COL9A3, COL14A1, COL23A1, CRISPLD2, CTSB, CXCL1, CXCL6, EDA, ELFN2, EREG, F7, FBLN7, FBN2, FGF5, FGL2, FRAS1, HAPLN3, IL-6, IL11, KAL1, LIF, LOXL3, LOXL4, MMP10, OGN, PCOLCE2, PCSK5, PDGFB, PRG4, RSPO1, SEMA3C, SEMA3E, SRPX2, TGFB3, THBS4, USH2A, WISP1, WNT5A
BP: animal organ morphogenesis (4.14E−19)	ACTC1, ADAMTS5, ALDH1A2, ALPL, AR, BMP6, CELSR1, CELSR2, COL7A1, COL14A1, DIO3, DLL4, EDA, EMX2, EPHB2, EREG, ETV4, EYA4, FBN2, FOXF1, FRAS1, HEYL, HHEX, HAND2, HMGA2, HOTTIP, HOXB3, HOXB7, HOXB8, HOXB13, HOXD3, HOXD9, HOXD10, HOXD11, HOXD13, ID3, IL-6, LIF, MAN2A1, NFIB, NKX3-2, NTRK2, PDGFB, PLAG1, RPS6KA1, SEMA3C, SHANK1, SHROOM2, SOBP, SOX8, TACSTD2, TBX2, TBX3, TBX15, TFAP2A, TFAP2B, TGFB3, TMEM100,TSHR, TSPAN12, USH2A, WNT5A, ZFPM2
MF: transcription regulatory region sequence-specific DNA binding (5.82E−10)	AR, ATF5, EBF1, EGR1, EGR3, EMX2, EN1, ETV4, FOS, FOXF1, FOXQ1, GLIS1, HAND2, HEYL, HHEX, HMGA2, HOXB3, HOXB7, HOXC10, HOXD3, HOXD9, HOXD10, HOXD13, KLF5, KLF8, LIF, NFIB, NKX3-2, NFE2L3, PITX1, PLAG1, PRDM6, RUNX3, SOX8, SOX10, TBX2, TBX3, TBX15, TFAP2A, TFAP2B
CC: ECM (1.53E-10)	ADAMTSL1, ADAMTS5, ALPL, CLEC14A, COL7A1, COL9A3, COL14A1, COL23A1, CRISPLD2, CTSB, ELFN2, F7, FBN2, FGL2, FRAS1, HAPLN3, KAL1, L1CAM, MMP10, OGN, PCOLCE2, PDGFB, PRG4, PTPRZ1, SEMA3C, SRPX2, TGFB3, THBS4, TLR3, USH2A, WISP1, WNT5A
CC: Integral component of plasma membrane (2.38E−08)	ADORA2B, ADRA1D, CADM1, CD9, CD70, CD74, CELSR1, CHRM3, CMKLR1, DLL4, DRD1, EDA, EPHB2, EREG, F2RL1, GPR17, GPR133, HLA-DQA1, HRH2, IL-6, ITGB7, KCNN4, LAT, LGR5, LPHN2, LRFN5, NOTCH3, MSR1, MST1R, NTRK2, NTSR1, PPAP2A, PPAP2B, PODXL, PROCR, PTGER2, PTPRD, PTPRZ1, REEP2, ROS1, SCARA5, SCN1A, SEMA3C, SEMA3E, SHANK1, SLC14A1, SLC16A2, SLC16A5, SYT7, TLR3, TRHDE, TSHR, TSPAN12, TSPAN18

This systems level analysis was performed in ToppFun using the 308 DE genes identified in lcSSc twins and validated with unrelated controls.
The p value for each pathway and ontology is shown in parenthesis. Genes in red are upregulated in lcSSc twins, and in blue downregulated.
BP; biological process; CC, cellular component; DE, differentially expressed; ECM, extracellular matrix; lcSSc, limited cutaneous SSc; MF, molecular function; PW, pathway; SSc, systemic sclerosis.

and dcSSc twins, we determined that 10 predicted targets were present as DE genes in our RNAseq dataset: in lcSSc twins *ALPL*, *ANK1*, *CADM1*, *HOXB3*, *HOXD10*, *SOBP*, *TFAP2A* and *VASH1*, and in dcSSc twins *HOXD10*, *TBX5* and *UNC5B* (figure 2D). Silencing of both *miR-10a-5p* and *miR-10b-5p* in normal dFBs (single or combined) significantly increased *TFAP2A* and *TBX5* mRNA levels (figure 2E), while only the silencing of *miR-10b-5p* induced a significant increase in *HOXD10* expression levels. This is consistent with previous reports stating that *miR-10b* targets HOXD10 for silencing.[17–19] In a complementary approach, transfection of SSc dFBs with *miR-10a-5p* and *miR-10b-5p* mimics (individually or combined) significantly reduced HOXD10 protein abundance (figure 2F), confirming the efficacy of the mimics and showing that *miR-10a* is also able to decrease HOXD10 in SSc dFBs. The combination of both mimics also reduced the TFAP2A, TBX5 and COL1A1 protein levels, and the *miR-10a-5p* and *miR-10b-5p* mimics individually exerted a significant translational silencing of TBX5. These results suggest that *miR-10a-5p* and *miR-10b-5p*, whether individually or in combination, modulate HOXD10, TFAP2A, TBX5 and COL1A1 levels in SSc.

Table 2 Functional enrichment in dcSSc twins

Enrichment	Hit in query list
BP: tube development (1.12E−08)	ALX1, ANGPT4, CD24, COL4A3, COMP, HOXD11, IL-6, KLF5, LEP, LIF, MIR27B, MYLK, NRXN3, RAMP1, RGCC, SPINT2, TBX5, TNS3, UNC5B
BP: cell adhesion (8.28E−07)	CD24, CD200R1, CDH8, COL4A3, COMP, EPDR1, IL-6, JAM2, LEP, MIR27B, NEGR1, NRXN3, PCDH19, PYCARD, RGCC, S100A10, SORBS1, SPINT2
MF: calcium ion binding (3.15E−05)	CDH8, COMP, EPDR1, MATN2, PADI2, PCDH19, PLN, S100A10, SCARA3, SYT12, VLDLR
MF: IL-6 receptor binding (2.33E−04)	IL-6, PYCARD
MF: ECM structural constituent	COL4A3, COL11A1, COMP, MATN2, SCARA3

This systems level analysis was performed in ToppFun using the 76 DE genes identified in dcSSc twins.
The p value for each ontology is shown in parenthesis. Genes in red are upregulated in dcSSc twins, and in blue downregulated.
BP, biological process; dcSSc, diffuse cutaneous SSc; DE, differentially expressed; ECM, extracellular matrix; lcSSc, limited cutaneous SSc; MF, molecular function; SSc, systemic sclerosis.

In early dcSSc, TBX5 and TFAP2A regulate *HOX* genes via KLF4, NANOG and POU5F1 pathway

Given that *HOX* genes encode TFs involved in cellular differentiation during embryogenesis,[20] and that myofibroblast differentiation is central to SSc and fibrosis pathogenesis,[14] a review of *TGFβ* and *HOX* gene regulation led us to examine the embryonic stem cell (ESC) TFs *SOX2*, *KLF4*, *NANOG* and *POU5F1* (aka *OCT4*).[21] In lcSSc twins, *SOX2* was significantly upregulated while *KLF4* was downregulated compared with unrelated controls (figure 1B; online supplemental figure 5A and table 2). Additionally, *POU5F1* was downregulated in several samples, although not significantly, and *NANOG* was not detected. In dcSSc and healthy twins, the expression levels of these four ESC TFs were different by samples, not by experimental groups (online supplemental figure 5B and table 5). Since the patients with lcSSc and dcSSc in the twin cohort had variable disease duration (online supplemental file 1), we quantified these ESC TFs in non-twin patients with early dcSSc with disease duration of 8–24 months. Upregulation of *SOX2* was observed in early dcSSc dFBs along with downregulation of *KLF4*, *NANOG* and *POU5F1* (figure 3A). *TFAP2A* and *TBX5* were also upregulated in these cells.

In discordant dcSSc twins, *KLF4* expression levels were noticeably, although not significantly, reduced as compared with healthy twins (figure 3B). We performed a disease duration

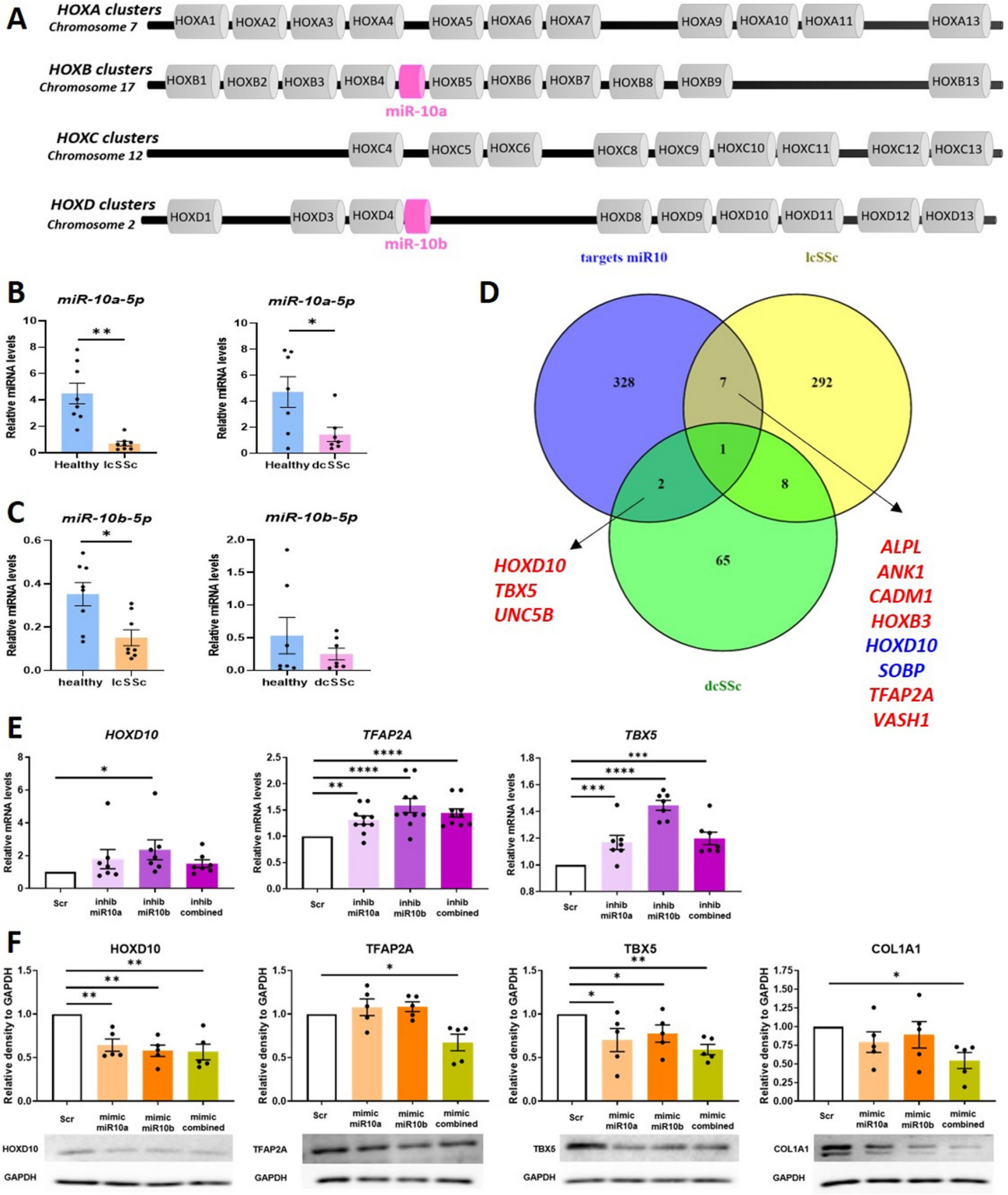

Figure 2 Both miR-10a and miR-10b mediate the dysregulation of TFAP2A, TBX5 and COL1A1 in SSc dFBs. (A) miR-10a and miR-10b are encoded from the Hox clusters, upstream of HOXB4 (chromosome 17) and HOXD4 (chromosome 2) respectively. (B) Levels of expression of miR-10a-5p in lcSSc and dcSSc dFBs compared with healthy dFBs. (C) Levels of expression of miR-10b-5p in lcSSc and dcSSc dFBs compared with healthy dFBs. (D) Comparison of predicted miR-10a/miR-10b target genes (obtained from TargetScan database) and validated DE genes in lcSSc twins. Genes in red font are upregulated, and genes in blue are downregulated. Note that HOXD10 is downregulated in lcSSc twins while upregulated in dcSSc twins. (E) Effect of antagomiR-mediated miR-10a-5p (inhib miR-10a) and miR-10b-5p (inhib miR-10b) silencing on HOXD10 (n=7), TFAP2A (n=10) and TBX5 (n=7) expression levels in normal dFBs transfected for 48 hours. Combined inhibition (inhib combined) was induced by simultaneous delivery of both miR-10a-5p and miR-10b-5p antagomiRs. SCR: scramble antagomiR. (F) Effect of miR-10a and miR-10b mimics in SSc dFBs on HOXD10, TFAP2A, TBX5 and COL1A1 protein abundance by immunoblotting (n=5). The concentration of mimic transfection was 50 mM for individual mimic and 25 mM for each mimic when transfected together (mimic combined). p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Error bars=SEM. dcSSc, diffuse cutaneous SSc; DE, differentially expressed; dFBs, dermal fibroblasts; SCR, scramble mimic control; SSc, systemic sclerosis.

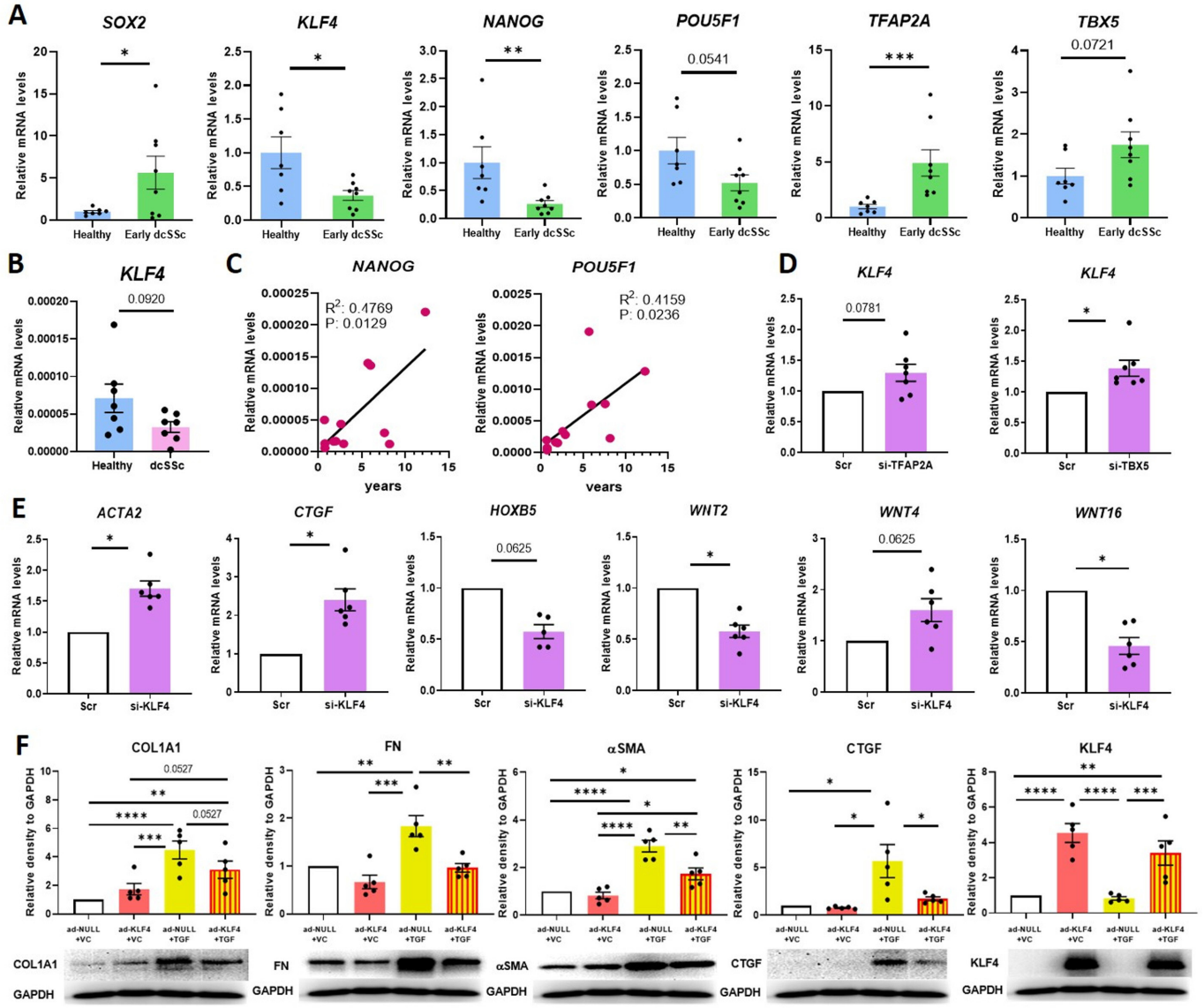

Figure 3 Validation in early disease and regulation of KLF4 function. (A) Expression levels of ESC pluripotency TFs and other genes of interest in early dcSSc dFBs (n=8, disease duration 8–24 months) compared with levels in normal dFBs from healthy twins (n=7, aged-matched to patients with early dcSSc). (B) KLF4 expression levels in dcSSc discordant twins (n=7). (C) Disease duration correlation in dcSSc for NANOG and POU5F1 expression levels. All dcSSc twins (n=7) and non-twin patients with dcSSc selected for the early disease study (n=5) were used in this linear regression analysis. (D) Effect of TFAP2A and TBX5 silencing (siRNA) on KLF4 expression levels in early dcSSc dFBs (n=7) transfected for 24 hours. (E) Effect of KLF4 silencing (siRNA) on ACTA2, CTGF, HOXB5, WNT2, WNT4 and WNT16 expression levels in normal dFBs (n=5–6) transfected for 72 hours. (F) Effect of adenoviral overexpression of KLF4 (ad-KLF4) in normal dFBs infected for 24 hours and stimulated with TGFβ (TGF) or VC for 72 hours on protein abundance of fibrotic genes (n=5). *p<0.05, **p<0.01, ***p<0.001. Error bars=SEM. ad-NULL, null adenovirus; CTGF, connective tissue growth factor; dcSSc, diffuse cutaneous SSc; dFBs, dermal fibroblasts; ESC, embryonic stem cell; SCR, scramble siRNA; SSc, systemic sclerosis; TFs, transcription factors; VC, vehicle control.

correlation analysis (figure 3C and online supplemental figure 5C) using data from all dcSSc twins (representing intermediate and late disease stages) and patients with early dcSSc and identified a strong positive correlation between disease duration and relative mRNA levels of *NANOG* (p=0.0129, R^2=0.4769) and *POU5F1* (p=0.0236, R^2=0.4159).

KLF4 is protective against fibrosis

KLF4 is a master regulator of skin and stem cell biology.[22] Silencing of *TFAP2A* and *TBX5* in early dcSSc dFBs increased *KLF4* expression levels (figure 3D). In turn, *KLF4* silencing in normal dFBs (figure 3E) significantly increased smooth muscle actin alpha 2 (*ACTA2*), an indicator of myofibroblast differentiation,[14] and connective tissue growth factor (CTGF), a central profibrotic factor,[23] while decreasing *HOXB5* levels, an expression profile mirroring that in SSc. We also examined the effect of *KLF4* silencing on the expression levels of several key molecules in the WNT pathway and determined that *KLF4* positively regulates *WNT2* and *WNT16* and negatively regulates *WNT4* (figure 3E). Normal dFBs overexpressing *KLF4* and stimulated with TGFβ showed a significant decrease in COL1A1, FN, αSMA and CTGF protein abundance as compared with TGFβ alone, confirming that KLF4 protects against TGFβ-induced fibrogenesis (figure 3F). These findings were further validated in human skin in organ culture as it is the relevant tissue for SSc and lends direct relevance to the human disease. Similarly to its

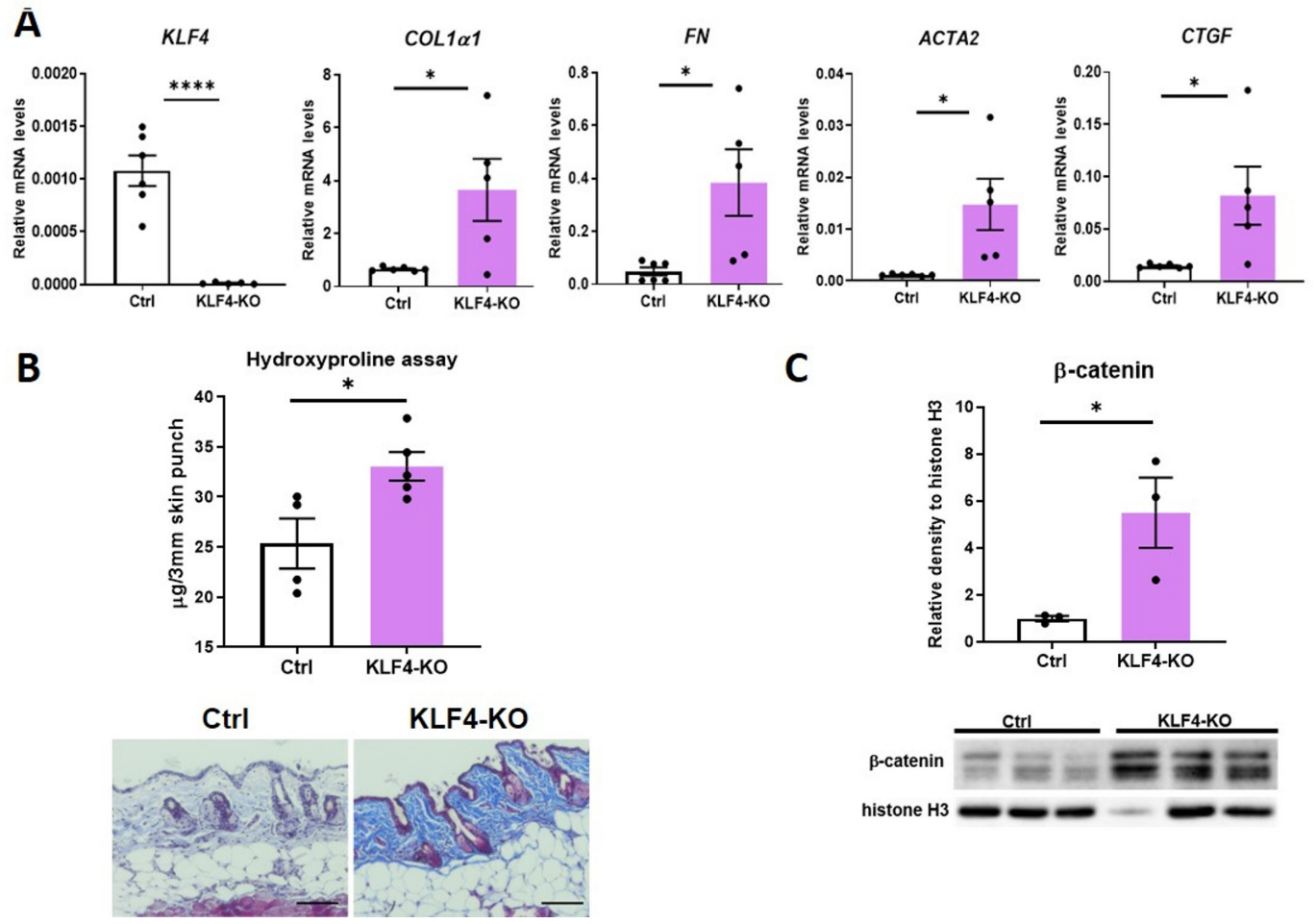

Figure 4 Antifibrotic properties of KLF4. (A) Effect of KLF4 conditional knock-out (KLF4-KO) on the expression levels of fibrotic genes in murine dFBs. Control KLF4-flox mice (Ctrl) received corn oil. (B) Hydroxyproline levels in 3 mm skin punches of control and inducible KLF4-KO mice. Representative images of skin sections stained with Masson's trichrome showing collagen in blue. The scale bar indicates 100 µm. (C) Protein abundance of β-catenin in the nuclear chromatin fraction of control and KLF4-KO dFBs relative to histone H3. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Error bars=SEM.

effect in dFBs, KLF4 reduced the TGFβ1 response in human skin (online supplemental figure 6). To extend our findings in vivo, we induced fibroblast-specific knock-down of KLF4 in mice (KLF4-KO). In KLF4-KO dFBs, fibrotic genes were highly expressed (figure 4A). Furthermore, hydroxyproline content was significantly higher in the skin of KLF4-KO mice as compared with control mice (figure 4B). Since KLF4 regulated WNT gene expression (figure 3E), we examined its effect on β-catenin. Loss of KLF4 resulted in a significant increase in nuclear chromatin levels of β-catenin, a key downstream component of the canonical Wnt signalling pathway, in dFBs of KLF4-KO mice (figure 4C). This suggests that the imbalance of WNT gene expression resulted in an activation status in mouse dFBs.

Together our data highlight *KLF4* as a key antifibrotic factor that is downregulated by TFAP2A and TBX5 early in disease progression, leading to fibroblast activation and fibrosis. KLF4 could be a candidate for the development of targeted therapies against fibrosis that would aim to maintain increased levels of this antifibrotic gene.

DISCUSSION

This study is the first to examine genome-wide DNA methylation profiling in combination with RNAseq in dFBs from a unique cohort of twins discordant for SSc categorised by disease subtypes. *HOX* genes belong to a family of evolutionarily highly conserved genes that encode TFs involved in stem cell differentiation.[20] In addition to driving stem cells towards their corresponding lineages, the HOX code is also important during adulthood to repair and maintain proper tissue and organ function, and provide cellular positional memory that is crucial to inform the location and cell lineage of new epidermal cells during turnover, a process that is mediated in adult dFBs via *HOXA13–WNT5A* pathway.[24] Our data show that both *HOXA13* and *WNT5A* are upregulated in lcSSc dFBs, implicating this pathway in SSc. Even though *HOXA13* function is required for *WNT5A* expression, it does not directly target *WNT5A*, which led Rinn *et al* to speculate that HOXA13 activates *WNT5A* directly via distant enhancer sequences, or indirectly via *HOXB6*.[24] Rinn *et al*[25] examined the HOX signature in primary human dFBs that delineates distal and proximal sections of limbs and showed that HOXA13 is exclusively expressed in adult dFBs isolated from distal sites such as hands, feet and foreskin. Our qRT-PCR data are consistent with Rinn *et al*[25] in that HOXA13 was upregulated in the lcSSc dFBs as compared with unrelated controls. Our data suggest that deregulation of the HOX zip code, including HOXA, HOXB, HOXC and HOXD genes, is dependent on SSc skin involvement and independent from biopsy location since the expression of HOXB genes is

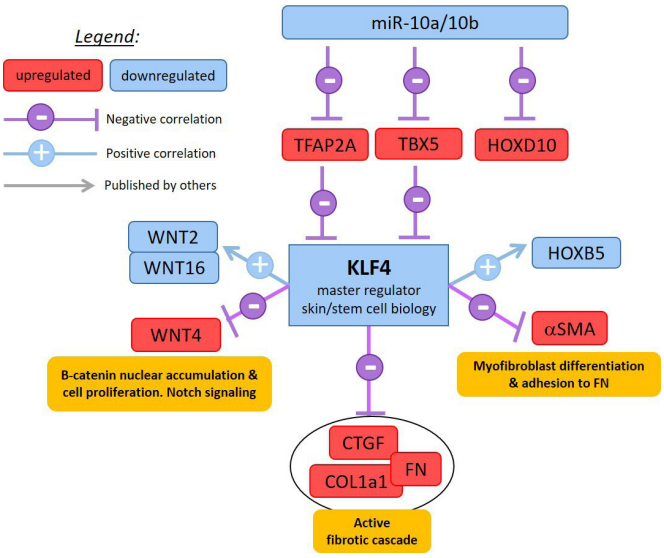

Figure 5 Summary model of the regulatory network. In SSc dFBs, miR-10a and miR-10b expression levels are low, allowing for the upregulation of two silencing targets TFAP2A and TBX5. KLF4 levels are repressed by TFAP2A and TBX5, which leads to high expression of ACTA2, COL1A1, CTGF and FN, enhancing myofibroblast differentiation and fibrosis. Low levels of KLF4 also correlate with low levels of HOXB5, WNT2 and WNT16, and high levels of WNT4, suggesting that KLF4 regulates the Wnt signalling pathway. HOXB5 is positively correlated with KLF4 levels, and has been shown to play a role in myofibroblast adhesion to FN. CTGF, connective tissue growth factor; dFBs, dermal fibroblasts; FN, fibronectin.

limited to the trunk and non-dermal samples while HOXD4 and HOXD8 are expressed exclusively in the trunk and proximal samples in healthy donors.[25]

Decreased promoter DNA methylation often leads to increased gene expression as is the case with *HOXB8* and *HOXC10* in lcSSc twins, and *TBX5* and *UNC5B* in dcSSc twins. In the same reasoning, gene silencing is expected with increased methylation of CpG islands, however, this classic paradigm does not always apply, as illustrated by *HOXB3* and *TFAP2A* which were more methylated yet upregulated in lcSSc twins. Mucientes *et al* also observed differential expression of *HOX* genes independent of their methylation status in stem cells of patients with osteoarthritis.[26] This suggests that other regulatory and epigenetic mechanisms are implicated in the regulation of these *HOX* genes, including sequential activation of *HOX* genes with 3′ to 5′ polarity.[27–29] Our data highlight that perturbation of homeotic genes drives developmental reprogramming in SSc dFBs. Interestingly, *TBX5* is located on chromosome 12, the same chromosome as the *HOXC* cluster (online supplemental figure 4A), and is a direct target of *HOX* genes.[30] *TBX5* showed reduced methylation status and upregulated gene expression in dcSSc dFBs in our study (online supplemental figure 4B), a state also observed in the activation of rheumatoid arthritis synovial fibroblasts.[31]

Our data showed that *TFAP2A* and *TBX5* negatively regulate *KLF4*, an upstream regulator of the ESC factors *NANOG, SOX2* and *POU5F1*[32] that control cellular differentiation into extraembryonic, endodermal, mesodermal and ectodermal lineages, and also regulate *HOX* genes[33] (figure 5). We determined that *KLF4* is a negative regulator of *ACTA2*, *CTGF and WNT4* in dFBs, indicative of active fibrotic cascade, and a positive regulator of *HOXB5*, *WNT2* and *WNT16*. Interestingly, binding motifs for *KLF4*, *POU5F1* and *SOX2* exist in the *WNT2* promoter region, and *WNT2* has been shown to play a role in the nuclear accumulation of β-catenin and cell proliferation in early stages of embryonic fibroblast reprogramming.[34] Beazley *et al*[35] showed that TGFβ inhibits *WNT16* expression, leading to the deactivation of Notch signalling in vascular smooth muscle cells. Considering that inhibition of Notch signalling limits both fibrosis and autoimmune activation[36] and even reversed established fibrosis in a murine model of SSc,[37] the role of downregulation of the KLF4–WNT16–Notch signalling axis in dFBs in the development of SSc warrants additional investigation. Due to functional redundancy among HOX5 paralogs, it is important to consider HOXA5 and HOXC5 when assessing the role of HOXB5. Loss of HOX5 leads to downregulation of WNT2,[38] consistent with our KLF4 silencing assay in which both WNT2 and HOXB5 were downregulated. Together this suggests that both KLF4 and HOXB5 regulate WNT/β-catenin signalling. Additionally, HOX5 paralogs modulate Th2 inflammation during chronic allergic reaction,[39] a role that is unexplored in SSc.

