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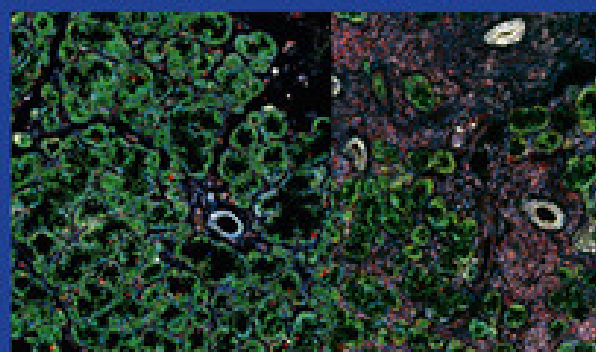
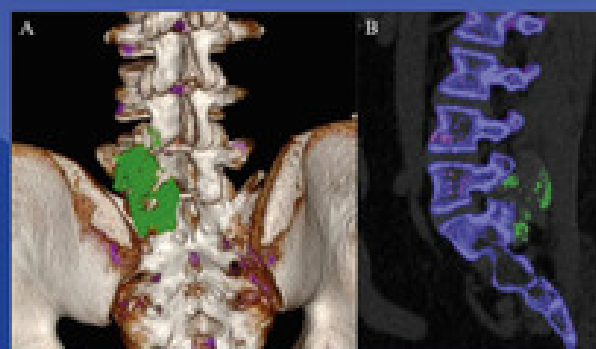
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Criteria

EULAR/ACR risk stratification criteria for development of rheumatoid arthritis in the risk stage of arthralgia

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ABSTRACT

Objectives: The field of rheumatoid arthritis (RA) is moving towards identification of and intervention in people at risk of RA, but a validated risk stratification method is lacking. This work was undertaken to develop a risk stratification method for persons presenting with arthralgia considered to be at risk of RA.

Methods: A joint European Alliance of Associations for Rheumatology (EULAR)/American College of Rheumatology (ACR) expert committee was established. Risk factor and outcome data from 10 arthralgia cohorts (including clinically suspect arthralgia and autoantibody-positive arthralgia) were studied. The work focused on differentiating the risk of progression to clinically apparent inflammatory arthritis (IA) within 1 year, using clinical and serologic variables, without and with subclinical joint inflammation detected by ultrasound (US) or magnetic resonance imaging (MRI). Developing RA according to the 2010 EULAR/ACR criteria within 1 year was a secondary outcome. A set of validated risk stratification criteria was developed.

Results: Using data from 2293 symptomatic at-risk individuals, a stratification method was derived consisting of 6 clinical and serologic variables (morning stiffness, patient-reported joint swelling, difficulty making a fist, C-reactive protein, rheumatoid factor, and anti-citrullinated peptide antibody) yielding an area under the curve (AUC) of 0.80 (95% CI, 0.77–0.83) for IA development. The inclusion of US variables did not increase the discriminative ability. When MRI-detected subclinical inflammation variables were included, the AUC was 0.87 (95% CI, 0.82–0.90). In the presence of clinical, serologic, and MRI variables, a sensitivity and specificity of >75% was achieved. For RA development, the AUC of the criteria with MRI was 0.93 (95% CI, 0.90–0.97).

Conclusions: EULAR/ACR risk stratification criteria have been developed for people with arthralgia in secondary care who are considered at risk for RA. They can be applied in the absence or presence of imaging data and have been developed to define homogeneous risk groups for future prevention trials.

INTRODUCTION

Rheumatoid arthritis (RA) is among the most common chronic autoimmune diseases. The presence of clinically apparent synovitis (swollen joints) is critical to diagnose and classify RA [1]. It has been established that early therapeutic interventions for RA improve clinical outcomes and reduce the progression of joint damage and disability [2]. Furthermore, it is well recognised that autoimmune processes may be aberrant long before clinical arthritis first develops [3–5]. This has led to a focus on the prearthritis stages of RA, under the hypothesis that these early stages are more amenable to disease-modifying interventions.

The transition from ‘health’ to RA has been divided into several stages: genetic and environmental risk factors, evidence of

systemic autoimmunity, and symptoms, followed by clinically apparent inflammatory arthritis (IA) onset [6]. The symptomatic risk stage was defined using a data-driven approach with clinical expertise for reference, resulting in the European Alliance of Associations for Rheumatology (EULAR) definition of arthralgia suspicious for progression to RA [7,8]. This definition (consisting of symptoms and signs) is useful to distinguish arthralgia suspicious for progression to RA from other kinds of arthralgia. Many groups have initiated observational cohort studies of arthralgia at risk for RA, either in persons with clinically suspected arthralgia (CSA) or in persons with autoantibodies and arthralgia/musculoskeletal (MSK) symptoms. A recent EULAR taskforce summarised existing biomarker data and concluded that there was not yet consensus on which combination of predictors was most informative and

that proper validation was lacking [9,10]. A uniform, accurate, and validated method for risk stratification in individuals with arthralgia is therefore warranted.

This need is fuelled by the results of recent randomised placebo-controlled trials that suggest that therapeutic interventions in CSA or autoantibody-positive arthralgia can modify the disease course [11–13]. To help design future trials efficiently, effective risk stratification is important to identify homogeneous groups when evaluating the effectiveness of interventions. In addition, at-risk individuals themselves have indicated that reliable risk information is pivotal for their interpretation of symptoms and decision making [14–16]. Finally, accurate and accepted risk stratification is required by regulatory agencies. Therefore, to support and homogenise further studies in the pre-RA field, a EULAR and American College of Rheumatology (ACR) expert committee was formed to develop an accurate and validated risk stratification algorithm. An important consideration was that the method should be applicable regardless of the method used to identify symptomatic persons at risk (clinical grounds [CSA] or autoantibody-positive arthralgia) and regardless of the availability of imaging modalities for detecting subclinical joint inflammation. Second, it was considered important to ensure that the generated risk stratification criteria would not be misinterpreted as indicating the presence of a disease or as classification criteria. With these overarching principles, an expert committee developed EULAR/ACR risk stratification criteria.

METHODS

Expert committee

In 2020, a EULAR committee of experts in the field of ‘arthralgia at risk of RA’ was established. To pool existing cohort data and develop consensus, members were invited because they were the principal investigator of an arthralgia cohort or because of their expertise in this area. In 2023, it was decided by ACR and EULAR to include ACR experts and to continue as a joint EULAR/ACR initiative. The final expert committee consisted of 20 rheumatologists, 2 fellows, 1 EMEUNET representative, 4 patient research partners, 2 allied health professionals, 1 methodologist, and 2 statisticians, recruited from 10 European countries and North America.

Data sharing procedure

An inventory of available data in at-risk cohorts was made (including inclusion criteria, endpoints, and risk variables). At the first meeting (June 2021), consensus was reached on the variables to be shared and on the primary endpoint. At the second meeting (June 2022), data transfer agreements were signed and some data were shared. The final datasets were shared in October 2022.

Study population

Some cohorts included persons defined as being at risk for RA because of autoantibody positivity with any arthralgia/MSK symptoms; others were selected based on the rheumatologists’ expert opinion that the person had arthralgia characteristics that put them at high risk of progressing to RA (ie, CSA). The expert committee agreed to include both approaches. Additionally, it was decided to exclude cohorts in which at least some individuals were treated with disease-modifying antirheumatic

drugs in the phase of arthralgia/MSK symptoms. The cohorts are described in [Supplemental Tables S1](#) and [S2](#). By definition, all at-risk individuals studied did not have clinically apparent IA at inclusion in the cohorts.

Endpoint

The primary endpoint was the development of clinically apparent IA, identified by physical examination by rheumatologists, at 1-year follow-up. This was collected across all cohorts. Secondary endpoints were fulfilling of the 2010 EULAR/ACR criteria for RA [1] at 1 year and developing IA at 2 years follow-up.

Risk variables

Three categories of predictor variables were studied: clinical and serologic (available in all cohorts), and, where available, subclinical inflammation detected by ultrasound (US) or magnetic resonance imaging (MRI) ([Supplemental Table S1](#)). Clinical variables were studied as available (31 variables, [Supplemental Fig S1A](#)). The presence of CSA, based on clinical opinion or EULAR definition [7], was not included as a variable because it was missing from autoantibody-positive arthralgia cohorts. However, data on several clinical items from the EULAR definition were available and examined. Imaging protocols differed between the cohorts ([Supplemental Table S3](#) for US, [Supplemental File S1](#) for MRI). Anatomical sites and characteristics that were reasonably similar across cohorts were included (92 variables for US and 62 for MRI, [Supplemental Fig S1B, C](#)). The presence of subclinical inflammation by US was defined as grey scale (GS) ≥ 2 (except for metatarsophalangeal [MTP] joints 1–3, where a threshold of ≥ 3 was chosen because of the prevalence of GS ≤ 2 in healthy persons [17]) or power Doppler ≥ 1 . For MRI, subclinical inflammation was considered present if the Rheumatoid Arthritis Magnetic Resonance Imaging Score at a joint/bone/tendon sheath was present in $<5\%$ of age-matched symptom-free persons at the same location [18,19].

Statistics

A statistical analysis plan was developed and approved.

Derivation

Missing variables were imputed using the total dataset (including available clinical, serologic, imaging, and outcome data). Twenty completed datasets were created using multiple imputation by chained equation.

Because of a high number of variables in relation to the number of events, the number of variables was reduced prior to the main analyses to prevent overfitting. Thirty-one clinical items were present. Some were not included, eg, because of absence of association in univariable logistic analyses of the primary endpoint, leaving 24 clinical and serologic variables for the main analyses ([Supplemental Fig S1A](#)). For US, data from 92 variables were present across the cohorts ([Supplemental Fig S1B](#)). Summation reduced this to 10 US variables without loss of discriminatory capacity (area under the receiver operating characteristic curve [AUC]) ([Supplemental Fig S1B](#)). Similarly, 62 MRI variables were summed into 12 variables ([Supplemental Fig S1C](#)).

For the main analyses, least absolute shrinkage and selection operator (Lasso) penalised regression was used. First, a Lasso logistic regression model was built with 24 clinical and serologic variables and the primary endpoint as outcome in the total dataset of all cohorts for application in the absence of imaging data.

The regression coefficients of the selected variables were fixed into a linear predictor (ie, clinical and serologic variables with fixed coefficients). Subsequently, this linear predictor was included in 2 other analyses, including 10 baseline US or 12 MRI variables. These analyses assessed the incremental value of US-/MRI-detected inflammation added to the information from the clinical and serologic variables.

Cohort heterogeneity was evaluated by adding a cohort variable as an adjustment variable. Two cohort variables were studied: grouped in 2 categories (identification of being at risk based on autoantibody-positive arthralgia/MSK symptoms or CSA) or 5 categories (grouping by geography/region and identification method).

A risk stratification algorithm was derived by summing the coefficients of the identified items with scaling and rounding for better applicability. This consisted of a section with clinical and serologic variables, and if US or MRI data were available, these additional variables could be used. The predicted risks were plotted against the risk scores. Test characteristics (sensitivity and specificity) and predictive values (positive and negative predictive values) were determined for different cutoffs of risk scores.

Discriminatory capacity of the 3 models (clinical and serologic, +US, and +MRI) was assessed primarily by the AUC, with higher AUCs indicating better performance. We evaluated whether a sensitivity and specificity of ~80% (a priori selected criterion) was achieved.

We assessed whether risk stratification could be simplified without loss of performance (AUC). Additionally, we compared the percentage of participants with low, intermediate, or high risk where risk categories were arbitrarily defined (low risk <25% development of clinically apparent IA; intermediate risk 25%–75%; high risk ≥75%). The goal was to have as few people as possible in the intermediate risk group. Statistical methods are further described in [Supplemental File S2](#).

Validation

We applied a cross-validation procedure with 200 bootstrap replications. In addition, the total dataset was split into 2 sets

comprising two-thirds and one-third; the split was performed at the cohort and outcome levels. Finally, the performance per each of 5 groups of cohorts was evaluated.

Final consensus

The results were presented at the third meeting (in-person, at EULAR congress 2023) and at the final online meeting (June 2023). Consensus was then assessed by voting according to the EULAR standardised operating procedures [20].

RESULTS

Population

Of the 14 cohorts in the inventory phase, 10 cohorts generated data from a total of 2583 symptomatic at-risk persons ([Supplemental Fig S2](#), [Supplemental Table S1](#)). These data came from observational cohorts from Amsterdam (the Netherlands), Birmingham (UK), Erlangen (Germany), Leeds (UK), Leiden (Netherlands), Rotterdam (Netherlands), Rome (Italy), and Vienna (Austria) and the placebo arm of the Dutch multicentre TREAT EARLIER trial. Two cohorts were excluded because of disease-modifying antirheumatic drug treatment, leaving 2293 persons for analysis ([Table 1](#), [Supplemental File S2](#)).

Primary endpoint

Clinically apparent IA developed in 389 persons (17%) (282 anti-citrullinated peptide antibody [ACPA]-positive, 107 ACPA-negative persons) within 1 year. Clinical, serologic, and imaging factors were studied in relation to this endpoint.

Clinical and serologic risk factors

From the 24 variables, 7 were strongly associated with the primary endpoint ([Table 2](#)). These were difficulty to make a fist, patient-reported joint swelling, increased C-reactive protein (CRP), rheumatoid factor (RF), and ACPA, and the cohort

Table 1
Characteristics at baseline and frequency of the primary endpoint

	Total population (n = 2293)	Population with US (n = 835)	Population with MRI (n = 730)	Population without imaging (n = 728)
Baseline characteristics				
Female, n (%)	1733 (76)	630 (75)	558 (76)	545 (75)
Age, y, mean (SD)	47 (13)	49 (13)	44 (12)	49 (12)
Symptom duration, wk, median (IQR)	43 (19–104)	51 (26–110)	21 (11–46)	52 (28–156)
Presence of hand symptoms, n (%)	987 (84)	327 (80)	582 (86)	78 (83)
Morning stiffness ≥60 min, n (%)	521 (26)	180 (22)	234 (34)	107 (21)
TJC44, median (IQR)	2 (0–6)	2 (0–6)	4 (2–9)	0 (0–3)
Increased CRP, n (%)	372 (17)	113 (14)	163 (22)	96 (14)
RF positivity, n (%)	949 (41)	357 (44)	150 (20)	431 (61)
Low-positive	455 (20)	155 (19)	59 (8)	241 (34)
High-positive	483 (21)	202 (25)	91 (12)	190 (27)
ACPA positivity, n (%)	1103 (48)	531 (65)	102 (14)	470 (66)
Low-positive	391 (17)	188 (23)	17 (2)	186 (26)
High-positive	712 (31)	343 (42)	85 (12)	284 (40)
Primary identification method for RA risk, n (%)				
Autoantibody positivity with arthralgia/MSK symptoms	1242 (54)	597 (71)	0	645 (89)
CSA	1051 (46)	238 (29)	730 (100)	83 (11)
Primary endpoint				
Clinically apparent inflammatory arthritis within 1 y, n (%)	389 (17)	153 (18)	102 (14)	134 (18)

ACPA, anti-citrullinated peptide antibody; CRP, C-reactive protein; CSA, clinically suspect arthralgia; MRI, magnetic resonance imaging; MSK, musculoskeletal; RA, rheumatoid arthritis; RF, rheumatoid factor; TJC, tender joint count; US, ultrasound.

Table 2

Results from Lasso regression including clinical and serologic variables plus additional ultrasound- or MRI-detected subclinical joint inflammation

Variable	Clinical and serologic (n = 2293)	Clinical and serologic + ultrasound (n = 835)	Clinical and serologic + MRI (n = 730)
	OR	OR	OR
Morning stiffness			
30-60 min	1.5	1.5	1.5
≥60 min	2.2	2.2	2.2
Patient-reported joint swelling	2.5	2.5	2.5
Difficulty making a fist	3.7	3.7	3.7
Increased CRP	1.3	1.3	1.3
RF			
Low-positive	1.5	1.5	1.5
High-positive	2.4	2.4	2.4
ACPA			
Low-positive	2.9	2.9	2.9
High-positive	8.2	8.2	8.2
2-category cohort variable ^a	1.7	1.7	1.7
Ultrasound			
PD synovitis PIPs	—	2.8	—
PD tenosynovitis	—	1.7	—
PD synovitis MTPs	—	1.4	—
GS synovitis wrist	—	1.2	—
GS tenosynovitis	—	1.2	—
MRI			
Tenosynovitis flexors wrist	—	—	3.3
Tenosynovitis extensors MCPs	—	—	2.7
Tenosynovitis extensors MTPs	—	—	1.9
Tenosynovitis extensors wrist	—	—	1.3
Osteitis wrist	—	—	1.1
Synovitis MCPs	—	—	1.1
AUC (95% CI)	0.80 (0.77-0.83)	0.80 (0.75-0.84)	0.87 (0.82-0.90)

ACPA, anti-citrullinated peptide antibody; AUC, area under the curve; CRP, C-reactive protein; CSA, clinically suspect arthralgia; GS, grey scale; Lasso, least absolute shrinkage and selection operator; MCP, metacarpophalangeal joint; MRI, magnetic resonance imaging; MTP, metatarsophalangeal joint; OR, odds ratio; PD, power Doppler, PIP, proximal interphalangeal joint; RA, rheumatoid arthritis; RF, rheumatoid factor.

Presented is the OR derived from the regression coefficient. The regression coefficient was used to derive the weights per variable in Figure 1. As Lasso shrinks the coefficient to zero, measures of variations are not informative. The AUC of the clinical and serologic Lasso model in the n = 835 and n = 730 groups were 0.78 (95% CI, 0.72-0.83) and 0.84 (95% CI, 0.79-0.88), respectively.

^a Identification of RA risk based on CSA or autoantibody-positive arthralgia/musculoskeletal symptoms.

variable that was included to adjust for cohort heterogeneity (2-category variable, being at risk based on autoantibody-positive arthralgia/CSA). The AUC was 0.80. When the 5-category cohort variable (5 groups based on geography and autoantibody-positive arthralgia/CSA) was used to adjust for cohort heterogeneity, the results were similar (Supplemental Table S4). The 2-category cohort variable was used as an adjustment factor in further analyses.

US-detected subclinical joint inflammation

A model with only the 10 aggregated US variables (n = 835 with US data) yielded an AUC of 0.63 (Supplemental Table S5). Including these to the panel of fixed clinical and serologic variables identified 5 US variables that were associated with the endpoint (Table 2). This model had an AUC of 0.80.