The fact that *TFAP2A* overexpression suppressed the expression of the matrix genes collagen types II and X, and aggrecan,[40] and *KLF4* negatively regulated the expression of mesenchymal markers *ACTA2* and fibroblast specific protein 1,[41] suggests that the *TFAP2A–KLF4* axis likely regulates myofibroblast differentiation and fibrosis. Furthermore, the decreased expression levels of *POU5F1* and *NANOG* in early dcSSc parallel those of *KLF4* and may reflect a state of reduced plasticity that points to cell fate reprogramming in dFBs.[42]

MiRNAs of the *miR-10* family target *TFAP2A*,[43] *TBX5*,[44] *KLF4* and several *HOX* genes for silencing.[16] Both *miR-10a-5p* and *miR-10b-5p* are active elements in mediating cancer metastasis[45 46] and the fibrogenic response.[47] Our silencing assay showed that they mediate *TFAP2A*, *TBX5* and *HOXD10* dysregulation in dFBs. Our data suggest that it is a combination of decreased DNA methylation and reduced miRNA silencing that maintain high levels of *TBX5* in dcSSc dFBs. However, the epigenetic regulation of *TFAP2A*, which was upregulated with increased methylation status, may be more driven by miRNAs. Even though *miR-10a*/*miR-10b* target several DE genes in our dataset, not all of these targets exhibited increased expression levels when *miR-10a and miR-10b* were downregulated, as is the case for *HOXD10* and *SOBP* in lcSSc twins, which could be due to the complex way these genes are epigenetically or post-transcriptionally regulated.

Our findings suggest that developmental programmes deregulated in lcSSc are similarly observed in the early phase of dcSSc. This may be due to the fact that skin involvement in lcSSc, although minimal, remains active as the disease progresses, whereas skin involvement regresses in dcSSc approximately 3 years after disease onset. This highlights the importance of including patients with lcSSc in studies involving gene expression profiling, whether in response to potential therapies or to monitor disease progression.

Some genes linked to DM CpG sites reported in individual SSc studies did not show evidence of differential methylation in this discordant twin design. This may be due to simple random sampling variation in combination with the power to detect more modest effects. Some may reflect differences in tissues assayed (type, physical location). But some of these genes may not replicate because of the robustness of a discordant twin study design where the test of association is between the twins, eliminating the confounding effects of genetic heterogeneity across samples that cause inflation of the test statistics (ie, lead to false positive).

This study provides insights into the interplay between different epigenetic mechanisms in SSc-associated dermal fibrosis

and implicate TFs and miRNAs in disease pathogenesis. Given that epigenetic regulation is reversible, this suggests that epigenetic modifying agents or CRISPR-based epigenome editing to ameliorate fibrosis may be of relevance in SSc.[48]

Author affiliations
[1]Medicine, Medical University of South Carolina, Charleston, South Carolina, USA
[2]Biostatistical Sciences and Center for Public Health Genomics, Wake Forest School of Medicine, Winston-Salem, North Carolina, USA
[3]School of Biological Sciences, Institute for Global Food Security, Queen's University Belfast, Belfast, UK
[4]Public Health Sciences, Medical University of South Carolina, Charleston, South Carolina, USA
[5]Science, Bay Path University, Longmeadow, Massachusetts, USA
[6]Internal Medicine, University of Michigan Michigan Medicine, Ann Arbor, Michigan, USA
[7]Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA

Twitter Paula S Ramos @PSRamos_PhD

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Contributors CAF-B is the guarantor for this study. CAF-B designed and coordinated the study. CAF-B and TAM recruited the patients. TAM did history and physical examinations and disease subtyping of patients. CAF-B and NT processed the skin biopsies, cultured fibroblasts and extracted DNA and RNA. SH confirmed the zygosity of the twins. MM and NT conducted the qPCR and gain/loss of function experiments. KDZ, PSR, WAdS, GH, NT, LRP, BW and CDL analysed the data. MP-G and LRP generated the KLF4 conditional knock out mice. MM, LR, PSR, and CFB wrote the manuscript. All authors were involved in critical review, editing, revision and approval of the final manuscript.

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ORCID iDs
Ludivine Renaud http://orcid.org/0000-0002-9300-5032
Paula S Ramos http://orcid.org/0000-0002-9433-6634
Carol A Feghali-Bostwick http://orcid.org/0000-0002-6750-6407

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TRANSLATIONAL SCIENCE

Whole gut virome analysis of 476 Japanese revealed a link between phage and autoimmune disease

Yoshihiko Tomofuji,[1] Toshihiro Kishikawa,[2,3] Yuichi Maeda ,[4,5,6] Kotaro Ogawa,[7] Takuro Nii,[4,5] Tatsusada Okuno,[7] Eri Oguro-Igashira ,[4,5] Makoto Kinoshita,[7] Kenichi Yamamoto,[1,8,9] Kyuto Sonehara,[1,6] Mayu Yagita,[4,5] Akiko Hosokawa,[10] Daisuke Motooka,[11] Yuki Matsumoto,[11] Hidetoshi Matsuoka,[12] Maiko Yoshimura,[12] Shiro Ohshima,[12] Shota Nakamura,[11] Hidenori Inohara,[2] Hideki Mochizuki,[7] Kiyoshi Takeda,[5,13] Atsushi Kumanogoh,[4,6,14] Yukinori Okada [1,6,9,15]

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For numbered affiliations see end of article.

Correspondence to
Dr Yukinori Okada, Department of Statistical Genetics, Osaka University Graduate School of Medicine, Suita, Japan; yokada@sg.med.osaka-u.ac.jp

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ABSTRACT

Objective The relationship between autoimmune diseases and the gut microbiome has been intensively studied, and several autoimmunity-associated bacterial taxa have been identified. However, much less is known about the roles of the gut virome in autoimmune diseases.

Methods Here, we performed a whole gut virome analysis based on the shotgun sequencing of 476 Japanese which included patients with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), multiple sclerosis and healthy control subjects.

Results Our case–control comparison of the viral abundance revealed that crAss-like phages, which are one of the main components of a healthy gut virome, significantly decreased in the gut of the patients with autoimmune disease, specifically the patients with RA and SLE. In addition, *Podoviridae* significantly decreased in the gut of the patients with SLE. To understand how these viruses affected the bacteriome, we performed a quantitative virus–bacterium association analysis and clustered regularly interspaced short palindromic repeat-based virus–bacterium interaction analysis. We identified a symbiosis between *Podoviridae* and *Faecalibacterium*. In addition, multiple bacterial targets of crAss-like phages were identified (eg, *Ruminococcus* spp).

Conclusion Our data suggest that the gut virome can affect our body either directly or via bacteria. Our analyses have elucidated a previously missing part of the autoimmunity-associated gut microbiome and presented new candidates that contribute to the development of autoimmune diseases.

Key messages

What is already known about this subject?

- Alteration of the gut microbiome has been linked to the pathogenesis of autoimmune diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and multiple sclerosis. However, much less is known about the roles of the gut virome in the development of these diseases.

What does this study add?

- Our case–control comparison of the viral abundance revealed that crAss-like phages, which are one of the main components of a healthy gut virome, significantly decreased in the gut of the patients with autoimmune disease, mainly in the patients with RA and SLE.
- Multiple bacterial targets of crAss-like phages were identified (eg, *Ruminococcus* spp) from the quantitative virus–bacterium association analysis and clustered regularly interspaced short palindromic repeat based virus–bacterium interaction analysis.
- *Podoviridae*, which has a symbiotic relationship to *Faecalibacterium*, significantly decreased in the gut of the patients with SLE.

How might this impact on clinical practice or future developments?

- Our analyses have elucidated a previously missing part of the autoimmunity-associated gut microbiome and presented new candidates that contribute to the development of autoimmune diseases.

INTRODUCTION

Despite the recent advancements in medicine, autoimmune diseases are increasing in prevalence and cause significant chronic morbidity and disability.[1 2] Thus, their prevention and treatment are considered important goals for modern medicine. Many autoimmune diseases are caused by complex genetic and environmental factors and their interactions. Genetic factors of autoimmune diseases are being revealed through various studies, such as the genome-wide association study.[3 4] Meanwhile, environmental factors have also been identified; however, much remain to be elucidated.

The gut microbiome, which refers to the microbial communities inhabiting our gut, substantially influences our health via the immune and metabolic systems.[5] Accompanied by advances in analytical methods, many studies have been conducted to reveal the complex relationships between the gut microbiome and human diseases such as metabolic disease,[6] cancer,[7] and intestinal disease.[8] As for autoimmune diseases, the gut microbiome has been considered one of the most significant

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environmental contributors in their development. The reduced diversity of the gut microbiome has been reported in various autoimmune diseases, including rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE).[9] In addition, *Prevotella copri* contributes to the pathogenesis of RA by activating the immune response via Th17 cells.[10]

A large proportion of the gut microbiome is composed of viruses.[11] The most predominant component of the gut virome is bacteriophage. Bacteriophages infect bacteria and regulate the gut bacteriome by either lysing their hosts or altering their physiological function.[12] In the patients with *Clostridium difficile* infection, reconstruction of a healthy gut microbiome through faecal transplantation was associated with the viral composition of the donor's faeces,[13–15] supporting the significant role of bacteriophages in the maintenance of the gut microbiome. In addition to their mediating effects, recent studies have suggested that bacteriophages can directly affect our body via the immune system.[16 17] Although it is evident that the gut virome has a significant impact on the host's physiology, many previous metagenomic studies have ignored the viral component of the gut microbiome due to technical difficulties.

As viruses require a host for growth and only a limited fraction of the gut bacteriome can be cultured, most of the gut virome cannot be evaluated through conventional laboratory approaches. Although high-throughput sequencing technologies have enabled us to analyse unculturable microbes, its application in virome analysis is not as straightforward. As viruses do not have universal taxonomic markers such as bacterial 16S ribosomal RNA (rRNA), gut virome studies have used virus-like particle (VLP) sequencing or whole-metagenome shotgun sequencing that require complex data processing and computational resources. In addition, the quantification methods used in bacterial analysis are not transferable to virome analysis because the current reference viral genome databases cover only a limited fraction of the actual gut virome populations.[11] Although previous studies have quantified the gut viral abundance by nucleotide-level alignment of individual reads or assembled contigs to viral sequence databases, they could only evaluate only a minority of the gut virome populations.[18 19] Owing to the extensive efforts in the development of computational methods and algorithms optimised for viruses, a comprehensive view of the gut virome in the physiological condition started to be revealed.[20–23] However, the utilisation of such viral-optimised analysis methods remains limited.

In addition to the quantification of the gut viral abundance, the identification of the viral host is necessary to reveal the effect of bacteriophages on the gut microbial community. Although the coabundance analysis was useful in conferring virus–host interaction, it was not sufficient to show the evidence of viral infections.[24] The whole-metagenome shotgun sequencing technology has enabled us to identify infectious targets of the bacteriophages using clustered regularly interspaced short palindromic repeat (CRISPR) sequences in the bacterial contigs. CRISPR and CRISPR-associated (Cas) proteins comprise the CRISPR–Cas system, a bacterial adaptive immune system against predators such as bacteriophages.[25] The CRISPR–Cas system intakes virus-derived sequences as CRISPR spacers to efficiently eject the virus during a subsequent infection. Thus, spacer sequences in the CRISPR loci are the immune memory of bacteria and footprints of a viral infection. Although the CRISPR analysis can provide direct evidence of a viral infection, its application in a large-scale virome analysis remains limited,[21 22] possibly due to the high sequencing and computational cost of a whole-metagenome shotgun sequencing analysis.

A case–control discrepancy of the gut virome was reported for only a limited number of intestinal,[19 26] and metabolic diseases,[27–29] which included inflammatory bowel diseases (IBD).[18 30] In IBD, the individual diversity of the gut virome and gut bacteriome increased and decreased compared with those of healthy subjects, respectively, suggesting the contribution of the gut virome to the disease pathogenesis. Most of the current virome case–control studies were conducted in Western countries,[18 30 31] or China.[26–28] Given the differences in the metagenomic landscape among different populations, the association between the gut virome and various diseases in diverse populations should be studied. In addition, limited numbers of studied diseases have hindered us from understanding the virus-associated disease aetiologies and the role of bacteriophages as healthy components of the gut microbiome. Although the importance of the gut microbial environment in systemic autoimmune diseases such as RA, SLE and multiple sclerosis (MS) has been well established,[9 32 33] there are few studies that have identified case–control discrepancies of the gut virome in these diseases.[31] Therefore, a whole-virome analysis to reveal the associations between bacteriophages and autoimmune diseases is warranted.

Here, we performed a whole gut virome analysis of 476 Japanese which included the patients with RA (N = 111), SLE (N = 47) and MS (N = 29) and healthy control (HC) subjects (N = 289). This is the largest case–control comparison of the viral abundance to date. We evaluated changes in the gut virome and whether it was specific to each autoimmune disease or shared across multiple autoimmune diseases. In addition, we performed a virus–bacterium association analysis based on the abundance data and a CRISPR-based virus–bacterium interaction analysis to reveal the virus–bacterium interaction mediated by disease-associated viruses identified in this study.

METHODS

Patient participation

The study included 112 patients with RA, 48 patients with SLE, 29 patients with MS, and 296 HC subjects. Most of these subjects were derived either from previous studies,[32 33] or a recently conducted SLE metagenome study.[34] The patients with RA were enrolled at the Osaka University Hospital, National Hospital Organization Osaka Minami Medical Center, and Daini Osaka Police Hospital. The patients with SLE were enrolled at the Osaka University Hospital and National Hospital Organization Osaka Minami Medical Center. The patients with MS were enrolled at the Osaka University Hospital. The HC subjects were enrolled at the Osaka University Graduate School of Medicine, Osaka University Hospital, and National Hospital Organization Osaka Minami Medical Center. The patients with RA were diagnosed by physicians according to the American College of Rheumatology and the European League Against Rheumatism 2010 criteria for RA.[35] The patients with SLE were diagnosed by physicians according to the Systemic Lupus International Collaborating Clinics classification criteria.[36] The patients with MS were diagnosed by physicians according to the McDonald 2010 criteria.[37] The Disease Activity Score 28 using C reactive protein,[38] SLE Disease Activity Index[39] and Expanded Disability Status Scale,[40] were calculated to evaluate the activity of each disease. The HC subjects had no personal history of the immune-related diseases.

Participants with extreme diets (eg, strict vegetarians) were not included in the dataset. All subjects provided written informed consent before participation. Those who took antibiotics within a month was reported as the patients treated with antibiotics.

The characteristics of the subjects are shown in online supplemental table 1.

Sample collection and DNA extraction

Faecal samples were collected in tubes containing RNAlater (Ambion). After the weights of the samples were measured, RNAlater was added to produce 10-fold dilutions of homogenates. Faecal samples were stored at −80°C within 24 hours after collection. Bacterial DNA was extracted according to a previously described method. Briefly, 0.3 g glass beads (diameter: 0.1 mm) (BioSpec) and 500 µL EDTA-Tris-saturated phenol were added to the suspension, and the mixture was vortexed vigorously using a FastPrep-24 (MP Biomedicals) at 5.0 power level for 30 s. After centrifugation at 20 000 g for 5 min at 4°C, 400 µL of supernatant was collected. Subsequently, phenol–chloroform extraction was performed, and 250 µL of supernatant was subjected to isopropanol precipitation. Finally, DNAs were suspended in 100 µL EDTA-Tris buffer and stored at −20°C.

Whole-genome shotgun sequencing

A shotgun sequencing library was constructed using the KAPA Hyper Prep Kit (KAPA Biosystems), and 150 bp paired-end reads were generated on HiSeq 3000 and NovaSeq 6000 for sequencing batches 1–4 and batch 5, respectively. The sequencing data from the patients with RA in sequencing batch 3 were newly obtained for this study, while other sequencing data were derived from previous studies,[32 33] or a recently conducted SLE metagenome study.[34] The sequence reads were converted to FASTQ format using bcl2fastq (V.2.19).

Quality control of sequencing reads

We followed a series of steps to maximise the quality of the datasets. The main steps in the quality control (QC) process were as follows: (1) trimming of low-quality bases, (2) identification and masking of human reads and (3) removal of duplicated reads. We marked duplicate reads using PRINSEQ-lite (V.0.20.4; -derep 1). We trimmed the raw reads to clip Illumina adapters and cut-off low-quality bases at both ends using the Trimmomatic (V.0.39; parameters: ILLUMINACLIP:TruSeq3-PE-2.fa:2:30:10:8:true LEADING:20 TRAILING:20 SLIDINGWINDOW:3:15 MINLEN:60). We discarded reads less than 60 bp in length after trimming. Next, we performed duplicate removal by retaining only the longest read among the duplicates with the same sequences. As a final QC step, we aligned the quality-filtered reads to the human reference genome (hg38) using bowtie2 (V.2.3.5) with default parameters and BMTagger (V.3.101). We kept only the reads of which both paired ends failed to align in either tool. The average QC-passed total sequencing base pairs were 25.5, 7.8, 6.7, 7.0 and 8.3 Gb for sequencing batches 1–5.

Viral contig assembly and identification

The de novo assembly of the filtered paired-end reads into contigs was conducted using MEGAHIT (V.1.2.9; parameters: -min-contig-len 1500). Following assembly, contigs whose 5' and 3' terminals have ≥50 bp overlap were marked as circular contigs. Linear contigs of ≥5 kbp and circular contigs of ≥1.5 kbp were subjected to VirSorter (V.1.0.6),[41] and VirFinder (V.1.1).[42] VirSorter was performed using both RefSeqABVir (–db 1) and Viromes (–db 2) databases, and sequences sorted as viruses with the 'most confident' prediction (category 1) or 'likely' prediction (category 2) were extracted for further analysis. Candidate viral sequences which were sorted as viruses with 'possible' prediction (category 3) by VirSorter were extracted for further analysis if they had a VirFinder score of ≥0.7. The remaining contigs were extracted for further analysis if they had a VirFinder score of ≥0.9. To minimise the contamination of bacterial sequences, we assessed the level of bacterial and viral gene enrichments as previously described by Gregory *et al*.[22] We used bacterial single-copy orthologs v4 (BUSCOv4; V.4.1.2),[43] to search the 124 bacterial single-copy orthologs registered in BUSCO's database and then used the BUSCO provided HMM score cutoffs to filter our results. Viral gene enrichment was assessed using hmmsearch (V.3.1b2) of viral contigs against the curated viral protein family (VPF) modules (https://portal.nersc.gov/dna/microbial/prokpubs/EarthVirome_DP/),[44] and any matches with E-value of <0.05 were defined as hits. We set a threshold for contamination of bacterial genome at BUSCO hit/number of VPF hits of >0.05. These procedures resulted in 93 254 total viral populations with an average length of 21.1 kbp.

Taxonomic annotation

We first classified viral contigs using complete viral RefSeq genomes (downloaded in June 2020 containing 12 696 genomes; https://www.ncbi.nlm.nih.gov/labs/virus/vssi/#/) as reference. Although the taxonomic information of viral genomes was based on NCBI taxonomy data, family-level classification of *crAssphage* (NC_024711.1) was modified to crAss-like phage according to recent publications.[24] The viral contig sequences were searched against the reference genome using megablast (ncbi-blast-plus V.2.10.1) with E-value of $<10^{-10}$, nucleotide identity of >95%, and coverage of contigs of ≥85%. Viral contigs were assigned to higher than or equal to species level taxonomy based on the megablast hit with the highest bit score. Contigs not assigned taxonomy in the previous step based on the nucleotide-level comparison proceeded to protein-level comparison for annotation of family-level taxonomy. First, we predicted open reading frames (ORFs) in viral contigs using MetaProdigal (V.2.6.3) with -p meta option. To detect crAss-like phages, we compared ORFs in viral contigs and crAss-like phage signature genes.[21 45] The protein sequences of the polymerase (UGP_018) and terminase (UGP_092) in *crAssphage* (NC_024711.1) were queried against the ORFs in viral contigs using blastp with E-value of $<10^{-5}$ and alignment length of ≥350. Viral contigs with blastp hits were assigned family-level taxonomic annotation as crAss-like phage. Then, the remaining unclassified contigs were proceed to taxonomic annotation by a voting system.[21 23] The ORFs in the viral contigs were searched against the RefSeq protein database (downloaded in June 2020, containing 420 609 proteins) using DIAMOND blastp (V.0.9.32.133) with E-value of $<10^{-5}$. Based on the DIAMOND blastp hits with the highest bit score, the ORFs were assigned taxonomic annotation. We then summarised all the taxonomic assignments of the ORFs for each contig, and assigned taxonomic information higher than or equal to family level based on the majority taxonomic assignment among the annotated ORFs. Viral contigs without a majority taxonomic assignment were regarded as unclassified viruses. Viral contigs with less than two annotated ORFs were also regarded as unclassified viruses.

Abundance quantification of viruses

To calculate the raw abundances of the different viral populations in each sample, the quality-checked reads from each sample were mapped to the viral contigs recovered from the sample using bowtie2 with default parameter. On average, 2.8% of the reads were mapped to viral contigs. CoverM filter (V.0.4.0) was used to remove the reads mapped at <95% nucleotide identity to the

contigs. Then, CoverM trimmed_mean (parameters: --trim-min 0.05 --trim-max 0.95) was used to calculate the average read depth across the viral populations. The read depth of each viral population was divided by the total sequenced length of each sample for normalisation. The normalised read depth of each viral population was summed up by each sample, and the normalised abundance of each clade was calculated at different taxonomic ranks. Then, we detected outlier samples using principal component analysis (PCA).

Abundance quantification of bacteria

After viral quantification, we extracted nonviral reads to obtain bacterial abundance. We used the previously constructed bacterial reference dataset composed of curated 7881 genomes.[32] The filtered paired-end reads were aligned to the reference genome dataset using bowtie2 with default parameters. For the multiple-mapped reads, only the best possible alignment was selected by the alignment scores. The number of reads that was mapped to each genome was divided by the length of the genome. The value of each genome was summed up by each sample, and the relative abundance of each clade was calculated at six levels (L2: phylum, L3: class, L4: order, L5: family, L6: genus, L7: species). Then, we detected outlier samples using PCA. We removed one RA sample, one SLE sample, and seven HC samples on the basis of the PCA of the viral or bacterial raw abundance. The samples that passed QC, including those derived from the 111 patients with RA, 47 patients with SLE, 29 patients with MS, and 289 HC subjects, were used in the subsequent analysis.

Association test of the viral abundance for age and sex

We normalised the viral abundances using log transformation. For family-level analysis, we retained only those clades that were detected in all the sequencing batches and ≥10% of the samples. The detection ratios of the QC-passed viral clades are indicated in online supplemental table 2. An association test of the viral abundance for age and sex was performed using the following formula: abundance of the virus ~ age + sex+phenotype + sequencing batch +total sequenced length. The lm() function in the R (V.4.0.1) was used for linear regression, and the effect size of the age and sex was evaluated.

Case–control comparison of the viral abundance

Case–control association tests were performed separately for each standardised clade abundance using the glm() function in the R (V.4.0.1), and the effect size of the viral abundance was evaluated. Sequencing batches 1, 2 and 3 were used in the analysis for RA (N_{case}=111 and $N_{control}$=111). Sequencing batches 2, 3 and 5 were used in the analysis for SLE (N_{case}=201 and $N_{control}$=47). Sequencing batch 4 was used in the analysis for MS (N_{case}=29 and $N_{control}$=74). We adopted sex, age, age^2, sequencing batch, and total sequenced length as covariates. To evaluate the effect of potential confounding factors, we performed sub-analysis with datasets from which (1) males were removed, (2) non-new onset patients were removed, (3) those treated with antibiotics were removed, (4) those treated with proton pump inhibitors were removed, (5) those treated with steroids were removed or (6) those treated for autoimmune diseases were removed. Case–control comparisons per sequencing batch were performed to evaluate the consistency of the results between the sequencing batches. In addition, comparisons between (1) the treated patients and the untreated patients, (2) the new onset patients and the non-new onset patients, and (3) those with high vs low disease activity were performed. For the meta-analysis, we used the metafor (V.3.0–2) package for R. As the case–control analyses for RA and SLE had significant overlap in control samples, we used only subjects from sequencing batch five for SLE case–control comparison. As for the random-effect model, 'REML' option was used for fitting parameters with restricted maximum likelihood estimation.

Virus–bacterium association analysis

We normalised the bacterial relative abundance profiles using log transformation. We only retained the clades that were detected in all the sequencing batches, in ≥20% of the samples, and with an average relative abundance of more than 0.001% of the total abundance. After selection, we assessed 802 clades (12 phyla, 24 classes, 35 orders, 74 families, 188 genera and 469 species).

Virus–bacterium association tests were performed separately for each virus–bacterium pair using the lm function in the R, and the effect size of the viral abundance was evaluated. We evaluated the association between eight viral families that passed clade QC described above and 802 bacterial clades. We adopted sex, age, age^2, sequencing batch, total sequenced length and the top 10 principal components of the normalised bacterial abundance as covariates. The false discovery ratio was calculated using the Benjamini-Hochberg procedure. To evaluate the effect of potential confounding factors, we performed subanalysis with datasets from which (1) male subjects were removed, (2) non-new-onset patients were removed, (3) only HC subjects were retained, (4) those treated with antibiotics were removed, (5) those treated with proton pump inhibitors were removed, (6) those treated with steroids were removed and (7) those treated for autoimmune diseases were removed. In addition, disease-specific virus–bacterium associations were evaluated by adding and evaluating virus × RA, virus × SLE, or virus × MS terms in the regression formula. The sample sets were defined as in the case–control comparison for each disease.

Virus–bacterium interaction analysis based on CRISPR spacers

We extracted contigs of ≥5 kbp that were not classified as viral ones. Then, we predicted the CRISPR sequences in these contigs with MinCED (V.0.4.2),[46] using the '-minNR 2' parameter as previously described.[22] Contigs with CRISPR sequences were queried against the bacterial reference genome using megablast with E-value of $<10^{-10}$, nucleotide identity of >95%, and coverage of contigs of ≥90%. Based on the megablast hit with the highest bit score, bacterial taxonomy was assigned to the contigs. Spacers within the predicted CRISPR sequences were queried against the viral contigs recovered from the same sample using blastn with E-value of $<10^{-5}$, nucleotide identity of >95%, and coverage of spacers of ≥90%. Based on the blastn hit with the highest bit score, viral taxonomy was assigned to the spacers. Then, we summarised the virus–bacterium pair based on taxonomy annotated to the spacers and the contigs, collapsed within each sample, and summarised across all the samples.

Patient and public involvement

This research was done without patient and public involvement. Patients and public were not invited to comment on the study design and were not consulted to develop patient relevant outcomes or interpret the results.

RESULTS

Obtaining viral abundance data from 476 individuals

An overview of our study is shown in figure 1A. We obtained the gut viral abundance from the shotgun sequencing data of 476

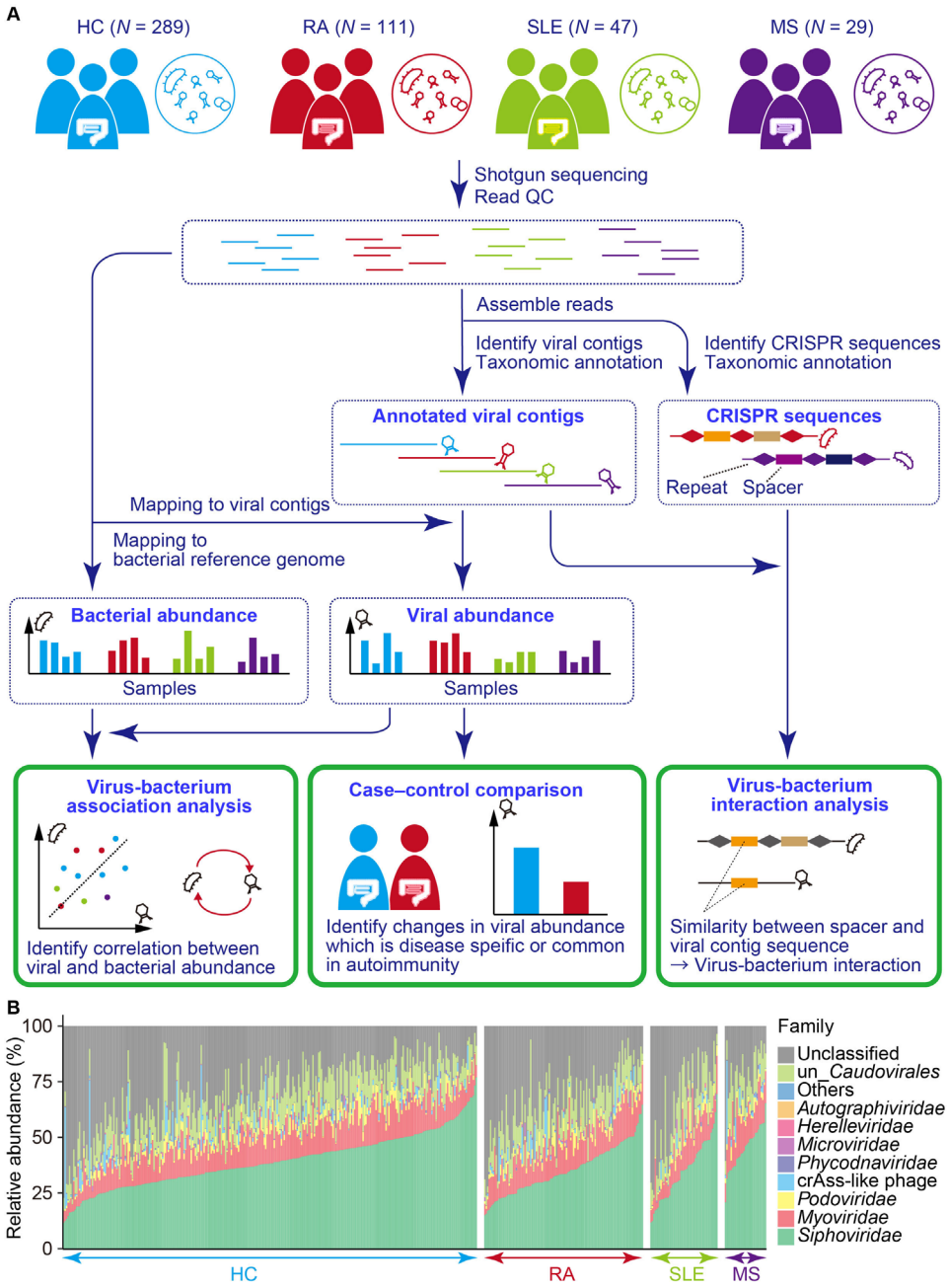

Figure 1 Overview of the whole gut virome analysis of the autoimmune diseases. (A) Schematic illustration of the study design. Shotgun sequencing data from HC subjects and patients with RA, SLE and MS were quality-checked and used for recovering viral contigs. The quality-checked reads were then mapped to the viral contigs recovered from the same sample to obtain the per sample viral abundance, followed by case–control comparison. Non-viral reads were mapped to a bacterial reference genome to obtain the per sample bacterial abundance. The viral and bacterial abundance data were integrated for virus–bacterium association analysis. CRISPR sequences in nonviral contigs were identified, and sequence similarity between spacer sequences and viral contigs recovered from the same sample was evaluated to identify the bacterial targets of the viruses. (B) Viral relative abundance at the family level. Relative abundance profiles were constructed using whole-genome shotgun sequencing (N_{HC}=289, N_{RA}=111, N_{SLE}=47, N_{MS}=29). CRISPR, clustered regularly interspaced short palindromic repeat; HC, healthy control; MS, multiple sclerosis; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; QC, quality control.

faecal DNA samples (289 HC subjects, 111 patients with RA, 47 patients with SLE, and 29 patients with MS), which passed stringent QC for sequencing reads and samples (online supplemental figure 1). Detailed characteristics of the participants are shown in online supplemental table 1). Contigs were assembled from the extracted QC sequencing reads and viral contigs. To extract the viral contigs from the whole-genome shotgun sequencing data, we first extracted candidate viral contigs using VirSorter[41] and VirFinder.[42] Subsequently, we removed potential bacterial contaminations using BUSCO,[43] and VPF,[44] which resulted in 93,254 viral contigs (on average, 195.9 viral contigs per sample). We then performed taxonomic annotation and quantification of each viral contig. Among the viral contigs, 65,201 (69.9%) were classified at the family level. Consistent with previous reports,[21–23] *Caudovirales* including *Siphoviridae*, *Myoviridae*, *Podoviridae*, and unclassified *Caudovirales* were highly abundant among the gut virome (average: 38.2%, 12.4%, 2.9% and 12.2%, respectively, figure 1B), suggesting that our

Table 1 Result of the case–control comparison of the viral abundance for RA, SLE and MS

	RA (N_{case}=111 and $N_{control}$=111)			SLE (N_{case}=47 and $N_{control}$=201)			MS (N_{case}=29 and $N_{control}$=74)		
Viruses	**Effect size**	**SE**	***P***	**Effect size**	**SE**	***P***	**Effect size**	**SE**	***P***
Autographiviridae	−0.051	0.293	0.86	0.257	0.338	0.45	−0.380	1.052	0.72
crAss-like phage	−0.476	0.173	0.0060**	−0.514	0.206	0.012*	0.425	0.488	0.38
Herelleviridae	−0.579	0.320	0.070	−0.109	0.311	0.73	1.730	1.278	0.18
Microviridae	0.155	0.231	0.50	0.265	0.279	0.34	0.729	0.781	0.35
Myoviridae	0.657	0.672	0.33	−0.753	0.858	0.38	−1.908	2.108	0.37
Phycodnaviridae	0.049	0.256	0.85	−0.513	0.273	0.060	−0.004	0.567	0.99
Podoviridae	−0.147	0.325	0.65	−0.947	0.330	0.0041**	0.893	1.041	0.39
Siphoviridae	−0.986	1.437	0.49	−3.710	1.487	0.013*	−0.667	3.417	0.85

*P<0.05, **P<0.01.
MS, multiple sclerosis; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.

pipeline successfully reconstructed the viral abundance from shotgun sequencing data. We performed QC of the family-level viral clades for subsequent case–control comparison. After clade QC, eight viral families were included in the association test. We evaluated the effects of age and sex on the viral abundance (online supplemental table 3). We found no significant association between age and sex, and the viral abundances ($P > 0.05 / 8 = 0.0063$).