MRI-detected subclinical joint inflammation

MRI data were analysed in a similar way as US data. MRI data alone resulted in a model with an AUC of 0.76 (Supplemental Table S6). Combining the aggregated 12 MRI variables with the fixed clinical and serologic risk factors revealed that 6 MRI variables were incrementally associated with the endpoint (Table 2). The AUC of this model was 0.87.

Development of extended risk stratification

Based on these results, an algorithm was derived using the 6 clinical and serologic variables, the cohort variable, and with the 5 US or 6 MRI variables (Fig 1). The possible scores ranged between 0 and 28 (clinical and serologic data), 0 and 40 (with US data), or 0 to 45 (with MRI data). The predicted risks per score are presented in Figure 1. Additionally, the test characteristics and predictive values for various cutoffs are presented.

Performance

The main statistics for performance were the AUC values, which were 0.80, 0.80, and 0.87 for the clinical and serologic data only, with additional US data, and with additional MRI data, respectively (Table 2). Sensitivity and specificity ~80% was only possible when including MRI data and with a risk score ≥12 (sensitivity 79%, specificity 78%) (Fig 1).

Validation

Internal validation showed little variability in the AUC (Table 3). Analyses in two-thirds and one-third of the population also showed only minor changes in AUC. Analysing the 5 groups of cohorts separately showed AUCs ranging from 0.75 to 0.84 (Supplemental Table S7).

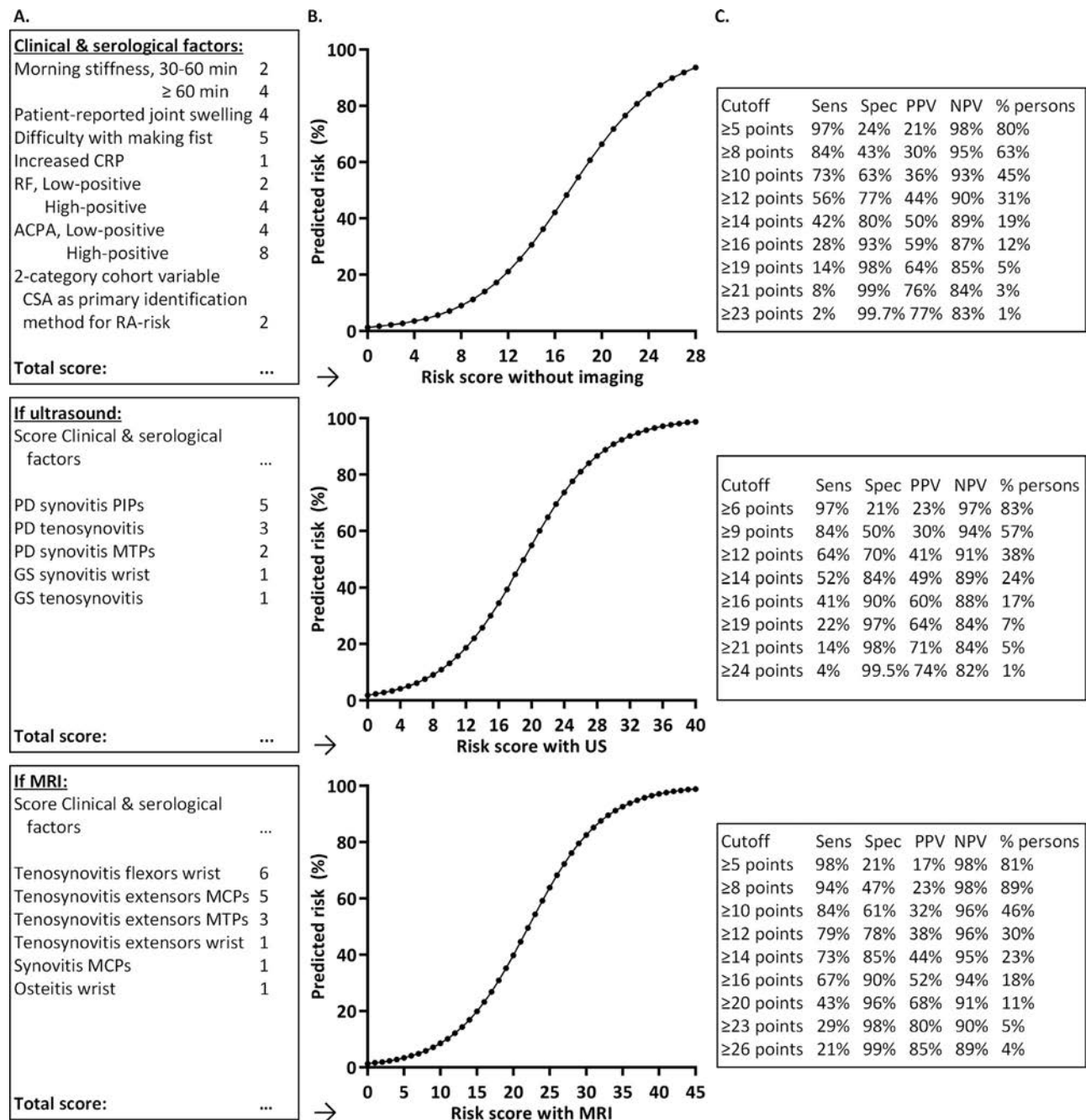


Figure 1. Risk stratification for development of inflammatory arthritis within 1 year using clinical and serologic data and with additional US- or MRI-detected subclinical joint inflammation in arthralgia at risk for RA (A), with predicted risks per risk scores (B), and test characteristics and predictive values for different risk score cutoffs (C). The risk score without imaging ranges from 0 to 28, the risk score with US from 0 to 40, and the risk score with MRI from 0 to 45. The cohort variable was included to adjust for heterogeneity between the cohorts. This was classified in a 2-category variable based on the primary identification method for RA risk, namely identification of being at risk based on autoantibody-positive arthralgia/musculoskeletal symptoms or CSA. ACPA, anti-citrullinated peptide antibody; CRP, C-reactive protein; CSA, clinically suspect arthralgia; GS, grey scale; MCP, metacarpophalangeal joint; MRI, magnetic resonance imaging; MTP, metatarsophalangeal joint; NPV, negative predictive value; PD, power Doppler; PIP, proximal interphalangeal joint; PPV, positive predictive value; RA, rheumatoid arthritis; RF, rheumatoid factor; Sens, sensitivity; Spec, specificity; US, ultrasound.

Secondary endpoints

Of the individuals, 12% (209 of 1708) developed RA at 1 year according to the 2010 EULAR/ACR classification criteria. The AUC for this secondary endpoint was 0.85 for the clinical and serologic data, 0.84 when US data were added, and 0.93 when MRI data were added (Table 3). Of the individuals, 21% (441 of 2055) developed clinically apparent IA within 2 years. The AUC values were roughly similar to those with this endpoint at 1 year follow-up (Table 3).

Simplified risk stratification

For ease of use, simplifications were applied for the algorithm with the primary endpoint. The cohort variable was omitted because it is an ‘artificial variable’ that was used to adjust risk estimates for cohort heterogeneity. US-detected inflammation data were omitted as their contribution was negligible in this analysis. For MRI, only tenosynovitis was included because the contribution of the other combined MRI variables was negligible.

Table 3
Performance of validation using bootstrapping and data split and for secondary endpoints

Included variables	Internal validation			Secondary endpoints	
	Bootstrapping	Data split		Clinical arthritis within 2 y	RA (2010 criteria) within 1 y
	AUC (95%CI)	² / ₃ AUC (95%CI)	¹ / ₃ AUC (95%CI)	AUC (95% CI)	AUC (95% CI)
Clinical and serologic	0.80 (0.77-0.83)	0.80 (0.77-0.83)	0.80 (0.77-0.83)	0.79 (0.77-0.82)	0.85 (0.83-0.88)
Clinical and serologic + US	0.79 (0.74-0.84)	0.80 (0.76-0.83)	0.79 (0.74-0.82)	0.78 (0.75-0.81)	0.84 (0.78-0.90)
Clinical and serologic + MRI	0.86 (0.81-0.89)	0.84 (0.81-0.86)	0.83 (0.80-0.85)	0.85 (0.81-0.89)	0.93 (0.90-0.97)

ACR, American College of Rheumatology; AUC, area under the curve; EULAR, European Alliance of Association for Rheumatology; MRI, magnetic resonance imaging; RA, rheumatoid arthritis; US, ultrasound.
A total of 2055 persons had 2-year follow-up data; of these, 441 (21.5%) progressed to clinical arthritis within 2 years. A total of 1708 persons had data on development of clinical arthritis and fulfilled the 2010 EULAR/ACR classification criteria for RA; of these, 209 (12.2%) developed RA within 1 year. Derived models were evaluated after a ²/₃-¹/₃ split that was performed at the cohort and outcome levels.

Therefore, the clinical and serologic variables in the final criteria were morning stiffness, presence of patient-reported joint swelling, difficulty making a fist, and increased CRP, RF, and ACPA status (Box). For imaging, the presence of tenosynovitis of the flexors of the wrist, the extensors of the wrist, the extensors of the metacarpophalangeal joints and the extensors of the MTP joints were included (Box). The clinical and serologic score ranged from 0 to 26; when MRI data was added, the score ranged from 0 to 42 (Box). The predicted risks were plotted per risk for RA score, both for the criteria without and with imaging (Fig 2). Test characteristics were presented for several cut-offs for the risk for RA score (Fig 2; Supplemental Tables S8 and S9).

The simplified clinical and serologic model had an AUC of 0.80; when adding MRI data, the AUC was 0.86. Calibration graphs are presented in Supplemental Figure S3. Sensitivity and specificity ≥75% was only possible with the criteria with MRI data; a risk score of ≥10 points corresponded to a sensitivity of 75% and specificity of 79%.

The percentages of participants classified as low, intermediate, and high risk (<25%, 25%-75%, and ≥75%, respectively) based on clinical and serologic data are shown for those who did and did not reach the endpoint (Fig 3). Patients who did not develop clinically apparent IA were almost exclusively in the low risk group. Patients who developed the endpoint were in all 3 groups. Exploration revealed that the converters classified as low risk were ACPA-positive with few symptoms or ACPA-negative with little subclinical joint inflammation (Supplemental Table S10). Importantly, the intermediate risk group, which should ideally be as small as possible, contained 18% of all studied at-risk individuals. This intermediate risk group was the smallest, 11%, when MRI data were included (Fig 3).

Consensus

During voting in the final meeting, 96% of task force members approved the stratification criteria. It was agreed to present both the extended and simplified versions, and agreement was reached on the target population defined in the eligibility criteria (Box).

DISCUSSION

We present EULAR/ACR risk stratification criteria for the development of clinically apparent IA and RA, representing the culmination of an international collaborative effort that included researchers curating arthralgia cohorts, expert

rheumatologists, health care professionals, and patient research partners across Europe and North America. The risk stratification criteria consist of symptoms (morning stiffness, patient-reported joint swelling), a sign (difficulty making a fist), serologic markers (CRP, RF, and ACPA), and MRI-detected tenosynovitis. This product has been developed with the key aim of supporting the inclusion of homogeneous risk groups in future prevention trials with individuals with arthralgia in secondary care in whom imminent RA is considered more likely than other causes of arthralgia. Importantly, risk of a disease is not the same as having a disease and therefore, the product is not described as classification criteria.

We aimed to derive an algorithm that is broadly applicable both in settings in which imaging modalities are available to detect subclinical joint inflammation and where such modalities are lacking. The statistical methodology was adapted accordingly, aiming to take maximum advantage of the information from clinical and serologic variables. Most importantly, the information from clinical and serologic data was fixed and prioritised. In the presence of both imaging-detected subclinical inflammation and clinical data, whereby the latter was related to inflammation (eg, morning stiffness is known to be related to subclinical synovitis [21] and difficulty making a fist to subclinical tenosynovitis [22]), multivariable analyses will generally select the imaging variable as the best predictor for RA development because of the most direct relationship. However, for practical application, clinical variables were given priority (set as fixed in the analyses). Consequently, the effect sizes of the clinical and serologic variables were larger than if such an approach had not been applied. In contrast, the effect sizes of imaging variables were lower than would have been the case without this prioritisation, and with this approach only revealed the incremental value of adding imaging to what was already known through the clinical and serologic variables. The value of imaging is therefore also lower than previously reported in studies in symptomatic at-risk individuals. This method led to an algorithm that is applicable also in the absence of imaging modalities. The criteria without imaging perform well because they use the underlying correlation of clinical with imaging variables in this population. Because this is a derivative, the criteria with imaging may be preferred for optimal trial design.

Importantly, the risk stratification criteria are derived from data collected in secondary care and as such are based on the correlation structure between the variables that exist in arthralgia patients in secondary care. For example, patient-reported swelling is related to subclinical joint inflammation in arthralgia at risk for RA [23]. In other situations, eg, the general population in which hand osteoarthritis

Box EULAR/ACR risk stratification criteria (after simplification) for individuals with arthralgia at risk for RA, to be used in presence or absence of imaging		
Target population (who should be tested?): persons with arthralgia in secondary care without clinical arthritis with the arthralgia not better explained by another disease/condition		
Clinical and serologic characteristics		Score
Morning stiffness ^a	0-30 min	0
	30-60 min	2
	30-60 min	4
Patient-reported joint swelling ^b	No	0
	Yes	4
Difficulty making a fist ^c	No	0
	Yes	5
Increased CRP ^d	No	0
	Yes	1
RF ^e	Negative	0
	Low-positive	2
	High-positive	4
ACPA ^e	Negative	0
	Low-positive	4
	High-positive	8
Clinical and serologic sum score		... See Figure 2 for risk
Imaging characteristics if also MRI		
Tenosynovitis flexors wrist ^f	Absent	0
	Present	6
Tenosynovitis extensors wrist ^g	Absent	0
	Present	2
Tenosynovitis extensors MCPs ^h	Absent	0
	Present	5
Tenosynovitis extensors MTPs ⁱ	Absent	0
	Present	3
Subscore MRI		...
Clinical, serologic, and imaging sum score		... See Figure 2 for risk
ACPA, anti-citrullinated peptide antibody; CRP, C-reactive protein; IU, international unit; MCP, metacarpophalangeal joint; MRI, magnetic resonance imaging; MTP, metatarsophalangeal joint; RAMRIS, Rheumatoid Arthritis Magnetic Resonance Imaging Score; RF, rheumatoid factor; ULN, upper limit of normal.		
The sum risk score without imaging ranges from 0 to 26 and with imaging from 0 to 42. Predicted risks and test characteristics for several cutoffs of risk scores are presented in Figure 2, with a more extensive list in Supplemental Table S8. The AUC of the criteria without imaging is 0.80 and with MRI data is 0.85. A sensitivity and specificity of ≥75% was only possible with the risk stratification criteria with imaging data and a score of ≥10 points.		
^a Morning stiffness refers to patient-reported duration of joint stiffness in the morning.		
^b Patient-reported joint swelling refers to presence of a swollen joint as reported by the patient.		
^c Difficulty with making a fist is defined as inability of ≥1 fist with incomplete closure (fingertips do not touch the palm at active closing).		
^d Increased CRP defined as above the local laboratory reference.		
^e For RF and ACPA, negative refers to IU values less than or equal to the ULN for the laboratory and assay; low-positive refers to IU values that are greater than the ULN but ≤3 times the ULN for the laboratory and assay; high-positive refers to IU values that are >3 times the ULN for the laboratory and assay.		
^f Tenosynovitis of the wrist flexors: presence (RAMRIS ≥1) Of any tenosynovitis of the following: flexor carpi ulnaris, ulnar bursa including flexor digitorum profundus and superficialis tendon quartets, flexor pollicis longus (tendon) in radial bursa, or flexor carpi radialis.		
^g Tenosynovitis of wrist extensors: presence (RAMRIS ≥1) Of any tenosynovitis of the 6 extensor compartments: (I) extensor pollicis brevis, abductor pollicis longus; (II) extensor carpi radialis brevis, extensor carpi radialis longus; (III) extensor pollicis longus; (IV) extensor digitorum communis, extensor indicis proprius; (V) extensor digiti quinti proprius; (VI) extensor carpi ulnaris.		
^h Tenosynovitis of the extensors of the MCPs: presence (RAMRIS ≥1) Of any tenosynovitis of the extensors of MCP2-5.		
ⁱ Tenosynovitis of the extensors of the MTPs: presence (RAMRIS ≥1) Of any tenosynovitis of the extensors of MTP1-5.		

is more prevalent than imminent RA, this relationship may be different. Consequently, this method cannot be extrapolated for trials in other populations (eg, primary care-based populations) or for triaging referrals from primary to secondary care. The developed criteria are expected to be widely applicable, but only in persons seen with arthralgia in secondary care, where impending RA is more likely than other causes of arthralgia.

The criteria were derived with the idea that the accuracy of data-driven risk stratification performs better than risk stratification by expert opinion of individual rheumatologists. Whether this assumption is true needs to be verified in future studies.

Performance was evaluated using the AUC (combining sensitivity and specificity). In line with the aim of promoting the inclusion of homogeneous risk groups in future studies, this

metric evaluates performance for groups of persons. At the design of the project, the intention was to achieve both specificity and sensitivity of ~80%. The algorithm using clinical and serologic data achieved a ‘best combination’ of sensitivity and specificity of approximately 65% to 70% (Figs 1 and 2). When MRI data were available, a specificity and sensitivity of almost 80% was achieved by a score ≥12 in the expanded version or ≥10 in the simplified version. Therefore, if a sensitivity and specificity of both >75% are desired, the imaging criteria and a cutoff of ≥10 points are appropriate. However, the expert committee did not choose one cutoff point because, depending on the clinical situation or intervention (with different risks of side effects), either a (very) high sensitivity or specificity may be preferred. With the current presentation, investigators can choose the test characteristics and corresponding cutoff that is

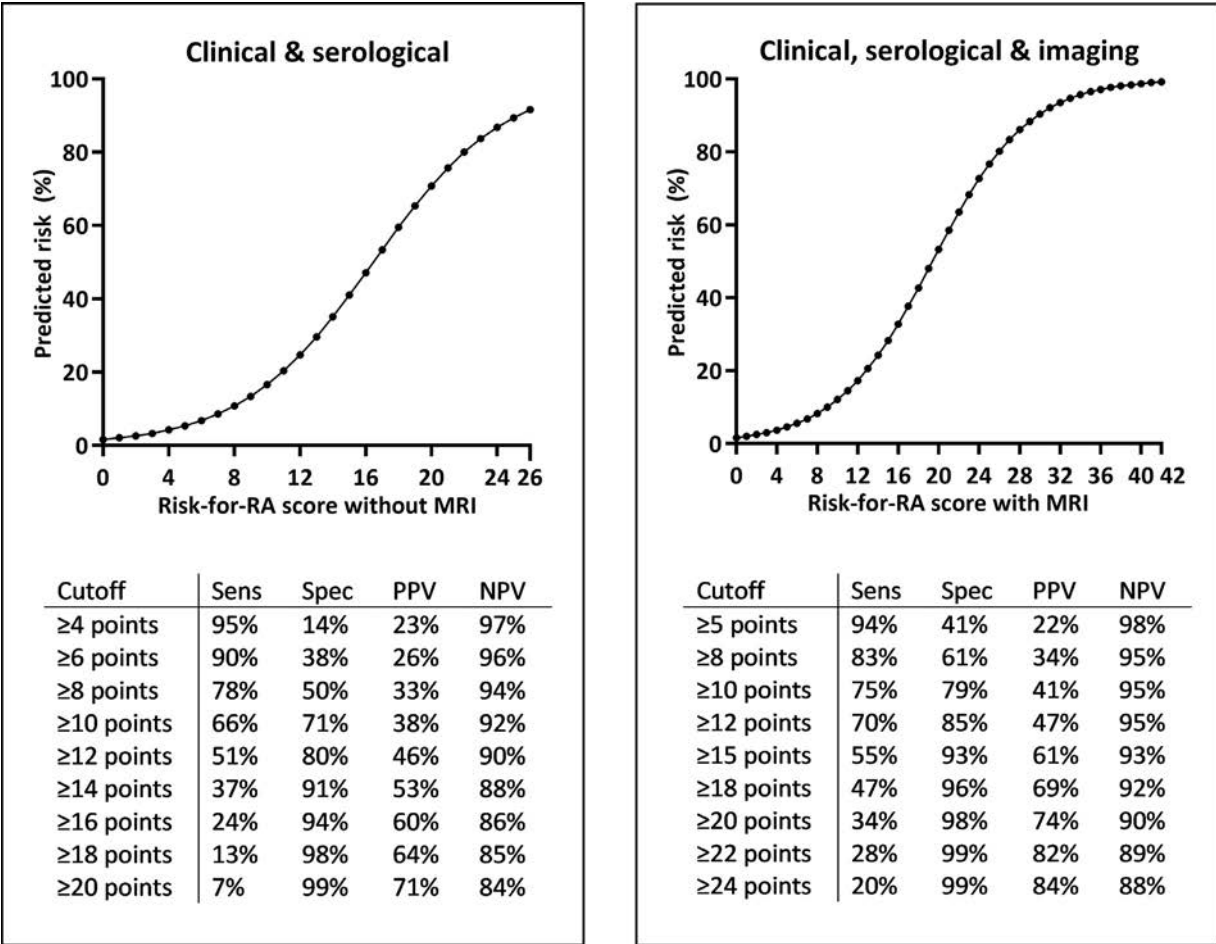


Figure 2. Predicted risks (graph) and test characteristics and predictive values for several score cutoffs (table) for the risk stratification criteria without or with imaging. Predicted risks for the sum scores obtained in the Box. Test characteristics and predictive values for several cutoffs of risk scores are shown; an extensive list of cutoffs with test characteristics and data on the percentage of persons with these cutoffs is presented in Supplemental Table S8. The area under the curve of the criteria without imaging is 0.80 and with MRI data is 0.85. MRI, magnetic resonance imaging; NPV, negative predictive value; PPV, positive predictive value; RA, rheumatoid arthritis; Sens, sensitivity; Spec, specificity.

felt acceptable by their team of rheumatologic and patient experts.

Importantly, it was not the intention to develop an instrument for individual patients in clinical practice. Absolute risk estimates are more difficult to generalise (than test characteristics) because they depend on prior risks of a disease (context-dependent prevalence). Interestingly, the prior risk of developing clinical arthritis within 1 year was quite similar in all at risk populations studied here (ranging from 14%–18%). Nonetheless, this may be different in other places, and the developed criteria are therefore not suitable for decision making in individual patients.

Subclinical joint inflammation (particularly tenosynovitis) is a known predictor of clinical arthritis and RA development [24], and data collected via US and MRI were assessed. MRI-detected subclinical joint inflammation showed some incremental value compared to clinical and serologic data alone. This was demonstrated by an increase in AUC and a higher combination of sensitivity and specificity. This did not occur for subclinical inflammation detected with US. This difference may be explained by previous findings that tenosynovitis is a powerful imaging predictor missed by US in up to 80% of tenosynovitis lesions compared to MRI [25,26]. Additionally, US scanning protocol and gradings that were used in the cohorts were different and could not be standardised in retrospect. The available

US data also included less tenosynovial sites than MRI (Supplemental Table S3). US-detected erosions were shared from 1 dataset (Rotterdam SONAR cohort) and were found to be nonpredictive. The taskforce had extensive discussions about the US results, and it was agreed that the data would be presented as they are, with the US in the extended algorithm. Furthermore, the US results presented here apply to the setting of individuals with arthralgia at risk for RA and are not relevant to the value of US in a broader context in the field of RA.

MRI also has disadvantages. Some subtle synovitis and osteitis is present when using contrast-enhanced MRI in the general symptom-free population, especially in older age and in specific joints and bones [18]. These variations need to be considered when defining an abnormal MRI result to prevent false-positive tests [19]. In contrast, tenosynovitis has been found to be rare in the general population [18] and was the only variable that remained in the simplified risk stratification criteria. Tenosynovitis is also relatively easy to detect reliably on MRI. Although MRI is already used for the classification of other rheumatic diseases [27], MRI may be considered impractical due to the need for intravenous contrast agents, long scan times, and resulting high costs. Recent data have emerged suggesting that a short modified Dixon MRI sequence correlates well with conventional contrast-enhanced MRI for scanning of hands/forefeet [28]. With a scan time of 5 minutes without the need for intravenous

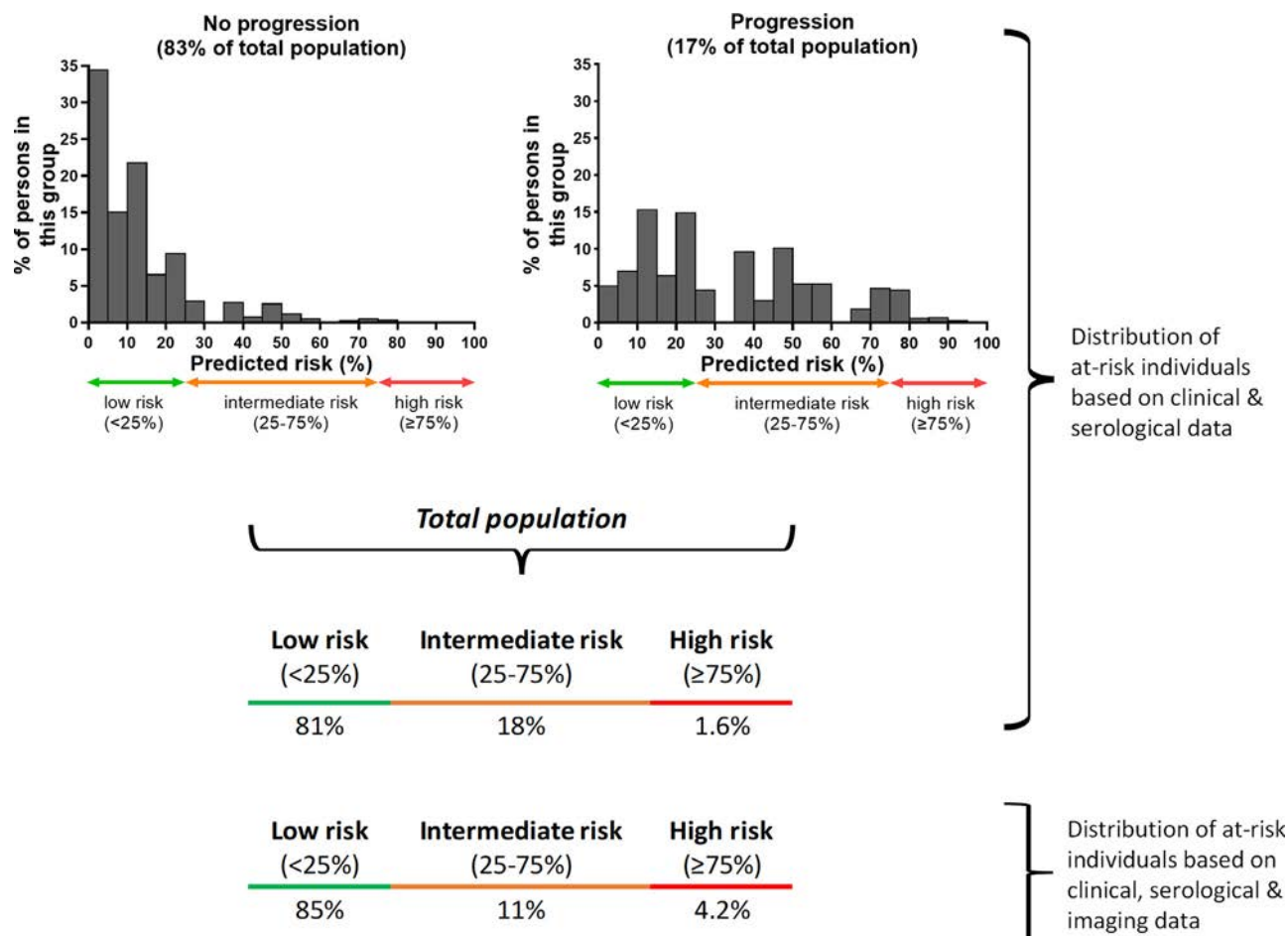


Figure 3. Distribution of subjects who did not and did reach the endpoint based on the predicted risks using the risk stratification criteria with clinical and serologic data in the Box. Subsequently, risk categories were defined as low (<25% risk), intermediate (25%-75%) and high (≥75%) in the total population based on clinical and serologic data (n = 2293). The same was done when additionally using MRI data (n = 703). Characteristics of persons who developed the endpoint and were categorised as low risk (8% of the total population) are presented in Supplemental Table S10, both for the setting without and with imaging data. MRI, magnetic resonance imaging.

contrast, this could make MRI more feasible. The value of this short MRI sequence and its cost-effectiveness are subjects for further research. If positive, this could have a significant impact on the assessment of the burden and costs of MRI compared to the incremental value in risk stratification.

The method for identifying persons at risk of RA varies between centres and countries. In some, the presence of a positive autoantibody test is the starting point, and the presence of any MSK symptom increases the risk. Alternatively, individuals can be identified by a combination of symptoms and signs that resemble RA but where clinical IA is absent, ie, by having CSA. Both identification methods alone have proven to be insufficient. Risk stratification, as shown previously [29–31] and also here, requires a combination of clinical and serologic features combined with imaging of joints. Although all participants in the CSA cohorts were reported to have CSA by their rheumatologists, for participants in the autoantibody-positive cohorts, it was unknown whether they had CSA (according to their rheumatologist), and data from some items of the EULAR definition of CSA [7] were absent/incomplete. Therefore, having CSA at an individual level could not be included in the analyses, but several clinical items that are part of the EULAR definition of CSA have entered the risk stratification criteria (morning stiffness and difficulty making a fist).

The primary endpoint, development of clinically apparent IA, was ascertained by consensus and was a pragmatic choice because data on the 2010 classification criteria for RA were

missing in 25%. RA was most likely the final diagnosis in the event of the development of clinical arthritis because at-risk individuals were selected based on RA-related autoantibodies or CSA. Previous studies of cohorts that are included here have demonstrated that diagnoses other than RA or undifferentiated arthritis are indeed rare [11,29,30]. Moreover, the performance for the development of RA was comparable to that for the primary endpoint. Thus, risk stratifications were made for developing IA, an acceptable proxy for RA in this setting.

Heterogeneity between cohorts (eg, individual characteristics, outcomes) poses a challenge when combining datasets [32,33]. Sex, age, and endpoint frequency were similar between the datasets, but there were also differences in clinical and serologic characteristics arising from the method of identifying individuals at risk (eg, the cohorts that included individuals with CSA had higher frequencies of tender joints and the cohorts that selected for autoantibodies showed a higher frequency of ACPA positivity). Unmeasured factors related to geography or other factors may also differ between the cohorts. Cohort heterogeneity was taken into account by including a cohort variable. This had an independent association with the endpoint, suggesting an influence. Although this may suggest some impact on generalizability, exclusion of the cohort variable in the simplification step did not affect performance.

Validation was performed with bootstrapping and a data split. The similarity in AUCs demonstrated robustness. Data from European populations were shared in the taskforce. We did

not find cohorts of arthralgia patients from the US, Canada, or Asia who were willing to share data. External validation in non-European populations should be conducted.

A limitation is that the selection of risk variables was based on consensus and availability. Some risk factors previously shown to be predictive in some cohorts (eg, human leukocyte antigen-shared epitope alleles [29,30,34], functional disability [35], and T cell subsets [30]), were not assessed in many cohorts and thus not included here. The additional value of these variables can be evaluated in future studies. Including additional variables would be valuable if, for example, the ACPA-negative progressing individuals that are currently classified as low risk would be recognised as high risk. This will not be easy because included ACPA-negative individuals with arthralgia had a prior risk of 9%, and posttest risks depend on prior risks. The high AUC also suggests that there is little room for improvement.

Patient research partners were involved during all phases of the project. Based on their advice, further research into the patients' perspective is needed. Understanding how risk information is perceived and relates to willingness to participate in trials is essential to inform future trials. Perceived risk status has been shown to affect tolerance for side effects of preventive treatments [16,36]. Furthermore, the proportion of at-risk individuals with 'intermediate' risk of RA was relatively small (11%), but their views of risk classification would need further investigation.

In conclusion, risk stratification criteria for arthralgia at risk for RA have been developed based on international cohort data and expert consensus. The developed risk stratification criteria are validated and intended to support the inclusion of homogeneous risk groups in future prevention trials.

Competing interests

The authors reported the following competing interests, outside the submitted work. SA—grants or contracts: Novartis, AbbVie; payment for lectures: AbbVie, Eli Lilly, Sandoz. CC—payment or honoraria for lectures: AbbVie, Amgen, Boehringer Ingelheim, Ewopharma, Lilly, Novartis, Pfizer, Sobi; leadership or fiduciary role in other board: President-elect of Romanian Society of Rheumatology, President of Romanian Registry of Rheumatic Diseases. SBC—grants or contracts: investigator and consultant Amgen, AbbVie, BMS, Pfizer; consulting fees: Amgen, AbbVie, BMS, Pfizer; payment or honoraria for lectures: Pfizer; participation on a data safety monitoring board (DSMB): Gilead. APC—grants or contracts: BMS, UCB; consulting fees: BMS, Galvani, Janssen, UCB, Lilly; payment or honoraria for lectures: BMS, Galapagos; leadership or fiduciary role in other board: chair EULAR Research Centre Clinical Research subcommittee. KD—payment or honoraria for lectures: Werfen, ThermoFisher; receipt of equipment, materials: Werfen. BE—grants or contracts: Boehringer Ingelheim; consulting fees: Boehringer Ingelheim. AK—consulting fees: Novartis Deutschland GmbH, Eli Lilly Deutschland GmbH, Gilead Sciences, Medac GmbH. KM—grants or contracts: Gilead, Lilly, Serac Healthcare, Astra Zeneca; consulting fees: Galapagos, Lilly, Serac Healthcare, Deepcure, Zura Bio; payment or honoraria for lectures: AbbVie, Galapagos; support for attending meetings and/or travel: Lilly; participation on a DSMB: Galapagos. LJON—consulting fees: Fresenius Kabi, UCB; payment or honoraria for lectures: AbbVie. AS—grants or contracts: FOREUM; consulting fees: AbbVie, UCB, Novartis; payment of honoraria for lectures: Lilly, AbbVie; support for attending meetings and/or travel: Janssen, Lilly; leadership or fiduciary role in other board: editor in chief ARP Rheumatology, associate editor RMD Open. JAPdS

—consulting fees: Amgen, Fresenius Kabi; payment or honoraria for lectures: UCB, Amgen; participation on a DSMB: Fresenius Kabi. JAS—grants or contracts: National Institute of Arthritis and Musculoskeletal and Skin Diseases, Rheumatology Research Foundation, R. Bruce and Joan M. Mickey Research Scholar Fund, Gordon and Llura Gund Foundation, BMS, Boehringer Ingelheim, Sonoma Biotherapeutics; consulting fees: AbbVie, Amgen, Boehringer Ingelheim, BMS, Gilead, Inova Diagnostics, Johnson & Johnson, MustangBio, Optum, Pfizer, ReCor, Sana, Sobi, UCB. PS—grants or contracts: FOREUM, Horizon/HADEA, AAL, EUREKA, Jubiläumsfond der Stadt Wien; consulting fees: AbbVie; payment or honoraria for lectures: AstraZeneca; support for attending meetings and/or travel: Janssen, Galapagos. RL—payment or honoraria for lectures: AbbVie, UCB, Pfizer, Galapagos, Janssen Pharma, Novartis, Lilly; participation on a DSMB: UCB SLE trial; leadership or fiduciary role in other board: ASA board; other financial or nonfinancial interests: director of Rheumatology Consultancy BV, director of Joint Imaging BV. KR—grants or contracts: BMS.

This criteria set has been approved by the American College of Rheumatology (ACR) Board of Directors and the EULAR Executive Committee. This signifies that the criteria set has been quantitatively validated using patient data, and it has undergone validation based on an independent data set. All ACR/EULAR-approved criteria sets are expected to undergo intermittent updates.

Classification criteria are essential in clinical and basic science research because they allow investigators to study relatively homogeneous populations of patients recruited from a single or multiple research sites. In clinical settings, diagnoses are made by health care professionals evaluating an individual patient's symptoms, signs, and results of laboratory and imaging studies in order to guide therapeutic recommendations. Patients diagnosed with a particular disease may or may not fulfill classification criteria for that disease. Classification criteria, in the hands of an experienced clinician with expertise in rheumatology, may inform a diagnostic evaluation, but improperly applied classification criteria may lead to misdiagnosis.

The ACR is an independent, professional, medical, and scientific society that does not guarantee, warrant, or endorse any commercial product or service.

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Contributors

All authors have contributed to this work, drafted the work or revised it critically and approved the final version. AHMvdHvM and HWvS are the guarantors of the work.

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Patient involvement

Patient research partners from EULAR and ACR were part of the taskforce and included as authors on this work.

Patient consent for publication

Not applicable.

Ethical approval

Is ensured by the individual cohorts.

Provenance and peer review

Not commissioned; externally peer reviewed.

Data availability statement

The principal investigators of the cohorts shared their cohort data for this project, but data will not be openly accessible to the general public.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ard.2025.01.021.