Case–control comparison of the viral abundance for RA, SLE and MS

We performed case–control comparison for the eight QC-passed viral families in each autoimmune disease (table 1) using logistic regression with adjustments for age, age^2, sex, sequencing batch and total sequencing length. We found that crAss-like phages significantly decreased in the metagenome of the patients with RA ($P < 0.05 / 8 = 0.0063$; figure 2A). As the medications of the patients with RA and male–female imbalance due to sex-biased prevalence could be confounding factors, we performed sub-analysis (online supplemental table 4). Even after removing the male subjects, non-new onset patients, or those who took medications such as proton pump inhibitors, steroids, and any therapeutics for RA, crAss-like phages still decreased in the gut metagenome of the patients with RA (effect size = −0.568, –0.385, −0.441, –0.475 and −0.520, respectively). A comparison within the case revealed that the abundance of crAss-like phages was not significantly affected by the treatment, timing of the onset, disease activity of RA, and an inflammation marker (effect size = −0.008, 0.313, 0.018, and 0.291; $P = 0.97$, 0.25, 0.93 and 0.20, respectively; online supplemental table 4). In the gut metagenome of the patients with SLE, *Podoviridae* significantly decreased ($P < 0.05 / 8 = 0.0063$; table 1), and crAss-like phages nominally decreased ($P < 0.05$; figure 2A, table 1). Similar to the patients with RA, we performed subanalysis. Although the p values increased in the subanalysis, which was possibly due to the decreased sample size, the effect size was consistent (online supplemental table 5). Abundances of crAss-like phages and *Podoviridae* in the gut metagenome of the patients with SLE were not significantly affected by the treatment, timing of the onset, and disease activity of SLE (effect size = −0.806, –0.593, and −1.147; $P = 0.15$, 0.31 and 0.070 for crAss-like phages; effect size = −0.289,–0.165 and 0.313; $P = 0.63$, 0.77 and 0.67 for *Podoviridae*, respectively; online supplemental table 5). None of the viral families, including crAss-like phages that decreased in the metagenome of both RA and SLE, were associated with MS (figure 2A, table 1).

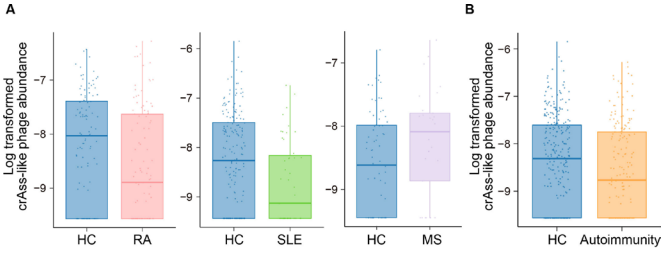

Figure 2 Case–control comparison of the crAss-like phage abundance. (A) Case–control comparison of the crAss-like phage abundance for RA (left), SLE (middle) and MS (right). Boxplots indicate the median values (centre lines) and IQRs (box edges), with the whiskers extending to the most extreme points within the range between (lower quantile−(1.5×IQR)) and (upper quantile+(1.5×IQR)). (B) Case–control comparison of the crAss-like phage abundance for autoimmunity. Boxplots indicate the median values (centre lines) and IQRs (box edges), with the whiskers extending to the most extreme points within the range between (lower quantile−(1.5×IQR)) and (upper quantile+(1.5×IQR)). HC, healthy control; MS, multiple sclerosis; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.

crAss-like phages decreased in the gut metagenome of combined autoimmune diseases (RA, SLE and MS)

Autoimmune diseases are known to have a shared aetiology, and a combined analysis of multiple autoimmune diseases is useful to reveal the hidden aetiology with increased statistical power.[47] A shared gut microbial component of the autoimmune diseases had been inferred from previous studies,[9] suggesting that there could also be a virus-related and shared aetiology among autoimmune diseases. Thus, we performed a combined case–control comparison of the viral abundance in RA, SLE and MS.

Through logistic regression of all the HC subjects and the combined autoimmune disease patients (RA, SLE and MS), we found that the abundance of crAss-like phages was significantly decreased in the combined autoimmune diseases (figure 2B, table 2). In addition, *Siphoviridae* nominally decreased in the combined autoimmune disease patients. We performed a sub-analysis for certifying the significant association between crAss-like phages and combined autoimmune diseases. Even after removing male subjects, non-new-onset patients, or those who took medications such as antibiotics, proton pump inhibitors, steroids, and any therapeutics for combined autoimmune diseases, crAss-like phages decreased with the consistent effect sizes in the gut metagenome of the combined autoimmune diseases patients (effect size = −0.464, –0.534, −0.387, –0.417, −0.406 and −0.549, respectively; online supplemental table 6). When sequencing batch 4 was removed, which was composed

Table 2 Results of the case–control comparison of the viral abundance for autoimmunity

Viruses	Effect size	SE	*P*
Autographiviridae	−0.048	0.216	0.82
crAss-like phage	−0.429	0.126	6.5×10^{-4}**
Herelleviridae	−0.253	0.220	0.25
Microviridae	0.193	0.176	0.27
Myoviridae	0.057	0.555	0.92
Phycodnaviridae	−0.101	0.163	0.53
Podoviridae	−0.304	0.219	0.17
Siphoviridae	−1.956	0.959	0.041*

*P<0.05, **P<0.01.

of the HC subjects and the patients with MS, the association became more significant compared with that of the analysis with all the sequencing batches (effect size = −0.499, $P = 2.6 \times 10^{-4}$), suggesting that the significant decrease in crAss-like phages was driven by RA and SLE. A comparison between the treated patients and the untreated patients, or the new-onset patients and the non-new onset patients showed no significant difference (effect size = −0.087 and −0.007; $P = 0.63$ and 0.97, respectively; online supplemental table 6). We also performed a stratified analysis for each sequencing batch (sequencing batch 1–5). Although the p values became less significant due to the decreased sample size, the directions of the effect were consistent in all the batches, except for the batch 4 (online supplemental table 6), suggesting that the detected association was not solely dependent on a specific dataset. To take into account of the heterogeneity among the patients with RA, SLE and MS, we performed a random-effect meta-analysis (online supplemental table 7). Again, we detected a significant association between the combined autoimmune diseases in a random-effect model (effect size = −0.392, $P = 0.0047$) with non-significant heterogeneity among the diseases ($Q = 3.03$ and P for $Q = 0.22$). The Association between crAss-like phages and combined autoimmune diseases was detected even when the viral abundance was transformed to the absence/presence state (effect size = −0.734, $P = 0.0046$; online supplemental table 8), which was independent of the quantification or normalisation method.

In our study, some crAss-like phages, which had nucleotide-level similarity to NC_024711.1, were identified. We performed a case–control comparison of NC_024711.1 and other crAss-like phages in combined autoimmune diseases, which revealed that both NC_024711.1 and other crAss-like phages were significantly associated with combined autoimmune diseases ($P = 0.0036$ and $P = 0.019$, respectively; online supplemental table 9). crAss-like phages are bacteriophages that were first identified in 2014 by the cross-assembly of metagenomic sequencing reads.[24] crAss-like phages occupy a significant part of the healthy gut virome, however, associations between crAss-like phages and human diseases have never been identified before.[48]

Virus–bacterium coabundance between *Podoviridae* and *Faecalibacterium*

Bacteriophages impact our health via virus–bacterium interactions by modifying the abundance or function of their bacterial hosts. Thus, a comprehensive study of virus–bacterium interactions is useful for revealing the virus-related pathologies of autoimmune diseases. We performed a virus–bacterium association analysis based on the abundance of viruses and bacteria of all samples. We first quantified bacterial abundance using nonviral reads (figure 1A, online supplemental figure 2). For

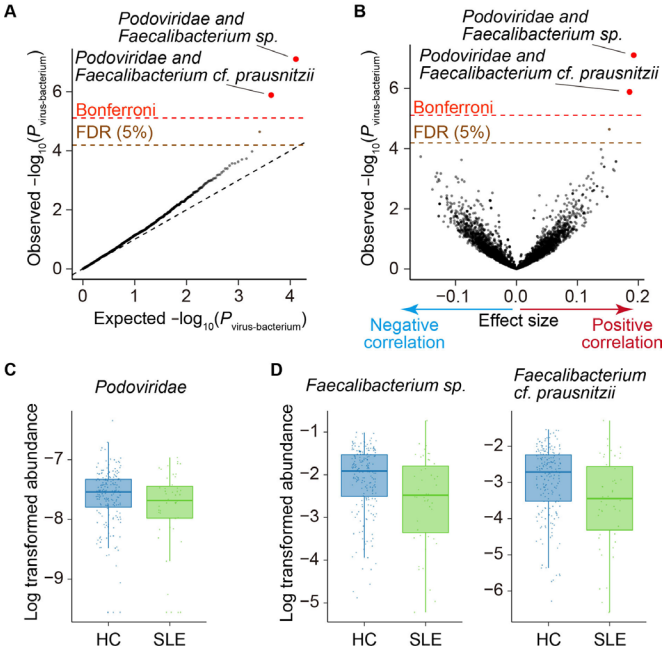

Figure 3 Virus–bacterium coabundances in the gut microbiome. (A) A quantile–quantile plot of the p values from the virus–bacterium association analysis ($P_{\text{virus-bacterium}}$). The x-axis indicates $-\log_{10}(P_{\text{virus-bacterium}})$ expected from uniform distribution. The y-axis indicates the observed $-\log_{10}(P_{\text{virus-bacterium}})$. The diagonal dashed line represents $y=x$, which corresponds to the null hypothesis. The horizontal red dashed line indicates the Bonferroni-corrected threshold (α=0.05), and the brown dashed line indicates the FDR threshold (FDR=0.05) calculated using the Benjamini–Hochberg method. The virus–bacterium pairs with p value of less than the Bonferroni thresholds are plotted as red dots. The virus–bacterium pairs with FDR of <0.05 are plotted as brown dots, and other virus–bacterium pairs are plotted as black dots. (B) A volcano plot. The x-axis indicates the effect sizes in linear regression. The y-axis, horizontal dashed lines and dot colours are the same as in (A). (C) Case–control comparison of the *Podoviridae* abundance for SLE. Boxplots indicate the median values (centre lines) and IQRs (box edges), with the whiskers extending to the most extreme points within the range between (lower quantile−(1.5×IQR)) and (upper quantile+(1.5×IQR)). (D Case–control comparison of the *Faecalibacterium* spp and *Faecalibacterium cf. prausnitzii* abundance for SLE. Boxplots indicate the median values (centre lines) and IQRs (box edges), with the whiskers extending to the most extreme points within the range between (lower quantile−(1.5×IQR)) and (upper quantile+(1.5×IQR)). FDR, false discovery rate; HC, healthy control; SLE, systemic lupus erythematosus.

the virus–bacterium association analysis, we performed bacterial clade QC. Afterward, 802 bacterial clades were included in the association test.

We then evaluated the association between 8 viral families and 802 bacterial clades. The abundances of two bacterial clades, *Faecalibacterium* spp and *Faecalibacterium* cf. *prausnitzii*, were positively correlated with the abundance of *Podoviridae* (effect size = 0.192, $P_{\text{virus-bacterium}} = 7.9 \times 10^{-8}$ for *Faecalibacterium* spp; effect size = 0.186, $P_{\text{virus-bacterium}} = 1.3 \times 10^{-6}$ for *Faecalibacterium*. cf. *prausnitzii*; figure 3A,B, online supplemental table 10, online supplemental file, satisfying a Bonferroni threshold ($\alpha < 0.05$; $P_{\text{virus-bacterium}} < 7.8 \times 10^{-6}$). Even after removing male subjects, non-new onset patients, patients with combined autoimmune disease, or those who took medications such as antibiotics, proton pump inhibitors, steroids and any therapeutics for

autoimmune diseases, the effect sizes of the association between *Faecalibacterium* and *Podoviridae* were consistent (online supplemental table 11). We performed a virus–bacterium association analysis with interaction term between diseases and viruses (online supplemental figure 3) but could not find significant disease-specific virus–bacterium associations.

In our case–control comparison of the viral abundance, *Podoviridae* significantly decreased in the gut metagenome of the patients with SLE (figure 3C, table 1). *Faecalibacterium* spp and *Faecalibacterium cf. prausnitzii*, which were positively associated with *Podoviridae*, also tended to decrease in the gut metagenome of the patients with SLE (figure 3D). In the bacterial microbiome-wide association study of the patients with SLE conducted with subsets of dataset,[34] (sequencing batch 1, 2 and 5), nonsignificant decreases of *Faecalibacterium* spp and *Faecalibacterium cf. prausnitzii* were observed (effect size = −0.04, P = 0.79; effect size = −0.11, P = 0.50, respectively). *Faecalibacterium* is a bacterial genus that produces short-chain fatty acids, which have anti-inflammatory activity.[49] Changes in the abundance of *Faecalibacterium* were frequently reported in immune-related diseases or conditions.[49–51] Thus, our results suggest that the symbiotic network of *Podoviridae* and *Faecalibacterium* is important to keep the homeostasis of the immune system.

Viral host detection based on the CRISPR spacer sequences

To further elucidate the virus–bacterium interaction, we analysed the CRISPR loci of bacterial contigs from all samples to identify infected viruses. We detected 28,347 CRISPR spacer sequences in total (601.6 CRISPR spacer sequences per sample) by MinCED,[46] and identified 834 unique taxonomically annotated virus–host interactions from 476 gut metagenomes (online supplemental figure 4).

Consistent to a previous report of healthy Japanese subjects,[21] virus–host interactions involving *Caudovirales* such as *Siphoviridae*, *Myoviridae* and *Podoviridae* were frequently observed. In addition, the preferred hosts for each viral family (eg, *Podoviridae* preferentially infected to *Actinobacteria*, including *Bifidobacterium*, while *Myoviridae* did not; *Microviridae* preferentially infected *Bacteroidetes*, including *Bacteroides* compared with other viral families.) were also consistent with the previous report.

To reveal the autoimmunity-associated virus–bacterium interaction, we searched the hosts of autoimmunity-associated viruses, crAss-like phages and *Podoviridae* (table 3). As for *Podoviridae*, interactions with *Faecalibacteriums* spp via the CRISPR–Cas system were identified from a sample. Manual inspection of a contig supporting this interaction revealed that this contig aligned to a common region across *Faecalibacterium* (*Faecalibacterium* spp, *Faecalibacterium cf. prausnitzii and Faecalibacterium prausnitzii*). This result supported the symbiotic relationship between *Podoviridae* and *Faecalibacterium*, which was suggested from the quantitative virus–bacterium association analysis.

It was reported that crAss-like phages, especially those closely related to the first identified *crAssphage* (NC_024711.1), infected to *Bacteroidetes* from multiple lines of evidence.[24 52] In our results, a predicted host of NC_024711.1 was *Bacteroides vulgatus*. Fujimoto *et al* reported that crAss-like phages, which had nucleotide-level similarity to NC_024711.1 dominantly infected to *Bacteroidetes*, while other crAss-like phages could also infect to *Firmicutes*.[21] In line with those results, crAss-like phages other than NC_024711.1 infected to various *Firmicutes* in our analysis. Among the virus–host interaction involving crAss-like phages, *Ruminococcus* spp infection was detected in three independent samples. Therefore, we detected several hosts of the bacteriophages which decreased in autoimmune diseases.

DISCUSSION

In this study, we revealed the disease-associated changes in the gut virome of Japanese patients with autoimmune disease. We discovered that crAss-like phages in the gut of the patients with RA and SLE decreased. In addition, *Podoviridae* also decreased in the gut of the patients with SLE. Our quantitative virus–bacterium association analyses revealed a positive correlation between *Podoviridae* and *Faecalibacterium* abundance, and a direct evidence of phage infection was provided by CRISPR-based virus–bacterium interaction analyses. *Bacteroidetes* and *Firmicutes*, including *Ruminococcus* spp, were detected as hosts of crAss-like phages, which were replicated in multiple samples.

We obtained the gut virome abundance data from the whole-metagenome shotgun sequencing reads. The resulting viral composition, characterised by highly abundant *Caudovirales* that included *Siphoviridae*, *Myoviridae* and *Podoviridae*, was mostly consistent with previous reports,[21–23] indicating that our pipeline successfully worked. This result indicated the advantage of whole-metagenome shotgun sequencing, which could be applied for both the bacteriome and virome analyses. Although the abundance of *Microviridae* was lower than that in previous studies performed with VLP-sequencing,[21 23] it could have been due to the difference in the sequencing and library preparation methods. In the gut, bacteriophages can present as both free viral particles and infecting phages, which include prophages that are integrated in the bacterial genome. Although VLP-sequencing mainly captures free viral particles, whole-metagenome shotgun sequencing mainly captures actively infecting viruses and integrated prophages.[11] In addition, the quantification of single-stranded DNA (ssDNA) viruses including *Microviridae* was strongly dependent on the library preparation method, specifically the multiple displacement amplification technique that is frequently used in VLP-sequencing and is known to show an upward bias of the ssDNA viral abundance.[11] Gregory *et al*

Table 3 Infectious targets of crAss-like phages and *Podoviridae* based on CRISPR spacers

Viruses	Bacteria
crAss-like phage	*Anaerobutyricum hallii*, Bacteroides vulgatus, *Blautia* spp, *Dorea longicatena*, *Eubacterium limosum*, Ruminococcus spp
Podoviridae	*Akkermansia muciniphila*, *Akkermansia* spp, *Bacteroides plebeius*, *Bacteroides* spp, *Bacteroides uniformis*, *Bifidobacterium adolescentis*, *Bifidobacterium bifidum*, *Bifidobacterium dentium*, *Bifidobacterium longum*, *Bifidobacterium pseudocatenulatum*, *Bifidobacterium* spp, *Blautia obeum*, *Blautia* spp, *Burkholderiales bacterium*, *Clostridium clostridioforme*, *Clostridium spp*, *Coprobacillus* spp, *Dialister invisus*, *Eubacterium rectale*, *Eubacterium ventriosum*, Faecalibacterium spp, *Firmicutes bacterium*, *Gemmiger formicilis*, *Holdemanella biformis*, *Parabacteroides distasonis*, *Parabacteroides merdae*, *Porphyromonas* spp, *Ruminococcus bromii*, *Ruminococcus* spp, *Streptococcus salivarius*, *Sutterella wadsworthensis*

The underlined bacteria have the following characteristics (see DISCUSSION): (1) *Bacteroides vulgatus* belongs to phylum *Bacteroidetes* which is a predicted host of crAss-like phages.[24] (2) *Ruminococcus* spp belongs to genus *Ruminococcus* which is reported to be associated with RA,[55] and SLE.[54] (3) *Faecalibacterium* spp is also associated with *Podoviridae* also in the quantitative virus–bacterium association analysis and reported to be associated with autoimmune diseases.[50 51]
CRISPR, clustered regularly interspaced short palindromic repeat.

compared the VLP-sequencing data with the shotgun sequencing data and revealed that there was a large difference in the reconstructed viral populations, and the composition of ssDNA viruses recovered from shotgun sequencing data was consistently lower than that of the VLP-sequencing data.[22] In this study, we used the whole-metagenome shotgun sequencing method to focus on actively infecting phages and prophages which occupied the majority of the gut virome and actively participated in virus–bacterium interaction. However, future integrative analysis with VLP-sequencing might result in a more comprehensive understanding of the gut virome.

crAssphage was first identified in 2014 through the cross-assembly of shotgun sequencing data from multiple faecal samples.[24] Although *crAssphage* frequently existed in the healthy gut virome, a large fraction of its proteins showed no similarity to the already existing viral proteins. Thus, *crAssphage* was considered as comprising a new taxonomic group (crAss-like phage). Since crAss-like phages were discovered, its biological features including taxonomy and infective hosts,[52 53] were extensively studied. Although the identification of the association between *crAssphage* and disease states have been warranted, there had been no reports on the relationships between *crAssphage* and diseases.[48] In this study, the case–control comparison of the viral abundance revealed that the amount of crAss-like phages decreased in the gut metagenome of the patients with autoimmune diseases, specifically RA and SLE. In our dataset, the sample size of the patients with MS was relatively smaller than those with RA and SLE due to the rarity of MS in Japan. Therefore, future larger-scale analyses are warranted to analyse the relationship between viruses and MS with more statistical power.

In our CRISPR-based virus–bacterium interaction analysis, *Bacteroides vulgatus* and various *Firmicutes* were detected as hosts of crAss-like phages. *Bacteroidetes* was originally predicted as a host of crAss-like phages by read co-occurrence analysis and CRISPR-based analysis.[24] Thus, the detection of *Bacteroides vulgatus* as a host of *crAssphage* (NC_024711.1) was reasonable. Although not detected in our CRISPR-based analysis, *Bacteroides intestinalis* was also a host of crAss-like phages, which was validated in vitro.[53] In our quantitative virus–bacterium interaction analysis, the abundance of crAss-like phages and *Bacteroides intestinalis* was nominally correlated (effect size = 0.083 and $P_{\text{virus-bacterium}} = 0.0078$), suggesting that the virus–host relationship between the two existed in our dataset. In previous bacterial case–control comparisons,[32 34] there was a nominally significant negative association between *Bacteroides intestinalis* and SLE (effect size = −0.412, $P = 0.033$), although the association was not significant for RA (effect size = 0.056, $P = 0.76$).

Among the virus–host interactions involving crAss-like phages, infection of *Ruminococcus* spp was detected in three independent samples. The 16S rRNA sequencing study of the patients with SLE,[54] and autoimmune arthritis model mice,[55] suggested that the genus *Ruminococcus* was related to the SLE and RA pathology.

Recently, Yutin *et al* performed a CRISPR-based virus–host association analysis for crAss-like phages,[56] and *Prevotella copri*, which associated with the pathogenesis of RA,[48] was also detected as a host of crAss-like phages. Collectively, these results have suggested that crAss-like phages are associated with the autoimmune diseases, possibly via the interaction with the host's immune system or the modulation of the biological properties of various bacteria. As the abundance of crAss-like phages is highly variable across different areas,[57] studies in other areas are warranted.

Podoviridae significantly decreased in the gut metagenome of the patients with SLE. Both the quantitative and CRISPR-based virus–bacterium interaction analyses suggested the symbiosis of *Podoviridae and Faecalibacterium*. *Faecalibacterium* is known beneficial bacteria as they produce short-chain fatty acids.[58] Among the *Faecalibacterium*, *Faecalibacterium prausnitzii* was the most intensively studied and is associated with many conditions, including autoimmune diseases.[50 51] While there is the possibility that the decreased *Podoviridae* affected *Faecalibacterium* and vice versa, the effect of *Podoviridae* on the biological property of *Faecalibacterium* should be experimentally tested in the future.

In summary, our whole gut virome case–control study revealed a previously unknown part of the link between the gut microbiome and autoimmune diseases. A comprehensive understating of the gut microbiome, including its virome, deepens the insights of the pathogenesis of autoimmune diseases.

URLs

bcl2fastq, https://support.illumina.com/sequencing/sequencing_software/bcl2fastq-conversion-software/downloads.html

Trimmomatic, http://www.usadellab.org/cms/?page=trimmomatic bowtie2, http://bowtie-bio.sourceforge.net/bowtie2/index.shtml

BMTagger, ftp://ftp.ncbi.nlm.nih.gov/pub/agarwala/bmtagger/

PRINSEQ, http://prinseq.sourceforge.net/

MEGAHIT, https://github.com/voutcn/megahit

VirSorter, https://github.com/simroux/VirSorter

VirFinder, https://github.com/jessieren/VirFinder

BUSCO, https://busco.ezlab.org hmmer, http://hmmer.org/download.html ncbi-blast-plus, https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastDocs&DOC_TYPE=Download

DIAMOND, https://github.com/bbuchfink/diamond coverM, https://github.com/wwood/CoverM

Prodigal, https://github.com/hyattpd/Prodigal

MinCED, https://github.com/ctSkennerton/minced

Samtools, http://www.htslib.org/download/ bedtools, https://github.com/arq5x/bedtools2

R, https://www.r-project.org

Python, https://www.python.org/downloads/release/python-376/

Seqkit, https://bioinf.shenwei.me/seqkit/download/

RefSeq (Virus), https://www.ncbi.nlm.nih.gov/labs/virus/vssi/#/

VPF, https://portal.nersc.gov/dna/microbial/prokpubs/EarthVirome_DP/

Author affiliations
[1]Department of Statistical Genetics, Osaka University Graduate School of Medicine, Suita, Osaka, Japan
[2]Department of Otorhinolaryngology-Head and Neck Surgery, Osaka University Graduate School of Medicine, Suita, Japan
[3]Department of Head and Neck Surgery, Aichi Cancer Center Hospital, Nagoya, Japan
[4]Department of Respiratory Medicine and Clinical Immunology, Osaka University Graduate School of Medicine, Suita, Japan
[5]Laboratory of Immune Regulation, Department of Microbiology and Immunology, Osaka University Graduate School of Medicine, Suita, Japan
[6]Integrated Frontier Research for Medical Science Division, Institute for Open and Transdisciplinary Research Initiatives, Osaka University, Suita, Japan
[7]Department of Neurology, Osaka University Graduate School of Medicine, Suita, Japan
[8]Department of Pediatrics, Osaka University Graduate School of Medicine, Suita, Osaka, Japan
[9]Laboratory of Statistical Immunology, Immunology Frontier Research Center (WPI-IFReC), Osaka University, Suita, Japan
[10]Department of Neurology, Suita Municipal Hospital, Suita, Japan

[11]Department of Infection Metagenomics, Research Institute for Microbial Diseases, Osaka University, Suita, Japan
[12]Rheumatology and Allergology, NHO Osaka Minami Medical Center, Kawachinaga, Japan
[13]WPI Immunology Frontier Research Center, Osaka University, Suita, Japan
[14]Department of Immunopathology, Immunology Frontier Research Center, Osaka University, Suita, Japan
[15]Laboratory for Systems Genetics, RIKEN Center for Integrative Medical Sciences, Yokohama, Japan

Twitter Yoshihiko Tomofuji @YTomofuji_1107

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Contributors YT, TK and YO designed the study, and conducted the data analysis. YT and YO wrote the manuscript. YT, TK, YMae, TN, EO-I, DM, YMat and SN conducted the experiments. YT, TK, YMae, KO, TN, TO, EO-I, MK, KY, KS, MYa, AH, HMa, MYo and SO collected the samples. HI, HMo, KT, AK and YO supervised the study. YO is responsible for the overall content as guarantor. All authors contributed to the article and approved the submitted version.

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Patient consent for publication Not applicable.

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Provenance and peer review Not commissioned; externally peer reviewed.

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ORCID iDs
Yuichi Maeda http://orcid.org/0000-0002-6831-8205
Eri Oguro-Igashira http://orcid.org/0000-0001-5863-0775
Yukinori Okada http://orcid.org/0000-0002-0311-8472

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Clinical characteristics and outcomes of COVID-19 breakthrough infections among vaccinated patients with systemic autoimmune rheumatic diseases

SARS-CoV-2 vaccines reduce the risk of COVID-19.[1–3] However, some disease-modifying anti-rheumatic drugs (DMARDs), particularly glucocorticoids, methotrexate, mycophenolate mofetil and rituximab, may blunt the immunological response to COVID-19 vaccination.[4] Little is known about the clinical efficacy of these vaccines at preventing COVID-19 infection in patients with systemic autoimmune rheumatic diseases (SARDs).

Mass General Brigham (MGB) is a large multicentre healthcare system in the Boston, Massachusetts, USA area. Patients with SARDs with a positive SARS-CoV-2 PCR or antigen test between 30 January 2020 and 30 July 2021 at MGB were identified using diagnostic billing codes or were referred by physicians, as previously described.[5] From this cohort, we identified breakthrough infections in fully vaccinated patients, defined as a positive test ≥14 days after the final vaccine dose.[6]

Of 786 SARD patients with COVID-19, 340 occurred after the initial emergency use authorisation for COVID-19 vaccination in the USA. Of these, 16 (4.7%) were breakthrough infections (online supplemental figure 1). Among the breakthrough infections, 12 (75%) were female, 11 (69%) were white, the median age was 50 years and 12 (75%) had ≥1 comorbidity (table 1). The most common SARDs included rheumatoid arthritis (6, 38%), inflammatory myositis (3, 19%) and systemic lupus erythematosus (3, 19%). Rituximab (5, 31%), glucocorticoids (5, 31%), mycophenolate mofetil or mycophenolic acid (4, 25%) and methotrexate (3, 19%) were the most frequent immunosuppressives recorded prior to first vaccine dose. One (6%) patient was on no DMARD or glucocorticoid at the time of his/her vaccine.

Seven (44%) patients received the BNT162b2 (Pfizer-BioNtech) vaccine, five (31%) received the mRNA-1273 (Moderna) vaccine and four (25%) received the AD26.COV2.S (Janssen/Johnson & Johnson) vaccine. The median time from final vaccine dose to infection was 54 days (table 1). Among the 16 breakthrough infections, 15 (93%) were symptomatic and 6 (38%) patients were hospitalised, during which 4 (25%) required supplemental oxygen and 1 (6%) required mechanical ventilation (online supplemental table 1). DMARDs used prior to infection among hospitalised patients included rituximab (4, 25%) and mycophenolate mofetil or mycophenolic acid (2, 13%). Two (13%) patients died; both deceased patients had received rituximab and had interstitial lung disease.

In conclusion, a small portion of COVID-19 cases among patients with SARDs in a large US healthcare system occurred among fully vaccinated patients. However, some patients required hospitalisation that ultimately culminated in death. The most common SARD treatments at the time of vaccination included those associated with blunted antibody responses to SARS-CoV-2 vaccination.[4] These findings suggest that the blunted SARS-CoV-2 antibody response following COVID-19 vaccination in certain DMARD users may be associated with an increased risk of breakthrough infections that may be severe and even fatal. Of note, the blunted response observed among glucocorticoid users is dose dependent, especially above 10 mg/day of prednisone. Some DMARD users may require

Table 1 Patient characteristics, vaccination details, medication use and infection details of COVID-19 breakthrough infections in fully vaccinated patients with SARDs (nN=16)

Patient characteristics	n, %
Female	12, 75
Age (median, IQR)	49.5 (38.0–64.5)
Race	
White	11, 68
Black	3, 20
Hispanic	2, 13
Rheumatic disease*	
Rheumatoid arthritis	6, 38
Rheumatoid arthritis-associated interstitial lung disease	1, 6
Dermatomyositis	3, 19
Myositis-associated interstitial lung disease	1, 6
Systemic lupus erythematosus	3, 19
Ankylosing spondylitis	2, 13
IgG4-related disease	1, 6
Mixed connective tissue disease	1, 6
Hypocomplementemic urticarial vasculitis	1, 6
Psoriatic arthritis	1, 6
Comorbidities*	
Hypertension	6, 38
Morbid obesity (BMI ≥40.0 kg/m^2)	3, 19
Interstitial lung disease	2, 13
End-stage renal disease	2, 13
Chronic obstructive pulmonary disease	1, 6
Asthma	1, 6
Diabetes	1, 6
Obesity (BMI ≥30.0 kg/m^2)	1, 6
Coronary artery disease	1, 6
Cancer	1, 6
Organ transplant	1, 6
Immunodeficiency	1, 6
Chronic neurological or neuromuscular disease	1, 6
Inflammatory bowel disease	1, 6
None	4, 25
Smoking status	
Current	1, 6
Former	7, 44
Never	6, 38
Unknown	2, 13
Vaccination details	**n, %**
Vaccine type	
Pfizer-BioNtech	7, 44
Moderna	5, 31
Janssen/Johnson & Johnson	4, 25
Disease activity at vaccination	
First vaccination	
Active	5, 31
Inactive	11, 69
Second vaccination	
Active	6, 38
Inactive	6, 38
Not applicable	4, 25
Rheumatic disease treatment use prior to first vaccine dose†	**n, %**
Rituximab	5, 31
Glucocorticoids	5, 31
Mycophenolate mofetil or mycophenolic acid	4, 25
Methotrexate	3, 19
Tacrolimus	2, 13
Adalimumab	1, 6

Continued

Table 1 Continued

Patient characteristics	n, %
Azathioprine	1, 6
Belimumab	1, 6
Hydroxychloroquine	1, 6
Intravenous immunoglobulin	1, 6
Sulfasalazine	1, 6
Tocilizumab	1, 6
Ustekinumab	1, 6
None	1, 6
COVID-19 infection details	**n, %**
Time (days) from second/final vaccine dose to infection (median, IQR)	54.0 (29.8–79.0)
Infection acquisition	
Close contact with confirmed or probable case of COVID-19	4, 25
Presence in a healthcare facility with COVID-19 cases	3, 19
Community acquired	3, 19
Unknown	8, 50
Treatment	
No treatment/supportive care only	7, 44
Remdesivir	6, 38
Glucocorticoids	3, 19
Neutralising monoclonal antibody	4, 25
Azithromycin	2, 13
Convalescent plasma	1, 6
Enrolled in clinical trial‡	1, 6
Any symptoms	
Yes	15, 93
No§	1, 6
Symptoms	
Fever	9, 56
Cough	7, 44
Malaise	6, 38
Myalgia	5, 31
Rhinorrhea	5, 31
Headache	4, 25
Shortness of breath	4, 25
Sore throat	4, 25
Diarrhoea/vomiting/nausea	3, 19
Anosmia	3, 19
Dysgeusia	3, 19
Chest pain	2, 13
Arthralgia	1, 6
Other	1, 6
Outcomes¶	**n, %**
Outpatient management alone	10, 63
Hospitalisation	6, 38
Ventilation	1, 6
Death	2, 13
Unresolved symptoms	2, 13

*Patients may have >1 SARD or comorbidity.
†One patient initiated methotrexate in between the first and second dose and one patient initiated rituximab between the second dose and infection.
‡NCT04501978; phase 3 randomised, blinded, trial assessing treatments for hospitalised patients with COVID-19. Intervention arms: investigational drug +standard of care (remdesivir) or placebo +standard of care (remdesivir).
§Diagnosed via pre-procedure PCR test, no reported symptoms in electronic health record.
¶Symptoms were unresolved (one active infection recent diagnosed, one reporting ongoing symptoms: fatigue/malaise) in two (13%) cases.
BMI, body mass index; SARD, systemic autoimmune rheumatic disease.

alternative risk mitigation strategies, including passive immunity or booster vaccines and may need to continue shielding practices.

Our study has certain limitations. First, we did not study the risk of breakthrough infections among a cohort of vaccinated patients with a known denominator. Therefore, we cannot estimate the rate of breakthrough infections among patients with SARDs. It is possible that the observed number of cases might be expected since no vaccine will prevent every infection. Second, the proportion of asymptomatic breakthrough infections observed in our study may be an underestimate because we only included patients who presented for testing. Third, we did not have SARS-CoV-2 antibody testing available for all patients and cannot rule out the possibility that SARD manifestations (eg, interstitial lung disease) commonly treated with these medications contributed to the severity of the presentation.

In light of our findings, additional studies are urgently needed to estimate the risk of breakthrough infections among patients with SARDs and to evaluate the efficacy of booster vaccines and other strategies for DMARD users with poor immunological response to COVID-19 vaccination.

Claire Cook,[1] Naomi J Patel,[1,2] Kristin M. D'Silva,[1,2] Tiffany Y -T Hsu ,[2,3] Michael Dilorio,[2,3] Lauren Prisco,[3] Lily W Martin,[3] Kathleen Vanni,[3] Alessandra Zaccardelli,[3] Derrick Todd,[2,3] Jeffrey A Sparks ,[2,3] Zachary Scott Wallace [1,2]

[1]Division of Rheumatology, Allergy, and Immunology and Clinical Epidemiology Program, Mongan Institute, Department of Medicine, Massachusetts General Hospital, Boston, Massachusetts, USA
[2]Harvard Medical School, Boston, Massachusetts, USA
[3]Division of Rheumatology, Inflammation, and Immunity, Brigham and Women's Hospital, Boston, Massachusetts, USA

Correspondence to Dr Zachary Scott Wallace, Division of Rheumatology, Allergy, and Immunology and Clinical Epidemiology Program, Mongan Institute, Department of Medicine, Massachusetts General Hospital, Boston, Massachusetts, USA; zswallace@partners.org

Handling editor Josef S Smolen

Twitter Jeffrey A Sparks @jeffsparks

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ORCID iDs
Tiffany Y -T Hsu http://orcid.org/0000-0003-1041-8040
Jeffrey A Sparks http://orcid.org/0000-0002-5556-4618
Zachary Scott Wallace http://orcid.org/0000-0003-4708-7038

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Booster-dose SARS-CoV-2 vaccination in patients with autoimmune disease: a case series

An attenuated humoral response to SARS-CoV-2 vaccination has been observed in some patients with autoimmune disease,[1 2] and immunosuppressed status has been associated with an increased risk of COVID-19 infection despite vaccination.[3] Recent studies have demonstrated enhanced humoral response to third-dose SARS-CoV-2 vaccination in immunosuppressed transplant patients,[4 5] but the immunogenicity of booster vaccination in other immunosuppressed populations is unknown. Thus, we sought to describe the humoral response in patients with autoimmune disease who received a booster SARS-CoV-2 vaccine.

Using our prospective cohort of patients with autoimmune disease,[5] we included patients who reported receipt of a single booster dose of SARS-CoV-2 mRNA or adenovirus vector vaccine between 10 April and 11 June 2021. We observed serial anti-spike antibody responses among these participants.

A total of 18 participants received a booster SARS-CoV-2 vaccine dose (table 1). Most (13/18) were women with median (IQR) age of 55 (44–63) years. The most common autoimmune diagnoses included myositis (n=6) and inflammatory arthritis (n=3). Most (14/18) were on antimetabolite therapy; mycophenolate was the most commonly reported immunosuppressive therapy (n=8), with a median (IQR) daily dose of 3000 mg (2500–3000 mg). Participants completed initial vaccination with either Pfizer (n=8), Moderna (n=6) or Johnson & Johnson/Janssen (J&J) Ad26.COV2.S (n=4).

Anti-spike antibodies, evaluated via Roche Elecsys anti-RBD pan-Ig were negative in 10 participants (anti-RBD <0.8 U/mL) and low-positive (anti-RBD 0.8–500 U/mL) in six participants at a median of 29 (IQR 28–33) days after completion of initial vaccine series with median anti-spike antibody level (IQR) of <0.4 (<0.4–222 U/mL).

Participants underwent booster vaccine at a median of 77 (IQR 54–94) days after completion of initial series. Booster vaccines included single dose of Moderna (n=8), J&J (n=6) or Pfizer (n=4). Nine participants obtained a different vaccine platform (mRNA vs adenovirus) for the additional dose, while the remainder received the same vaccine type.

Repeat anti-spike antibody testing was performed at a median 30 (IQR 27–36) days after booster dose. Eighty-nine per cent of participants had an augmented humoral response following booster vaccination, with median anti-spike antibody level (IQR) of 2500 (885–2500 U/mL) (online supplemental figure 1). Among those negative following initial vaccine series, 80% were positive following the booster dose. All low-positive participants demonstrated high titre response following booster vaccination. Two patients remained negative following booster dose; neither adjusted peri-vaccination immunosuppression and reported taking anti-CD20 therapy and mycophenolate respectively. Most participants (10/15) continued immunosuppression during the initial vaccine series, compared with the minority (5/18) who continued therapy peri-booster.

In this first published series of booster-dose SARS-CoV-2 vaccination in patients with autoimmune disease, augmented antibody response was observed in the majority of participants. De novo antibody response was observed in eight participants, while an additional eight participants demonstrated increased antibody levels.

Our findings of enhanced humoral response to booster-dose SARS-CoV-2 vaccine are similar to those in immunosuppressed solid organ transplant recipients[4 5]; our participants demonstrated a more robust augmentation of humoral response, which may be reflective of baseline immune dysregulation in patients with autoimmune disease as well as the impact of peri-vaccination immunosuppressive management.

This study is limited by observational design, small and inhomogenous sample as well as absence of data on memory B-cell and T-cell response. The augmented effect of booster dose may be confounded by peri-booster pause of immunosuppression. Baseline disease activity or severity was not routinely collected.

Evidence-based approaches to safely optimising immune responses to SARS-CoV-2 vaccination for vulnerable populations are urgently required. While no antibody titre has been defined to correlate with protection, booster dosing may be an option for patients with limited antibody responses to standard vaccine series. The SARS-CoV-2 vaccination schedule may require further refinement in immunosuppressed populations. Further studies are needed to address safety and efficacy of booster vaccination, as well as optimal adjustment in peri-vaccination timing of immunosuppressive therapies; this should be investigated further in a clinical trial setting.

PATIENT AND PUBLIC INVOLVEMENT

Patients were not involved in the design, conduct or dissemination of the study, though this study was motivated by questions frequently posed by patients. The study has a public website (https://vaccineresponse.org/) and email account where we welcomed participants and the public to contact the research team. Results of the study will be shared with national RMD organisations for dissemination to their patient communities once published.

Caoilfhionn M Connolly ,[1] **Mayan Teles,**[2] **Sarah Frey,**[2] **Brian J Boyarsky** ,[2] **Jennifer L Alejo** ,[2] **William A Werbel,**[3] **Jemima Albayda,**[1] **Lisa Christopher-Stine,**[1] **Jacqueline Garonzik-Wang,**[2] **Dorry L Segev,**[2,4] **Julie J Paik** [1]

[1]Division of Rheumatology, Johns Hopkins University, Baltimore, Maryland, USA
[2]Surgery, Johns Hopkins University, Baltimore, Maryland, USA
[3]Infectious Diseases, Johns Hopkins University, Baltimore, Maryland, USA

Table 1 Vaccines administered, autoimmune diagnoses, immunosuppression and peri-vaccination management with longitudinal anti-spike antibody responses

Age	Sex	Diagnosis	Immunosuppressive therapy	Initial vaccine series	Meds held during initial vaccine	Pre-booster antibody	Booster vaccine type	Days from initial to booster vaccine	Post-booster antibody	Therapy held peri-booster*
39	F	Multiple sclerosis	Ocrelizumab	Pfizer	Yes	<0.40	J&J	60	<0.40	No
56	F	Mucous membrane pemphigoid	Mycophenolate	Pfizer	No	<0.40	J&J	47	<0.40	No
43	F	Inflammatory bowel disease†	Mycophenolate Tacrolimus	Pfizer	No	<0.40	Moderna	72	8.9	No
54	F	Myositis	Mycophenolate	Moderna	Yes	<0.40	J&J	98	205	Yes
53	F	Myositis	Methotrexate Hydroxychloroquine Prednisone	Moderna	Yes	<0.40	J&J	86	1111	Yes
56	M	Sarcoidosis	Infliximab Mycophenolate Prednisone	Pfizer	NA‡	<0.40	Moderna	86	1276	Yes
44	F	SLE§	Belimumab Hydroxychloroquine Leflunomide Prednisone	J&J	No	<0.40	Moderna	91	2013	Yes
54	F	Sjogren's syndrome	Azathioprine	J&J	NA‡	<0.40	Pfizer	36	>2500	Yes
75	M	Myositis	Mycophenolate	Pfizer	No	<0.40	Moderna	56	>2500	Yes
66	F	Inflammatory arthritis¶	Abatacept	J&J	No	<0.40	Pfizer	94	>2500	Yes
38	F	Myositis	Azathioprine Prednisone Tacrolimus	Moderna	No	2.7	Moderna	95	>2500	No
59	F	Myositis/scleroderma overlap	Hydroxychloroquine Mycophenolate Prednisone	Moderna	No	8.8	J&J	54	>2500	Yes
53	M	Myositis/inflammatory arthritis overlap	Hydroxychloroquine Mycophenolate	J&J	No	18.6	Pfizer	NA‡	>2500	Yes
72	F	Inflammatory arthritis¶	Methotrexate	Pfizer	No	222.7	J&J	95	>2500	Yes
64	F	Autoimmune hepatitis	Azathioprine Tacrolimus	Moderna	Yes	260	Moderna	83	>2500	Yes
44	M	Inflammatory bowel disease†	Golimumab Methotrexate	Pfizer	NA‡	359.8	Pfizer	68	>2500	Yes
75	F	Autoimmune hepatitis	Mycophenolate	Moderna	No	825.8	Moderna	96	>2500	No
57	M	Inflammatory arthritis¶	Secukinumab	Pfizer	Yes	2418	Moderna	54	>2500	Yes

*Median number (IQR) doses held for mycophenolate 27 (25–28). Fourteen doses of leflunomide held by one patient. Two doses of azathioprine held by two participants. One dose held for abatacept, belimumab, methotrexate and secukinumab respectively.
†Denotes Crohn's disease or ulcerative colitis.
‡Denotes missing data.
§Systemic lupus erythematosus.
¶Rheumatoid arthritis, ankylosing spondylitis, psoriatic arthritis, reactive arthritis or inflammatory bowel disease associated arthritis.
**Roche Elecsys anti-RBD pan-Ig ≥0.8 U/mL is considered positive (upper limit reported as >2500).

[4]Johns Hopkins University Bloomberg School of Public Health, Baltimore, Maryland, USA

Correspondence to Dr Dorry L Segev, Surgery, Johns Hopkins, Baltimore, Maryland, USA; dorry@jhmi.edu

Handling editor Josef S Smolen

Twitter Caoilfhionn M Connolly @CaoilfhionnMD and Jennifer L Alejo @JenLAlejo

Contributors CMC, MT, SF, BJB, JLA, JA, LC-S, WAW, JG-W, DLS, JJP substantially contributed to the conception or design of the work; or the acquisition, analysis or interpretation of data for the work. CMC, MT, SF, BJB, JLA, JA, LC-S, WAW, JG-W, DLS, JJP contributed to drafting the work or revising it critically for important intellectual content. CMC, MT, SF, BJB, JLA, JA, LC-S, WAW, JG-W, DLS, JJP contributed to final approval of the version to be published. CMC, BJB, JLA, DLS, JJP contributed to agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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ORCID iDs
Caoilfhionn M Connolly http://orcid.org/0000-0002-1898-3530
Brian J Boyarsky http://orcid.org/0000-0001-6902-9854
Jennifer L Alejo http://orcid.org/0000-0003-3137-9271
Julie J Paik http://orcid.org/0000-0001-8436-1601

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Temporary hold of mycophenolate augments humoral response to SARS-CoV-2 vaccination in patients with rheumatic and musculoskeletal diseases: a case series

Mycophenolate is the mainstay of treatment for many organ and life-threatening manifestations of rheumatic and musculoskeletal diseases (RMD). In contrast to most patients with RMD, those taking mycophenolate have an attenuated humoral response to SARS-CoV-2 mRNA vaccination.[1 2] The American College of Rheumatology recently recommended withholding mycophenolate for 1 week after vaccination to enhance immunogenicity in this vulnerable population.[3] Thus, we sought to analyse the impact of withholding perivaccination mycophenolate in 24 patients with RMD.

We leveraged our observational prospective cohort of patients with RMD without prior COVID-19 who underwent SARS-CoV-2 vaccination between 17 December 2020 and 13 May 2021.[2] Information on demographics, diagnoses, immunosuppressive regimens and management of perivaccination immunosuppression was collected via electronic questionnaire. One month following vaccination, venipuncture samples were obtained and tested on the semiquantitative Roche Elecsys anti-SARS-CoV-2 S enzyme immunoassay which tests for antibodies against the receptor binding domain (RBD) of the SARS-CoV-2 spike protein; a consistent correlate of neutralising antibody.[4] We compared the percentage of participants with detectable anti-RBD antibody in the group that withheld mycophenolate (n=24) to the group that continued mycophenolate (n=171) using Fisher's exact test (online supplemental table 1). Crude and adjusted logistic regression analyses were performed to assess associations between antibody response and the primary variable of withholding mycophenolate, as well as after adjusting for clinical characteristics (age, sex, race, vaccine type (mRNA vs adenovirus vector), use of rituximab and glucocorticoids). Wilcoxon rank-sum test was used to compare anti-RBD titers of the patients who withheld therapy to those who continued therapy. This study was approved by the Johns Hopkins Institutional Review Board (IRB00248540).

We studied 24 patients who withheld mycophenolate (table 1). Most were female (96%) with a median (IQR) age 51 (40-58) years. Overall, 13% received the Janssen/Johnson and Johnson vaccine while the remainder completed two-dose Pfizer/BioNTech or Moderna mRNA series. The most common diagnoses were systemic lupus erythematosus (25%) and myositis (20%). Most participants reported two times per day dosing of mycophenolate, with a median (IQR) total daily dose of 2000 mg (1625–3000 mg). The median (IQR) number of doses held was 20 (8–34). Thirteen participants (54%) withheld before vaccination, nine (38%) withheld both before and after vaccination, while two (8%) withheld after vaccination. Among those who withheld both before and after vaccination, the majority (seven out of nine) held for the same duration before and after, while the remaining two participants held more doses after vaccination.

At a median (IQR) of 32 (28–35) days after vaccination, 22/24 participants who withheld mycophenolate had detectable antibody response compared with 112/171 who continued therapy (92% vs 65%, p=0.01). Those who withheld therapy were more likely to have a positive antibody response (OR 5.8, 95% CI 1.3 to 25.5 p=0.02). In the adjusted logistic regression model, the association between withholding mycophenolate and positive response remained statistically significant (aOR 7.24, 95% CI 1.72 to 44.31 p=0.01) (online supplemental table 2). Since the rare disease assumption was not met, this OR cannot be interpreted as a relative chance of a positive response. Median anti-RBD Ig titers in the withholding group were significantly higher than the group that continued therapy (125 vs 7 U/L, p=0.004) (online supplemental figure 1). Two participants reported flare of their underlying disease requiring treatment in the perivaccination period; these were treated with topical and oral glucocorticoids, respectively.

In this case series, we describe 24 patients with RMD who withheld mycophenolate in the perivaccination period of whom (92%) had a detectable humoral response, which was more frequent and robust than among participants who continued therapy.

The small sample size did not allow for evaluation of optimal duration of withholding therapy. Further limitations of this study include non-randomised design, lack of data on cellular response and limited information on dosing of other immunosuppressive agents.

These early results suggest that a temporary hold in mycophenolate therapy is safe and augments the humoral response to SARS-CoV-2 vaccination in diverse patients with RMD. Given

Table 1 Clinical characteristics of participants with rheumatic and musculoskeletal diseases who withheld perivaccination mycophenolate

Participant	Age	Sex	Race	Diagnosis	Vaccine type	Mycophenolate dose	Number of doses held	Concurrent therapy	Antibody titre *	Flare
1	36	M	White	CT-ILD†	Moderna	2000 mg	3	No	>250	No
2	62	F	White	CT-ILD†	Pfizer	500 mg	88	Prednisone	>250	No
3	19	F	White	IA‡	Pfizer	1000 mg	5	Abatacept	16	Yes
4	58	F	White	IA‡	Pfizer	2000 mg	28	Tofacitinib	>250	No
5	46	F	White	Myositis	J+J	2000 mg	NA	Prednisone	82	No
6	53	F	White	Myositis	J+J	2500 mg	56	Prednisone	206	No
7	46	F	White	Myositis	Pfizer	3000 mg	20	IVIG§, HCQ¶	40	No
8	54	F	White	Myositis	Pfizer	3000 mg	NA	No	<0.40	No
9	35	F	White	Myositis	Moderna	3000 mg	24	No	>250	No
10	71	F	White	Overlap CTD**	Moderna	2000 mg	4	Rituximab	9.0	No
11	58	F	White	Overlap CTD**	Moderna	2000 mg	9	HCQ¶, Prednisone	8	No
12	55	F	White	Overlap CTD**	Moderna	2000 mg	30	HCQ¶, Prednisone	8	No
13	64	F	White	Overlap CTD**	Pfizer	500 mg	38	No	>250	No
14	70	M	White	Scleroderma	Moderna	3000 mg	42	Rituximab	<0.40	Yes
15	36	F	White	Scleroderma	Pfizer	3000 mg	14	No	35	No
16	40	F	White	Scleroderma	Pfizer	2500 mg	28	No	244	No
17	42	F	White	Scleroderma	Pfizer	3000 mg	8	Abatacept	22	No
18	63	F	White	Sjogren's	Pfizer	2500 mg	NA	No	12	No
19	49	F	White	SLE††	J+J	3000 mg	13	No	>250	No
20	54	F	White	SLE††	Moderna	1000 mg	10	HCQ‡	>250	No
21	50	F	Black	SLE††	Pfizer	3000 mg	98	Belimumab, Prednisone	>250	No
22	31	F	White	SLE††	Pfizer	2000 mg	10	HCQ‡, Prednisone	80	No
23	38	F	White	SLE††	Pfizer	1500 mg	20	Prednisone	168	No
24	51	F	White	SLE††	Moderna	1000 mg	5	Abatacept	>250	No

*The assay ranges from<0.4 to >250 units/mL. Positive antibody was defined as an anti-SARS-CoV-2 receptor binding domain antibody titre>0.79 units/mL.
†Denotes connective tissue disease-related ILD.
‡Rheumatoid arthritis, ankylosing spondylitis, psoriatic arthritis, reactive arthritis, or inflammatory bowel disease-associated arthritis.
§Intravenous immunoglobulin.
¶Hydroxychloroquine.
**Denotes a combination of two or more of the rheumatic conditions.
††Systemic lupus erythematosus.

the limited immunogenicity to SARS-CoV-2 vaccination in other immunosuppressed patients,[5] the generalisability of these preliminary findings warrants further investigation. Evidence-based, personalised approaches to perivaccination immunosuppression modulation will be key in safely optimising responses to SARS-CoV-2 vaccination for vulnerable populations.

PATIENT AND PUBLIC INVOLVEMENT

Patients were not involved in the design, conduct or dissemination of the study, though this study was motivated by questions frequently posed by patients. The study has a public website (https://vaccineresponse.org/) and email account where we welcomed participants and the public to contact the research team. Results of the study will be shared with national RMD organisations for dissemination to their patient communities once published.

Caoilfhionn M Connolly [1], **Teresa Po-Yu Chiang,**[2] **Brian J Boyarsky** [2], **Jake A Ruddy,**[2] **Mayan Teles,**[2] **Jennifer L Alejo** [2], **Allan Massie,**[2] **William A Werbel,**[3] **Ami A Shah,**[1] **Lisa Christopher-Stine,**[1] **Jacqueline Garonzik-Wang,**[2] **Dorry L Segev** [2,4], **Julie J Paik** [1]

[1]Rheumatology, Johns Hopkins University, Baltimore, Maryland, USA
[2]Surgery, Johns Hopkins University, Baltimore, Maryland, USA
[3]Infectious Diseases, Johns Hopkins University, Baltimore, Maryland, USA
[4]Epidemiology, Johns Hopkins University Bloomberg School of Public Health, Baltimore, Maryland, USA

Correspondence to Dr Dorry L Segev, Surgery, Johns Hopkins, Baltimore, Maryland 21205, USA; dorry@jhmi.edu

Handling editor Josef S Smolen

Twitter Caoilfhionn M Connolly @CaoilfhionnMD and Jennifer L Alejo @JenLAlejo

Contributors Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work: CMC, TP-YC, BJB, MT, JLA, AM, AAS, WAW, JG-W, DLS, JJP. Drafting the work or revising it critically for important intellectual content: CMC, TP-YC, JAR, BJB, MT, JLA, AM, LC-S, AAS, WAW, JG-W, DLS, JJP. Final approval of the version to be published: CMC, TP-YC, JAR, BJB, JLA, AM, LC-S, AAS, WAW, DLS, JG-W, JJP. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved: CMC, TP-YC, JAR, BJB, JLA, LC-S, WAW, JG-W, DLS, JJP.

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ORCID iDs
Caoilfhionn M Connolly http://orcid.org/0000-0002-1898-3530
Brian J Boyarsky http://orcid.org/0000-0001-6902-9854
Jennifer L Alejo http://orcid.org/0000-0003-3137-9271
Dorry L Segev http://orcid.org/0000-0002-1924-4801
Julie J Paik http://orcid.org/0000-0001-8436-1601

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Inactivated vaccines may not provide adequate protection in immunosuppressed patients with rheumatic diseases

Patients with autoimmune rheumatic diseases (AIRDs) are vulnerable to COVID-19 due to the presence of multiple comorbidities.[1] Moreover, patients on immunosuppressants have blunted responses to vaccination as compared with healthy people.[2 3] Persistence of the virus in such people may lead to the selection of more virulent mutants of SARS-CoV-2.[4] It is crucial that they are prioritised for the best possible vaccine. India has crossed 650 million vaccinations with predominantly two vaccines: the adenoviral vector-borne AZD1222 (ChAdOx1 nCoV-19) and the indigenous whole-virion β-propiolactone-inactivated BBV152. In our cohort of around 1500 patients with AIRD who are being followed up to assess the immunogenicity of COVID-19 vaccines, we identified 475 patients who have completed two doses of either vaccine. Serum was collected on median 30th (range 28–35) day after the second dose of vaccine with informed consent after ethics clearance. Titres of IgG antibodies to spike protein were estimated with the Elecsys kit (Roche, Switzerland). To check the neutralisation potential of the sera, age, sex and disease matched 80 BBV152 and 85 AZD1222 recipients were selected. Neutralisation potential of the sera was assessed using the SARS-CoV-2 sVNT Kit (GenScript). Analysis was done in R V.4.0.3. Shapiro-Wilk confirmed normality; antibody titres were compared with unpaired t-test after log transformation, while proportions were compared with Fisher's exact test.

Age was more in the AZD1222 group, but other baseline characteristics were the same as compared with the BBV152 group (online supplemental table 1). Seroconversion had occurred in 342 (93.9%) of the AZD1222 group and 45 (40.5%) of the BBV152 group ($p<0.001$). Similarly seroconversion of neutralising antibody, defined as a neutralisation activity of more than 30%, was seen in a higher proportion of AZD1222 group (48/80) than of the BVV152 group (25/85) (60%vs 29%, respectively; $p<0.001$). The antibody levels in the AZD1222 group were 10–100× higher than those in the BBV152 group (figure 1A), while the percentage of neutralisation activity of sera was also higher in the AZD1222 group (figure 1B). There was high correlation between antibody titres and neutralisation potential (Pearson's $R=0.755$) (figure 1C).

Our data show the poor immunogenicity of the whole-virion β-propiolactone-inactivated vaccine in immunosuppressed patients. This has been validated by estimating neutralisation activity after matching recipients of BBV152 with those of AZD1222. Inactivated vaccines have reduced immunogenicity as compared with other vaccines in healthy individuals.[5] However, in our patients on immunosuppressants, more than half of the BBV152 recipients failed to generate a humoral immune response, exposing them to higher risk of COVID-19 and its complications.

The limitations of this study include a non-random sample and the lack of T-cell immunogenicity data. However, these are pragmatic data, and statistical differences between the inactivated and vector-borne vaccine have been very clearly demonstrated. Peak titre neutralisation antibodies and anti-spike antibodies have been shown to correlate well with protection from symptomatic COVID-19 infection.[6] So lack of antibody response at 1 month when the peak response is expected is likely to translate to lack of protection in this high-risk population.

Thus, the humoral responses to the BBV152 vaccine is inferior to that of the AZD1222 (ChAdOx1) vaccine in immunosuppressed patients. This is mirrored in neutralisation assays that are a surrogate for real-world protection. This implies a pressing need to update vaccine policies so that such patients on immunosuppressants receive vaccines other than inactivated ones.

Padmanabha Shenoy ,[1] **Sakir Ahmed** ,[2] **Aby Paul**,[1] **Somy Cherian**,[1] **Anuroopa Vijayan**,[1] **Sageer AS Babu**,[1] **Aswathy Sukumaran**[1]

[1]Center For Arthritis and Rheumatism Excellence, Sree Sudheendra Medical Mission, Kochi, Kerala, India
[2]Clinical Immunology and Rheumatology, Kalinga Institute of Medical Sciences, Bhubaneswar, Odisha, India

Correspondence to Dr Padmanabha Shenoy, Center For Arthritis and Rheumatism Excellence, Sree Sudheendra Medical Mission, Kochi, Kerala, India; drdpshenoy@gmail.com

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Contributors PS and SA conceptualised and drafted the paper. All authors approved the final version.