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Criteria

EULAR/ACR classification criteria for paediatric chronic nonbacterial osteomyelitis (CNO)

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ABSTRACT

Objectives: To develop and validate classification criteria for paediatric chronic nonbacterial osteomyelitis (CNO) jointly supported by the European Alliance of Associations for Rheumatology (EULAR) and the American College of Rheumatology (ACR).

Methods: This international initiative had 4 phases: (1) candidate items were proposed in a survey of paediatric rheumatologists, (2) criteria definition and reduction by Delphi and nominal group technique exercises, (3) criteria weighting using multicriteria decision analysis, and (4) refinement of weights and threshold score in a development cohort of 441 patients and validation in another cohort of 514 patients.

Results: The new EULAR/ACR classification criteria for CNO require typical radiographic or magnetic resonance imaging findings and bone pain as an obligatory entry criterion and exclusion criteria of malignancy, infection, vitamin C deficiency, and hypophosphatasia, followed by additive weighted criteria in 5 clinical (site of bone lesions, pattern of bone lesions, age at onset, coexisting conditions, fever) and 4 pathology/laboratory domains (bone biopsy findings if done, anaemia, C-reactive protein level, and erythrocyte sedimentation rate). A total score ≥ 55 is

required for classification as CNO. The new criteria had a sensitivity of 82% and specificity of 98% in the validation cohort.

Conclusions: These new classification criteria for paediatric CNO developed with international input reflect current views about CNO, have high specificity and good sensitivity, and provide a key foundation for future CNO research.

INTRODUCTION

Chronic nonbacterial osteomyelitis (CNO) is a rare autoimmune inflammatory bone disease of unknown cause with an annual incidence of 0.4 to 2.3 per 100,000 children [1–3]. CNO may significantly impact patients' quality of life and result in permanent bone damage, long-term disability, and deformity if left untreated [4–7]. The term chronic recurrent multifocal osteomyelitis is used when multiple bones are affected over time. We use CNO throughout this article for consistency. CNO is difficult to characterise and diagnose because of its insidious onset, variable bone location and symptoms at presentation, and lack of high-quality clinical research describing the disease spectrum. These factors, together with limited awareness among physicians about CNO, often result in misdiagnosis [1] and misclassification for research.

There are no previous classification criteria for CNO. Classification criteria are essential to identify homogeneous groups of patients to better characterise the epidemiology and natural history of CNO and to enrol in clinical trials. In the absence of classification criteria, diagnostic criteria proposed for clinical use have been used as inclusion criteria for research in CNO. CNO diagnostic criteria have been proposed by 3 groups: King et al [8] and Manson et al [8,9], Jansson et al [10], and Roderick et al [11]. These diagnostic criteria were based on clinical experience from single centre cohorts with fewer than 100 patients each and have not been validated in independent cohorts. Thus, with the lack of evidence on the validity of these diagnostic criteria, they should not be used as entry criteria for clinical trials or observational studies.

The roles of advanced imaging methods and bone biopsy need to be carefully evaluated in classification criteria for CNO. Previous CNO diagnostic criteria required presence of lytic and/or sclerotic lesions in radiographs or hyperintensity within bone marrow in fluid-sensitive magnetic resonance imaging (MRI) sequences. Symmetric bone lesions and multifocal lesions occur much more frequently than unifocal lesions [5,10]. Whole-body MRI (WBMRI) is a relatively new modality that better defines patterns of lesion distribution and often identifies clinically 'silent' lesions that may affect the vertebral spine, which can impact treatment choice [2,12–14].

Bone biopsy may be needed to exclude differential diagnoses of CNO, especially infections and malignancy [10,11]. However, there are no pathognomonic biopsy features for CNO. Common histologic findings include acute and chronic inflammation, marrow fibrosis, or osteonecrosis, and sometimes biopsy reveals only normal bone, likely due to sampling error [10,15–18]. Fibrosis in bone biopsy is present in children with prolonged duration of disease [19]. Microbiological investigations including special stains, cultures, or polymerase chain reaction tests are negative for bacteria, fungus, and mycobacteria, except for occasional contaminant organisms [20].

We aimed to develop the first classification criteria for paediatric CNO combining international expert consensus and data-driven methods and including the patient's perspective [21,22].

METHODS

Study overview

As in prior European Alliance of Associations for Rheumatology (EULAR)/American College of Rheumatology (ACR) classification criteria [23–27], the study had 4 phases: (1) generation of candidate criteria items, (2) item reduction and definition of items, (3) criteria weighting and identification of threshold for CNO classification, and (4) refinement and validation.

Investigators

The study was led by a core group (CH, SO, AS, HG, FD, MO, YZ, PJF, EW, RN, AH, and KC) and overseen by a steering committee of 20 members (core group plus AVR, AJ, AR, RML, SS, LP, AT, and LV) from North America, Europe (including Turkey), and New Zealand (Supplement S1). Steering committee members were nominated based on CNO expertise or methodological experience. In addition to academic paediatric rheumatologists (7 from Europe and 7 from North America), the steering committee included 1 patient with CNO, 1 parent of a patient with CNO, a physical therapist, methodologists, and a biostatistician. The CNO workgroup of the Childhood Arthritis and Rheumatology Research Alliance (CARRA) assisted with item reduction, and additional international collaborators participated by submitting cases of CNO and mimicking conditions used in the development and validation cohorts for the study.

Phase I. Item generation

Candidate items were identified using a free-text survey sent to members of CARRA, the German Society for Pediatric Rheumatology, the Eurofever registry, and the Dr Peter Dent paediatric rheumatology listserv between September 2017 and May 2018. Responders were asked to list features relevant to CNO and individually rank them in order of importance for classification.

Phase II. Item reduction and definition

Candidate items generated from phase I were reduced and defined in a CARRA CNO work group meeting held in Denver, CO, USA in April 2018 followed by a postmeeting survey. Potential items were retained or removed if 80% agreement of all participants at the meeting was achieved using a nominal group technique. In the postmeeting survey, members of the CARRA CNO work group were asked to score each item as follows: +3 (increases the likelihood of CNO the most), +2 (increases the likelihood of CNO moderately), +1 (increases the likelihood of CNO slightly), 0 (no difference), –1 (decreases the likelihood of CNO slightly), –2 (decreases the likelihood of CNO moderately), –3 (decreases the likelihood of CNO the most). Survey results were discussed by the core group, and no threshold was set to include/exclude these items.

The items remaining, and the items in previously proposed diagnostic criteria [10,11], were included in a case report form (CRF; Supplement S2) used to collect data on CNO cases and mimickers to assemble a development and a validation cohort [10,11]. Paediatric rheumatologists in North America, Europe, Australia, Africa, and South America used the CRF to submit cases and mimickers to an online central database using REDCap software [28]. Seattle Children's Hospital Institution Review Board approved the study as the coordination centre (#1115), and each participating site obtained approval from their institutions. Data received between December 2018 and August 2020 formed the development cohort and data received between August 2020 and May 2021 formed the validation cohort. Submitted CNO cases and mimickers were required to have bone pain, ≥ 1 imaging study (ie, x-ray and/or MRI), and ≥ 12 months of follow-up (except for mimickers with a confirmed laboratory or pathologic diagnosis) because of the concern of inadequate observation for mistakenly diagnosed CNO or mimickers. Institutional Review Board or ethics committee approval was obtained at each site. Collaborators submitting CRFs had no prior knowledge of the items likely to advance to final consideration as classification criteria and provided an assessment of their degree of confidence in the cases being CNO or a mimicker, rated from +3 to -3.

Once the development cohort was assembled in 2020, candidate items were further reduced using the prevalence ratio (PR) of each item as a predictor of 'CNO'. PRs were calculated using only cases with moderate to high confidence (ie, +2 or +3 or -2 or -3) according to the submitting physicians. PRs >2 or <0.5 suggested distinguishing features for or against CNO, and a P value <0.05 was considered significant.

The core group met every other week for 24 weeks to consider PRs, discuss example cases or mimickers, further define criteria, and group them in domains and levels within a domain. Thirty ambiguous cases from the development cohort with low confidence levels by the submitting physician (ie, -1 to +1 scores) representing as many clinical features as possible were used by the core group to help establish hierarchical levels within each domain in preparation for multicriteria decision analysis (MCDA).

The CNO classification draft domains prepared by the core group were sent to the steering committee. Members were asked to either agree or disagree with each item and level based on the statistical analysis, clinical feasibility, and summary of discussions from the core group. Responses were reviewed at the first steering committee meeting.

Phase III. Criteria definition and weighting

Ten virtual consensus meetings of the steering committee were held between March 2021 and October 2023 to refine the draft prepared by the core group and assign preliminary weights using MCDA facilitated by 1000minds software (1000minds decision-making and conjoint analysis software, <https://www.1000minds.com>). The steering committee was presented with hypothetical pairs of cases contrasting 2 domains at a time (Supplement S3) and asked to decide individually and anonymously which case of the pair they were more likely to classify as CNO. Importantly voters were to assume the 2 cases were equal in all other features.

The participants then reviewed the tally of votes together. One hundred percent agreement was considered consensus without further discussion. Incomplete agreement prompted further discussion, moderated by a methodologist (AH), to achieve

consensus as to which case was more likely to be classified as CNO. Discussion was encouraged and facilitated until all members reached consensus. Complex choices in which consensus was not readily achieved were deferred for additional voting rounds. As a result of these forced choices between the alternative pairs, candidate items were weighted and ranked using 1000minds decision-making software.

Threshold identification

The draft weights derived from MCDA were applied to each case of the development cohort. A different set of 30 CNO cases and mimickers that covered the entire range of confidence from very likely to very unlikely CNO, based on the opinion of the submitting clinician, were selected by the statistician, and the case/mimicker status was confirmed by a 3-expert group (SO, AS, and YZ). The total scores for these 30 cases and mimickers were used to examine the performance of the draft weights and to determine a threshold for classification.

The submitting physician's diagnosis was considered the gold standard unless there was disagreement from the 3-expert group. This contrary opinion and its rationale were shared with the submitting physician and they were asked to review their diagnosis. The submitting physician's subsequent diagnosis after review of the expert commentary became the final diagnosis.

An initial threshold score for CNO was identified by examining the additive scores of all cases ranked from high to low. A receiver operating characteristic (ROC) curve analysis was conducted using the additive scores for all cases in the development cohort. This process identified an optimal threshold to distinguish CNO cases from mimickers.

Phase IV. Refinement and validation

The final criteria domains were examined by the steering committee based on the misclassified cases to ensure the clarity of the definitions for the differentially weighted classification system. The updated definitions with minimal change aimed to enhance the ease of application, sensitivity, and specificity of the classification system.

The final criteria were then applied to every CNO case or mimicker confirmed by adjudicators in the validation cohort to obtain their final score and their classification as CNO or not, relative to the agreed threshold. Adjudicators consisted of 5 rheumatologists who did not participate in the steering committee or submit any cases. They adjudicated 600 to 660 cases to ensure triple coverage of each case to verify the diagnosis of cases within the development cohort and exclude disagreed cases from the validation cohort. Sensitivity and specificity of the criteria were calculated and contrasted with the sensitivity and specificity of previously proposed diagnostic criteria [10,11]. Because the original criteria by Jansson et al [10] did not include MRI findings, we tested a version of the Jansson criteria modified by adding 'typical MRI findings' defined in the same way as for the new EULAR/ACR classification criteria.

Patient and public involvement

Caregivers of children with CNO and adults who were paediatric patients of CNO were involved in setting the research question and they were intimately involved in design and consensus meetings.

RESULTS

Phase I. Item generation

Overall, 259/865 (30%) of surveyed international paediatric rheumatologists responded and provided candidate items for CNO classification. Among responders, 132 (51%) practiced in North America, 77 (30%) in Europe, and 50 (19%) in other continents (South America 9, Asia 8, Australia/New Zealand 9, and other 14). A total of 138 (53%) responders had >10 years of practicing experience, and 108 (42%) had diagnosed and treated >10 CNO patients. The 33 items proposed in free-text survey responses were categorised into 6 domains (clinical presentation, physical examination, laboratory findings, imaging findings, bone biopsy, and treatment response). This is a smaller number of items than in recent criteria for other rheumatic disorders [24,27]. The following 8 items were ranked most important by survey respondents: (1) exclusion of malignancy and infection by bone biopsy, (2) multifocal bone lesions, (3) presence of bone pain or swelling, (4) signs of fibrosis or inflammation in bone biopsy, (5) bone lesions in typical locations (clavicle, metaphysis of long bones, mandible, vertebra), (6) presence of CNO-related comorbidities, (7) normal or mildly elevated C-reactive protein (CRP) or erythrocyte sedimentation rate (ESR), and (8) MRI findings ‘typical’ of CNO.

Phase II. Item reduction and definition

At the 2018 CARRA CNO workgroup meeting, 39 paediatric rheumatologists (35 from North America, 4 from Europe) and 7 parents of patients with CNO participated in item reduction. Twelve of the 33 items were thoroughly discussed, and the rest deferred to a postmeeting survey. Consensus was reached to exclude 8 items (antinuclear antibody, human lymphocyte antigen B27, lactate dehydrogenase [LDH], uric acid, and alkaline phosphatase [ALP] tests, fever, arthritis, and good clinical response to nonsteroidal anti-inflammatory drugs). LDH, low ALP, and fever were reconsidered in phase III. Physical examination findings were split into 3 items, and ESR and CRP values were expanded from 2 to 6 items to capture the range more accurately. Thus, the total number of items was only modestly reduced from 33 to 31.

In the postmeeting survey of all members of the CARRA CNO work group, 77/82 members (94%) scored the 31 items from +3 (increases the likelihood of CNO the most) to –3 (decreases the likelihood of CNO the most). Respondents included 62 paediatric rheumatologists, 9 paediatric rheumatology trainees, 2 adult rheumatologists, and 2 patient/parent representatives. Seventy percent of practitioners had >6 years of experience, and 34% managed >10 CNO patients every year. [Supplement S4](#) shows the average scores for the 31 items. The items with the highest scores were ‘multifocal lesions on imaging’, ‘ruling out malignancy and infection by biopsy’, and ‘typical location on imaging’. The item with the lowest scores (decreases likelihood of CNO the most) was ‘CRP and ESR >3 times upper limit of normal’. Results were presented to the steering committee at their first meeting.

CNO cases and mimickers

Demographic information for cases included in the development and validation cohorts is summarised in [Table 1](#). The diagnoses of mimicker cases are reported in [Table 2](#). Among 441 CRFs included in the development cohort, 264/285 (93%) CNO cases and 145/156 (93%) mimickers had moderate or greater confidence in the diagnosis reported by the submitting physician

and were used for calculation of PRs ([Supplement S5](#)). Adjudicators disagreed with the diagnosis of 2 CNO cases and 2 mimicker cases that were included in PR calculations. Data missing rates were also assessed. CNO cases more frequently had intermittent pain, clavicular swelling, symmetric bone lesions, and involvement of the thoracic spine, clavicle, sternum/manubrium, pelvic bones, bilateral femur, bilateral tibia, unilateral fibula, and foot bones. Mimickers more frequently had continuous pain, fever, active arthritis (examination findings or imaging findings of arthritis), or imaging findings suggesting infection or malignancy. Finally, complete and sustained response to antibiotic treatment was more frequent in mimickers.

Based on the calculated PRs and discussions of ambiguous cases by the core group (those cases with less than moderate confidence in the diagnosis as reported by the submitting physician, $n = 30$), candidate criteria were further reduced from 31 to 14, and the levels or options within each domain were defined. Findings from physical exams, disease durations, and pain patterns were among the 17 items removed after reviewing PRs, which were between 0.5 and 2. LDH, fever, and axial arthritis as part of coexisting conditions were added back to the criteria because of their PRs and incidence of >5% in the total population.

Phase III. Criteria definition and weighting

Phase IIIa. Criteria definition

Based on discussions by the steering committee, further refinements were made to the definitions for bone lesion pattern and distribution with consensus. The most specific bone sites were determined as the mandible and clavicle, and the least specific sites for CNO were the skull and hands. LDH levels were removed from the classification domains and ‘pathological LDH concerning for malignancy’ was added as exclusion criteria instead. Although an LDH >700 international units/L had a significant PR of <0.5 to distinguish CNO and mimickers, there was variability across laboratory reporting systems and a lack of expert agreement to establish a cutoff value. Platelet levels were also removed from the classification domains and ‘platelets <100,000/mm³’ was added as exclusion criteria instead due to concerns for malignancy. Family history of associated conditions, defined as reported axial arthritis, inflammatory bowel disease, psoriasis in a first-degree relative, and an ESR/platelet ratio ≥ 0.2 (relative thrombocytopenia during inflammation) were later removed due to insignificant weight relative to the other criteria. The remaining criteria were hierarchically organised, and definitions for the domains and their levels were further refined by the steering committee. The final 9 domains include sites of bone lesions and distribution patterns of these lesions based on imaging, bone biopsy pathological findings, age at the onset of symptoms, coexisting conditions, haemoglobin level, associated fever history, ESR, and CRP ([Table 3](#)).