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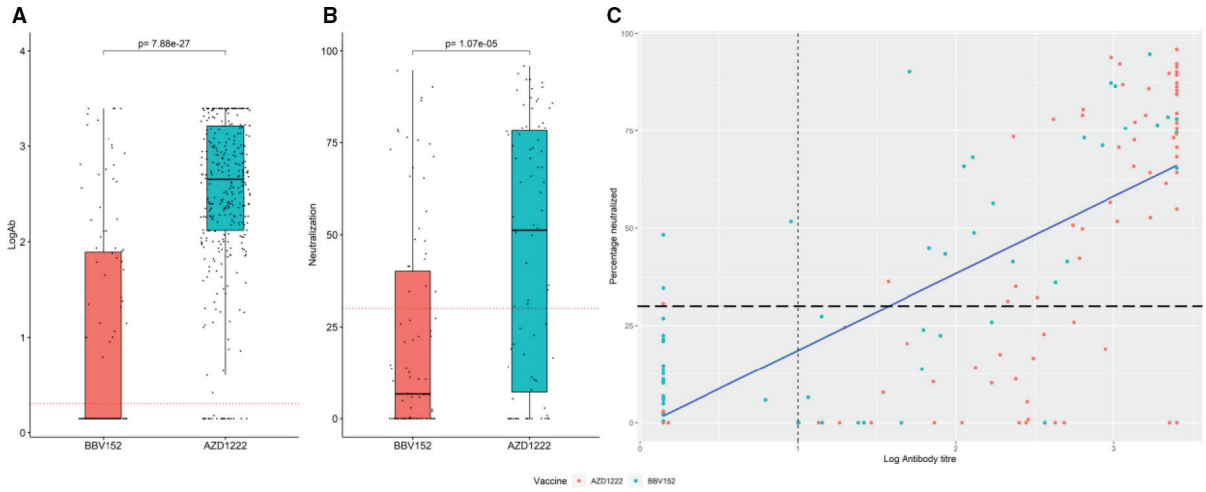

Figure 1 (A) Antibody levels in the AZD1222 group (n=364) versus those in the BVV152 group (n=111) in log 10 scale. (B) Neutralisation activity of the sera of matched patients from the AZD1222 group (n=80) versus those in the BVV152 group (n=85). (C) Scatterplot showing the correlation between antibody levels and neutralisation activity. The linear regression line is coloured blue. The vertical line with smaller dashes is the cut-off for antibody positivity, while the horizontal line with the larger dashes represents the 30% cut-off for minimum neutralisation activity.

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ORCID iDs
Padmanabha Shenoy http://orcid.org/0000-0002-7666-1361
Sakir Ahmed http://orcid.org/0000-0003-4631-311X

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Impact of sustaining SDAI remission for preventing incident of bone fragility fracture in patient with rheumatoid arthritis

It is well known that rheumatoid arthritis (RA) has a determinant risk for bone fragility, and patient with RA has a seriously high risk for bone fragility fracture (BFF).[1] On the other hand, the BFF risks in the patient with RA owe disease activity.[2 3] We hypothesised that if disease activity is successfully controlled and achieved clinical remission, adverse effects on bone metabolism would be minimised, resulting in a lower incidence of BFF.[4] We therefore statistically examined whether there was a difference in the incidence of BFF between patients with and without RA who achieved clinical remission.

Patients who matched the European League Against Rheumatism/American College of Rheumatology (EULAR/ACR) classification criteria under the T2T since August 2010, have been treating RA and were measured bone mineral density (BMD) with dual-energy X-ray absorptiometry, were recruited. The initial target of therapy is the attainment of remission with a Simplified Disease Activity Index (SDAI) within 6 months of initiation.[5] The primary outcome was incident BFF. Follow-up started at BMD measurement (baseline) and continued until the development of the first fracture or censoring at death, loss to follow-up or end of the study. Kaplan-Meier survival curves were determined for incident BFF incidence up to the last observation. Risks for the incidence of BFF were classified as RA specific and general candidate. In general candidate, comorbidities that might affect the incidence of BFF, such as lifestyle-related diseases,[6] and increased ability to fall or a disorder were included. Candidate risk factors are shown

Table 1 Baseline and follow-up characteristics of candidate risk factors in patients in the RA group, its subgroups; RA-rem and RA-nonrem, and the non-RA group

Candidate risk factors		RA group (n=278)	RA-rem (n=147)	RA-nonrem (n=131)	non-RA (n=278)	P value			
						non-RA to RA	non-RA to RA-rem	non-RA to RA-nonrem	RA-rem to RA-nonrem
General	Women, %	86	85	87	86.3	0.985	0.9	0.661	0.532
	Age, years	74.1 (10.7)	74.0 (10.5)	74.2 (10.9)	75.0 (10.8)	0.119	0.183	0.236	0.846
	BMI, kg/m^2	22.6 (4.2)	23.3 (3.8)	21.6 (4.6)	23.5 (4.1)	0.187	0.897	0.029	0.087
	Lifestyle-related diseases, %	80.9	79.6	82.4	75.9	0.131	0.307	0.098	0.468
	Type 2 DM, %	23.4	21.1	26	28.4	0.17	0.103	0.568	0.325
	COPD, %	10.4	9.5	11.5	7.6	0.238	0.564	0.228	0.588
	Hypertension, %	57.2	52.4	62.6	54.3	0.526	0.681	0.097	0.076
	Hyperlipidaemia, %	34.2	36.1	62.1	34.2	0.976	0.736	0.695	0.516
	Chronic heart failure, %	20.9	19	22.9	19.4	0.69	0.87	0.427	0.414
	CKD ≥stage2, %	49.5	28.7	35.3	49.6	0.955	0.01	0.214	0.272
	Insomnia, %	23.4	19.7	27.5	23.3	0.939	0.316	0.417	0.121
	Fall-ability, %	63.3	63.3	63.3	69.4	0.115	0.192	0.278	0.922
	MADS, %	15.8	13.6	18.3	21.9	0.064	0.033	0.397	0.273
	Osteoarthritis, %	55.4	57.1	53.4	60.1	0.288	0.542	0.244	0.586
	Disuse, %	9	8.2	9.9	4.7	0.051	0.198	0.059	0.598
	Contractures, %	13.6	32.9	36.1	10.1	0.19	0.563	0.146	0.452
	Parkinsonism, %	2.5	2	3.1	2.2	0.798	0.763	0.74	0.588
	Cognitive impairment, %	9.4	9.5	9.2	8.3	0.649	0.752	0.828	0.935
	T-score	−1.73 (1.17)	−1.61 (1.09)	−1.87 (1.26)	−1.71 (0.97)	0.805	0.281	0.11	0.05
	pr-BFF, %	50.4	51	49.6	55.8	0.22	0.338	0.283	0.867
	Anti-osteoporotic drug,* %	61.2	61.2	61.1	58.6	0.581	0.627	0.572	0.958
	GCS administration until baseline, %	55.8	63.3	49	13.3	0.034	<0.001	<0.001	0.014
	GCS mean dosage,† mg/day	2.7 (3.4)	2.7 (3.4)	2.1 (1.9)	5.9 (7.7)	<0.001	0.034	0.028	0.495
	GCS total dose ever,† mg	2460 (6481)	3640 (8445)	1408 (3678)	859 (4972)	<0.001	<0.001	<0.001	0.006
RA specific	Disease duration of RA, years	7.6 (8.8)	6.7 (8.4)	8.5 (9.1)					0.072
	ACPA titre at BL, U/mL	176.6 (478.9)	180.3 (360.5)	173.7 (558.2)					0.622
	ACPA positive, %	77.7	75.6	80					0.479
	RF titre at BL, IU/mL	93.2 (214.0)	107.7 (189.9)	80.6 (233.5)					0.051
	SDAI score at BL	5.67 (7.91)	2.29 (3.92)	9.47 (9.44)					<0.001
	CDAI score at BL	5.03 (6.78)	1.99 (3.43)	8.35 (7.89)					<0.001
	CRP at BL, mg/L	7.2 (19.6)	3.6 (9.6)	11.1 (26.1)					0.074
	HAQ-DI at BL	0.548 (0.656)	0.382 (0.530)	0.733 (0.735)					<0.001
	SHS at BL	60.3 (73.6)	42.6 (50.9)	78.8 (88.2)					0.011
	JSN at BL	28.3 (32.8)	21.0 (23.4)	36.1 (38.9)					0.064
	BE at BL	33.0 (42.9)	22.6 (29.5)	44.7 (51.3)					0.002
	Mean SDAI score after BL	4.39 (4.38)	1.69 (0.88)	7.43 (4.75)					<0.001
	Mean CDAI score after BL	3.82 (3.90)	1.38 (0.85)	6.47 (4.18)					<0.001
	Mean CRP level after BL, mg/L	6.23 (10.67)	3.49 (4.14)	9.30 (14.35)					<0.001
	Mean SDAI remission rate after BL, %	39.5 (32.5)	85.6 (13.2)	32.3 (23.3)					<0.001
	Mean CDAI remission rate after BL, %	58.5 (32.9)	81.6 (19.3)	32.1 (24.0)					<0.001

The values are presented as mean (SD) unless indicated otherwise.
*Anti-osteoporotic drugs included selective oestrogen receptor modulators, bisphosphonates, denosumab, teriparatide and romosozumab.
†As prednisone dose equivalent.
ACPA, anti-cyclic citrullinated polypeptide antibodies ; BE, Bone Erosion Score; BFF, bone fragility fracture ; BL, baseline ; BMI, body mass index ; CDAI, Clinical Disease Activity Index; CKD, chronic kidney disfunction ; COPD, chronic obstructive pulmonary diseases ; CRP, C reactive protein ; DM, diabetes mellitus ; FRAX, fracture risk assessment tool; GCS, glucocorticoid steroids ; HAQ-DI, Health Assessment Questionnaire Disability Index ; JSN, Joint Space Narrowing score ; LSD, lifestyle-related diseases; MADS, musculoskeletal ambulation disfunction complex ; MOF, major osteoporotic fractures ; RF, rheumatoid factor ; SDAI, Simplified Disease Activity Index; SHS, Sharp/van der Heijde Score .

in table 1. Each evidence was evaluated using Cox regression analysis to identify significantly higher risk factors within 5% in univariate models and to evaluate using multivariate model.

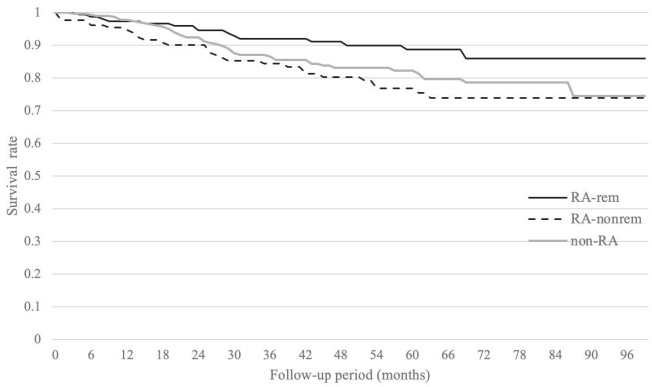
In a preliminary study with our dataset, we examined the incidence of bone erosion score (BE) and BFF, and BE correlated with the incidence of BMD and pr-BFF, but not with the incidence of new BFF using linear and binary logistic regression analyses (online supplemental table 1).

We compared the incidence of BFF between RA and non-RA groups, in setting as a control group consisting of

A

	univariate model		multivariate model	
Candidate risk factors	risk ratio	95% CI	risk ratio	95% CI
General				
female	6.42	0.88 – 46.70		
older age	1.01	0.99 – 1.04		
lower T-score	1.33	1.02 – 1.74	0.836	0.591 – 1.182
prevalent bone fragility fractures	5.85	2.60 – 13.12	4.877	1.880 – 12.651
lifestyle-related diseases	4.83	1.17 – 20.0	2.579	0.335 – 19.858
Fall-ability	2.75	1.28 –5.91	1.887	0.790 – 4.503
cognitive impairment	2.23	1.04 – 4.80	0.684	0.242 – 1.931
anti-osteoporotic drug administration	0.92	0.50 – 1.72		
GCS administration	3.01	1.45 – 6.27	1.421	0.610 – 3.310
greater GCS total dose ever	0.99	0.99 – 1.00		
RA specific				
longer disease duration of RA	0.98	0.939 – 1.020		
higher ACPA titer	1.00	1.0001 – 1.001	1.0007	1.0002 – 1.0011
higher ACPA positivity	0.96	0.489– 1.867		
higher RF titer	1.00	0.998 – 1.002		
higher SDAI score at baseline	1.02	0.99 – 1.05		
higher CDAI score at baseline	1.05	0.99 – 1.11		
higher CRP at baseline	1.16	0.95 – 1.42		
higher HAQ-DI at baseline	1.42	0.93 – 2.14		
higher SHS at baseline	1.0007	0.99 – 1.00		
higher JSN at baseline	1.004	0.995 – 1.01		
higher BE at baseline	1.00	0.995 – 1.01		
higher mean SDAI score after baseline	1.05	1.0004 – 1.09	0.942	0.807 – 1.100
higher mean CDAI score after baseline	1.05	0.999 – 1.11		
higher CRP level after baseline	1.16	0.953 – 1.422		
lower mean SDAI remission after baseline	4.22	1.75 – 10.19	6.676	1.005 – 44.361
lower mean CDAI remission after baseline	2.16	0.89 – 5.25		

B



C

	Hazard ratio	95%CI
RA-rem/non-RA	0.583	0.345 – 0.983
RA-nonrem/non-RA	1.265	0.783 – 2.045
RA/non-RA	0.901	0.596 – 1.362
RA-rem/RA-nonrem	0.451	0.249 – 0.814

D

Fracture data	RA group (n=278)	RA-rem (N=147)	RA-nonrem (N=131)	non-RA (n=278)	non-RA to RA	non-RA to RA-rem	non-RA to RA-nonrem	RA-rem to RA-nonrem
follow-up period, months	85.1 (26.2)	89.1 (21.5)	80.6 (30.2)	85.0 (25.8)	0.974	0.061	0.194	0.011
actual incidence of BFF, %	15.8	10.2	22.1	16.5	0.833	0.074	0.183	0.006
10-year probability of MOF, %	27.0 (18.4)	25.2 (18.3)	29.1 (18.3)	16.1 (10.7)	< 0.001	< 0.001	< 0.001	0.048
					p-value			

Figure 1 (A) Cox regression analysis results of the candidate risk factors with respect to the incidence of bone fragility fractures. Factors with statistically significance within 5% using multivariate model are shown in bold style. Lifestyle-related diseases include comorbidities such as type 2 diabetes mellitus (DM), chronic obstructive pulmonary disease (COPD), hypertension, hyperlipidaemia, chronic heart failure, chronic kidney disfunction (CKD) ≥stage 2 and insomnia. Fall-ability (increased ability to fall or a disorder) include comorbidities such as musculoskeletal ambulation disability complex (MADS), osteoarthritis of the lower extremities (OA), joint contractures of the trunk or lower extremities (Contractures), disuse syndrome (Disuse), parkinsonism and neuromuscular disorders (Parkinsonism). ACPA, anti-cyclic citrullinated polypeptide antibodies; BE, bone erosion score; CDAI, clinical disease activity index score; CRP, C reactive protein; HAQ-DI, Health Assessment Questionnaire Disability Index; JSN, Joint Space Narrowing score; RF, rheumatoid factor; SDAI, Simplified Disease Activity Score, SHS, Sharp/van der Heijde Score. (B) Kaplan-Meier survival curve of incident bone fragility fracture with respect to having rheumatoid arthritis, and remission status of disease activity after baseline. Non-RA, a patient group who does not have rheumatoid arthritis; RA-rem, a patient group with rheumatoid arthritis, and achieved average SDAI score ≤3.3 after baseline; RA-nonrem, a patient group with rheumatoid arthritis, and the average SDAI score was more than 3.3 after baseline. (C) HR in comparison with each pair of the RA-rem and the non-RA, the RA-nonrem and the non-RA, the RA and the non-RA, and the RA-rem and the RA-nonrem groups. (D) Average follow-up period, actual incidence of bone fragility fracture, and the 10-year probability of major osteoporotic fracture (MOF) in each group, and their p values.

patients without RA and matched for general candidate risk factors. Furtherly, mean 10-year probability of major osteoporotic fracture (MOF) calculated with fracture assessment tool (FRAX) in the two groups was compared using Mann-Whitney U test.

Patients in the RA group were classified according to the mean SDAI score ≤3.3 or >3.3 (RA-rem and RA-nonrem). The incidence of BFF among the non-RA, the RA-rem and the RA-nonrem groups were compared using Kaplan-Meier survival curve analysis.

Factors with significantly higher risk ratios in the RA group were higher anti-cyclic citrullinated polypeptide antibodies (ACPA) titre, higher mean SDAI score, lower mean SDAI remission rate, presence of lifestyle-related diseases (LSD), presence of Fall-ability, glucocorticoid steroid (GCS) administration, lower T-score and presence of prevalent fracture (pr-BFF). Among these, ACPA, mean SDAI remission rate and pr-BFF showed a significantly higher risk ratio in the multivariate model (figure 1A).

The incidence rate of BFF was significantly lower in the RA-rem group than in the RA-nonrem and the non-RA group, whereas no significant difference between the non-RA and the RA-nonrem groups as well as HRs were shown (figure 1B,C). These trends were also shown when SDAI was replaced by the clinical disease activity index (CDAI) (online supplemental figure 1). The 10-year MOF in both of the RA groups were significantly higher than in the non-RA group (figure 1D).

These results suggest that disease activity control is the most important factor in the prevention of BFF. The incidence of fractures increases in patients with RA in whom SDAI or CDAI remission is difficult to achieve, or unless it is maintained above a certain level during SDAI or CDAI remission. Higher SDAI or CDAI remission rate is desirable to prevent BFF.

PATIENT AND PUBLIC INVOLVEMENT

This study was carried out without patient and public involvement.

Ichiro Yoshii ,[1] **Naoya Sawada** ,[2] **Tatsumi Chijiwa** ,[3] **Shohei Kokei**[4]

[1]Musculoskeletal Medicine, Yoshii Hospital, Shimanto-City, Kochi, Japan
[2]Department of Rheumatology, Dohgo-Onsen Hospital Rheumatology Center, Matsuyama, Ehime, Japan
[3]Department of Rheumatology, Kochi Memorial Hospital, Kochi, Kochi, Japan
[4]Department of Internal Medicine, Yoshii Hospital, Shimanto-City, Kochi, Japan

Correspondence to Dr Ichiro Yoshii, Orthopaedics, Yoshii Hospital, Shimanto-City, Kochi 787-0033, Japan; ichiroyo@giga.ocn.ne.jp

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Patient consent for publication Not required.

Ethics approval This study was approved by Yoshii Hospital ethics committee (approval number: Y-OP-2020-3) in accordance with the ethical standards laid down in 1964 Declaration of Helsinki and its later amendments. In addition, anonymity was ensured for all patients and their families who participated in this study, and no names, and/or addresses were issued that could help identify these individuals.

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ORCID iDs
Ichiro Yoshii http://orcid.org/0000-0002-9123-4624
Naoya Sawada http://orcid.org/0000-0001-9551-3252
Tatsumi Chijiwa http://orcid.org/0000-0002-2104-7014

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Fat mass and response to TNFα blockers in early axial spondyloarthritis: an analysis of the DESIR cohort

Some studies have showed that obesity, as determined by a body mass index (BMI) ≥30 kg/m^2, hampers treatment response to tumour necrosis factor-alpha blockers (TNFb) in patients with axial spondyloarthritis (axSpA).[1] However, no study has specifically determined the impact of abdominal adiposity as assessed by waist circumference (WC), on treatment response to TNFb in early axSpA. As compared with BMI, WC is more strongly associated with the metabolic disturbances thought to underlie many of the obesity-related conditions. Notably, it is associated with low-grade inflammation and all-cause and cardiovascular mortality with or without adjustment for BMI.[2] Therefore, we hypothesise that WC could be also an independent factor for TNF b response.

We conducted an analysis to assess the impact of abdominal adiposity on the treatment effect of TNFb in real life in an early axSpA population. Devenir des Spondylarthropathies Indifférenciées Récentes cohort[3] is a multicenter, observational, prospective cohort of 708 patients with early (<3 years) inflammatory back pain suggestive of axSpA. Demographics (weight, height, BMI and WC) and disease characteristics (including treatment) were collected every 6 months. Patients receiving a TNFb for the first 2 years of following were matched to their nearest neighbour (according to their propensity score value) to patients receiving any other treatment (usual care, including non-steroidal anti-inflammatory drugs, steroids or conventional DMARDs but no other biologics). The propensity score included all the available variables at baseline (n=48), including demographics, disease activity, disease severity, function and NSAID treatment. The goodness of fit of the model was very good (AUC=0.9). More details on the propensity score matching procedure have been published previously.[4] The primary outcome was the ASAS 40 response after at least 12 weeks of TNFb initiation. An abnormal WC was defined as a WC≥94 cm and ≥80 cm in men and women, respectively, according to international consensus, including WHO, to be applied in European populations.[5–8]

To evaluate the impact of abdominal adiposity on such TNFb response, the interaction between WC and treatment was evaluated. At baseline, 680 patients (313 men and 367 women) had a WC available: mean WC was 84.1±11.4 cm (82.0±11.5 cm and 86.5±10.7 cm in women and men, respectively); adiposity was present in 74 (22.6%) men and 205 (53.8%) women, respectively.

Table 1 summarises the patients and disease characteristics of patients included in this analysis. Patients with abnormal WC were significantly older, less frequently males and the prevalence of inflammatory bowel disease was significantly lower in this group. At baseline, these patients had poorer disease activity and function scores.

Overall, 197 patients were treated by TNFb during the first 2 years of follow-up and had information allowing to estimate the primary outcome (ie, ASAS 40) and were matched to 197 controls according to a propensity score. The percentage of ASAS 40 responders to TNFb was comparable in both groups (31.0% vs 32.7% in the abnormal and normal WC groups, respectively). Interactions analyses between therapeutic response and WC did not show any impact of WC on TNFb treatment response. Similar results were found when we assessed the interaction

Table 1 Demographics and disease characteristics of patients based on waist circumference (WC)

	Normal WC, n=402	Abnormal* WC, n=278	P value
Age (years)	32.2 (8.1)	36.0 (9.0)	<0.001
Gender (male)	239/402 (59.5%)	74/278 (26.6%)	<0.001
Symptom duration (years)	1.5 (0.9)	1.6 (0.8)	NS
Past articular peripheral involvement	221/402 (55.0%)	170/278 (61.2%)	NS
Past peripheral enthesitic involvement	201/402 (50.0%)	138/278 (49.6%)	NS
History of dactylitis	56/402 (13.9%)	35/278 (12.6%)	NS
History of personal of extra-articular involvement			
► Skin psoriasis	68/402 (16.9%)	40/278 (14.3%)	NS
► Uveitis	32/402 (8.0%)	26/278 (9.3%)	NS
► IBD	45/402 (11.2%)	16/278 (5.8%)	0.02
HLA-B27+	250/401 (62.3%)	147/278 (52.9%)	0.016
Radiographic sacroiliitis	70/365 (19.7%)	39/244 (16.0%)	NS
MRI sacroiliitis	141/395 (35.7%)	86/272 (31.6%)	NS
CRP abnormality	91/388 (23.5%)	92/271 (33.9%)	0.004
BASDAI (0–100)	42.8 (19.8)	47.7 (20.0)	0.002
BASFI (0–100)	27.4 (22.0)	34.9 (23.4)	<0.001
Body mass index (kg/m^2)	22.0 (2.55)	26.7 (4.20)	<0.001

*Defined as a WC≥94 cm and ≥80 cm in men and women, respectively.
BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; BASFI, Bath Ankylosing Spondylitis Functional Index; CRP, C reactive protein; HLA, human leucocyte antigen; IBD, inflammatory bowel disease; NS, not significant.

between obesity (BMI ≥30 kg/m^2) and TNFb response (data not shown).

We confirm that abdominal adiposity and obesity associate with low-grade inflammation, functional disability and pain in axSpA. However, we did not observe a poorer response to TNF in adipose or obese patients with early axSpA in a usual care setting. It is worth noticing that in our analysis, among the 197 patients treated by TNFb, only 4 patients were treated with infliximab, the only TNFb prescribed by body weight. Nevertheless, even in the absence of impact on the treatment response to TNFb, educational initiatives and interventions aiming at preventing/reducing adiposity and obesity in patients with axSpA should be encouraged.

Anna Molto ,[1,2] **Solange Tang**,[3] **Bernard Combe**,[4] **Maxime Dougados**,[5] **Pascal Richette** [3,6]

[1]Rheumatology, Hospital Cochin, Paris, France
[2]ECAMO team, INSERM U1153, Paris, France
[3]Service de Rhumatologie, Hopital Lariboisiere Centre Viggo Petersen, Paris, France
[4]Rheumatology, University Montpellier 1, Montpellier, Languedoc-Roussillon, France
[5]Rheumatology, Hopital Cochin, Université de Paris, Paris, France
[6]Inserm UMR1132 Bioscar, Universite Paris Diderot UFR de Medecine, Paris, France

Correspondence to Dr Anna Molto, Rheumatology, Hospital Cochin, Paris, France; anna.molto@aphp.fr

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Twitter Anna Molto @annamolto

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ORCID iDs
Anna Molto http://orcid.org/0000-0003-2246-1986
Pascal Richette http://orcid.org/0000-0003-2132-4074

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Anti-ANP32A antibodies in systemic sclerosis

Numerous studies have identified autoantibody status as an important biomarker of cancer risk around the time of systemic sclerosis (SSc) onset. For example, anti-RNA polymerase III antibody positivity associates with increased cancer risk near SSc onset, while the presence of anticentromere or anti-Th/To antibodies may be protective.[1–4]

In this context, we selected serum from a well-characterised patient with SSc with squamous cell skin cancer and a short cancer–scleroderma interval to discover cancer-associated autoantibodies. Immunoprecipitations performed with this serum using 624 melanoma cell lysates were subjected to on bead digestion and liquid chromatography tandem mass spectrometry peptide sequencing. This identified antibodies targeting the tumour suppressor gene, acidic leucine-rich nuclear phosphoprotein (ANP32A), which migrates on SDS-polyacrylamide gels at ~28.5 kDa. Antibodies against ANP32A were validated in this serum by immunoprecipitation using ^{35}S-methionine labelled ANP32A generated by in vitro transcription/translation from DNA (Origene) as described.[5]

To further explore the association between anti-ANP32A antibodies and cancer in patients with SSc, sera from patients with (n=213) and without cancer (n=190) were randomly selected from the Johns Hopkins Scleroderma Center Research Registry for anti-ANP32A antibody testing. The Registry prospectively collects demographic information, cancer diagnoses and longitudinal disease metrics.[3] Patients were classified as without cancer if they were cancer free >5 years after SSc onset. An ELISA was set up to screen patient sera. Briefly, 96-well ELISA plates were coated with 50 ng/well of recombinant human ANP32A (Sino Biological cat # 11942-H20E). Sera were screened (1:200 dilution), followed by incubation with HRP-labelled antihuman IgG (Jackson ImmunoResearch) diluted 1:10 000. Colour was developed with SureBlue peroxidase reagent (KPL). The initial serum sample was included as a reference in every ELISA, and optical density (OD) readings were calibrated relative to it. The antibody positivity cut-off was determined by assaying sera from 31 healthy controls. The mean + 3SD of these was used as the cut-off. This value was 0.1807. ELISAs performed with patient sera were assigned a negative value if their calibrated ODs were <0.1807. The range of OD values for anti-ANP32A-positive sera in the scleroderma cohort was 0.197–1.368. Two disease control groups were also tested for these antibodies. These consisted of sera from patients with systemic lupus erythematosus (SLE) (n=30) and primary Sjogrens syndrome (n=30). Anti-ANP32A antibodies were not detected in any of the disease control samples. The ELISA results were also validated by immunoblotting purified recombinant ANP32A as shown in online supplemental figure 1. The Johns Hopkins IRB approved this study protocol.

Statistical analyses were performed using Stata.[6] We evaluated whether anti-ANP32A antibodies conferred increased cancer risk. Secondary analyses evaluated phenotypic differences between anti-ANP32A positive and negative patients. For comparison between groups, χ^2 or Fisher's exact test were used for binary variables. Student's t-tests and Wilcoxon rank sum tests were used for comparison of continuous variables. As these analyses were exploratory, adjustment for multiple comparisons was not performed.

Of the 403 patients with SSc, 17 (4%) patients were anti-ANP32A positive (table 1). The majority of patients were female with a median follow-up of >10 years. The primary analysis did not show an association between anti-ANP32A positivity and cancer (71% vs 52%; p=0.134). Similar results were obtained when non-melanoma skin cancers were excluded. Comparing anti-ANP32A positive and negative patients phenotypically, the former were more likely to have echocardiographic evidence of pulmonary hypertension (69% vs 37%; p=0.012), but have less severe Raynaud's (frequency of digital pits, ulcers or gangrene 35% vs 60%; p=0.042). There was no significant association between anti-ANP32A and other common SSc autoantibodies, including antitopoisomerase I, anti-RNA polymerase III and anticentromere antibodies.

In this study, we describe a new autoantibody specificity, anti-ANP32A, and evaluate the prevalence of these antibodies in a

Table 1 Demographic and phenotypic characteristics of patients with SSc by anti-ANP32 status

	Anti-ANP32A+	Anti-ANP32A–	P value
Overall, no (%)	17 (4%)	386 (96%)	
ACR 2013 criteria	15 (88%)	369 (96%)	0.188
Sex (female), no (%)	15 (88%)	323 (84%)	1.000
Black, no (%)	0 (0%)	51 (13%)	0.146
Ever smoker, no (%)	9 (53%)	199/382 (52%)	0.945
Age of SSc onset (years), mean (SD)	49.9 (18.3)	43.5 (15.2)	0.095
Disease duration at entry, median (IQR)	5.0 (2.0–14.6)	5.5 (1.9–15.1)	0.973
Years of follow-up, median (IQR)	10.0 (5.8–20.0)	14.1 (8.8–23.1)	0.292
Diffuse, no (%)	3 (18%)	142 (37%)	0.126
Limited no (%)	13 (76%)	233 (60%)	
Sine no (%)	1 (6%)	11 (3%)	
First mRSS, median (IQR)	3.0 (2.0–4.0)	4.0 (2.0–12.0)	0.113
Maximum mRSS, median (IQR)	4.0 (3.0–9.0)	6.0 (4.0–17.0)	0.057
History of Raynaud phenomenon, no (%)	16 (94%)	382 (99%)	0.195
Tendon friction rubs at baseline, no (%)	0 (0%)	39/384 (10%)	0.392
Calcinosis, no (%)	7 (41%)	167/385 (43%)	0.858
Telangiectasia, no (%)	16 (94%)	375/385 (97%)	0.382
Synovitis ever, no (%)	5 (29%)	81 (21%)	0.407
History of renal crisis, no (%)	1 (6%)	21 (5%)	1.000
Pulmonary hypertension,† no (%)	11/16 (69%)	127/339 (37%)	**0.012**
Pulmonary function			
Baseline FVC (% pred), mean (SD)	79.7 (20.9)	80.8 (19.0)	0.821
Baseline DLCO (% pred), mean (SD)	70.7 (27.9)	76.1 (23.3)	0.453
Minimum FVC (% pred), mean (SD)	72.9 (18.8)	69.7 (21.2)	0.542
Minimum DLCO (% pred), mean (SD)	62.4 (28.1)	58.0 (25.4)	0.505
Moderate-to-severe weight loss/anaemia,‡ no (%)	6 (35%)	125 (32%)	0.802
Moderate-to-severe Raynaud's, no (%)	6 (35%)	232 (60%)	**0.042**
Moderate-to-severe lung disease, no (%)	12 (71%)	264/367 (72%)	0.904
Moderate-to-severe heart disease, no (%)	2 (12%)	120/366 (33%)	0.107
Moderate-to-severe GI involvement, no (%)	8 (47%)	228 (59%)	0.325
Moderate-to-severe kidney disease, no (%)	2 (12%)	27/373 (7%)	0.366
Moderate-to-severe muscle disease, no (%)	0 (0%)	28/375 (7%)	0.622
Deceased during study, no (%)	8 (47%)	144/382 (38%)	0.437
Cancer,* no (%)	12 (71%)	201 (52%)	0.134
Cancer-first symptom interval, mean (SD)	12.4 (16.6)	8.6 (15.0)	0.402
ANA with titre ≥1/80, no (%)	15 (88%)	313/336 (93%)	0.343
Anticentromere, no (%)	5 (29%)	122 (32%)	0.849
Antitopoisomerase I, no (%)	2 (12%)	75 (19%)	0.751
Anti-RNA polymerase III, no (%)	2 (12%)	75 (19%)	0.751

Values <0.05 are in bold to denote statistical significance.
*Cancer includes non-melanoma skin cancers (there are two non-melanoma skin cancers in the anti-ANP32A+ group and 97 non-melanoma skin cancers in the anti-ANP32A– group). Results were unchanged when excluding non-melanoma skin cancers.
†Pulmonary hypertension defined by estimated RVSP ≥45 mm Hg.
‡Moderate-to-severe weight loss/anaemia = weight loss ≥ 10.0 kg or haematocrit ≤ 32.9; moderate-to-severe Raynaud's = digital pits, ulceration or gangrene; moderate-to-severe lung = FVC and/or DLCO <70% predicted, mild-to-severe PH or oxygen dependence; moderate-to-severe heart = left ventricular ejection fraction < 45%, clinical signs of left or right heart failure or sustained clinically important arrhythmia; moderate-to-severe GI = high-dose acid reflux medications, antibiotics for bacterial overgrowth, malabsorption syndrome, episodes of pseudo-obstruction or total parental nutrition requirement; moderate-to-severe kidney= serum creatinine ≥ 1.7 mg/dL or at least 2+ urine protein; moderate-to-severe muscle = strength of ≤ 3/5 in the upper or lower extremities or the requirement of ambulatory aids.
ACR, American College of Rheumatology; ANA, anti-nuclear antibody; DLCO, diffusing capacity of the lungs for carbon monoxide; Echo, echocardiogram; FVC, forced vital capacity; GI, gastrointestinal; ILD, interstitial lung disease; mRSS, modified Rodnan skin score; no, number; pred, predicted; RP, Raynaud's phenomenon; RVSP, right ventricular systolic pressure; SSc, systemic sclerosis.

large sample of carefully phenotyped patients with SSc. Anti-ANP32A antibodies were observed in 4% of patients. While there was no association with malignancy, we found that patients who were anti-ANP32A positive may be more likely to develop pulmonary hypertension and less likely to have severe Raynaud's phenomenon. Further study is warranted to assess anti-ANP32A as a biomarker of pulmonary hypertension risk.

Rachel Wallwork , **Livia Casciola-Rosen, Ami A Shah**

Department of Medicine/Rheumatology, Johns Hopkins School of Medicine, Baltimore, Maryland, USA

Correspondence to Dr Ami A Shah, Medicine/Rheumatology, Johns Hopkins School of Medicine, Baltimore, Maryland, USA; Ami.Shah@jhmi.edu

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ORCID iD
Rachel Wallwork http://orcid.org/0000-0002-0073-7438

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The cost of arthralgia 'pretreatment' to prevent rheumatoid arthritis

The article by Helm-van Mil and Landewé[1] represents the tip of the iceberg. Justifying initiation of medications, in the absence of clinical or radiological evidence because an individual might at some time develop a particular disease is a speculative approach. We are now in an era of evidence-based medicine and such evidence is currently lacking for recognition of who will develop rheumatoid arthritis (RA). Actually, even controlled studies of undifferentiated arthritis failed to reveal progression to RA.[2–4] If progression to RA was not evidenced in those individuals who actually initially had arthritis, why would we have higher expectations for arthralgia? That speculation seems fallacious.

We have no idea what percentage, if any, of individuals with arthralgia will go on to develop RA. Even if medicinal interventions routinely utilised in the treatment of RA were without harmful side effects, their fiscal impact is substantial: cost of medications and safety monitoring, time loss (related to office visits and laboratory testing) and lifestyle complications (eg, avoiding contact with virally infected or freshly (live) vaccinated individuals). However, medical interventions are not without side effects. They are potentially significant and costly. Without evidence of what percentage of individuals with arthralgia, we cannot even conduct a cost-benefit analysis. All we can determine is the cost.

So, one must question the justification of initiating interventions (that are not without risk) because of the unverified speculation that the individual might at some time develop a particular disease (eg, RA). Helm-van Mil and Landewé[1] suggested that 'disease activity can be so well suppressed in most RA patients, too early treatment may do more harm than good'. That observation holds doubly for those individuals whose complaint (arthralgia) is limited joint pain without clinical evidence of arthritis.

Bruce M Rothschild [1,2]

[1]Department of Medicine, IU Health, Muncie, Indiana, USA

[2]Department of Vertebrate Paleontology, Carnegie Museums of Pittsburgh, Pittsburgh, Pennsylvania, USA

Correspondence to Professor Bruce M Rothschild, Medicine, IU Health, Muncie, IN 47303, USA; Spondylair@gmail.com

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ORCID iD
Bruce M Rothschild http://orcid.org/0000-0003-1327-6615

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Response to: 'The cost of arthralgia 'pretreatment' to prevent rheumatoid arthritis' by Rothschild

We read the letter from Rothschild with interest.[1] We agree that we need more data and better evidence on: (1) how to determine the risk of patients with arthralgia progressing to 'true RA' and on (2) whether or not Disease Modifying AntiRheumatic Drug (DMARD) treatment is better than placebo in this phase. Both points were discussed in our Viewpoint.[2] Cost–benefit analyses can only be performed afterwards.

Rothschild seems to agree with us not to condone treating patients presenting with arthralgia with DMARDs, pending further evidence. Still, there are different opinions in the field. This is illustrated by a recent study from the UK, in which rheumatologists were asked about their management in clinical practice of arthralgia patients with positive anti-cyclic citrullinated peptide antibodies antibodies and signs of synovitis on power Doppler in at least one joint, but in the absence of clinically apparent arthritis. Seventy-one per cent of consultants said to start DMARD treatment, 16% would treat with glucocorticoids only, 8% considered inclusion in a clinical trial and only 3% replied to wait and see without immediate initiation of DMARD treatment.[3]

We believe it may be harmful if the rheumatic field gets too comfortable with initiating DMARD treatment already in arthralgia patients with a certain risk of developing true rheumatoid arthritis (RA) without solid proof. Such a behaviour hampers the course of observational studies to properly determine the risk of RA in individual patients. Hindering the natural course of patients in observational studies with DMARD treatment means that we will never be able to know which patients are being overtreated. Importantly, the regular use of DMARDs in this setting may also hinder the inclusion of arthralgia patients in ongoing and future placebo-controlled trials, as this will then be considered increasingly counterintuitive or unethical because physicians in daily clinical practice may increasingly consider DMARD treatment standard of therapy.

Annette van der Helm-van Mil [1,2] **Robert BM Landewé**[3,4]

[1]Rheumatology, Leiden University Medical Center, Leiden, The Netherlands
[2]Rheumatology, Erasmus Medical Center, Rotterdam, The Netherlands
[3]Amsterdam Rheumatology Center, AMC, Amsterdam, The Netherlands
[4]Rheumatology, Zuyderland MC, Heerlen, The Netherlands

Correspondence to Professor Annette van der Helm-van Mil, Rheumatology, Leiden University Medical Center, 2333 ZA Leiden, The Netherlands; A.H.M.van_der_Helm@lumc.nl

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ORCID iD
Annette van der Helm-van Mil http://orcid.org/0000-0001-8572-1437

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Effectiveness and safety of ruxolitinib for the treatment of refractory systemic idiopathic juvenile arthritis like associated with interstitial lung disease : a case report

We read with interest the report of a series of 61 patients with systemic juvenile idiopathic arthritis (s-JIA) or s-JIA-like associated with high-fatality lung diseases.[1] Lung disease was associated with digital clubbing, a high frequency of anaphylactic reactions to tocilizumab, and macrophage activation syndrome (MAS). Because of the low 5-year survival probability of 42%, there is an urgent need to identify efficient drugs to treat such patients. Herein we report the effectiveness and safety of ruxolitinib in one patient demonstrating clinical and radiological characteristics consistent to those reported by Saper *et al*

A 2-year-old girl born to non-consanguineous parents presented with s-JIA-like since the age of 12 months, including recurring episodes of unexplained fever, urticaria, arthralgia, poor general health status, leukocytosis and elevated serum C-reactive protein (CRP). The use of corticosteroids resulted in a complete remission but the patient relapsed when prednisone was tapered below 0.5 mg/kg/day. Owing to this corticosteroid dependence, the patient received several lines of biological agents from the age 15–22 months, none of which was either effective nor tolerated. Anakinra had no benefit on the patient's features, and was replaced by canakinumab—but which resulted in a probable drug reaction with eosinophilia and systemic symptoms and a MAS after the third injection. Finally, a third line with tocilizumab led to severe anaphylactic reactions after the second infusion. At age 34 months, the patient developed acute digital clubbing without any respiratory symptoms. Chest CT scan showed a diffuse interstitial disease with interlobular septal thickenings, bronchovascular bundles thickenings, ground glass opacities with a peripheral and lower lobes predominance, and enlargement of mediastinal lymph nodes (figure 1). Bronchoalveolar lavage (BAL) fluid showed 2 50 000 cells /mL (macrophages: 66%, neutrophils: 27%, lymphocytes: 7%). Microbiological investigations were all negative. A whole exome sequencing did not identify any causal mutation.

The expression of interferon (IFN)-stimulated genes in the whole blood of the patient was normal on two occasions, and increased once while the patient received anti-interleukin-1 treatment. Immunophenotyping showed normal counts of T cell, B cell and natural killer (NK) lymphocytes. Further study showed that, among memory $CD4^+$ T cells, the patient had frequencies of Th1 cells well above the control range, and decreased frequencies of Th17 cells (figure 2).

At the age of 4, the patient was treated with the Janus kinase 1/2 selective inhibitor ruxolitinib (1 mg/kg/day) in association with oral prednisone (0.5 mg/kg/day) and 3 monthly intravenous infusions

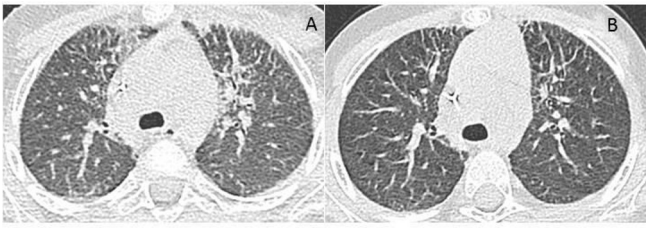

Figure 1 Radiological response to JAK1/JAK2 blockade with ruxolitininb . (A) Chest CT scan before the initiation of ruxolitinib. (B) Chest CT scan 12 months after the initiation of ruxolitinib.

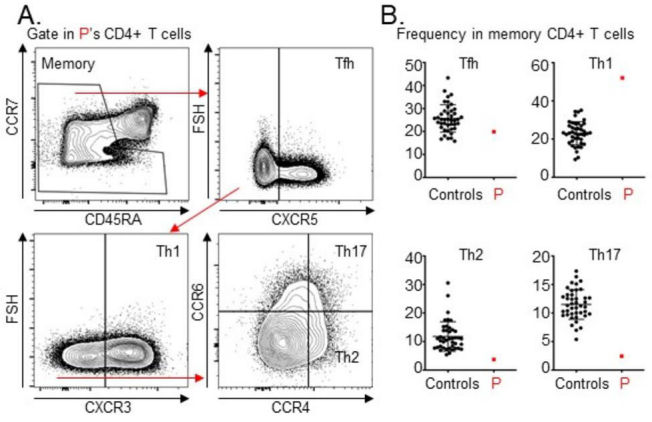

Figure 2 T helper immunophenotyping. (A) Gating strategy in the patient's peripheral blood mononuclear cells (PBMCs) for T helper immunophenotyping. (B) Representative fluorescence activated cell sorting (FACS) plots are presented. The horizontal bars represent the mean+/-SD. Frequencies of T helper subsets within the $CD4^+$ memory compartment in controls and P. Subsets were defined as follows: Th1 ($CXCR5^-CXCR3^+CCR4^-CCR6^-$), Th2 ($CXCR5^-CXCR3^-CCR4^+CCR6^-$), Th17 ($CXCR5^-CXCR3^-CCR4^+CCR6^+$) and Tfh ($CXCR5^+$).

of methylprednisolone (600 mg/m^2). At last follow-up, 15 months post initiation of ruxolitinib, febrile attacks remitted, CRP value was normal, prednisone was tapered to 0.1 mg/kg/day, and methylprednisolone dosage was progressively decreased to one infusion (300 mg/m^2) every 6 weeks. Chest CT scan abnormalities decreased (figure 1). Oxygen saturation increased from 92% to 100%. There was a catch-up growth with an improvement of the height Z-scores from −3 to −2. Ruxolitinib was well tolerated.

Our patient demonstrates features similar to those reported in the series of Saper *et al*. An upregulation of genes related to the IFNγ reponse both in the (BAL) fluid and lung tissue was demonstrated in some patients from this series.[2] In line with a putative pathogenic role of IFNγ signaling, a high frequency of MAS was reported. The observation in our patient of (1) elevated frequency of Th1 cells, main producers of IFNγ[3], and (2) a clinical response to ruxolitinib, blocking the IFNγ signalling, although not selectively,[4 5] is consistent with this hypothesis. Although no definite conclusion can be drawn from this single case, our report suggests that ruxolitinib may represent a valid therapeutic option to be tested early in patients with s-AJI associated with severe early-onset lung disease.

Brigitte Bader-Meunier ,[1,2] **Alice Hadchouel**,[3] **Laureline Berteloot**,[4] **Laura Polivka**,[5] **Vivien Béziat**,[6,7] **Jean-Laurent Casanova**,[1,6,7,8,9] **Romain Lévy**[1,6,7]

[1]Pediatric Hematology-Immunology and Rheumatology Unit, Hôpital Necker-Enfants Malades, AP-HP Centre, Paris, France
[2]INSERM UMR 1163, Laboratory of Immunogenetics of Paediatric Autoimmunity, Institut Imagine, Paris, France
[3]Pediatric Pneumology Unit, Hôpital Necker-Enfants Malades, AP-HP Centre, Paris, France
[4]Pediatric Radiology Unit, Hôpital Necker-Enfants Malades, AP-HP Centre, Paris, France
[5]Dermatology and Pediatric Dermatology Unit, Hôpital Necker-Enfants Malades, AP-HP Centre, Paris, France
[6]Laboratory of Human Genetics of Infectious Diseases, Necker Branch, INSERM UMR 1163, Paris, France
[7]Paris Descartes University, Imagine Institute, Paris, France
[8]St Giles Laboratory of Human Genetics of Infectious Diseases, Rockefeller Branch, The Rockefeller University, New York, NY, USA
[9]Howard Hughes Medical Institute, New York, NY, USA

Correspondence to Dr Brigitte Bader-Meunier, Service d'Immunologie-Hematologie et Rhumatologie Pediatrique, Hopital universitaire Necker-Enfants malades, Paris 75015, France; brigitte.bader-meunier@aphp.fr

Contributors LP and BB-M collected clinical data. VB, J-LC and RL performed the immunological experiments and analysed data. BB-M, LP, AH and LB wrote the paper. AH and LB analysed the pulmonary clinical and radiological data. RL and BB-M supervised the study. All authors have read final approval of the version published.

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ORCID iD
Brigitte Bader-Meunier http://orcid.org/0000-0001-8476-8196

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Response to: 'Effectiveness and safety of ruxolitinib for the treatment of refractory systemic idiopathic juvenile arthritis like associated with interstitial lung disease: case report' by Bader-Meunier *et al*

We were very interested to read the correspondence from Dr Bader-Meunier and colleagues, who report a case of systemic juvenile idiopathic arthritis (sJIA) with associated lung disease.[1] This case indeed matches the description of and provides further evidence for the striking and unusual clinical characteristics we recently detailed in an international case series of such patients.[2] We thank the authors for this communication.

One aspect of particular importance in the reported case is the development of drug reaction with eosinophilia and systemic symptoms (DReSS), a delayed hypersensitivity reaction, to canakinumab (monoclonal antibody to interleukin (IL)-1β). As we noted, DReSS is a possibly under-recognised event preceding the clinical recognition or development of this lung disorder in at least a subset of patients.[2] The authors' correspondence, along with Drs Bader-Meunier and Povlika's prior publication of two cases of probable DReSS in association with IL-1 inhibitors,[3] highlights the need to consider this type of drug hypersensitivity.

Further studies will be required to evaluate the potential benefit of ruxolitinib for sJIA and lung disease and will be of considerable interest to clinicians caring for children with this condition. In addition, and importantly, removal of the IL-1 inhibitor in this case may also have contributed to the improved lung status. Early cessation of the suspect drug is a key to controlling DReSS progression, and DReSS can continue for a period of time subsequent to withdrawal of the causative medication.[4] High-dose steroids, well-known to be efficacious in active sJIA, also may have provided broader clinical benefit,[4] including for lung disease. Indeed, the apparent increased incidence of parenchymal lung disease in children with sJIA occurred concurrent with changes in sJIA management that typically involve reduced steroid use.[2]

The patient in this report showed increased expression of interferon (IFN)-stimulated genes during anti-IL-1 treatment, consistent with evidence that a subset of patients on cytokine inhibition for sJIA developed an IFN signature/heightened response.[5 6] Together with the observation of elevated circulating Th1 cells, the findings raise the possibility that IFN-γ signalling is a target of ruxolitinib in this patient. There may be other targets, as the authors acknowledge. A role for IFN-γ in lung disease in sJIA has been proposed[7] and requires more investigation. IFN-γ is strongly implicated in macrophage activation syndrome (MAS).[8 9] In sJIA cases with the constellation of lung disease and unusual clinical features, we looked at the occurrence of MAS in relation to lung disease. We found that, whereas 33% had MAS before lung disease detection (most at disease onset and responsive to treatment), 78% had MAS at or during lung disease, 52% being overt MAS and 26% being subclinical (table 1). More research is needed to determine whether MAS in sJIA with lung disease is triggered by DReSS[10] or by lung disease, or reflects the immune dysfunction that causes lung disease.

Based on these observations, it is very difficult to assign the sources of improvement in this case with certainty. Nonetheless, as the authors suggest, further testing of the safety and effectiveness of ruxolitinib or other Janus kinase inhibitors in sJIA with lung disease appears warranted.

Table 1 MAS in relation to lung disease

MAS before lung disease*†		MAS at or during lung disease‡		P value
Total MAS	15 (33%)§	Total MAS	36 (78%)	<0.0001
Overt	15	Overt	24	0.059
Subclinical only	0	Subclinical only	12	0.016
At sJIA onset	12 (26%)	First MAS episode	20 (43%)	
>1 episode	6 (13%)			

*MAS definitions were per Ravelli *et al*.[11]
†Before lung disease: up to 6 months before diagnosis of lung disease, because true onset of lung disease is not known.
‡At or during lung disease: from 6 months before diagnosis of lung disease and thereafter.
§n=46 patients with sJIA, parenchymal lung disease and exposure to IL-1/IL-6 inhibition.
IL, interleukin; MAS, macrophage activation syndrome; sJIA, systemic juvenile idiopathic arthritis.

Vivian E Saper,[1] Guangbo Chen,[2] Purvesh Khatri,[2] Elizabeth D Mellins [3]

[1]Department of Pediatrics, Stanford University, Stanford, California, USA
[2]Institute for Immunity, Transplantation and Infection, Center for Biomedical Informatics, Medicine, Stanford University, Stanford, California, USA
[3]Department of Pediatrics, Program in Immunology, Stanford University, Stanford, California, USA

Correspondence to Dr Elizabeth D Mellins, Department of Pediatrics, Program in Immunology, Stanford University, Stanford, CA 94305, USA; mellins@stanford.edu

Handling editor Josef S Smolen

Contributors VES, GC, PK and EDM all contributed to and agreed on the final version of this response to correspondence.

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ORCID iD
Elizabeth D Mellins http://orcid.org/0000-0003-2577-139X

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Do 2019 European League against rheumatism/American College of Rheumatology classification criteria for systemic lupus erythematosus also indicate the disease activity?

The 2019 European League against rheumatism/American College of Rheumatology classification criteria (2019 criteria) for systemic lupus erythematosus (SLE) has introduced a new scoring system to classify SLE.[1] It is a thrill for rheumatologists to get the new SLE classification criteria, which has both excellent sensitivity and specificity, and further demonstrated by other studies to be effective in the early SLE diagnosis and distinguish patients with SLE from patients with primary Sjögren's syndrome.[2 3] It has also been reported that higher scores of 2019 criteria were associated with higher rates of organ damage.[4] While using antinuclear antibody (ANA) as an entry criterion, these hierarchically clustered and weighted criteria made a significant breakthrough compared with the past several criteria. However, based on the thinking of weighted criteria, we are curious as to whether they can reflect the disease activity of SLE? Because assessing tools such as SLE disease activity index (SLEDAI) and revised systemic lupus activity measure (SLAM-R) also use weighted scores widely, could these weighted items in the new criteria have the same trend?

Thus, we enrolled 96 consecutive and hospitalised new-onset SLE patients in the Department of Rheumatology and Immunology of Ruijin Hospital from August 2016 to June 2018. The data were collected through the electronic medical records. Two qualified senior rheumatologists (JT and CY) confirmed the diagnosis of SLE according to the 2019 criteria. SLEDAI and SLAM-R were recorded when the patients were hospitalised and confirmed by another two qualified rheumatologists (JY and ZZ). British Isles Lupus Assessment Group was not used in this study. Because it should be compared with the previous visit's disease activity, while, there is only one visit available for new-onset patients. This study was approved by the Ethics Committee of Ruijin Hospital.

As a result, the mean age was 41±16 years and the mean duration was 19±53 months. Eighty-three (86%) were female and 13 (14%) were male. The detailed distribution of clinical characteristics according to 2019 criteria were shown in table 1. It was interesting to find out that 2019 criteria correlated positively with SLEDAI ($p<0.0001$, $r^2=0.65$) and SLAM-R ($p<0.0001$, $r^2=0.37$) (figure 1). Compared with SLAM-R, SLEDAI were more convergent and correlated better with the scores of 2019 criteria. Both correlations provided some clues that the score of 2019 criteria might also indicate the disease activity.

Table 1 The clinical characteristics of new-onset SLE patients diagnosed with 2019 criteria

Clinical criteria	Positive/total patients (%)
N	96
Fever	40/96 (42)
Cutaneous domain	
Acute cutaneous lupus	24/96 (25)
Subacute cutaneous lupus or discord rash	25/96 (26)
Non-scaring alopecia	19/96 (20)
Oral ulcer	18/96 (19)
Arthritis domain	51/96 (53)
Serositis domain	25/96 (26)
Pleural or pericardial effusion	21/96 (22)
Acute pericarditis	4/96 (4)
Renal domain	37/96 (39)
Protein >0.5 g/24 hours	37/96 (39)
Class II or V lupus nephritis	0/96 (0)
Class III or IV lupus nephritis	0/96 (0)
Neurologic domain	
Seizure	0/96 (0)
Psychosis	0/96 (0)
Delirium	0/96 (0)
Haematologic domain	
Leucopenia	61/96 (64)
Thrombocytopenia	31/96 (32)
Autoimmune haemolysis	57/96 (59)
Laboratory criteria	Positive/total patients (%)
Immunologic	
Anticardiolipin antibodies	10/96 (10)
Antibeta2-glycoprotein antibodies	23/96 (24)
Lupus anticoagulant	26/96 (27)
Complement domain	86/96 (90)
Highly specific antibodies domain	
Anti-dsDNA antibody	87/96 (91)
Anti-Sm antibody	20/96 (21)

Anti-dsDNA, antibodies to double-stranded DNA; Anti-Sm, anti-Smith; SLE, systemic lupus erythematosus.

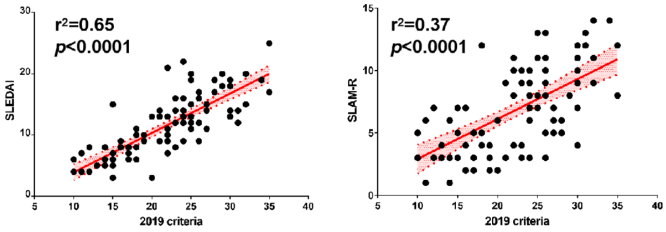

Figure 1 The correlation of 2019 criteria with SLEDAI and SLAM-R. 2019 criteria correlated positively with SLEDAI ($p<0.0001$, $r^2=0.65$) and SLAM-R ($p<0.0001$, $r^2=0.37$). SLAM-R, revised systemic lupus activity measure; SLEDAI, SLE disease activity index.

In our study, it is the first attempt to associate 2019 criteria with SLEDAI and SLAM-R. SLEDAI correlates better with 2019 criteria, partly because items in 2019 criteria are similar to those in SLEDAI. However, in 2019 criteria, with each domain, only the highest weighted criterion is counted, while, in SLEDAI, they are counted separately.[1 5] SLAM-R has items that exist neither in 2019 criteria nor in SLEDAI,which may finally lead to the difference in the analysis of correlation.

Besides, when using 2019 criteria in the clinic, it increases burden for rheumatologists to calculate scores, and then to calculate SLEDAI or SLAM-R. Considering the efforts for the rheumatologists to memorise 2019 criteria and two more disease activity score systems, it raises a question whether it is possible in the future we will have new criteria that could be used both as disease activity and classifying criteria?

Jialin Teng, Zhuochao Zhou, Fan Wang, Yue Sun, Hong-Lei Liu, Xiaobing Cheng, Yutong Su, Hui Shi, Chengde Yang, Junna Ye

Department of Rheumatology and Immunology, Ruijin Hospital,Shanghai Jiao Tong University School of Medicine, Shanghai, China

Correspondence to Junna Ye, Shanghai, China; yjn0912@qq.com

Contributors Concept and writing: JY and JT; revising: YS and YD; acquisition of data: ZZ, FW and YS; analysis and interpretation of data: LL, XC and HS.

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ORCID iDs
Jialin Teng http://orcid.org/0000-0003-1016-9064
Yutong Su http://orcid.org/0000-0003-0488-2939
Chengde Yang http://orcid.org/0000-0002-3720-634X

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Response to: 'Do 2019 European League against rheumatism/American College of Rheumatology classification criteria for systemic lupus erythematosus also indicate the disease activity?' by Teng *et al*

The letter by Dr Teng and colleagues[1] describes an interesting intellectual exercise. In showing that the scores of the European League Against Rheumatism (EULAR)/American College of Rheumatology (ACR) 2019 classification criteria for systemic lupus erythematosus (SLE)[2 3] correlate very well (r=0.81) with the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) in their SLE patient cohort, they have added disease activity to the concepts inter-related to the new SLE criteria. These relationships, which were discussed for SLE disease severity or 'ominousity' early in the criteria development process,[2 3] may have more overlap than we usually think based on theoretical concepts. In this way, the data by Dr Teng *et al*[1] add to the proof of face validity of the EULAR/ACR criteria.

While the tools to assess criteria versus disease activity may share certain domains and weight, there are relevant distinctions for capturing disease activity. In particular, sensitivity to change of individual domains and its relation to damage accrual appears to be crucial and requires different validation than classification.

However, the fact that the SLEDAI correlates with the EULAR/ACR criteria better than the revised systemic lupus activity measure (r=0.61) may also point to construct criteria the EULAR/ACR criteria share with the SLEDAI.[4] Both have weighted items, even though the weights are based on different reasoning. Both count each individual item in a categorical way. Both have limited themselves to a feasible number of items. Moreover, while not so obvious an overlap, a useful SLE activity score should have significant specificity for SLE.

For the EULAR/ACR 2019 classification criteria, it is also obvious that most of the criteria items are indeed associated with activity. This is true for all clinical items, which are only present in active disease, but also for low complements and for antibodies to double-stranded DNA (dsDNA). The arguable exceptions are anti-Sm antibodies and antiphospholipid antibodies, but anti-Sm antibodies without concomitant anti-dsDNA antibodies are relatively uncommon, and antiphospholipid antibodies carry a low weight in the new criteria system.[2 3] This makes the correlation understandably robust.

At the same time, and this is of importance, the SLEDAI is based on counting only currently active disease. In contrast, the classification criteria count items not only on a given day but also historically present.[2 3 5] For classification, it is sufficient to have had this organ manifestation once.[2 3 6 7] This is a major and important difference to the approach taken by Dr Teng and colleagues.[1] Their clever approach is revealing as an intellectual exercise, which we highly appreciate. However, it should not lead to the erroneous idea that this was a legitimate use of the EULAR/ACR classification criteria. Modifying important parameters, such as an item having been present to be sufficient for classification, would change outcomes.

Martin Aringer ,[1] **Karen Costenbader**,[2] **Thomas Dörner** ,[3] **Sindhu R Johnson** [4]

[1]Division of Rheumatology, Department of Medicine III, University Medical Center and Faculty of Medicine at the TU Dresden, Dresden, Germany
[2]Division of Rheumatology and Immunology, Department of Medicine, Brigham and Womens Hospital, Harvard Medical School, Boston, Massachusetts, USA
[3]Department of Rheumatology and Clinical Immunology, Charité – Universitätsmedizin Berlin, Corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany
[4]Division of Rheumatology, Department of Medicine, Toronto Western Hospital, Mount Sinai Hospital; Institute of Health Policy, Management and Evaluation, University of Toronto, Toronto, Ontario, Canada

Correspondence to Professor Martin Aringer, Internal Medicine III, Medical Faculty, Technical University of Dresden, Dresden 01307, Germany; martin.aringer@uniklinikum-dresden.de

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ORCID iDs
Martin Aringer http://orcid.org/0000-0003-4471-8375
Thomas Dörner http://orcid.org/0000-0002-6478-7725
Sindhu R Johnson http://orcid.org/0000-0003-0591-2976

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Dupilumab as a *potential* steroid-sparing treatment for IgG4-related disease

We read with interest the article from Simpson *et al* on the efficacy of dupilumab—an anti-IL-4 receptor alfa monoclonal antibody—in a patient with multi-organ IgG4-related disease (IgG4-RD) involving the retroperitoneum and, apparently, the prostate and the parotid glands.[1] According to the case presentation, the patient refused immunosuppressive agents due to the risk of adverse events, and treating physicians decided to start him on 40 mg oral prednisone. Subcutaneous dupilumab was added based on multiple concomitant poorly controlled atopic manifestations including asthma, dermatitis and periorbital angioedema. Prednisone was tapered over 2 months and then withdrawn. Dupilumab was administered subcutaneously at initial dose of 600 mg, followed by 300 mg injections every other week for 12 months. Three months later, amelioration of all manifestations was observed, and after 12 months on dupilumab, retroperitoneal fibrosis was dramatically improved. Sensible decrease in serum IgG4 was also reported after 3 months of treatment. Based on these findings the authors conclude that dupilumab is effective in IgG4-RD and represents a novel steroid sparing treatment for this multifaceted condition.

Although we strongly support the idea that, by interfering with IL-4 and IL-13 pathways, dupilumab might represent a promising biologic therapy for IgG4-RD, we would like to share with the authors our perplexities about the conclusions drawn from this case report.[2]

First and foremost, we think that concomitant use of glucocorticoids in the first months prevents from claiming that dupilumab is effective in IgG4-RD. Prompt response to corticosteroids is, in fact, a hallmark of IgG4-RD.[3] As such, the rapid improvement of the retroperitoneal fibrosis as well as the reduction of serum IgG4 levels were expected after induction of remission therapy with glucocorticoids. Indeed, failure to respond to an appropriate dose of glucocorticoids is considered an exclusion criterion by the recently released American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) Classification Criteria for IgG4-RD.[4]

Second, the authors provide a follow-up abdominal magnetic resonance after 12 months of treatment with dupilumab showing marked improvement compared with baseline. A comparison with the evolution of the retroperitoneal fibrosis at 3 months, after corticosteroid treatment, would have been more useful to provide definitive proof of the additional benefit of dupilumab over steroid therapy. Response of IgG4-RD to glucocorticoids, in fact, can last for up to 2–3 years before disease relapses, and a 12-month follow-up is, to our mind, too short to conclude that dupilumab alone maintains remission.[5]

Finally, magnetic resonance findings appear difficult to compare because the images captured before and after treatment are not representative of the same anatomical structures, and the contrast enhanced sequences used are different. In addition, while different organs affected by IgG4-RD can respond differently to immunosuppressive treatments, the authors do not provide evidence of disease response in the prostate and salivary glands.