The steering committee reached consensus on entry and exclusion criteria to be applied before scoring the 9 domains (5 clinical and 4 pathology/laboratory domains) ([Table 3](#)). Entry criteria are as follows: (1) aged <18 years at symptom onset, (2) presence of bone pain and/or functional musculoskeletal limitation for ≥ 6 weeks, and (3) abnormal radiographic or MRI imaging findings at nonarthritic bone sites. Nonarthritic bone sites refer to bones whose endings, if forming a joint, do not demonstrate signs of arthritis on physical examination or imaging (ie, no synovial thickening, contrast enhancement, or moderate to large joint effusions). Exclusion criteria are as follows: (1) confirmatory evidence of mutually exclusive mimicker diseases such as malignancy, infection, scurvy, or hypophosphatasia; (2)

Table 1
Demographic, clinical, and laboratory characteristics of subjects included in development and validation cohorts

Variables	Development cohort		Validation cohort	
	CNO (n = 285)	Mimickers (n = 156)	CNO (n = 382)	Mimickers (n = 132)
Age at onset (y)	9.5 (3.3)	8.6 (5.1)	9.2 (3.3)	7.3 (5.5)
Time to diagnosis (mo)	13.0 (21.1)	4.6 (8.0)	13.6 (20.5)	6.1 (17.3)
Male sex at birth	115 (40.4%)	96 (61.5%)	159 (42.1%)	84 (63.6%)
Geographic locations				
North America	141 (49.5%)	94 (60.3%)	216 (56.5%)	74 (56.1%)
South America	0 (0%)	0 (0%)	13 (3.4%)	0 (0%)
Europe	117 (41.1%)	59 (37.8%)	147 (38.5%)	55 (41.7%)
Asia	18 (6.3%)	1 (0.6%)	1 (0.3%)	1 (0.8%)
Africa	0 (0%)	0 (0%)	3 (0.8%)	1 (0.8%)
Oceania	9 (3.2%)	2 (1.3%)	0 (0%)	1 (0.8%)
Other	0 (0%)	0 (0%)	2 (0.5%)	0 (0%)
Race/ethnicity				
Asian	12 (4.2%)	8 (5.1%)	21 (5.5%)	6 (4.5%)
Black	5 (1.8%)	8 (5.1%)	9 (2.4%)	6 (4.5%)
Hispanic/Latino	6 (2.1%)	8 (5.1%)	9 (2.4%)	4 (3.0%)
Middle Eastern/North African	5 (1.8%)	4 (2.6%)	6 (1.6%)	2 (1.5%)
Native American	0 (0%)	4 (2.6%)	3 (0.8%)	1 (0.8%)
Native Hawaiian/Pacific Islander	0 (0%)	0 (0%)	0 (0%)	2 (1.5%)
White	246 (86.3%)	105 (67.3%)	310 (81.2%)	100 (75.8%)
Multiracial	5 (1.8%)	3 (1.9%)	10 (2.6%)	3 (2.3%)
Unknown	6 (2.1%)	16 (10.3%)	14 (3.7%)	8 (6.1%)
Bone biopsy results available	187 (65.6%)	93 (59.6%)	241 (63.1%)	114 (86.4%)
Haemoglobin (g/dL)	12.5 (11.8–13.2)	11.6 (10.3–13.0)	12.3 (11.4–13.2)	11.6 (10.0–12.9)
Platelets (10 ⁹ /L)	345 (293–414)	333 (255–427)	330 (275–395)	300 (253–395)
ESR (mm/h)	21 (11–44)	33 (15–73)	24 (10–43)	25 (10–47)
C-reactive protein (mg/L)	8 (3–16)	12 (6–44)	8 (5–15)	12 (3–44)

CNO, chronic nonbacterial osteomyelitis; ESR, erythrocyte sedimentation rate.

Variables are presented as mean (SD), median (IQR), or n (%). The development cohort included 3 CNO cases and 3 mimicker cases that adjudicators disagreed with submitting physicians. The validation cohort excluded 5 CNO cases that adjudicators disagreed with submitting physicians.

Table 2
Detailed distribution of mimicker cases within development and validation cohorts

Mimicker diagnosis	Development (n = 156)	Validation (n = 132)
Infection	49 (31%)	57 ^a (43%)
Malignancy	61 (39%)	61 (46%)
Inflammatory arthritis	21 (13%)	4 (3%)
Other mimickers	25 (16%)	10 (8%)
Vitamin C deficiency	10	2
Benign bone tumour	7	4
Hypophosphatasia	1	2
Amplified pain syndrome	2	0
Fracture	1	0
Hyperostosis hypophosphatemia	1	0
NEMO deficiency syndrome	1	0
Traumatic injury	1	0
Unicameral bone cyst	1	0
Other autoinflammatory disease	0	1
Vascular malformation	0	1

NEMO, nuclear factor kappa B essential modulator.

The development cohort included 1 case of inflammatory arthritis, 1 case of hyperostosis hypophosphatemia, and 1 case of amplified pain syndrome that adjudicators disagreed with submitting physician.

^a Includes 33 patients with bone tuberculosis.

pathological LDH concerning for malignancy; (3) platelets <100,000/mm³; and (4) complete and sustained clinical and laboratory response to antimicrobial treatment alone.

Phase IIIb. Criteria weighting

During 8 virtual consensus meetings of the steering committee, 59 MCDA pairwise trade-off decisions were made. Criteria weights were calculated by the 1000minds software based on

these decisions. The final list of criteria and their assigned weighted scores are shown in Table 3.

Phase IV. Refinement and validation

During the initial weighting, LDH, response to antibiotics, family history, and ESR/platelet ratio were included. LDH and complete response to antibiotics were moved to exclusion criteria due to the difficulty to define absolute threshold and >50% missing data. The latter 2 domains were removed due to low weight (1%–2%). The optimal threshold for CNO classification, based on Youden index of the ROC curve analysis in the development cohort (Supplement S6), was identified as 55 of a maximum of 100 when inclusion criteria and the composite domain score were applied. Cases that would have met exclusion criteria were kept in the pool for the exercise of threshold setting. There was a mixed cluster of CNO cases and mimicker cases in the development cohort with weighted scores between 52 and 54. These CNO cases did not have a bone biopsy, and it was probable that biopsy findings could have moved their weighted score above the threshold for classification. After discussion, the steering committee agreed that a patient should be classified as having CNO if their score was ≥55. This cutoff value corresponded to a sensitivity of 92% and a specificity of 99% in the development cohort.

The final CNO criteria were then applied to the validation cohort to determine their performance. Given that no previous CNO classification criteria exist, their performance was compared to proposed diagnostic criteria by Jansson et al [10] and Roderick et al [11]. The demographic information of the validation cohort (n = 382 CNO patients) is summarised in Table 1, and the distribution of mimickers (n = 132) is presented in

Table 3
EULAR/ACR classification criteria for paediatric chronic nonbacterial osteomyelitis

Step 1, Verify entry criteria (ALL should be present):	
1. Bone pain and/or musculoskeletal functional limitation ≥6 wk	
2. Age of onset <18 y	
3. Abnormal findings from radiograph and/or advanced imaging including MRI, CT, bone scintigraphy at nonarthritic bone sites ^a	
Step 2, Verify exclusion criteria (NONE should be present):	
1. Confirmatory evidence of mutually exclusive mimicker diseases ^b	
2. Platelet <100,000/mm ³	
3. Pathological LDH concerning for malignancy ^c	
4. Complete and sustained clinical and laboratory response to antimicrobial treatment alone	
Step 3, Add the scores: Add the highest value from each of the 9 domains below. A score of ≥55 is required to classify a patient as having CNO.	
Criteria domains / levels	Score
Bone biopsy^d	
• Signs of inflammation ^e AND fibrosis	17
• Signs of fibrosis only	11
• Signs of inflammation only	9
• No signs of inflammation or fibrosis	0
Age at the onset of symptoms	
• ≥3 y	17
• <3 y	0
Sites of bone lesions based on imaging	
• Clavicle and/or mandible	16
• Sites other than clavicle, mandible, skull, or hand	9
• Skull or hand without clavicle or mandible	0
Distribution pattern of bone lesions based on imaging	
• Multifocal lesions (in ≥2 bones) with symmetrical pattern (bilateral involvement of at least one bone)	9
• Multifocal without any symmetrical pattern of bone involvement	7
• Unifocal without whole-body imaging such as WBMRI, PET/CT performed	3
• Unifocal with whole-body imaging performed	0
Coexisting conditions prior to the diagnosis of CNO	
• Inflammatory bowel disease (IBD)	9
• Cutaneous condition ^f without IBD	8
• Axial arthritis ^g without cutaneous condition or IBD	5
• None	0
Haemoglobin (normal range varies by age)	
• ≥10 g/dL	8
• <10 g/dL	0
Fever (oral/temporal temperature above 38°C and not related to common infections)	
• Absence of fever	8
• Presence of fever	0
Erythrocyte sedimentation rate (normal range: 0-20 mm/h)	
• <60 mm/h	8
• ≥60 mm/h	0
C-reactive protein (normal range: 0-10 mg/L)	
• <30 mg/L	8
• ≥30 mg/L	0

ACR, American College of Rheumatology; CNO, chronic nonbacterial osteomyelitis; CT, computed tomography; EULAR, European Alliance of Associations for Rheumatology; LDH, lactate dehydrogenase; MRI, magnetic resonance imaging; PET, positron emission tomography; WBMRI, whole-body MRI.

Fever is defined as temperature >38°C without common infections including clinical symptoms of upper airway infection or urinary tract infection or abscess. It does not need to be present throughout 6 wk but any stage between the onset and the diagnosis.

^a Typical imaging findings include but are not limited to lytic, sclerotic lesions with or without periosteal reaction, hyperostosis on radiograph or CT, bone marrow oedema or hyperintensity in a fluid-sensitive sequence of MRI. Nonarthritic bone sites refer to bones whose joint-forming ends do not demonstrate imaging signs of arthritis including synovial thickening, enhancement, and/or moderate to large joint effusions. See Supplement S7.

^b Primary or metastatic malignancy in bone, leukaemia, lymphoma, neuroblastoma, infectious osteomyelitis, metabolic bone disease, vitamin C deficiency, hypophosphatasia, and monogenic bone diseases including Majeed or deficiency of interleukin-1 receptor antagonist (DIRA).

^c Due to differences in laboratory assays across institutions, no absolute threshold is defined. Levels ≥700 IU or >2 times the upper normal limit may be considered pathological.

^d Bone biopsy is not required before applying these criteria.

^e Defined as the presence of immune cells including neutrophils, monocytes, lymphocytes, and/or plasma cells.

^f Cutaneous conditions include psoriasis, palmoplantar pustulosis, pyoderma gangrenosum, acne fulminans, and hidradenitis suppurativa.

^g Axial arthritis is defined as imaging confirmation of inflammation within the sacroiliac joint or intervertebral joint.

Table 2. The new EULAR/ACR CNO classification criteria had a sensitivity of 82% (95% CI, 78-86) and specificity of 98% (95% CI, 94-100) in the validation cohort. Two false positive cases had benign bone tumours. Sensitivity compared favourably to the Roderick diagnostic criteria (63%) and similarly to the modified Jansson criteria (85%) while specificity was similar to the Roderick criteria and superior to the Jansson criteria (Table 4)

[10,11]. Since all 3 criteria include bone biopsy results, a bone biopsy showing evidence of infection or malignancy is considered an exclusion for all 3 criteria, ie, the case is not classified as CNO if such evidence is found. However, the other exclusions given in Table 3 are only applied to the EULAR/ACR classification criteria because they are not specified as exclusions in the other 2 criteria. In our dataset, confirmatory evidence of

Table 4
Sensitivity and specificity of EULAR/ACR classification criteria compared to previously published diagnostic criteria using validation cohort data

Criteria	Sensitivity	Specificity
EULAR/ACR Classification criteria	82% (78%–86%)	98% (94%–100%)
Modified Jansson diagnostic criteria ^a	85% (81%–89%)	80% (72%–87%)
Roderick diagnostic criteria ^b	63% (58%–68%)	95% (89%–98%)

ACR, American College of Rheumatology; EULAR, European Alliance of Associations for Rheumatology.

^a Jansson diagnostic criteria require 2 of 4 major criteria or 1 of 4 major criteria and 3 of 6 minor criteria to diagnose chronic nonbacterial osteomyelitis [10].

^b Roderick diagnostic criteria require the presence of typical clinical findings and typical radiological findings and 1 of 2 criteria depending on bone sites and the number of lesions as well as the level of C-reactive protein [11].

mutually exclusive mimicker diseases was based on vitamin C measurement, bone marrow aspiration biopsy, blood culture, and test for tuberculosis or atypical mycobacteria, in addition to bone biopsy, which was the source of such evidence in most mimicker cases. In the validation cohort, 88% of mimicker cases met one of the exclusion criteria in Table 3, including 69% with a bone biopsy showing evidence of infection or malignancy. That said, a bone biopsy is not required to apply the classification criteria, and approximately one-third of all CNO cases had no bone biopsy.

DISCUSSION

The EULAR/ACR endorsed classification criteria for paediatric CNO presented here represent a milestone in CNO care and research. Through a 4-phase, iterative process, an international collaborative group defined weighted criteria to classify CNO, distinguishing it from mimicker conditions. The excellent specificity (98%) and sensitivity (82%) of these criteria will help define homogenous CNO cohorts for research studies. A key strength of the criteria is that patients can be classified accurately as having CNO, even in the absence of bone biopsies. However, it is important to reiterate that classification criteria are not diagnostic criteria and that biopsies may be essential in certain settings to exclude differential diagnoses of CNO.

These EULAR/ACR paediatric CNO classification criteria were built on a robust and widely recognised multiphase methodological approach that defined entry and exclusion criteria. The absolute exclusion criteria cover important mimickers of paediatric CNO such as malignancy, infection, and metabolic bone disease. Scurvy and hypophosphatasia can be diagnosed via laboratory testing and may present with a clinical picture resembling CNO, and it is important for researchers to exclude these 2 conditions prior to classifying a patient as CNO. In prospective use of the criteria to select subjects for future studies, exclusion criteria are to be applied during case review before attempting to add criteria weights. If confirmatory evidence of an alternative diagnosis such as malignancy or vitamin C deficiency is already available, if the patient had received monotherapy with antibiotics that fully resolved the symptoms, or if thrombocytopenia or marked elevation of LDH are documented, the patient should not be classified as CNO. These exclusion criteria do not mean that every subject needs to have bone biopsies or have received full courses of antibiotics before they can be classified as CNO. If none of these is documented, then proceed to add criteria weights.

Some patients with a clinical diagnosis of paediatric CNO will not fulfil classification criteria. We focused classification criteria

development efforts on patients with ‘typical’ and ‘common’ manifestations with the goal of enrolling homogeneous populations into observational studies and clinical trials. As reported here, some patients with a diagnosis of CNO without bone biopsy did not accrue sufficient points to be classified as CNO. It is possible that if a biopsy was performed, those patients would have had sufficient points for classification. That said, in the hands of experienced clinicians, biopsy may not be necessary in all cases.

Differential weighting of criteria represents their relative contribution to an individual child’s classification as having CNO. For CNO, having a lesion at a typical site, such as the mandible and clavicle, in the absence of lesions at rarely affected sites, such as the skull and hands, carries the most weight. While the clavicle was proposed as a pathognomonic site by Roderick et al [11], based on the personal experience of steering committee members, mimicker diseases, such as Langerhans cell histiocytosis and primary bone malignancy, may also present with clavicular lesions [29]. For this reason, clavicular lesions were not chosen as a sufficient criterion to classify as CNO. Mandibular CNO lesions have been reported extensively by oral maxillofacial surgeons [30–37], and it is important to recognise their usefulness for classifying an individual as having CNO.

The pattern of bone lesions (unifocal, multifocal, symmetrical) is also very important for CNO classification. Unifocal lesions confirmed by whole-body imaging are not characteristic of CNO. If whole-body imaging was not performed, multifocal or symmetrical lesions may be missed. Bone biopsy findings are not sufficient by themselves for CNO classification. This may suggest consideration of obtaining WBMRI before deciding on a bone biopsy.

Several items that may be useful for recognising CNO in clinical practice, such as family history of associated diseases and ESR/platelet ratio, were not included in the criteria because they carried very low weight in the 1000minds exercise. Coexisting conditions such as inflammatory arthritis may pose a challenge in classifying CNO. Some children with inflammatory arthritis may subsequently develop CNO, and future longitudinal studies may help determine the prevalence of such occurrence by applying our CNO criteria.

Our study has several strengths. First, a large cohort of nearly 1000 patients with CNO or a mimicking condition was assembled by an international group of investigators. These criteria can be generalised to patients worldwide regardless of racial composition. Second, the experts involved in the consensus exercise and decision analysis (MCDA) also had international representation.

While it represents a significant step forward in planning randomised controlled trials and improving the quality of clinical and laboratory studies, this study also has limitations. First, the laboratory, imaging, and pathological findings were not measured or confirmed centrally, and detailed imaging findings were not available. Second, not all the cases had received all diagnostic tests considered within the inclusion criteria, including ESR, CRP, and haemoglobin. Therefore, the authors had to score ‘not available’ as ‘0’. Finally, ascorbic acid (vitamin C) deficiency, benign bone tumours, and hypophosphatasia are difficult to distinguish from CNO without applying exclusion criteria, which highlights the importance of obtaining appropriate history and laboratory results prior to applying classification criteria. The sensitivity and specificity results should be interpreted cautiously for 3 reasons. First, there is no true gold standard; the sensitivity and specificity presented reflect how well each of the classification rules agrees with the diagnosis given by the

physician who submitted the case and confirmed by independent adjudicators. Second, specificity may be different if different mimicking conditions are included; the results presented here apply to the mix of mimicker conditions in our data and may be different with a different mix. Third, patients who had incomplete data may have been misclassified.

CONCLUSION

These EULAR/ACR endorsed classification criteria for paediatric CNO are the first criteria set developed through international collaboration using large development and validation cohorts and rigorous methodology. The criteria had excellent specificity (98%) and good sensitivity (82%). These criteria are easy to apply in a paediatric population because clinical, laboratory, and imaging findings are incorporated into the classification without the absolute requirement for a bone biopsy. The criteria will help researchers to easily identify a homogeneous study population for future clinical and laboratory studies.

Competing interests

YZ: ACR, EULAR, CARRA (Grants to Institution, Finance Committee), Bristol-Myer Squibbs (Grants to Institution), Rheumatology Research Foundation (Grants to Institution), CTIP (Grants to Institution), UpToDate CRMO/CNO (Royalties/Licence), Novartis (Consulting Fees to Self), U.S. Provisional Application No. 63/356977 (Patent Issued), Seattle Children's CRMO Warriors Guild (Leadership/Fiduciary Role), American Board of Pediatrics, Pediatric Rheumatology Subboard (Leadership/Fiduciary Role). Melissa SO: CARRA (AF-Small Grants from 2022 to 2024, Total: \$48,399.00). AS: EULAR (Fellow with Funding of €15,000.00, Grants or Contracts). JDA: Novartis (Participation on Advisory Board 2023). MLB: Sjogren Foundation (Grants to Institution), Childhood Arthritis and Rheumatology Research Alliance (Grants to Institution). SKL: Time Hearst Foundation Physician Scientist Award (Grants), ACR (Support for Attending Meetings), Hackensack Meridian Health (Support for Attending Meetings), CARRA PFAPA/Autoinflammatory (Work Group Leader), APMC (Pediatrics Committee). TCL: Crohns & Colitis Congress Talk 2024 (Honoraria for Self, Support for Travel), Packard Children's Health Alliance Board of Directors (Board Member, Unpaid), Lupus Foundation of Northern California (Board Member, Unpaid). AL: National Institution of Health Award Number K23AR082966 (Grants to Institution). TSS: Invicor (Consulting Fees to Institution), Hippo Education (Payment to Self). GS: Sobi (Consulting Fees), Novartis (Consulting Fees), AbbVie (Consulting Fees, Payment/Honoraria). LBT: Cassie & Friends Society (Board Member, Unpaid). LFV: Trinds 2022-2025 (Contract for Trials, Payment to Self), Wolters Kluwer 2021 (Payment for Authorship to Self), Scholar Rock 2024 (Support for Travel). SC-YW: Arthritis Foundation Great West Region (Medical Advisory Board, Unpaid), Aicardi Goutieres Syndrome Advocacy Association (ECHO Faculty, Unpaid). KB: Czech Ministry of Health (DRO FNOL, 00098892, Support for the Manuscript). AMH: Seattle Children's Research Institute (Support for the Manuscript, Consulting Fees). FD: UpToDate (Royalties or Licences), ISSAID (Support for Attending Meetings in 2023, Education Committee Chair). CMH: Merck (Unrestricted Research Grant), Novartis (Unrestricted Research Grant Ended in 2022, Advisory Board on the Use of IL-1 blockers in CNO). RML: UpToDate (Royalties or Licences), Elsevier (Royalties or Licences), Sanofi (Consulting Fees to Institution, Participation on Board), Eli Lilly Canada (Consulting Fees to Self,

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This criteria set has been approved by the American College of Rheumatology (ACR) Board of Directors and the EULAR Executive Committee. This signifies that the criteria set has been quantitatively validated using patient data, and it has undergone validation based on an independent data set. All ACR/EULAR-approved criteria sets are expected to undergo intermittent updates.

Classification criteria are essential in clinical and basic science research because they allow investigators to study relatively homogeneous populations of patients recruited from a single or multiple research sites. In clinical settings, diagnoses are made by health care professionals evaluating an individual patient's symptoms, signs, and results of laboratory and imaging studies in order to guide therapeutic recommendations. Patients diagnosed with a particular disease may or may not fulfill classification criteria for that disease. Classification criteria, in the hands of an experienced clinician with expertise in rheumatology, may inform a diagnostic evaluation, but improperly applied classification criteria may lead to misdiagnosis.

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Contributors

YZ is the guarantor. YZ, PJF, CMH, RN, KCC, and SO designed the study. KCC conducted the statistical analysis. AMH led the 1000minds exercises. YZ, MO, and AS drafted the paper. YZ, MO, AS, EW, AFJ, AVR, AR, SMS, ACT, LFV, AMH, KCC, HJG, FD, CMH, RML, PJF, and SO contributed to the 1000minds expert meetings. LP designed artwork for CRF. RSI and TSS contributed to the design of radiology portion of CRF. SL

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Provenance and peer review

Not commissioned; externally peer reviewed

Data availability statement

The data that support the findings of this study are available from the corresponding author, YZ, upon reasonable request.

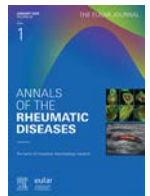
Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ard.2024.11.006.

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Rheumatoid arthritis

Systematic review and independent validation of genetic factors of radiographic outcome in rheumatoid arthritis identifies a genome-wide association with *CARD9*

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ABSTRACT

Objectives: This study aimed to investigate non-HLA genetic mechanisms underlying radiographic severity in rheumatoid arthritis (RA).

Methods: A systematic review of publications reporting non-HLA genetic associations with radiographic severity in RA across ancestries was undertaken. Experimental validation was performed in the Norfolk Arthritis Register, comprising 1407 patients with available genetic and treatment data followed prospectively for up to 10 years, with 2198 longitudinal radiographs. Genome-wide genotyping was performed with Illumina Human Core Exome Array. Radiographic outcomes (presence of erosions; Larsen score) were modelled longitudinally. Fine mapping and functional annotations to refine associations to potential causative loci were undertaken using FUMA, PolyPhen2, and RegulomeDB.

Results: The systematic review identified 102 publications reporting 139 independent associations with radiographic outcome. Association with 15 independent polymorphisms were replicated in the Norfolk Arthritis Register data set, implicating adaptive immune processes (Th1, Th2, and Th17 pathways), cytokine regulation, and osteoclast differentiation. Notably, we refined the association of rs59902911 at the *CARD9* locus to an intronic polymorphism within an active enhancer (rs78892335), achieving genome-wide significance and with an effect size exceeding the minimal clinically important difference for each copy of the minor allele (4.78

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Larsen units/copy; 95% CI, 3.15–6.41; $p = 9.01 \times 10^{-9}$). This polymorphism is associated with the expression of *CARD9* in immune cells, including B cells.

Conclusions: We provide a comprehensive list of validated genetic associations with RA outcome and demonstrate that non-*HLA* polymorphisms can associate with radiographic severity independently of disease susceptibility. This highlights the importance of dedicated genetic outcome studies for patient stratification in precision medicine for RA.

WHAT IS ALREADY KNOWN ON THIS TOPIC

- The genetic architecture of prognosis and outcomes in immune-mediated diseases remains poorly understood compared to that of susceptibility.

WHAT THIS STUDY ADDS

- We provide an extensive review of the literature on genetic associations with radiographic severity in rheumatoid arthritis (RA) summarising key findings including cohort details, effect sizes, and study designs, generating an important resource for future research.
- rs78892335 located within the *ENTR1* gene, is associated with radiographic outcome, reaching genome-wide significance. This single-nucleotide polymorphism regulates expression of *CARD9*, which plays a role in inflammatory cytokine production, and suggests pathways that could be key drivers of RA severity and a target for future therapies.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE, OR POLICY

- Genetic drivers of prognosis in RA can differ from those influencing disease susceptibility, particularly in non-*HLA* loci. Our findings emphasise the need to study RA progression pathways independently of susceptibility mechanisms.

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disorder characterised by flares of inflammation of synovial joints. This leads to pain and swelling and, if left uncontrolled, can result in permanent disability. Determinants of prognosis in RA and other immune-mediated diseases remain poorly understood.

In RA, patients may develop radiographic damage comprises characteristic erosive changes, periarticular osteopenia, and loss of joint space [1]. Some individuals exhibit a particularly aggressive, erosive phenotype [2]. There are some established predictors of radiographic severity, for example, anticyclic citrullinated peptide (anti-CCP) positivity [3]. Radiographic damage is the result of the cumulative burden of uncontrolled inflammation. However, only 32% of the total variance of joint destruction has been attributed to known risk factors, which is not enough in isolation to sufficiently predict clinical course [4]. The genetic contribution to joint destruction likely accounts for some of this with heritability estimates of ~50% [5].

Genome-wide association studies (GWAS) have focussed on identifying susceptibility markers, leaving the genetic architecture of prognosis relatively understudied. Approximately 150 loci have been associated with susceptibility to RA, with the *HLA* region accounting for the largest effect sizes [6–8]. In contrast, identification of robust genetic markers of prognosis has been hampered by several, significant challenges. These include confounders such as treatments, as well as lack of large, well-characterised cohorts with sufficient follow-up data available.

Despite these challenges, it is clear that at least some genetic mechanisms involved in susceptibility to RA, unsurprisingly,

overlap with those involved in disease severity. In particular, *HLA-DRB1* haplotypes as defined by amino acids at positions 11/13, 71, and 74 have been associated with susceptibility and radiographic severity, as well as all-cause and cardiovascular disease-related mortality [2,9]. Outside the *HLA* region, results are much more conflicting. Although there is a large body of literature reporting potential non-*HLA* associations with radiographic severity, most have been identified in small studies and have shown inconsistent results across cohorts, with small effect sizes. Only a minority have been replicated, including single-nucleotide polymorphisms (SNPs) near the *TRAF1* gene, a known susceptibility locus [10].

It is beginning to emerge that some loci which are associated with prognosis, are not associated with susceptibility [6]. A notable example is an SNP in the *FOXO3A* gene, where the minor allele of rs12212067 has been associated with lower radiographic severity of RA and lower severity of Crohn disease, but this locus is not associated with disease susceptibility for either condition [6,11]. The minor allele is associated with reduced inflammatory response secondary to increased monocyte production of interleukin (IL)-10 and reduced production of tumour necrosis factor (TNF) [6,11,12]. This implies that specific genetic factors, although not involved in the initiation of the disease process, could be involved in potentiation and maintenance of the inflammatory response once it has begun and has been noted in other complex diseases, such as multiple sclerosis [13].

In this study, we aimed to explore genetic mechanisms driving radiographic severity in RA, moving beyond the well-established association with the *HLA* region. By reviewing the existing literature and using a large, well-characterised inception cohort of patients, our goal was to identify non-*HLA* loci that warrant prioritisation for future research and clinical application.

PATIENTS AND METHODS

Identification of SNPs

A systematic literature review was performed by 1 reviewer (SDS) according to the updated PRISMA 2020 guideline [14] to identify all SNPs that have previously been reported to associate with radiographic outcome in RA ($P < .05$), including reports until November 12, 2022. A PRISMA checklist is available as a [Supplementary File](#). Methods of evaluation are included in [Supplementary Methods](#) including search strategy, PICOS (population, intervention, comparison, outcome, and study design) criteria and inclusion/exclusion criteria ([Supplementary Tables S1–S3](#)). Relevant information from each study was extracted ([Supplementary File S2, Tab 1](#)).

Patients and cohorts

The Norfolk Arthritis Register (NOAR) is an inception cohort of patients with inflammatory polyarthritis (IP) recruited since 1989 and followed prospectively for up to 20 years. IP was defined as the presence of 2 or more swollen joints for a

minimum of 4 weeks duration. The majority of patients (84%) were recruited within 2 years of disease onset. Patients with disease duration of over 5 years at time of registration were excluded. All patients were recruited after informed consent. Ethical approval was granted by the Norwich Research Committee (NOAR – REC Ref 2003/075, December 18, 2003, Norwich Local Research Committee [NHS]).

A case note review of patients in the NOAR cohort was undertaken, during which the subsequent clinical diagnosis made by a consultant rheumatologist at the last available follow-up was recorded. As the target population for the study was RA, patients with other confirmed diagnoses were excluded ($n=159$). A flowchart of patients illustrating patients retained for final analysis is depicted in [Supplementary Figure S1](#).

Radiographic outcome

During the first 10 years of follow up, radiographs of hands and feet were performed at regular intervals. Each radiograph was scored using the method by Larsen et al [15], a validated tool to determine radiographic severity of RA. In brief, a score from 0 to 5 is attributed to each small joint of the hands or feet, where 0 signifies no joint damage and 5 is complete destruction of the joint. A 2.0-mm or larger cortical break was labelled as an erosion and was assigned a score of 2 or greater. The sum of scores for each individual joint represents a score per patient radiograph, ranging from a Larsen score of 0 to 200. Each radiograph was scored in this way by 2 medically qualified staff, with a third investigator arbitrating in cases of disagreement.

Genotyping, quality control, and imputation

Patients from the NOAR cohort with available DNA and one set of hands and feet X-rays available at least at one follow-up time point were genotyped in 3 independent batches. Further details are available in [Supplementary Methods](#).

Statistical analysis

Radiographs were not performed systematically at every time point. Therefore, longitudinal models were used. Analysis was performed consistent with and published technical details [2].

Analysis was carried out on the cohort with genetic, treatment, and radiographic data available. *LDlink* (National Institutes of Health; <https://ldlink.nih.gov/>; the CEU European reference panel) was used to determine the linkage disequilibrium (LD) between chromosome 6 SNPs and rs660895, a SNP tagging HLA alleles associated with RA susceptibility and severity [16]. Those SNPs with evidence of LD (R^2 or D' > 0.4) with HLA susceptibility alleles were excluded from further analysis ([Supplementary Table S1](#)). SNPs were all tested for association with presence of erosions of the hands and feet as a binary variable using a generalised estimating equation model with logit-link function and an exchangeable within-subject correlation structure. We report results as odds ratios (ORs) with 95% confidence intervals (CIs).

Subsequently, association of SNPs with Larsen score was tested as a longitudinal variable. As this variable was nonnormally distributed, statistical association testing was performed by fitting a generalised linear latent and mixed model (GLLAMM) with discrete random effects and 3 latent classes [2,11]. Results are reported as β coefficients (representing change in Larsen score for every copy of the minor allele), with 95% CIs.

All generalised estimating equation and GLLAMM models were adjusted for age of onset, disease duration and the square of disease duration to model the nonlinear progression of radiographic damage over time in NOAR. Disease duration was calculated for each radiograph and each patient as the sum of disease duration at the time of recruitment plus the duration of follow up at the time of the radiograph. GLLAMM models were additionally adjusted for square of age of onset. The rationale for appropriateness of fitting a quadratic term in this cohort has previously been described [2]. All models were tested under an additive genetic model (SNPs coded as 0, 1 and 2 for the number of copies of the minor allele carried by each patient). Each individual treatment at recruitment and at every follow up, even when no radiographs were taken, has been recorded for each patient. A categorical dummy variable (presence/absence) of treatment with a corticosteroid was created, alongside an independent categorical variable for treatment with any disease-modifying antirheumatic drug (DMARD), for every follow up time point. Consequently, these variables capture the on/off effect of treatment longitudinally. All longitudinal models were adjusted for these 2 time-varying covariates to remove the effect of cumulative exposure to DMARD and corticosteroid treatments to time of radiograph.

Because this study was designed to replicate previously reported associations, 1-sided P values are presented, as we specifically tested for effects in the same direction as reported. Results are reported without correction for multiple testing. The Benjamini–Hochberg false discovery rate at 0.05 was applied to correct for multiple comparisons, and associations that remained significant after this adjustment are highlighted.

Other analysis

As many studies in the literature have previously used a dominant genetic model, results using this model are also reported in [Supplementary File S2](#). Stratified analysis was performed by anti-CCP status, and this is reported alongside analysis of the whole cohort ([Supplementary File S2, Tabs 2 and 3](#)). Models adjusted for anti-CCP status are presented in [Supplementary File S2, Tabs 4 and 5](#); association testing restricted to patients who met the ACR 1987 criteria in [Supplementary File S2, Tabs 6 and 7](#); and those who met the ACR 2010 criteria in [Supplementary File S2, Tabs 8 and 9](#). Mediation analysis was performed to determine the effect of anti-CCP status for SNPs associated with radiographic severity ([Supplementary File S2, Tab 10](#)).

Fine mapping analysis and functional annotation

For SNPs nominally associated in the NOAR data set ($P < .025$), association with radiographic severity with all SNPs within a 500-kb window of each of these SNPs was tested, and 95% credible single-nucleotide polymorphism sets (CSSs) were determined [17]. CSSs use a Bayesian approach to prioritise likely causative SNPs through assignment of probability, based on statistical evidence and LD. Plots of P values (2-sided) for regions of interests were generated in R using package *LocusZoomR* [18,19].

FUMA was used as a basis for functional mapping and annotation in order to explore the functional impact of potential causative genetic loci implicated in radiographic severity [20]. FUMA combines data from databases such as CADD (combined annotation–dependent deletion) alongside plots to identify likely causal SNPs. A high CADD score indicates a variant is

likely to have significant functional impact, based on integrated data from multiple sources such as evolutionary conservation, protein structure and gene regulation. Scores range from 1 to 99. RegulomeDB scores were obtained from the website. RegulomeDB provides information on the likelihood of a variant to affect gene regulation, where a higher score suggests a lower likelihood of regulatory impact [21–23]. Scores range from 1 (highly likely to have functional impact) to 7 (least likelihood of functional impact). Open Targets Genetics was also used to prioritise variants based on published information such as gene expression data [24]. Likely functional consequences of exonic SNPs were predicted using PolyPhen2 [25] and SIFT [26].

Gene ontology pathway analysis

Gene ontology (GO) pathway analysis was used to identify pathways enriched for genes associated with radiographic outcome in NOAR [27].

RESULTS

Literature review of previous non-HLA associations with radiographic severity

In total, 1162 records were identified from Medline and screened for eligibility. Figure 1 is a flowchart that summarises reasons for exclusion at each stage. A total of 102 articles fulfilled the inclusion criteria.

Figure 2

We identified 139 independent SNPs (European ancestry LD < 0.8) associated with radiographic outcome in RA from the literature (Table 1) [28–126]. Ancestry of the cohort is shown alongside the radiographic outcome used in the study (Supplementary File S2, Tab 1). Further information extracted from each individual article is shown in Supplementary File S2, Tab 1. Two TNF SNPs (rs1800629 and rs361525) were excluded from further analysis due to LD with the HLA (Supplementary Table S4) [127].

Cohort characteristics

Experimental validation was performed in the NOAR cohort (clinical characteristics in Table 2). There was 1407 patients with IP with at least 1 radiograph, genetic, and treatment data available. NOAR is an inception cohort; patients included in this study were recruited early in their disease course between 1989 and 2008. The majority of patients were not treated with any DMARD, and <4% were on biologic drug treatment.

In total, 144 SNPs were identified, 139 of which were independent (LD < 0.8). Gene names are those referenced in the original publication, which in most cases represents closest gene. For further information please, see Supplementary File S1, Tab 1. This includes information on the following: title, year, authors, journal, URL, ancestry, whether known susceptibility locus, chromosomal position, minor and major alleles and frequency in both NOAR cohort and in Europeans (dbVar), gene model used in original publication, sample size, radiographic outcome used, direction of effect, effect sizes, and cohort characteristics from original publication (percentage rheumatoid factor positive, anti-CCP positive, female, and mean age).