All together, although we think that no clear conclusions can be drawn on the efficacy of dupilumab in IgG4-RD from this case, we also believe that the experience reported by Simpson and colleagues is of value to highlight the importance of exploring novel therapeutic targets for IgG4-RD. Current available treatments for IgG4-RD including glucocorticoids and B-cell depleting agents, in fact, are associated with many potential side effects, and their long-term use can become problematic in a disease that frequently affects middle-aged to elderly individuals.[6–8] Given the central role of IL-4 and IL-13 in IgG4 class switch and tissue fibrosis, targeting these pathways has strong rationale in IgG4-RD.[2] Whether dupilumab might find ideal application in disease phenotypes characterised by peripheral eosinophilia, elevation of serum IgG4 or IgE levels, or atopic manifestations remains speculative and deserves confirmation in randomised clinical trials.[9]

Emanuel Della-Torre [1,2] **Marco Lanzillotta,**[1,2] **Mona-Rita Yacoub**[1,2]

[1]Università Vita-Salute San Raffaele, San Raffaele Scientific Institute, Milan, Italy
[2]Unit of Immunology, Rheumatology, Allergy, and Rare Diseases (UniRAR), San Raffaele Scientific Institute, Milan, Italy

Correspondence to Dr Emanuel Della-Torre, Università Vita-Salute San Raffaele, San Raffaele Scientific Institute, 20132 Milano, Italy; dellatorre.emanuel@hsr.it

Contributors ED-T: conceived the hypothesis, analysed data and drafted themanuscript. ML: conceived the hypothesis, analysed data, critically revised the manuscript and gave the final approval. M-NY: analysed data, critically revised the manuscript and gave the final approval.

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ORCID iD
Emanuel Della-Torre http://orcid.org/0000-0002-9192-4270

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Response to: 'Dupilumab as a *potential* steroid-sparing treatment for IgG4-related disease' by Della-Torre *et al*

We thank Della-Torre *et al* for their interest in our paper and for providing their thoughts through correspondence.[1 2] As they have summarised, our case described a patient whom was prescribed a 40 mg daily dose of prednisone to treat his IgG4-related disease (IgG4-RD); however, the patient declined to pursue additional adjunct immunosuppressants due to the concern of adverse effects.[1] To hopefully mitigate such concerns and control his disease, we proposed that dupilumab, a monoclonal anti-interleukin 4 (IL-4) and IL-13 antibody, would be able to treat the patient's condition mechanistically through two pathways. First, because IL-4 is the signal which plays a pivotal role in class switching from IgM to IgG, we theorised that dupilumab would be able to reduce the amount of serum IgG and IgG4, which is a hallmark of IgG4-RD pathology. Second, because dupilumab inhibits IL-13, which is implicated in the activation of fibroblasts causing fibrosis,[3] we believed dupilumab would also be able to address this component of the IgG4-RD mechanism of disease. Upon initiation of dupilumab treatment, we observed remission of the patient's condition with improvements starting as soon as 3 months post-dupilumab treatment.[1] As such, our theory is that IgG4-RD is an example of type 2 inflammation, similar to other conditions such as chronic spontaneous urticaria (CSU) that have been misclassified as non-type 2 inflammation in the past.[4] This is what we believe allows dupilumab to be an effective treatment.[4]

To address Della-Torre *et al*'s first point with respect to their concern that the remission in the patient's IgG4-RD was actually due to a delayed response to glucocorticoids, at the time of publication, his records from rheumatology stated that the patient was on 40 mg prednisone daily prior to initiating dupilumab treatment. However, from a recent correspondence with the patient postpublication (on 29 December 2019), it has actually come to light that the patient was non-compliant and did not take a single dose of the prednisone that was prescribed. This oversight was due to discordance of clinical report and patient compliance. The absence of any corticosteroids however, greatly strengthens the role of dupilumab in reversing IgG4-RD. As such, we conclude that the improvements in the patient's condition can actually be fully attributed to effectiveness of dupilumab therapy alone.

Second, Della-Torre *et al* expressed concerns with respect to the timelines of the MRI provided.[2] Although it would have been ideal to implement MRI more frequently over the course of treatment, there is no current accepted consensus on how frequently imaging should take place to monitor IgG4-RD progression and such factors such as availability and cost should be taken into consideration as limiting factors.[5] Specifically, in our jurisdiction of Ontario, Canada, MRI booking is triaged based on priority and wait times can vary greatly based on this necessity.

With regards to Della-Torre *et al*'s point that 12 months post-treatment is too soon to pronounce disease remission in our case, we recognise that because IgG4-RD has a relapsing-remitting disease progression, it is inherently difficult to determine if a patient will remain in remission. We can state his manifestations both externally and internally are controlled and from the patient's complete resolution of IgG4-RD-related symptoms including fibrosis, atopic dermatitis, asthma, parotitis, prostatitis and sinusitis, along with his decrease in serum IgG and IgG4 levels, we believe we have sufficient evidence to conclude that there is remission and/or control. Moreover, there is no consensus guideline to assess IgG4-RD remission.[5] The IgG4-RD responder index, which is a tool that can help in assessing IgG4-RD remission, was not done over the course of treatment as we did not have publication in mind at the time, but when done retrospectively for the 12 months post-treatment timepoint, the patient's organ site activity score was 0 and his serum IgG4 concentration was 11.43 g/L. This is significantly improved from his previous multiple organ involvement pretreatment and his serum IgG4 concentration of 20.60 g/L showing compelling evidence of remission. Due to the fact that IgG4-RD does exhibit a remitting-relapsing disease progression, we continue to monitor the patient's successful improvements on dupilumab and report that we have not observed any relapses or adverse effects since the initiation of treatment or since the time of publication.

Finally, we thank Della-Torre *et al* for pointing out the discrepancies in figure 1. Unfortunately, an error occurred in which the lower section of figure 1B was omitted during the publication process of our paper. We have thus included in this response to show the respective anatomical structures such as the prostate for comparison against figure 1A (figure 1). The imaging slices were selected as they show a similar region and were recommended by our radiologist. The same procedure and contrast (gadolinium) were used for both images. The initial findings pretreatment that revealed parotitis were found through a CT of the neck, chest, abdomen and pelvis. However, the 12-month postdupilumab treatment MRI was only done of the lumbar plexus region to prioritise the most pressing symptoms and reduce healthcare burden as the patient was asymptomatic with regards to parotitis at 12 months postdupilumab treatment and showed no further signs of parotitis on physical examination. As can be seen in the 12-month post-treatment lumbar plexus MRI (figure 1), prostatitis improved significantly and this was noted in the report from radiology.

Due to the fact that Della-Torre *et al*'s concerns lie predominantly around the belief that a delayed response to glucocorticoids confounds our result of dupilumab being an effective treatment for IgG4-RD, we believe that such issues are no longer applicable to our case considering it has recently been brought to our attention that the patient never commenced prednisone

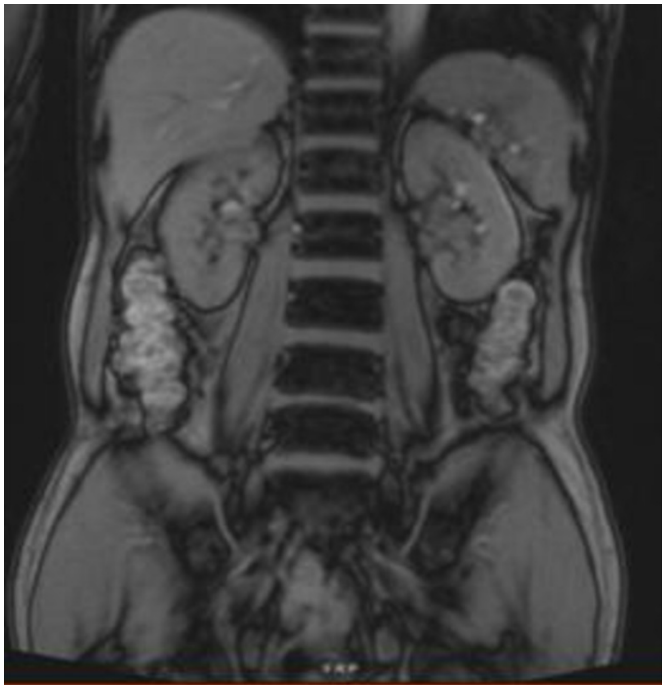

Figure 1 MRI taken approximately 1-year postdupilumab treatment showed dramatic resolution of fibrosis.

treatment out of fear of side effects and failed to disclose this to their healthcare provider. However, we recognise the fact that we have observed the remission of IgG4-RD in only a single patient and that larger randomised control trials would ideally provide the most robust evidence to conclude the safety and efficacy of dupilumab for IgG4-RD. Due to the fact that IgG4-RD is a rare condition in which the prevalence is actually unknown because it is commonly misdiagnosed or unrecognised,[6] this can be a profound limiting factor to recruitment in such trials. We advocate for these randomised control trials to be done and believe that our case provides strong rationale to further explore the uses of dupilumab for IgG4-RD.

Rachel S Simpson , Jason K Lee

Department of Allergy and Immunology, Toronto Allergists, Toronto, M5G 1E2, Canada

Correspondence to Ms Rachel S Simpson, Toronto Allergists, Toronto, M5G 1E2, Canada; rachel.simpson@queensu.ca

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ORCID iD
Rachel S Simpson http://orcid.org/0000-0003-1779-4049

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Correspondence on: 'Dupilumab as a novel steroid-sparing treatment for IgG_4-related disease' by Simpson *et al*

We read with interest the letter by Simpson *et al* reporting the use of dupilumab in a patient with IgG_4-related disease (IgG_4-RD).[1] Indeed blocking the IL-4/IL-13 pathway is tempting, considering the roles of these cytokines and the physiopathological changes observed during IgG_4-RD.[2 3]

We have also treated with dupilumab a 51-year-old man with IgG_4-related dacryoadenitis and sialadenitis (bilateral lacrimal, parotid, sublingual and submandibular involvement, pattern of Mikulicz syndrome). Left submandibular salivary gland and two cervical lymph nodes biopsies concluded, respectively, to lymphoplasmacytic polyclonal infiltration and reactive hyperplasia.

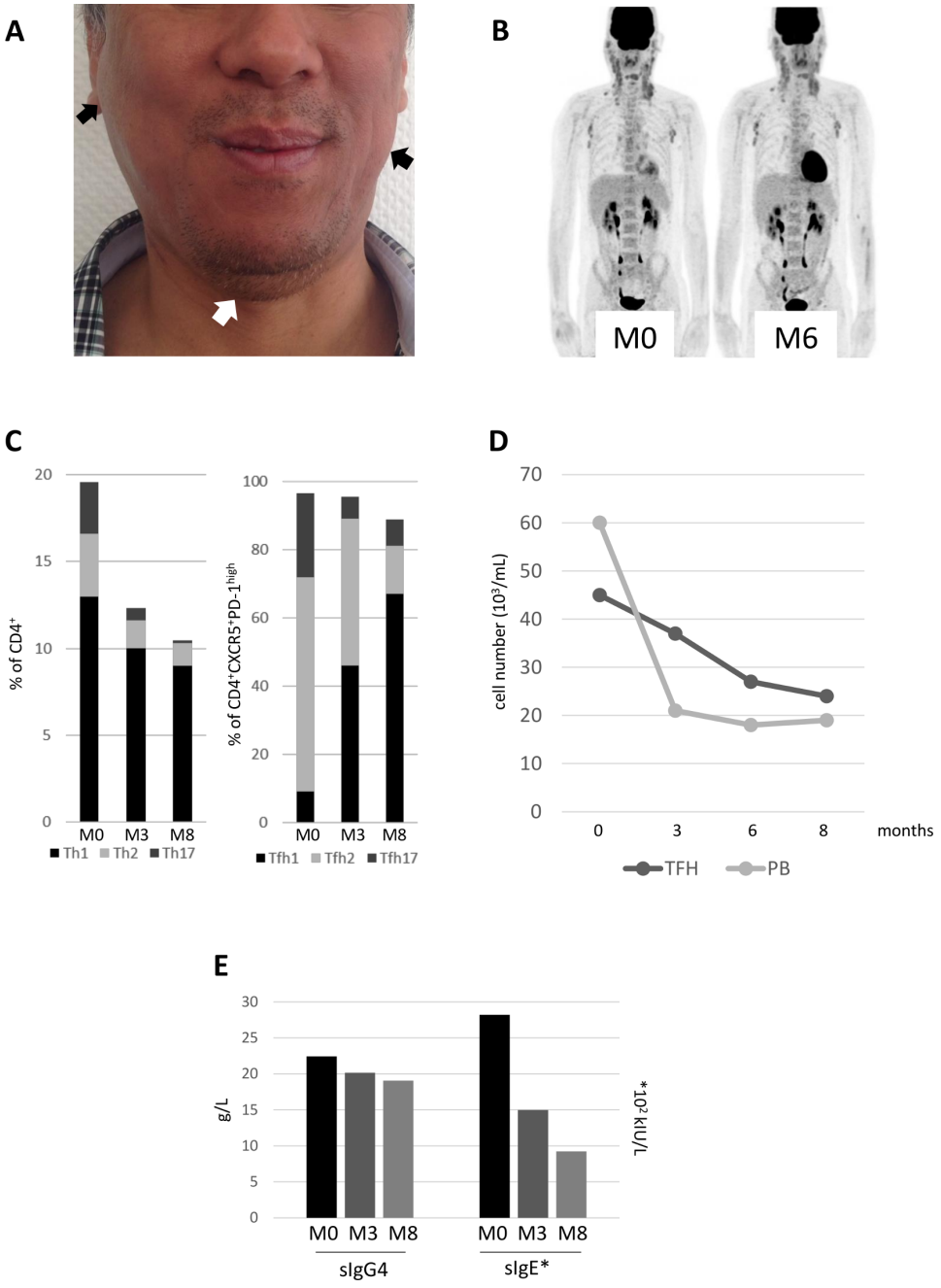

Figure 1 (A) Patient's initial clinical findings before dupilumab treatment showed bilateral parotid (black arrows), sublingual (white arrow), submandibular glands and cervical lymph nodes involvements. (B) ^{18}FDG-PET/CT scan before and 6 months after dupilumab treatment alone showed no improvement of salivary glands and lymph nodes involvements. (C) Proportion over time under dupilumab of T helper (Th)1 ($IFNg^+$), Th2 ($IL4^+$) and Th17 ($IL17^+$) cells among T $CD3^+CD4^+$ cells; and proportion over time under dupilumab of T follicular helper (Tfh)1 ($CD4^+CXCR5^+PD\text{-}1^{high}CXCR3^+CCR6^-$), Tfh2 ($CD4^+CXCR5^+PD\text{-}1^{high}CXCR3^-CCR6^-$) and Tfh17 ($CD4^+CXCR5^+PD\text{-}1^{high}CXCR3^-CCR6^+$) cells among Tfh ($CD4^+CXCR5^+PD\text{-}1^{high}$) cells. (D) Absolute plasmablast (PB) ($CD19^+CD27^{high}CD38^{high}$) and Tfh ($CD4^+CXCR5^+PD\text{-}1^{high}$) count ($10^3$/mL) at baseline (month 0) and under dupilumab (months 3, 6 and 8). (E) Serum IgG_4 (g/L) and IgE (10^2 kIU/L) levels at baseline (month 0) and under dupilumab (months 3 and 8). IFNg, interferon gamma; IL4, interleukin-4.

Immunohistochemistry was only available on lymph nodes, showing an $IgG_4^+/CD138^+$ plasma cells ratio over 40% with 40 IgG_4^+ plasma cells/high power field. Serum IgG_4 and IgE levels were found to be 17.7 g/L (normal range <0.8 g/L) and 3499 kIU/L (normal range <100 kIU/L), respectively, with normal complete blood count, liver and renal function tests. According to the 2019 American College of Rheumatology/European League Against Rheumatism classification criteria for IgG_4-RD, entry criteria were met, no exclusion criteria were present, and the total points score was 29 (≥20).[4] Clinical and radiological evaluation revealed no other IgG_4-related involvement, except for cervical and axillary lymph nodes (figure 1A,B). The patient had cervical marked discomfort without sicca syndrome. Salivary glands and lymph nodes involvement improved after a 3 months steroid course but relapsed. A second course of steroids was associated with rituximab (two infusions of 1000 mg, 15 days apart) with clinical, biological (serum IgG_4 level reduced to 7.59 g/L) and radiological response, but a new relapse occurred 1 year later. Because the patient also presented chronic rhinosinusitis with nasal polyps, a medical history of asthma and urticarial manifestations, a treatment with dupilumab was introduced without any other treatment, with an initial 600 mg subcutaneous injection, followed by a 300 mg subcutaneous injection every other week for 8 months. Rapidly the patient reported improvement of allergic rhinitis manifestations, but after an 8 months treatment course with dupilumab, salivary gland and lymph nodes involvements remained unchanged on clinical and radiological evaluation (figure 1B). Blood evaluation showed under dupilumab a decrease of total T helper 2 cells, T follicular helper (Tfh) cells, Tfh2 cells and plasmablasts after 3 and 8 months of treatment (figure 1C,D). Serum IgE significantly declined, but serum IgG_4 only slightly decreased during the treatment (figure 1E).

Glucocorticoids are the first line agent for remission induction in patients with IgG_4-RD, with a high rate of response but frequent relapse during tapering or after discontinuation.[5] Different maintenance therapy has been proposed, such as low-dose steroids, conventional steroid-sparing medications (azathioprine, mycophenolate mofetil, methotrexate) or rituximab, with different profiles of efficacy and side effects. In the case reported by Simpson *et al*, prednisone was administered initially during 2 months. We think that the initial clinical and biological response reported by Simpson *et al* could be attributable to steroids rather than to dupilumab, and that the radiological response presented at 12 months (and not assessed at 3 months) should be analysed cautiously. Indeed, in our patient, we observed no clinical nor radiological improvement after an 8-month course of dupilumab alone without steroids. However, we observed significant and interesting immunological improvements, with a marked decrease of Tfh2 cells and plasmablasts, which have been both correlated with disease activity,[2 3 6] despite the fact that serum IgG_4 remained fairly stable. We think that these observations taken together suggest that dupilumab could be an interesting and well tolerated therapeutic approach in IgG_4-RD, but rather as relapse-free maintenance therapy after induction treatment. This strategy should be further evaluated in a dedicated clinical trial.

Mikael Ebbo ,[1,2] **Benjamin De Sainte-Marie,**[1] **Romain Muller,**[1] **Christelle Piperoglou,**[3] **Aurélie Grados,**[4] **Frédéric Vély,**[2,3] **Nicolas Schleinitz**[1,2]

[1]Internal Medicine, AP-HM, Hôpital de la Timone, Marseille, France
[2]Aix Marseille Univ, CNRS, INSERM, CIML, Marseille, France
[3]Immunology, AP-HM, Hôpital de la Timone, Marseille, France
[4]Internal Medicine, Centre hospitalier de Niort, Niort, France

Correspondence to Dr Mikael Ebbo, Internal Medicine, AP-HM, Hôpital de la Timone, Marseille, France; mikael.ebbo@ap-hm.fr

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ORCID iD
Mikael Ebbo http://orcid.org/0000-0002-1179-8113

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Response to: 'Correspondence on: 'Dupilumab as a novel steroid-sparing treatment for IgG4-related disease' by Simpson *et al*' by Ebbo *et al*

We thank Ebbo *et al* for their interest in our case report[1] and for providing their case of a patient with IgG4-related disease (IgG4-RD) whom they have also treated with dupilumab.[2] Notably, Ebbo *et al* reported variability in the response to dupilumab compared with our patient. Our patient did have different clinical manifestations than that of Ebbo *et al*'s patient, as our patient had profound retroperitoneal and genitourinary fibrosis along with atopic dermatitis, asthma, allergic rhinoconjunctivitis, parotitis, and sinusitis while Ebbo *et al*'s patient presented with dacryoadenitis and sialadenitis in conjunction with allergic manifestations such as chronic rhinosinusitis with nasal polyps, asthma and urticaria. We are curious also to ask if the relationships between the variables presented in Figures C, D and E were statistically significant from their data.

In response to Ebbo *et al*'s belief that the response we have seen is confounded by initial use of prednisone, we would like to clarify that it has come to our attention post publication (29 December 2019), that the patient in our case did not take a single dose of prednisone due to undisclosed non-compliance, therefore making dupilumab the first-line treatment that was used. Due to the fact that the patient Ebbo *et al* described underwent rituximab therapy prior to dupilumab therapy, it is also possible that this added to the variability in the observed patient's response compared with ours. It should also be noted that because Ebbo *et al*'s patient was on glucocorticoids for 3 months and then approximately 1 month a year before dupilumab treatment, the same argument can be made that perhaps this confounds their results. However, due to the variability in both clinical manifestations and prior treatments that can be seen in the IgG4-RD patient presented in comparison to ours, it can be understood why perhaps Ebbo *et al*'s patient may have not shown such complete resolution to their disease after dupilumab treatment.

We do find it interesting that Ebbo *et al* have reported their case with respect to a suboptimal clinical and radiological response to dupilumab 8 months after treatment. We believe it would be useful to provide information with respect to the 2019 American College of Rheumatology/European League Against Rheumatism classification criteria for IgG4-related involvement post-dupilumab treatment to compare to the pre-treatment score. We do believe Ebbo *et al*'s report shows valuable data with regards to the variability in responses to dupilumab that may take place in IgG4-RD and we wonder if it would be valuable to study IgG4-RD response to dupilumab in two separate groups through a large randomised clinical trial: dupilumab as a first-line treatment for IgG4-RD and dupilumab for patients that are refractory to conventional treatments such as glucocorticoids, immunosuppressants and/or rituximab for IgG4-RD. By studying both groups fully using well-detailed inclusion and exclusion criteria, we believe that the optimal place for dupilumab in the IgG4-RD treatment algorithm will be able to be identified, as well as dupilumab's safety and efficacy for this condition will be thoroughly investigated.

Rachel S Simpson , **Jason K Lee**

Toronto Allergists, Toronto, Ontario, Canada

Correspondence to Rachel S Simpson, Toronto Allergists, Toronto, Ontario, Canada; rachel.simpson@queensu.ca

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ORCID iD
Rachel S Simpson http://orcid.org/0000-0003-1779-4049

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Interleukin-4 as an emerging therapeutic target for IgG4-related disease

We read the manuscript reported by Simpson *et al* with great interest.[1] They showed remarkable effectiveness of dupilumab, a monoclonal antibody targeting the interleukin-4 (IL-4) receptor alpha, in immunoglobulin G4-related disease (IgG4-RD) complicated with retroperitoneal fibrosis for the first time. Considering the frequent relapse in patients with IgG4-RD during glucocorticoid tapering and the difficulty in glucocorticoid withdrawal,[2] it is of great value that their patient could discontinue prednisone within 2 months after starting dupilumab and even remain stable without any relapse for 12 months.[1] They also showed no significant adverse effects of dupilumab for the duration. We have previously revealed that IL-4 plays an important role in the pathogenesis of IgG4-RD.[3–5] In particular, IL-4-producing follicular helper T cells contribute to IgG4 class-switching and plasmablast differentiation in the disease.[3–5] The case reported by Simpson *et al* connects the basic research findings with the translational application and sheds light on the possibility of dupilumab as a glucocorticoid-sparing and relapse-suppressing agent in patients with IgG4-RD. We are pleased to read their great report and would like to ask several questions for clarification.

The first is regarding the diagnosis of IgG4-RD. Antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis can mimick IgG4-RD as patients with ANCA-associated vasculitis frequently show elevated levels of serum IgG4 and IgG4-positive B-cell infiltration along with fibrosis in the inflamed tissues, whereas neutrophilic inflammation, granulomatous changes, necrotising lesions and multinucleated giant cells are not usual findings in IgG4-RD.[6] Therefore, the findings of serum IgG4 levels and pathological IgG4-positive B-cell infiltration might not be sufficient for differentiating the two diseases. And, while retroperitoneal fibrosis is one of the major organ involvements in patients with IgG4-RD, ANCA-positive retroperitoneal fibrosis has also been reported.[6] Clinical characteristics of ANCA-positive retroperitoneal fibrosis are very similar to those of IgG4-RD in terms of elderly and male predominance. Collectively, careful exclusion of ANCA-associated vasculitis is needed before the final diagnosis with IgG4-RD. In the context, we would like to ask if the patient had any indication of ANCA-associated vasculitis rather than IgG4-RD such as fever, elevated blood neutrophil counts and serum C reactive protein levels, and ANCA positivity as well as neutrophilic infiltration, granulomatous formation, necrotising vasculitis and multinucleated giant cells in the prostate tissues. Of note, the similar case of granulomatosis with polyangiitis complicated with rhinitis, retroperitoneal fibrosis and pathologically confirmed granulomatous prostatitis along with PR3-ANCA positivity was reported.[7] Furthermore, prostatitis is the most common extrarenal urogenital manifestation of granulomatosis with polyangiitis.[8]

Second, the authors noted that the patient also had the eye swelling and parotitis at initial presentation, however, it is unclear whether those manifestations were derived from IgG4-RD. We are curious about the laterality of his lacrimal and parotid gland enlargement and the improvement in those organs after dupilumab therapy.

Third, why did the patient get a prostate biopsy for pathological confirmation of IgG4-RD? Was the enlargement of prostate symptomatic or just found by images? In addition, the information about whether tertiary lymphoid organs (also known as 'ectopic lymphoid organs', 'ectopic germinal centres' or 'lymphoid aggregates') were observed in the prostate biopsy specimen is important as those lymphoid organ-like structures frequently observed in the lesions of IgG4-RD are associated with IL-4-producing follicular helper T-cell infiltration.[6 9]

We believe that answers to our questions can further confirm the promising prospects of dupilumab as the treatment of IgG4-RD. Further randomised studies are warranted to determine the benefits of dupilumab in terms of its glucocorticoid-sparing effect and reduction of relapse or even remission induction in patients with IgG4-RD.

Mitsuhiro Akiyama , Yuko Kaneko, Tsutomu Takeuchi

Division of Rheumatology, Department of Internal Medicine, Keio University School of Medicine, Shinjuku-ku, Tokyo, Japan

Correspondence to Dr Yuko Kaneko, Division of Rheumatology, Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo, Japan; ykaneko.z6@keio.jp

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ORCID iD
Mitsuhiro Akiyama http://orcid.org/0000-0001-5075-8977

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Response to : 'Interleukin-4 as a emerging target for IgG4-related disease' by Akiyama *et al*

We thank Akiyama *et al*, for their kind words regarding our manuscript.[1] We agree that IgG4-related disease (IgG4-RD) relapse frequently occurs in patients tapering glucocorticoids, and we wanted to offer a novel solution to our patient to potentially mitigate or all together avoid this. As communicated in our previous correspondence response[2] it has actually come to our attention post-publication (December 29th, 2019) that the patient noted in our report never took a dose of prednisone that was prescribed to him due to undisclosed non-compliance. Therefore, the remission of IgG4-RD can be completely attributed to dupilumab treatment. We are happy to report our findings and hope that our report will propel this treatment to be studied further for the treatment of this disease which otherwise, as you mentioned, is poorly controlled by glucocorticoids especially when tapering,[3] and when other agents such as chemotherapeutic immunosuppressants come with profound adverse effects.[4] Although this disease has a low prevalence, so low that it is actually unknown due to being underrecognized or underreported,[1] it does have a significant mortality rate, showing the importance of developing novel therapies to control this disease. It is our conceptual understanding that some IgG4-RD is an extreme example of unabated type II inflammation, which we have seen similar pathology in chronic spontaneous urticaria.[5]

We thank Akiyama *et al* for their interest in the exclusion of ANCA-associated vasculitides, including granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA), and eosinophilic granulomatosis with polyangiitis (EGPA) in the differential diagnosis of this patient. When the patient originally presented, atypical EGPA was part of the differential diagnosis due to the reasons Akiyama *et al* stated and was excluded before the diagnosis of IgG4-RD was made. This was a consideration given his lower airway symptoms. The suspicion of small vessel vasculitis diminished due to the fact that the patient did not have a fever, nor elevated neutrophil counts nor elevated serum C-reactive protein levels. Regarding the prostate biopsy, there was no neutrophilic infiltration, granulomatous formation, necrotizing vasculitis, or multinucleated giant cells. ANCA testing was negative; however, it should be noted that many patients with ANCA-associated vasculitides actually do not test positive for P-ANCA and C-ANCA.[6] Although IgG4-RD and ANCA-associated vasculitides can overlap in clinical presentations, we further believe it is unlikely that the patient had an ANCA-associated vasculitis such as EGPA because dupilumab can actually raise eosinophil levels due to changes in VCAM-1 expression and eosinophilia is central to the pathophysiology of EGPA.[7] We observed that as eosinophil levels became raised from the baseline level of 1.4×10^9 cells/L to 2.0×10^9 cells/L (normal range 0–0.5×10^9 cells/L) at 4 months into dupilumab treatment likely due to the aforementioned indirect cell trafficking changes from dupilumab. It was noted that the patient's symptoms continued to improve in spite of the increased eosinophilia.

To address Akiyama *et al*.'s second question, we believe the eye swelling was most likely caused by atopic dermatitis as this was a concurrent condition the patient presented with; however, it is possible this was a manifestation of IgG4-RD as this cannot be ruled out completely. His eye swelling completely resolved at 3 months post-dupilumab treatment. With regards to parotitis, this manifestation was resolved at 12 months post dupilumab treatment, as determined by physical examination as MRI was only done of the lumbar plexus region.[1]

In regard to Akiyama's third point, the original MRI of the pelvis (pictured as figure 1A in our original paper)[4] was ordered by a gastroenterologist in order to rule out pathologies associated with normocytic anaemia which the patient was being worked up for. When this MRI revealed profound retroperitoneal and genitourinary thickening, the radiologist suggested follow-up with a urologist whom recommended the prostate biopsy to rule out malignancy and also to obtain a tissue diagnosis for suspected IgG4-RD as the patient had extensive retroperitoneal thickening and elevated IgG4 levels. The prostate was asymptotic at the time this fibrosis was found and was an incidental finding in the work-up for normocytic anaemia. Once malignancy was ruled out by the biopsy, urology deemed the case to most likely be IgG4-RD and therefore referred the patient to rheumatology.

Once again, we would like to thank Akiyama *et al* for their interest in our paper and providing their questions through correspondence. We believe dupilumab to be a promising monotherapy and/or addon for IgG4-RD and look forward it being studied further through randomised control trials to investigate large-scale safety and efficacy of this novel treatment.

Rachel S Simpson , **Jason K Lee**

Toronto Allergists, Toronto, Ontario, Canada

Correspondence to Ms Rachel S Simpson, Toronto Allergists, Toronto, ON M5G 1E2, Canada; rachel.simpson@queensu.ca

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Contributors RSS undertook the primary duties in writing the manuscript. JKL oversaw the manuscript writing and edited the manuscript.