Associations with radiographic outcome

Of the 139 SNPs identified from the systematic review, 130 could be tested in NOAR (9 were not available in the imputed data set). Effect sizes for presence of erosions and Larsen score were heavily correlated ($P = 2.22 \times 10^{-16}$) (Supplementary Figure S18). There were 15 independent SNPs (~12%), which reached a P value of <.05, including 4 SNPs with $P < .005$ (Table 3). Of the 15 SNPs, 14 are not located in known susceptibility regions. One SNP (rs59902911) located in exon 5 of CARD9 retained statistical significance following correction for multiple testing. The minor allele (minor allele frequency [MAF] = 0.03) was associated with increased risk of erosions (OR, 1.66; 95% CI, 1.05-2.62; $P = 1.51 \times 10^{-2}$) and with increased Larsen score ($\beta = 4.65$; 95% CI, 2.87-6.42; $P = 1.46 \times 10^{-7}$). This effect size is equivalent to approximately

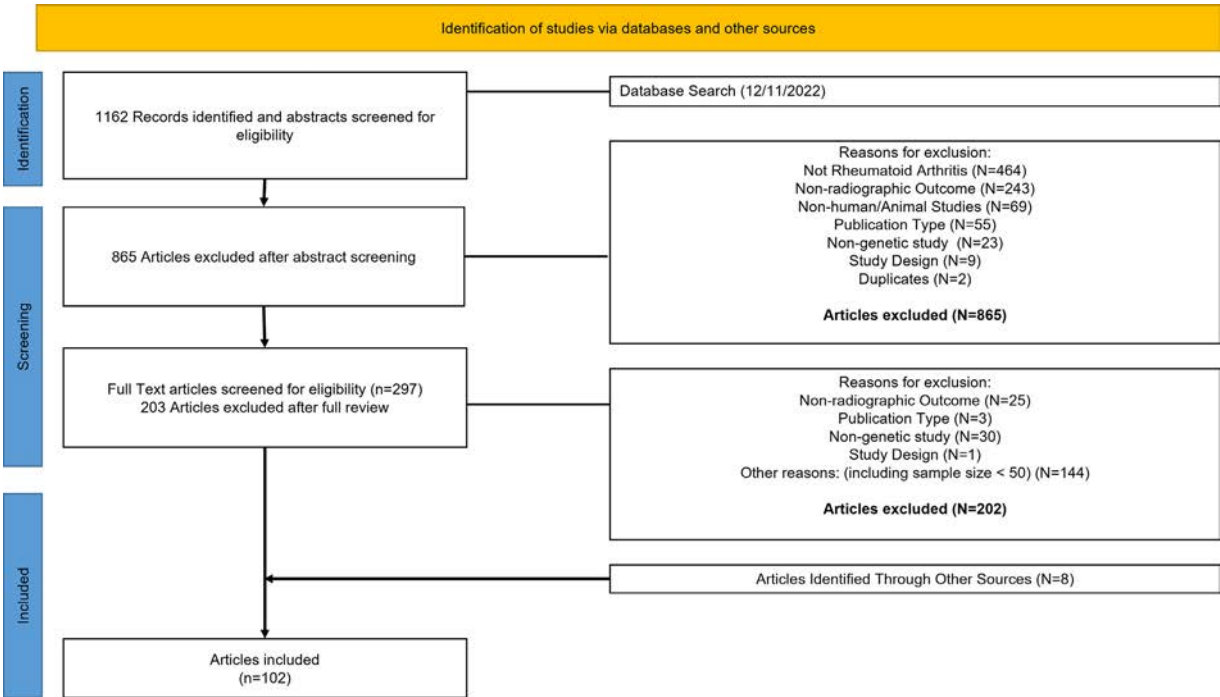


Figure 1. Flowchart of literature review, depicting literature review and reasons for exclusion.

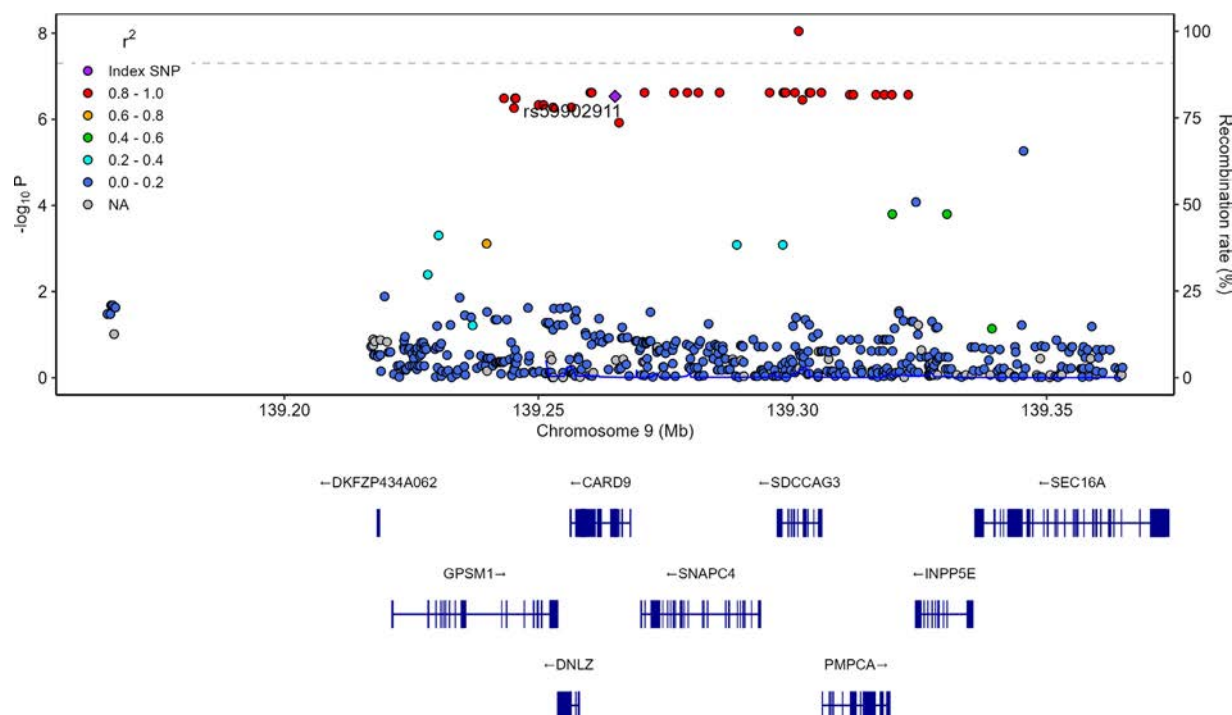


Figure 2. Fine mapping of association with radiographic outcome at the *CARD9* locus, with a LocusZoom plot generated using the LocusZoomR [18,19]. The index SNP, rs59902911, is highlighted in purple. The dotted line refers to genome-wide significance. Each dot represents a SNP, colour-coded for the linkage disequilibrium to the index SNP, and y axis represents the absolute value of the $\log_{10}$ of the *P* value for the association with Larsen score. Chromosomal positions are presented on the x axis, with the genetic structure of the region (genes, exons, introns, direction of transcription, and gene names). Note *ENTR1* gene is also known as *SDCCAG3*.

twice the minimal clinically important difference (2.3 units) for each copy of the minor allele [15]. Table 3 shows association results in NOAR for 13 additional SNPs from Table 1, which were previously replicated in at least 2 independent cohorts and were reported in 2 independent publications. These SNPs did not formally reach the threshold for significance in this study in NOAR.

Effect of anti-CCP-status on non-HLA associations with radiographic outcome

Genetic mechanisms underlying RA severity may differ by anti-CCP status. To explore this, stratified analyses were conducted by anti-CCP status (Supplementary File S2, Tabs 2 and 3). Additionally, some SNPs may influence severity indirectly by increasing the likelihood of anti-CCP positivity. Recognising this distinction is crucial for understanding the mechanisms driving these associations and assessing their clinical relevance. To address this, subanalysis was performed with adjustment for anti-CCP status. (Supplementary File S2, Tabs 4 and 5).

For SNPs associated with radiographic severity in the overall cohort (Table 3), mediation analysis (Supplementary File S2, Tab 10) was performed to determine whether these effects were independent of anti-CCP status. One SNP, rs10760130 (*TRAF1*), was associated with anti-CCP positivity, and the association with radiographic severity disappeared after adjusting for anti-CCP status. This suggests that this SNP may influence radiographic severity primarily through their effect on anti-CCP positivity.

Of those associated with radiographic severity in overall cohort, 2 SNPs – rs6887695 (*IL12β*) and rs59902911 (*CARD9*) – showed greater effect sizes in anti-CCP-positive patients. However, CIs between anti-CCP-positive and anti-CCP-negative patients overlapped for rs6887695. In the case of

rs59902911, the minor allele (T) of rs59902911 was significantly associated with higher Larsen scores in anti-CCP-positive patients (7.94; 95% CI, 4.00–11.88; $P = 3.9 \times 10^{-5}$) but showed no significant association in anti-CCP-negative patients (1.21; 95% CI, −0.77 to 3.20; $P = 0.12$). This suggests that this SNP may exert a more pronounced effect on radiographic severity in the context of anti-CCP-related mechanisms, potentially interacting with pathways specific to anti-CCP-positive RA.

Fine mapping analysis

For radiographic severity SNPs previously identified in the literature, which replicated in NOAR (Table 3), fine mapping analysis was performed to identify potential causal SNPs within the genomic vicinity (for those SNPs $P < .025$ in NOAR). Visual results are depicted using LocusZoom plots (Figure 2 and Supplementary Figures S2–S12).

CSS analysis was used to prioritise likely causative SNPs. For the majority of 10 regions explored, CSSs were broad, which is reflective of small effect sizes in outcome studies. However, for rs1805011 (*IL4R*) and rs506746 (*NALCN/ITGBL1*), the CSSs were unusually small containing only 1 SNP and 3 SNPs, respectively. These SNPs were, however, in low LD to rs1805011 ($R^2 = 0.0003$) and rs506746 ($R^2 = 0.0001$). The absence of these candidate SNPs from the 95% CSS suggests limited evidence supporting their direct causal role within this analysis framework.

Based on the results of their statistical associations, LD patterns, and potential functional relevance, FUMA analysis was applied to rs2243250 (*IL4*), rs6887695 (*IL12B*), and rs59902911 (*CARD9*). Results are shown in Supplementary File S2, Tabs 11–13, respectively. LocusZoom plots representing SNPs within a 500-kb window or an LD window ($R^2 > 0.6$),

Table 1
A summary of published genetic associations with radiographic outcome in RA identified from literature review (until November 2022)

CHR	Gene	SNP	Reference
1	<i>FCGR2B</i>	rs1050501	[28,29]
1	<i>CRP</i>	rs2808630	[30]
1	<i>Cyclooxygenase-2</i>	rs20417	[31]
1	<i>FCRL3</i>	rs7528684	[32–35]
1	<i>PARP1</i>	rs1805413	[36]
1	<i>IL10</i>	rs1800872	[37]
1	<i>IL10</i>	rs1800896	[37–39]
1	<i>FcγR3A</i>	rs396991	[40–42]
1	<i>IL10</i>	rs6703630	[43]
1	<i>IL6R</i>	rs4845374	[44]
1	<i>IL6R</i>	rs4845618	[44]
1	<i>KIAA1107/C1orf146</i>	rs80105455	[45]
1	<i>PADI2</i>	rs1005753	[46]
1	<i>PADI4</i>	rs1748033	[47]
1	<i>PADI4</i>	rs2240340	[48,49]
1	<i>PCSK9</i>	rs644000	[45]
1	<i>PTPN22</i>	rs2476601	[10,37,50,51]
1	<i>TNFRSF9</i>	rs228702	[52]
2	<i>AFF3</i>	rs11676922	[53]
2	<i>CTLA4</i>	rs73055463	[52]
2	<i>CYP1B1</i>	rs10012	[40]
2	<i>IL1A</i>	rs3783550	[54]
2	<i>IL1B</i>	rs1143634	[55]
2	<i>IL1B</i>	rs16944	[56]
2	<i>IL-1 RN</i>	rs419598	[57,58]
2	<i>RASGRP3</i>	rs13014054	[52]
2	<i>STAT4</i>	rs7574865	[59,60]
2	<i>SPAG16</i>	rs7607479	[61]
3	<i>CCR5</i>	rs1799987	[62]
3	<i>GHRL</i>	rs696217	[63]
3	<i>OGG1</i>	rs3219008	[64,65]
4	<i>HMGB1P28/TMEM33</i>	rs7655001	[45]
4	<i>IL15</i>	rs4371699/rs6821171/ rs7665842/rs7667746	[66]
4	<i>SPP1</i>	rs4754/rs9138	[67]
4	<i>TLR10</i>	rs11466657	[68]
5	<i>C5orf30</i>	rs26232	[69]
5	<i>CAPSL</i>	rs13157282/rs7445819	[45]
5	<i>IL-12B</i>	rs6887695	[70]
5	<i>IL13</i>	rs1800925	[71]
5	<i>IL4R</i>	rs2243250	[72]
5	<i>LOC100132524/RAI14</i>	rs10043548/rs10058554	[45]
5	<i>MiRNA-146a</i>	rs2910164	[73]
5	<i>PTGER</i>	rs6896969/rs76523431	[74]
5	<i>SLC22A4</i>	rs2073838	[75]
6	<i>6q23</i>	rs13207033	[76]
6	<i>CDKAL1</i>	rs981042	[45]
6	<i>ESR1</i>	rs1801132	[40]
6	<i>TNFAIP3-OLIG3</i>	rs675520	[77]
6	<i>FOXO3</i>	rs12212067	[11,12,78]
6	<i>IL17</i>	rs3804513	[79]
6	<i>MnSOD</i>	rs4880	[80]
6	<i>PSORS1C1</i>	rs2233945	[81]
6	<i>TAGAP</i>	rs394581	[10]
6	<i>THEMIS/PTPRK</i>	rs77843069	[45]
6	<i>TNF</i>	rs1800610	[82]
6	<i>TNF</i>	rs1800629	[83–88]
6	<i>TNF</i>	rs361525	[89]
6	<i>TNFAIP3</i>	rs6920220	[90]
6	<i>TNFAIP3</i>	rs9376293	[91]
7	<i>CDK6</i>	rs42041	[92]
7	<i>CYP3A4</i>	rs2740574	[40]
7	<i>IL6</i>	rs1800795	[37,93]
7	<i>IRF5</i>	rs2004640	[94]
8	<i>BLK</i>	rs13277113	[53]
8	<i>OPG</i>	rs1485305	[95]
9	<i>CARD9</i>	rs59902911	[45]
9	<i>MOB3B</i>	rs774351	[45]
9	<i>TRAF1-C5</i>	rs7034499	[52]
9	<i>TRAF1</i>	rs10760130	[96]

(continued)

Table 1 (Continued)

CHR	Gene	SNP	Reference
9	<i>TRAF1</i>	rs10818488 ^a	[60,97]
9	<i>TRAF1</i>	rs2900180	[10,96,98]
10	<i>CYP2C9</i>	rs1057910/rs1799853	[40]
10	<i>DKK1</i>	rs10762715/rs1194750/ rs1441124/rs1528873/ rs1896367/rs1896368	[99]
10	<i>DKK1</i>	rs1896367	[99,100]
10	<i>IL2RA</i>	rs2104286	[53]
10	<i>IL2RA</i>	rs7077067/rs2104286	[52]
10	<i>MBL</i>	rs1800450	[101]
10	<i>SDF-1</i>	rs1801157	[102]
11	<i>MMP3</i>	rs3025082	[103]
11	<i>ETS1</i>	rs4362159/rs7108537	[52]
11	<i>IL18</i>	rs1946518	[104]
11	<i>LRP5</i>	rs3736228/rs2306862/ rs4988321	[105]
11	<i>MMP12</i>	rs2276109	[106]
11	<i>MMP13</i>	rs2252070	[106]
11	<i>MMP7</i>	rs11568818	[106]
11	<i>TMEM80</i>	rs7101785 ^b	[52]
12	<i>KIF5A</i>	rs775241	[10]
12	<i>NK10</i>	rs2617169	[107]
12	<i>NK9</i>	rs2246809 ^c	[107]
13	<i>NALCN/ITGBL1</i>	rs506746	[52]
13	<i>RANKL</i>	rs2277438	[79,108]
14	<i>ESR2</i>	rs1271572	[40]
14	<i>Granzyme B</i>	rs12433772/rs8192916	[109]
14	<i>RAD51B</i>	rs911263	[110]
14	<i>ZFP36L1 / C14orf181</i>	rs189866/rs451066 ^d	[111]
16	<i>IL4R</i>	rs1805010	[112–114]
16	<i>IL4R</i>	rs1119132/rs1801275	[115]
16	<i>IL4R</i>	rs1805011/rs1805015/ rs4787423/rs6498016/ rs7191188	[114]
17	<i>LGALS9</i>	rs3763959	[116]
17	<i>MAP3K14</i>	rs7216796	[45]
17	<i>p53</i>	rs1042522	[117]
17	<i>SHGB</i>	rs6259	[40]
17	<i>SOST</i>	rs4792909/rs6503475	[99]
18	<i>PTPN2</i>	rs2542151	[81]
19	<i>TGF-β1</i>	rs1982073	[118–120]
19	<i>LILRA3</i>	rs103294	[121]
19	<i>TGF-β1</i>	rs1800469	[122]
19	<i>XRCC1</i>	rs25487/rs25489	[64]
20	<i>CD40</i>	rs4810485	[92]
20	<i>DNMT3B</i>	rs6087990	[123]
20	<i>MiRNA-499</i>	rs3746444	[47]
20	<i>MMP9</i>	rs11908352	[111]
21	<i>HLCS</i>	rs6517385	[45]
21	<i>HUNK/SCAF4</i>	rs2833522	[124]
22	<i>MIF</i>	rs755622	[125]
22	<i>NF2</i>	rs2158619	[45]
22	<i>NIPSNAP1</i>	rs62227968 ^e	[45]
X	<i>IRAK</i>	rs1059703	[126]

CHR, chromosome; LD, linkage disequilibrium; SNP, single-nucleotide polymorphism.

^a Variant in LD with rs10760130 ($R^2 = 0.972$).

^b Variant in LD with rs4362159 ($R^2 = 0.9902$).

^c Variant in LD with rs2617169 ($R^2 = 1.0$).

^d Variant in LD with rs189866 ($R^2 = 1.0$).

^e Variant in LD with rs2158619 ($R^2 = 0.8574$).

alongside CADD scores/RegulomeDB scores, are shown in [Supplementary Figures S13 to S17](#).

In the region of rs2243250 (*IL4*), 623 SNPs were in the 95% CSS with 35 in LD ($R^2 > 0.7$) with rs2243250 ([Supplementary File S2, Tab 11](#)) These SNPs spanned the *IL4-KIF3A* region. One SNP was exonic (*KIF3A*) but functional prediction by SIFT suggested this is well tolerated. Many SNPs in this region [20] have strong evidence for involvement in regulatory function (RegulomeDB scores 1a-1f).