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ORCID iD
Rachel S Simpson http://orcid.org/0000-0003-1779-4049

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Total adenosine deaminase highly correlated with adenosine deaminase 2 activity in serum

We read with great interest the article published by Lee and colleagues[1] presenting the potential diagnostic value of adenosine deaminase 2 (ADA2) in systemic juvenile idiopathic arthritis (sJIA) with macrophage activation syndrome (MAS). Their study examined ADA2 activity in children with inflammatory and immune-mediated diseases. By comparing normal ranges of ADA2 in healthy individuals, their finding identified the utility of plasma ADA2 activity as a biomarker of MAS. ADA2 activity were measures by a spectrophotometric assay in the presence of a selective inhibitor of ADA1, erytro-9-amino-β-hexyl-α-methyl-9-ethanol hydrochloride (EHNA). We support the views expressed by the authors that ADA2 detection could be helpful in the diagnosis of MAS in sJIA. Here, we would like to share with the authors our data of ADA activity detection.

As we know, ADA contains two isoenzymes: ADA1 an ADA2. In plasma, ADA2 is a major component of total adenosine deaminase (tADA).[2 3] tADA activity detection has been carried out in the clinical laboratory for many years, which was usually used for differential diagnosis of benign and malignant effusions.[4] The measurement method of tADA activity is simpler than ADA2 activity. Here, we report the correlation between tADA and ADA2 activity in the serum of healthy individuals and patients with immune-mediated diseases. Serum tADA activity was measured with an enzymatic method kit (Sichuan Maccura Biotechnology, China), adapted to the automated biochemistry analyser (Hitachi 7600, Japan).[5] Serum ADA2 activity was measured in the presence of 0.1 mM EHNA. We determined serum tADA and ADA2 activity in 386 healthy individuals and 430 patients with different immune-mediated diseases, including rheumatoid arthritis, systemic lupus erythematosus, ankylosing spondylitis, myasthenia gravis and autoimmune liver diseases.

First, we found that tADA levels highly correlated with ADA2 in 816 individuals (figure 1A; r=0.921, p<0.0001). Notably, the correlation coefficient between tADA and ADA2 in patients with immune-mediated diseases (figure 1B; r=0.947, p<0.0001) was higher than that in healthy individuals (figure 1C; r=0.860, p<0.0001). Thus, like ADA2 activity, tADA activity might also be a biomarker of MAS. Due to convenient detection, tADA activity would be more suitable for clinical application than ADA2.

Second, in the study of Lee *et al*,[1] they showed that ADA2 activity was higher in children than in adults (age 18 years and older), with an overall negative correlation with age (r=−0.250, p<0.0001). Here, we analysed the distribution of tADA and ADA2 activity in adults. The results showed that tADA and ADA2 activity were higher in elderly people (age ≥60 years) than young adults (figure 2A,B; p<0.01), with an overall positive correlation with age (figure 2C,D; p<0.0001). Because ADA plays an important role in the immune system, these combined data indicated the potential difference of the immune system status between children, elderly people and other adults.

In conclusion, the high correlation between tADA and ADA2 activity supports the clinical application value of tADA detection. Further studies are needed to validate the effect and mechanism of higher level of ADA activity in children and elderly people.

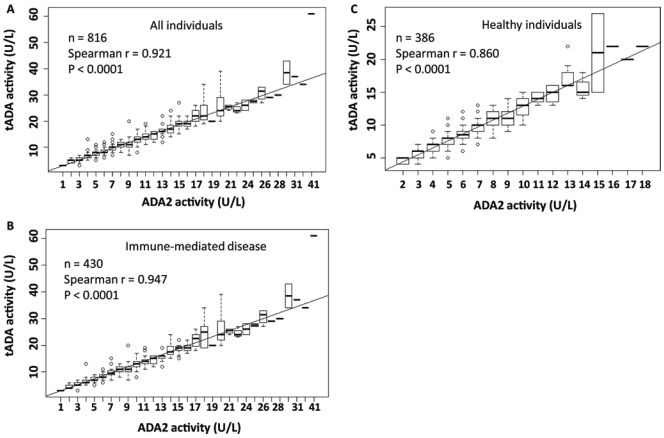

Figure 1 Correlation between serum ADA2 and TadA activity was calculated by Spearman's rank correlation analysis. (A–C) Correlation between ADA2 and TadA in all 816 individuals, 430 patients with immune-mediated diseases and 385 healthy individuals. ADA2, adenosine deaminase 2; TadA, total adenosine deaminase.

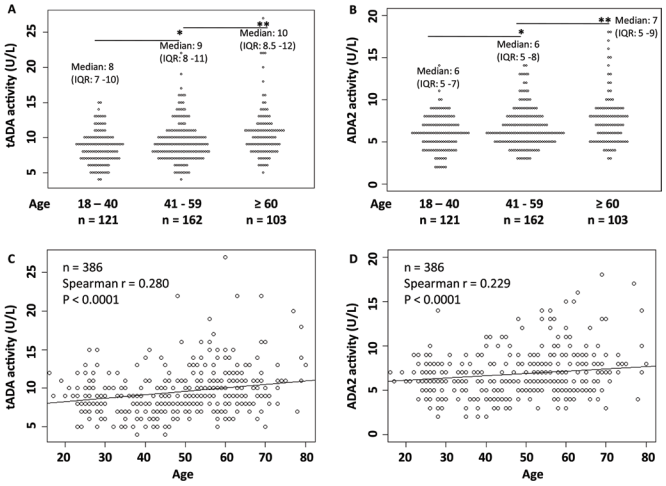

Figure 2 Determination of serum TadA and ADA2 activity in healthy adults. (A,B) Plot comparing serum TadA and ADA2 activity in adults stratified by age. (C,D) Correlation between serum TadA, ADA2 activity and age. *p<0.05, **p<0.01. ADA2, adenosine deaminase 2; TadA, total adenosine deaminase.

Zhao-Wei Gao , **Xi Wang, Fang Lin, Ke Dong**

Department of Clinical Laboratories, Fourth Military Medical University, Xi'an, China

Correspondence to Dr Fang Lin and Dr Ke Dong, Department of Clinical Diagnosis, Tangdu Hospital, Air Force Medical University, Xinsi Road, Xi'an, Shanxi 710038, China; linfang168@hotmail.com, tdjyk3@fmmu.edu.cn

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ORCID iD
Zhao-Wei Gao http://orcid.org/0000-0003-4477-0966

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Response to: 'Total adenosine deaminase highly correlated with adenosine deaminase 2 activity in serum' by Gao *et al*

We thank Gao and colleagues for their interest in our recent study on adenosine deaminase 2 (ADA2) as a biomarker of macrophage activation syndrome.[1] In their letter, the authors demonstrated a strong correlation between total adenosine deaminase (tADA) and ADA2 levels in the peripheral blood of healthy controls and patients with immune-mediated diseases.[2] They suggest that, as it is easier to measure tADA than ADA2 activity, tADA activity alone would be suitable as a diagnostic marker. Because tADA is the sum of ADA1 and ADA2 activity, the biology of both ADA isoforms should be considered in evaluating this claim.

Among several differences between ADA1 and ADA2, two are relevant to their assay in clinical laboratories: the affinity of ADA1 for adenosine is 100-fold greater than that of ADA2, and ADA1 is inhibited by the analogue erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), but ADA2 is not.[3] Plasma ADA2 activity is therefore performed at a saturating adenosine concentration that is ~10 000-fold higher than physiological, and in the presence of EHNA to inhibit ADA1. Under these in vitro conditions, ADA2 accounts for the majority of measured ADA activity in plasma. The correlation between ADA2 and tADA activity observed by Gao *et al* has previously been recognised.[4] We observed a similar association in our measurement of 294 healthy individuals comprised 174 children and 120 adults (figure 1A). As expected, ADA2 activity was generally higher than ADA1 activity (median 11.8 U/L (IQR 9.5–15.4) vs 3.6 U/L (1.9–4.9); figure 1B), although considerable variability among individuals was seen in both parameters. We observed that ADA1 levels were twofold higher in children compared with adults (median 4.3 U/L (IQR 3.4–5.5) vs 2.1 U/L (1.0–3.4), p<0.0001; figure 1C). Taking into account the higher ADA2 levels in children we previously described,[1] the fraction of tADA represented by ADA1 remained elevated in children compared with adults (median 25% (IQR 18–33) vs 17% (8 – 24), p<0.0001; figure 1D).

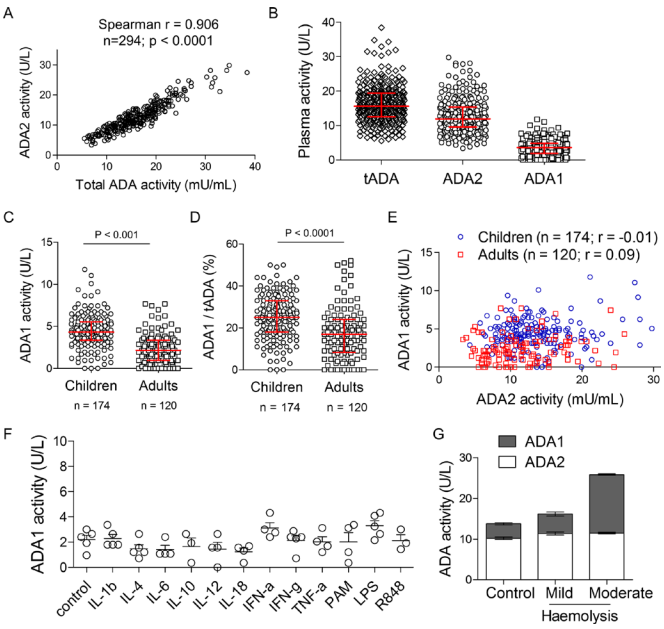

Figure 1 Evaluation of ADA1, ADA2 and tADA in healthy subjects. (A) Correlation between plasma ADA2 activity and tADA activity in 294 healthy individuals (174 children under age 18 years and 120 adults). (B) Comparison of ADA1, ADA2 and tADA in 294 healthy individuals. Median and IQR are displayed in scatter dot plots. (C) Comparison of plasma ADA1 levels and (D) fraction of ADA1 in tADA (ADA1/tADAx100%) in children and adults. Median and IQR are displayed in scatter dot plots. (E) Correlation between plasma ADA1 activity and ADA2 activity in children (blue; n=174) and adults (red; n=120). (F) ADA1 activity in the supernatant of healthy donor PBMC stimulated with cytokines or Toll-like receptor ligands for 5 days. Dots represent results from three to five healthy donors per condition. (G) Impact of haemolysis on plasma ADA1, ADA2 and tADA levels. Bars represent mean and error bars present SD of duplicate samples. ADA, adenosine deaminase; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; PAM, Pam3CSK4; PBMC, peripheral blood mononuclear cells; tADA, total adenosine deaminase; TNF, tumour necrosis factor.

How ADA1 levels are regulated has not been well studied. Whereas ADA2 is primarily secreted by monocytes/macrophages, ADA1 is an intracellular enzyme present in all cells including erythrocytes.[3] The ADA isoforms have distinct physiological functions; genetic deficiency of ADA1 results in severe combined immunodeficiency while deficiency of ADA2 causes systemic vasculitis and bone marrow failure.[5] In line with this view, ADA1 and ADA2 levels did not correlate in either the paediatric group or adult group (figure 1E). Cytokines that induce ADA2 secretion in vitro, including interleukin (IL)-12, IL-18 and interferon-γ, did not affect ADA1 activity (figure 1F).

Importantly, we have observed that blood samples with visible haemolysis tend to have higher levels of ADA1. As haemolysis during phlebotomy is more common in young children,[6] the release of ADA1 from damaged erythrocytes may explain higher ADA1 levels in children. Indeed, when we simulated haemolysis by passing whole blood through a 30-gauge needle (one passage for mild haemolysis, two for moderate haemolysis), plasma ADA1 activity and tADA activity showed a stepwise increase, while ADA2 activity remained stable (figure 1F).

We agree with Gao and colleagues that tADA activity provides a reasonable proxy for ADA2 activity under some conditions. However, intrinsic differences in ADA1 levels among individuals (including disease-related changes still to be defined) and extrinsic factors such as haemolysis can sometimes give rise to substantial discrepancy between these values. Specific measurement of ADA2 is particularly important in the paediatric population where haemolysis associated with phlebotomy is more common. The confounding effects of ADA1 can be eliminated by simply adding the inhibitor EHNA to the assay without other changes to the protocol, which is neither technically challenging nor costly. Therefore, given the incomplete understanding of ADA biology, we suggest the use of ADA2 activity as a more informative and specific biomarker compared with tADA.

Pui Y Lee [1,2] **Zhengping Huang,**[2,3] **Michael S Hershfield,**[4] **Peter A Nigrovic** [1,2]

[1]Division of Immunology, Boston Children's Hospital, Boston, Massachusetts, USA
[2]Division of Rheumatology, Inflammation and Immunity, Brigham and Women's Hospital, Boston, Massachusetts, USA
[3]Department of Rheumatology and Immunology, Guangdong Second Provincial General Hospital, Guangzhou, China
[4]Department of Medicine and Biochemistry, Duke University School of Medicine, Durham, North Carolina, USA

Correspondence to Dr Pui Y Lee, Division of Immunology, Boston Children's Hospital, Boston, MA 02115, USA; pui.lee@childrens.harvard.edu and Dr Peter A Nigrovic, Division of Immunology, Boston Children's Hospital, Boston, MA, United States; pnigrovic@bwh.harvard.edu

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ORCID iDs
Pui Y Lee http://orcid.org/0000-0002-5779-4193
Peter A Nigrovic http://orcid.org/0000-0002-2126-3702

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Rheumatic disorders associated with immune checkpoint inhibitors: what about myositis? An analysis of the WHO's adverse drug reactions database

As shown by Kostine *et al*,[1] and recently underlined by Ceccarelli *et al*,[2] rheumatic inflammatory disorders induced by anticancer therapy are becoming a major concern for rheumatologists at the era of immune checkpoint inhibitors (ICIs). Beyond inflammatory arthritis, which may concern 10%–20% of patients, myositis represents a rare (<1%) but potentially life-threatening event. We thus aimed at investigating the risk of ICI-related myositis in real-life setting using VigiBase, the WHO's pharmacovigilance database.

First, we analysed the myositis case associated with ICIs (Anti-Programmed Death (PD)-1, anti-Programmed Death Ligand (PDL)-1, and anti-Cytotoxic-T-Lymphocyte-Associated Protein (CTLA)-4 agents) reported to VigiBase. From 14 786 263 adverse drug reactions (ADRs) recorded between 1 January 2008 and 12 February 2019, we identified 54 085 ICI-related ADRs including 345 myositis (0.6%) (table 1). Among myositis cases, 85.2% occurred with anti-PD-1 or anti-CTLA-4 monotherapies, while 14.8% with combination therapy. Lung (34.8%) and skin cancers (34.2%) were the most frequent indications for ICI therapy. ICI-related myositis was more frequent in male patients, and over 65 years. The median time to onset was 4–5 weeks ranging from 1 day to 20 months, consistently with other reports.[3] Almost all ICI-related myositis (95.3%) were considered serious (ie, requiring at least a hospitalisation), with a fatality rate of 22.3%. Myocarditis and myasthenia were associated with ICI-related myositis in 11.3% and 11.9% of cases and resulted in death in 51.3% and 26.8%, respectively.

Second, using case/non-case analyses,[4] we found that myositis was reported more than 17 times with ICI agents than with any other drugs (reporting OR 17.3; 95% CI: 15.5 to 19.2). Moreover, myositis was more reported in the group of anti-PD-1/PD-L1 monotherapy compared with anti-CTLA-4 monotherapy (OR=2.4, 95% CI: 1.6 to 3.5). Myositis was also more frequently reported in patients using ICI combination therapy versus those using ICI monotherapy (OR=1.8, 95% CI: 1.3 to 2.4). Similar results were obtained after adjusting for potential confounders such as sex, age and reporter type.

Herein, we report a considerable outbreak of ICI-related myositis in the past years. VigiBase represents the largest medical postmarketing surveillance database allowing the study of ADRs in real conditions of use and facilitates the study of rare ADRs such as myositis, which is hardly observed in clinical trials or observational studies with limited sample size. A previous work by Anquetil *et al* identified 180 ICI-related myositis.[5] With 165 additional cases, we report the largest series of ICI-related myositis and further characterise this unique ICI-related ADR using disproportionality (case/non-case) analyses, a method designed for early detection of pharmacovigilance signals.[4] Although disproportionality estimates cannot be interpreted as risk estimates, they have been shown to be significantly correlated to risk estimates.[6]

Interestingly, ICI-related myositis seems to differ greatly in comparison to primitive inflammatory muscle disorders (IMDs), suggesting a novel and unique emerging autoimmune entity with specific concerns. Indeed, clinical manifestations in ICI-related myositis include frequent bulbar symptoms, ptosis and oculomotor impairment, whereas those are rare in primitive myositis, but may occur in myasthenia gravis, a disorder affecting neuromuscular junction. Hence, the association between immune-related myositis and myasthenia gravis may not be fortuitous as shown here and by others, even if specific antibodies against acetylcholine receptor may be lacking.[7–9] Interestingly, the great majority of myasthenia gravis cases was not observed with anti-CTLA-4 agents, but rather with anti-PD-1/PD-L1 agents, suggesting an increased risk with the latter, in line with our proper observations.[9]

Of note, the majority of the reports were merely qualified as 'myositis', even if some also referred to specific entities. This may originate from reporting difficulties but questions the complex

Table 1 Characteristics of immune checkpoint inhibitor (ICI)-exposed myositis cases

Characteristics	ICI-exposed myositis cases (n=345)
Age (years), median (P25–P75) (n=265)	71 (63–76)
Sex, n (%)	
Male	228 (69.7)
Female	99 (30.3)
Unknown	18
Reporter type, n (%)	
Health professional	293 (86.2)
Other	47 (13.8)
Unknown	5
Reporting year, n (%)	
2019	4 (1.2)
2018	184 (53.3)
2017	90 (26.1)
2016	47 (13.6)
2008–2015	20 (5.8)
Cancer type, n (%)	
Lung cancer	111 (34.8)
Skin cancer	109 (34.2)
Melanoma	102 (32.0)
Other cancers	32 (31%)
Exposure to ICIs	
Monotherapy, n (%)	294 (85.2)
Anti-PD-1	252 (73.0)
Nivolumab	154 (44.6)
Pembrolizumab	97 (28.1)
Nivolumab or pembrolizumab	1 (0.3)
Anti-PD-L1	15 (4.3)
Atezolizumab	7 (2.0)
Avelumab	3 (0.9)
Durvalumab	5 (1.4)
Anti-CTLA-4	27 (7.8)
Ipilimumab	27 (7.8)
Tremelimumab	0 (0.0)
Combination therapy	51 (14.8)
Nivolumab/ipilimumab	49 (14.2)
Pembrolizumab/ipilimumab	1 (0.3)
Durvalumab/tremelimumab	1 (0.3)
Time to onset (days) (n=97)	
Median (P25–P75)	33 (19–57)
Min–Max	1–606
Reported myositis term	
Myositis	276 (80.0)
Dermatomyositis	25 (7.2)
Polymyositis	20 (5.8)
Immune-mediated necrotising myopathy	13 (3.8)
Orbital myositis	8 (2.3)
Inclusion body myositis	2 (0.6)
Antisynthetase syndrome	1 (0.3)
Specific co-reported irAEs	
Myocarditis	39 (11.3%)
Myasthenia	41 (11.9%)
Death, n (%)	77 (22.3)

Anti-CTLA-4, Anti-Cytotoxic-T-Lymphocyte-Associated Protein (CTLA)-4; Anti-PD-1, Anti-Programmed-Death-1; anti-PD-L1, anti-Programmed-Death-Ligand-1; ICI, immune checkpoint inhibitor; irAEs, immune-related-adverse events.

nosology in IMD. Actually, the classification of IMD improved considerably in the last years and now distinguishes definite entities according to clinical presentation, specific auto-antibody profiles, histological patterns, associated with distinct prognoses. If these data may be lacking in VigiBase, case reports have characterised ICI-myositis as driven by T-CD8-lymphocyte and macrophages infiltrates together with fibre necrosis, a description close to necrotising myositis.[9] Reports have described circulating antibodies against antisynthetase, polymyositis-scleroderma PM-SCL), signal recognition particle (SRP) and transcription intermediary factor 1 gamma (TIF-1-gamma).[9] Noteworthily, the latter is strongly associated with cancer and considered as a paraneoplastic syndrome (PNS) in this context. By analogy with other pre-existing autoimmune conditions, the use of ICIs in the context of PNS may be associated with an increased risk for immune toxicity, including PNS flare.[10] Beyond these considerations, the association with other autoimmune entities may also be of interest, since myositis can also stand as a manifestation of other connective tissue disorders, such as lupus, scleroderma or Sjögren syndrome.[11]

Importantly, another major concern with ICI-related myositis is the strong association with myocarditis. In accordance with our results, other series revealed high prevalence of myocarditis (ranging from 15% to 32%) in patients with ICI-related myositis, together with high lethality (up to 50%). In another study on VigiBase, which focused on ICI-related myocarditis, musculoskeletal disorders were the most frequent concurrent complications occurring along with myocarditis.[12] Thus, the increased prevalence of myocarditis may explain the higher mortality rate in ICI-related myositis compared with primitive IMD, and prompts for a systematic cardiac screening in these patients.

In conclusion, despite the limitations inherent to pharmacovigilance studies which are concerned with under-reporting, we confirm a strong signal suggesting an increasing risk of myositis associated with ICIs, especially anti-PD-1/PD-L1 agents or when these drugs were used in combination with anti-CTLA-4 agents. Prospective studies will be necessary to better investigate this risk, and better define the place of ICI-related myositis within the spectrum of IMD. In the meantime, clinicians' awareness and vigilance are needed to improve early detection and management of this unique and severe complication. In this context, the specific risk of myocarditis, a life-threatening complication, prompts to a systematic screening.

Thủy Nguyễn,[1] Alexandre Thibault Jacques Maria ,[2,3] Chayma Ladhari,[1] Pascale Palassin,[1] Xavier Quantin,[4] Candice Lesage,[5] Guillaume Taïeb,[6] Xavier Ayrignac,[6] Patricia Rullier,[2] Dominique Hillaire-Buys,[1] Olivier Lambotte,[7] Philippe Guilpain,[2,3] Jean-Luc Faillie[1]

[1]Department of Medical Pharmacology and Toxicology, University of Montpellier, Faculty of Medicine, Montpellier, Languedoc-Roussillon, France
[2]Department of Internal Medicine: Multi-Organic Diseases, Local Referral Center for Systemic Autoimmune Diseases, Montpellier University Hospital, University of Montpellier, Medical School, Montpellier cedex 5, France
[3]IRMB Institute for Regenerative Medicine and Biotherapy, INSERM U1183, University of Montpellier, Montpellier, France
[4]Department of Medical Oncology, Montpellier Cancer Institute, University of Montpellier, Faculty of Medicine, Montpellier, Languedoc-Roussillon, France
[5]Department of Dermatology, University of Montpellier, Faculty of Medicine, Montpellier, Languedoc-Roussillon, France
[6]Department of Neurology, University of Montpellier, Faculty of Medicine, Montpellier, Languedoc-Roussillon, France
[7]Department of Internal Medicine, Hopital Bicetre, Le Kremlin-Bicetre, France

Correspondence to Dr Alexandre Thibault Jacques Maria, Internal Medicine: Multi-Organic Diseases, Local Referral Center for systemic autoimmune diseases, Montpellier University Hospital, Univ Montpellier, Medical School, Montpellier cedex 5, France; alexandremaria@hotmail.fr

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Contributors All authors meet the criteria for authorship, for their substantial contributions to the conception or design of the work, the acquisition and the interpretation of data. All authors have revised the manuscript critically and gave their final approval of the version published. They give agreement for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work.

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ORCID iD
Alexandre Thibault Jacques Maria http://orcid.org/0000-0002-0868-5804

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A case of episodic and refractory arthritis due to a novel variant of NLRP12

It was with great interest that I read the paper by Gupta *et al* describing novel NLRP12 variant is linked to familial cold autoimmunity syndrome phenotype.[1] Here, we reported a patient present with phenotypes of episodic and refractory arthritis due to another novel variant in NLRP12.

A 28-year-old man presented at the age of 20 years with episodic pain, swelling and erythematous skin rash in his wrists, metacarpophalangeal, interphalangeal, knee and ankle joints. His symptoms typically occur once per 1–2 months and sometimes with the increased frequency of episodes to three to four times in 1 week. No identifiable factors were found to trigger the onset. Symptoms lasted from several hours to 3–5 days and subsided spontaneously. His erythrocyte sedimentation rate and C-reactive protein level were elevated during periods of symptoms with normalisation during remission. He was negative for rheumatoid factor, antinuclear antibody and anticitrullinated peptide antibody. No synovitis was detected by ultrasound or MRI in the affected joints. Numerous empirical treatments including nonsteroidal antiinflammatory drugs, colchicine, glucocorticoid (prednisone 10 mg/day), methotrexate and hydroxychloroquine failed to reduce recurrent episodes or relief symptoms. Then, after a sudden onset, only a wait to see strategy can be used for him to wait for the spontaneous remission.

Whole exome sequencing revealed a novel heterozygous mutation in the exon 3 of the *NLRP12* gene (c.1771C>A: p.L591M) on chromosome 19. By using Sanger Sequencing, we did not find this mutation in his parents, indicating that it might be a de novo mutation. This missense variant is extremely rare and absent in >200 000 individuals who have been well-sequenced with high depth at the *NLRP12* region in gnomAD and TOPMed. It is predicted to be deleterious and probably damaging the protein function by different in silico computational tools including PANTHER, polyphen2 and SIFT. *NLRP12* is a novel member of the inflammasome complex and acts as a negative regulator of inflammation.[2 3] We found peripheral mononuclear cells (PBMC) from this patient showed markedly increased NF-κB activity and IL-1β production after tumour necrosis factor alpha (TNFα) stimulation, as compared with arthritis patients non-carrying this variant and healthy controls, supporting the functional significance of this heterozygous mutation in *NLRP12*.

Compared with the patient under discussion in this journal present with familial cold autoimmunity syndrome phenotype,[1 4] the distinct clinical manifestations in our case are recurrent transient episodes of arthritis and erythematous skin rash. This patient has ever been diagnosed as palindromic rheumatism and undifferentiated arthritis. A diagnosis of *NLRP12* associated systemic autoinflammatory disorder was therefore made. Due to non-availability of interleukin-1 inhibitor in China, the patient was treated with tofacitinib and had a modest benefit from it. Our case highlights the importance of screening autoinflammatory disorder and *NLRP12* in patients with unexplained episodic and refractory arthritis.

Jiajia Wang, Qian Zhang, Lingxiao Xu, Chengyin Lv, Rui Liu, Miaojia Zhang, Wenfeng Tan

Department of Rheumatology, The First Affiliated Hospital of Nanjing Medical University, China, Nanjing, Jiangsu, China

Correspondence to Dr Wenfeng Tan, Department of Rheumatology, The First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, China; tw2006@njmu.edu.cn

Contributors The conception and design of the correspondence, acquisition of data, and analysis and interpretation of data: WT, MZ and JW; Involved in care of the patient: JW and WT; Drafting the article: JW, LX and WT; Revising it critically for important intellectual content: JW, QZ, LX, RL and CL; Final approval of the version to be submitted: all authors; Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved: all authors.

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ORCID iD
Wenfeng Tan http://orcid.org/0000-0002-5160-2023

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Correction: *Significant weight loss in systemic sclerosis: a study from the EULAR Scleroderma Trials and Research (EUSTAR) database*

Hughes M, Heal C, Siegert E, *et al*. Significant weight loss in systemic sclerosis: a study from the EULAR Scleroderma Trials and Research (EUSTAR) database. *Ann Rheum Dis* 2020;79:1123–5. doi:10.1136/annrheumdis-2020-217035

In table 1, CRP for non-significant weight loss should be 2.1. Otherwise, the values are correct including the specified ranges and interpretation of the data. Also, to highlight, that disease duration is displayed in days.

Ann Rheum Dis 2022;**81**:e34. doi:10.1136/annrheumdis-2020-217035corr1

Correction: *Emergent high fatality lung disease in systemic juvenile arthritis*

Saper VE, Chen G, Deutsch GH, the Childhood Arthritis and Rheumatology Research Alliance Registry Investigators, *et al*. Emergent high fatality lung disease in systemic juvenile arthritis. *Ann Rheum Dis* 2019;78:1722–31.

The correct figure 3 and legends for figure 2 and 3 should be:

Figure 2. Panels A-E: Representative axial chest CT images: A. Multilobar, predominantly peripheral septal thickening, most marked in the lower lung, parahilar and/or anterior upper lobes with or without adjacent ground glass opacities. B. Crazy-paving C. Peripheral consolidations. D. Peribronchovascular consolidations. E. Predominantly ground-glass opacities. F. Hyper- enhancing lymph nodes on contrast-enhanced CT. Panels G-H: Histopathologic findings (hematoxylin & eosin staining) along the pulmonary alveolar proteinosis/endogenous lipoid pneumonia (PAP/ELP) spectrum. Alveolar filling with eosinophilic proteinaceous material (figure 2G left), admixed with a variable degree of ELP, indicated by cholesterol clefts (arrowheads) and foamy (lipid-containing) macrophages (figure 2G middle and right), as described.[76,77] Regions of PAP/ELP accompanied by type II alveolar epithelial cell hyperplasia (figure 2G, right insert, arrow), mild to moderate interstitial infiltration by inflammatory cells and lobular remodeling (airspace widening with increased interstitial smooth muscle). Typically, PAP/ELP findings were patchy, with involved areas juxtaposed to normal lung (figure 2G, left, arrow). Figure 2G, right shows pulmonary arterial wall thickening; a=artery. In A-G, # cases with pattern/# assessable cases are indicated. H. Electron micrograph showing normal lamellar bodies within type II cells (arrows) and macrophage (center), containing lamellar debris, lipid (*) and cholesterol clefts (arrowhead). Original magnification x 7000. 4 PAP/ELP cases (1 each: ABCA-3, CSF2RB variant), stained for surfactant proteins (SP-B, proSP-C, SP-D, ABCA-3, TTF-1), demonstrated robust immunoreactivity (not shown). ABCA-3, ATP binding cassette subfamily A member 3; SP-B, surfactant protein B; proSP-C, prosurfactant C; SP-D, surfactant protein D; TTF-1, thyroid transcription factor 1.

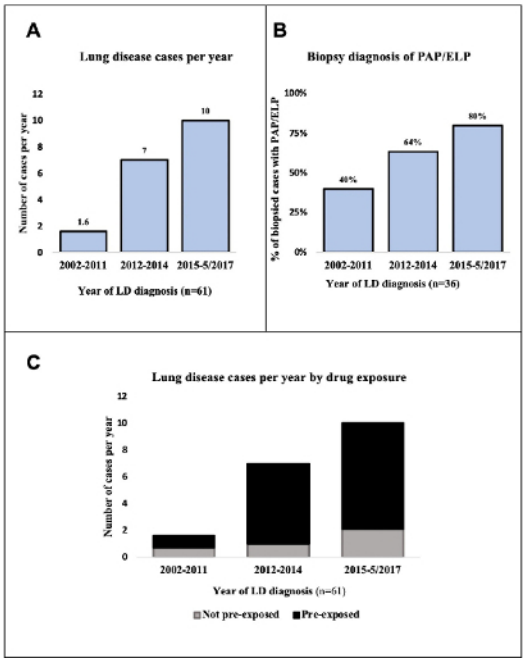

Figure 3. (A) Annual number of LD cases in this series (total n=61). (B) Percentage of biopsied LD cases (n=36) with PAP/ELP pathology, grouped by year of LD diagnosis. (C) Annual incidence of LD, indicating proportions exposed (black) or not (grey) to anti-IL-1/IL-6 inhibitors. LD, lung disease; PAP/ELP, pulmonary alveolar proteinosis/endogenous lipoid pneumonia; IL, interleukin.

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