Table 2
Cohort characteristics for the Norfolk Arthritis Register (NOAR)

	Total	Baseline ^a	Year 1 ^b	Year 2	Year 5	Year 10
No. of patients with available genotype, radiographic outcome, and recorded treatment	1407 ^c	415	802	252	550	144
Total No. of radiographs included in the study	2198					
Recruitment period	From 1989 to 2008					
Satisfy 1987 ACR criteria for RA during follow up, n/N (%)	1044/1407 (74)					
Satisfy 2010 ACR criteria for RA during follow up, n/N (%)	1112/1407 (79)					
Sex (male/female), n (%)	455/952 (32/68)					
Age at disease onset (y), median (IQR), % missing	56 (45;66), 0					
Disease duration at baseline (mo), median (IQR), % missing	6.2 (3.0;12.3), 0 ^d					
Ever tested positive (anti-CCP), n/N (%)	495/1311 (38)					
Ever tested positive (RF), n/N (%)	740/1402 (53)					
Erosive disease, n/N (%) ^e		203/415 (49)	351/811 (43)	88/263 (34)	241/550 (44)	136/146 (93)
Larsen score, median (IQR) ^f		4 (0-15)	4 (0-14)	2 (0-14)	5 (0-22)	36.5 (17-60)
Treatment, n/N (%)						
On steroids		158/1407 (11)	197/1325 (15)	147/1132 (13)	136/1077 (13)	71/632 (11)
On any DMARD or biologic treatment (excluding steroids)		447/1407 (32)	644/1325 (49)	564/1132 (50)	522/1077 (49)	300/632 (47)
Breakdown (DMARD or biologics) ^g , n/N (%)						
Methotrexate		229/1407 (16)	359/1325 (27)	317/1132 (28)	332/1077 (31)	193/632 (31)
Sulphasalazine		188/1407 (13)	247/1325 (19)	210/1132 (19)	160/1077 (15)	87/632 (14)
Chloroquine/hydroxychloroquine		29/1407 (2)	62/1325 (5)	67/1132 (6)	77/1077 (7)	48/632 (8)
Intramuscular gold		9/1407 (1)	20/1325 (2)	19/1132 (2)	10/1077 (1)	6/632 (1)
Cyclosporine		3/1407 (0)	7/1325 (1)	5/1132 (0)	10/1077 (1)	6/632 (1)
Penicillamine		3/1407 (0)	3/1325 (0)	4/1132 (0)	5/1077 (0)	2/632 (0)
Azathioprine		2/1407 (0)	3/1325 (0)	5/1132 (0)	5/1077 (0)	4/632 (1)
Etanercept		2/1407 (0)	6/1325 (0)	11/1132 (1)	14/1077 (1)	9/632 (1)
Auranofin		1/1407 (0)	1/1325 (0)	1/1132 (0)	0/1077 (0)	0/632 (0)
Adalimumab		1/1407 (0)	2/1325 (0)	9/1132 (1)	14/1077 (1)	12/632 (2)
Cyclophosphamide		0/1407 (0)	0/1325 (0)	0/1132 (0)	0/1077 (0)	0/632 (0)
Leflunomide		0/1407 (0)	6/1325 (0)	9/1132 (1)	16/1077 (1)	16/632 (3)
Mycophenolate mofetil		0/1407 (0)	0/1325 (0)	0/1132 (0)	0/1077 (0)	0/632 (0)
Anakinra		0/1407 (0)	0/1325 (0)	0/1132 (0)	0/1077 (0)	0/632 (0)
Infliximab		0/1407 (0)	1/1325 (0)	3/1132 (0)	4/1077 (0)	7/632 (1)
Rituximab		0/1407 (0)	0/1325 (0)	0/1132 (0)	2/1077 (0)	2/632 (0)
Other biologic drugs		0/1407 (0)	0/1325 (0)	0/1132 (0)	0/1077 (0)	3/632 (0)
Other (including drug trials)		2/1407 (0)	5/1325 (0)	6/1132 (1)	3/1077 (0)	1/632 (0)

ACR, American College of Rheumatology; DMARD, disease-modifying antirheumatic drug; RA, rheumatoid arthritis; RF, rheumatoid factor.

^a At recruitment.

^b This table shows selected time points. Patients were followed up every year during the first 5 y, and every 2 y between year 5 and 10.

^c At least at 1 time point with available radiographs and 1 time point with available treatment information during the first 10 y of follow-up. N over follow-ups do not add up to 1407, as not all patients had a radiographic assessment at the same time.

^d 22 patients of 1407 presented in this table had a disease duration of >5 y at recruitment and were excluded from the genetic association study.

^e Erosive disease was defined by the presence of at least 1 erosive joint (Larsen score ≥ 2 ; cortical break >2.0 mm).

^f Larsen score ranges from 0 to 200 where higher scores indicate a greater degree of joint damage [15].

^g Sorted by frequency at baseline.

LocusZoom (Supplementary Figure S5) showed potentially 2 distinct LD blocks associated with Larsen score in the region of rs6887695 (IL12 β); 862 SNPs were identified in the 95% CSS. Seventeen SNPs that were in LD ($R^2 > 0.6$) with rs6887695 and, in the second LD block, 37 SNPs, with LD ($R^2 > 0.6$), were given in reference to rs55840985. There were no exonic SNPs in either block. In the first LD block containing rs6887695, 6 SNPs have RegulomeDB scores (1a-1f), suggesting high likelihood of involvement in regulatory function, and 5 SNPs were GWAS hits for susceptibility to number of autoimmune diseases including psoriasis, Takayasu arteritis, and inflammatory bowel disease.

***R*s59902911, *CARD9* region**

R_s59902911 had 30 SNPs in the 95% CSS (Supplementary File S2, Tab 13) and the lead variant rs78892335 ($R^2 = .903$ with the index SNP rs59902911) reached genome-wide significance for its association with Larsen score ($\beta = 4.78$; 95% CI,

3.15-6.41; $P = 9.01 \times 10^{-9}$) (Supplementary File S2, Tab 13). This SNP was not directly genotyped, MAF was 3.4%, and only 1 patient was homozygous for the minor allele (AA). However, it imputed well ($R^2 = 0.949$), and there was low missingness in the data set (average call rate, 0.998), increasing confidence that this association was not the result of a technical artefact. Figure 3 shows the median Larsen score by genotype over follow-up (years). This intronic SNP is located within the *ENTR1* (also known as *SDCCAG3*) gene in an active enhancer and promoter region in B lymphocytes (RegulomeDB) [22]. Open Targets Genetics show this SNP is an expression quantitative trait loci for *CARD9* (minor allele increases expression) in B cells ($P = 2.8 \times 10^{-9}$) [128], CD4⁺ T cells ($P = 1.7 \times 10^{-60}$) [129], CD8⁺ T cells ($P = 3.3 \times 10^{-54}$) [129], monocytes ($P = 5.5 \times 10^{-65}$) [130] and neutrophils ($P = 1.5 \times 10^{-13}$) [131–133]. Figure 4 illustrates how this SNP may be implicated in RA prognosis [134,135].

There were 6 exonic SNPs within >0.6 LD of rs59902911, 3 of which were nonsynonymous (Table 4). One of these SNPs

Table 3
List of validated genetic associations with radiographic severity in rheumatoid arthritis

Chr	Position	Gene	SNP (rsID)	A1	A2	MAF	Erosions	Larsen score			
								OR (95% CI)	P	β coefficient (95% CI)	P
Previous non-HLA associations replicated in NOAR ^a											
2	113129630	IL1RN	rs419598 ^b	C	T	0.27	0.99 (0.84, 1.17)	4.63 × e ⁻¹	-0.64 (-1.32, 0.04) ^c	3.32 × e ⁻²	
4	141776015	IL15	rs6821171	G	T	0.27	0.86 (0.73, 1.02) ^c	4.42 × e ⁻²	-0.03 (-0.76, 0.71)	4.73 × e ⁻¹	
5	114305447	KCNN2	rs11958855	A	G	0.23	1.12 (0.94, 1.34)	1.03 × e ⁻¹	1.14 (0.38, 1.90)	1.50 × e ⁻³	
5	132673462	IL4	rs2243250	T	C	0.13	1.42 (1.14, 1.78)	1.01 × e ⁻³	1.24 (0.32, 2.17)	4.26 × e ⁻³	
5	159395637	IL12B	rs6887695	C	G	0.32	0.97 (0.82, 1.14)	3.45 × e ⁻¹	-0.72 (-1.37, -0.07)	1.48 × e ⁻²	
6	159061489	FCGR3A	rs394581 ^d	C	T	0.28	1.11(0.93, 1.31)	1.21 × e ⁻¹	0.65 (-0.03, 1.32) ^c	2.98 × e ⁻²	
6	108659993	FOXO3	rs12212067 ^d	G	T	0.09	0.78 (0.61, 1.01) ^c	2.94 × e ⁻²	-0.70 (-1.80, 0.40)	1.06 × e ⁻¹	
6	159692840	SOD2	rs4880 ^d	A	G	0.49	1.16 (1.00, 1.35)	2.73 × e ⁻²	0.86 (0.20, 1.52)	5.30 × e ⁻³	
9	120939712	TRAF1	rs10760130 ^e	G	A	0.46	1.17 (1.00, 1.36)	2.21 × e ⁻²	0.12 (-0.50, 0.74)	3.51 × e ⁻¹	
9	120942809	TRAF1/C5-OT1	rs10818488 ^e	A	G	0.46	1.18 (1.01, 1.37)	1.90 × e ⁻²	0.18 (-0.44, 0.81)	2.84 × e ⁻¹	
9	120944104	TRAF1/C5-OT1	rs2900180 ^f	T	C	0.38	1.16 (0.99, 1.35)	3.17 × e ⁻²	0.18 (-0.46, 0.82)	2.93 × e ⁻¹	
9	136370636	CARD9	rs59902911 ^g	T	C	0.03	1.66 (1.05, 2.62)	1.51 × e ⁻²	4.65 (2.87, 6.42)	1.46 × e ⁻⁷	
10	52309426	PARP1	rs1896367	T	C	0.41	1.15 (0.99, 1.34) ^c	3.76 × e ⁻²	0.23 (-0.40, 0.86)	2.35 × e ⁻¹	
11	112164735	IL18	rs1946518	T	G	0.40	0.92 (0.79, 1.08)	1.54 × e ⁻¹	-0.60 (-1.24, 0.04) ^c	3.30 × e ⁻²	
13	101329420	NALCN/ITGBL1	rs506746	T	C	0.15	0.82 (0.67, 1.02) ^c	3.52 × e ⁻²	-1.11 (-1.99, -0.23)	6.65 × e ⁻³	
16	27362551	IL4R	rs1805011 ^h	C	A	0.12	1.26 (0.99, 1.59) ^c	2.82 × e ⁻²	1.25 (0.25, 2.25)	7.10 × e ⁻³	
19	54293995	LILRA3	rs103294	T	C	0.21	0.83 (0.69, 1.00)	2.71 × e ⁻²	-0.39 (-1.19, 0.41)	1.70 × e ⁻¹	
Previously replicated non-HLA associations not replicating in NOAR ⁱ											
1		FCGR2B	rs1050501				Not available in the imputed NOAR data set				
1	157701026	FCRL3	rs7528684	G	A	0.46	1.10 (0.94, 1.28)	1.13 × e ⁻¹	-0.44 (-1.10, 0.22)	9.48 × e ⁻²	
1	206773552	IL10	rs1800896	T	C	0.49	0.92 (0.79, 1.06)	1.16 × e ⁻¹	-0.43 (-1.04, 0.17)	8.09 × e ⁻²	
1		FcγR3A	rs396991				Not available in the imputed NOAR data set				
1	17336144	PADI4	rs2240340	T	C	0.41	0.90 (0.77, 1.05)	9.36 × e ⁻²	0.06 (-0.58, 0.70)	4.27 × e ⁻¹	
1	113834946	PTPN22	rs2476601	A	G	0.12	1.04 (0.83, 1.32)	3.57 × e ⁻¹	0.23 (-0.71, 1.16)	3.19 × e ⁻¹	
2	191099907	STAT4	rs7574865	T	G	0.22	1.15 (0.96, 1.38)	6.47 × e ⁻²	0.01 (-0.74, 0.76)	4.92 × e ⁻¹	
3	9753859	OGG1	rs3219008	G	A	0.22	0.96 (0.80, 1.15)	3.22 × e ⁻¹	-0.12 (-0.88, 0.64)	3.81 × e ⁻¹	
7	22727026	IL6	rs1800795	C	G	0.44	1.06 (0.91, 1.23)	2.40 × e ⁻¹	-0.05 (-0.70, 0.61)	4.44 × e ⁻¹	
10	52309426	DKK1	rs1896367 ^j	T	C	0.41	1.15 (0.99, 1.34)	9.62 × e ⁻¹	0.23 (-0.40, 0.86)	2.34 × e ⁻¹	
13	42581032	RANKL/TNFSF11	rs2277438	G	A	0.17	1.00 (0.82, 1.22)	4.97 × e ⁻¹	-0.13 (-0.96, 0.70)	3.78 × e ⁻¹	
16	27344882	IL4R	rs1805010	G	A	0.45	1.12 (0.97, 1.31)	6.58 × e ⁻²	-0.26 (-0.86, 0.34)	1.94 × e ⁻¹	
19		TGFβ1	rs1982073				Not available in the imputed NOAR data set.				

Chr, chromosome; MAF, minor allele frequency; OR odds ratio.

^a Results for SNPs from Table 1 reaching a *P* of <.05 for their association with either erosions or Larsen score models in NOAR. We report uncorrected 1-sided *P* values to test for associations in a predefined direction based on previous evidence. Full results are available in Supplementary File S2, Tabs 2 and 3. Chromosome position is given in reference to Build38. A1 refers to the minor allele and A2 refers to the major allele. All reported effect sizes are given with reference to the minor allele. Effect sizes for erosions are given as odds ratios and effect sizes for Larsen score are given as β coefficients. All results are reported with 95% CIs.

ⁱ Results for SNPs from Table 1, which did not replicate in this study but did replicate in at least 2 independent cohorts and reported in at least 2 independent publications. Note that other criteria could be applied to the list of SNPs in Table 1 to extract additional associations likely to represent true positive associations (eg, replication in multiple cohorts within 1 single publication or the presence of additional functional data, like correlation with expression or functional *in vitro* data).

^b The C allele was previously found to be protective in a previous publication in keeping with NOAR [58]. However, another publication showed an opposite direction of effect [57].

^d Chromosome 6 SNPs were checked for linkage disequilibrium (LD) with the HLA, via tagging SNP rs660895: (Supplementary Table S4), and none of the above were found to be in LD with the HLA. All were also tested with the shared epitope in the final models, which in most cases did not substantially alter the results. However, rs394581 lost significance, although this was borderline and may be secondary to power.

^e In LD (*R*² > 0.7) with a known susceptibility SNP and with each other (*R*² = 0.97), therefore not representing an independent association.

^g Remained significant after correction for multiple testing in Larsen score model (Benjamin–Hochberg correction, 0.05 false discovery rate).

^h In the original publication, this SNP has a protective effect in Leiden EAC but was a risk allele in other 3 cohorts (Lund, Groningen and Sheffield, and NARAC). In NOAR, we found the effect direction consistent with risk allele as in other 3 cohorts [115].

^c 95% CI was around the mean, hence crossed 1 as 1-tailed *P* values were used, to test for associations in a predefined direction based on previous evidence. Bold-face values highlight associations which replicated in NOAR with *p*<0.05.

^f In LD, *R*² = 0.72 with rs10760130, therefore not representing an independent association.

^j This SNP was associated in NOAR with an effect in the opposite direction of the original publication [99]. Therefore, under the assumption of consistent direction of association across studies, the 1-sided *P* value calculated in this study as follows: 1 – (2-sided *P* value)/2, with the 2-sided *P* values taken from Supplementary File S2, Tab 2.

(rs3812553), located in DNLZ/CARD9, was predicted to be highly likely damaging by PolyPhen2 (score = 0.996) [25]. Whether this results in loss or gain of function, however, is unknown.

The abovementioned statistical and functional evidence strongly implicates a number of candidate SNPs within the CARD9 genomic region on chromosome 9 influencing radiographic trajectory of patients with RA through modulation of CARD9 expression in immune cells.

GO pathway analysis

Results of GO pathway analysis of genes in Table 3 are shown in Supplementary File S2, Tab 15. This points almost exclusively towards an involvement of immune cell types and pathways, in particular of the adaptive immune response (including Th1, Th2, and Th17 pathways) and the regulation of cytokine production (including the regulation of nuclear factor κB transcription factor activity). There are also a minority of significant

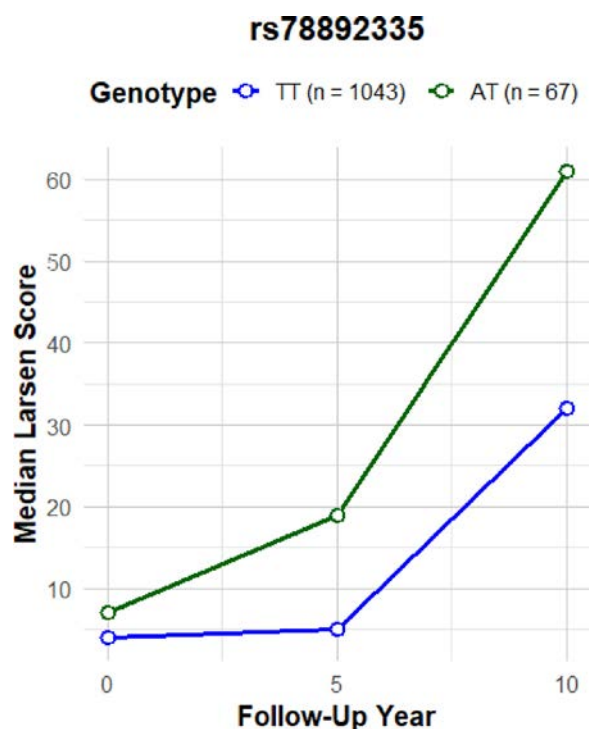


Figure 3. Median Larsen score according to genotype of rs78892335 *ENTR1* over time. Median Larsen score at follow-up years 0, 5, and 10 of patients with different genotypes of SNP rs78892335 *ENTR1* is shown. TT denotes those patients homozygous for the major allele, and AT are heterozygous. Only 1 patient was homozygous (AA) for the minor allele and had only baseline data; therefore, not included in this graph. Patients who had a copy of the minor allele (A) had a more severe radiographic changes compared with those without.

pathways, which suggest musculoskeletal and stromal processes, not directly related to the immune system. This includes regulation of smooth muscle cell apoptotic processes (GO:0034393; $P = .00695$) and smooth muscle proliferation (GO:0048660; $P = .0131$).

DISCUSSION

HLA polymorphisms associated with RA susceptibility are also associated with severity [6]. However, emerging evidence suggests that genetic mechanisms underpinning susceptibility to disease and its progression are different outside the HLA region. Despite a plethora of reports on genetic associations with radiographic outcome in RA, the characterisation of biological mechanisms leading to poor outcome has remained elusive, as most associations fail to replicate in independent cohorts. Our goal was to identify non-HLA loci that warrant prioritisation for future research and clinical application, through (1) review of all reports on radiographic severity in RA across ancestries, (2) independent validation in a large cohort, and (3) gaining biological insights into mechanisms of disease prognosis.

We identified 139 independent genetic associations with radiographic damage in RA reported in the literature between 1998 and 2022. Of those, 15 independent SNPs (Table 3) associated with radiographic progression in NOAR and only 1 of them mapped to a known susceptibility locus (*TRAF1/C5*). This suggests certain genetic factors may not trigger a disease process, but could be implicated in the perpetuation of an inflammatory response, and thus joint damage, after disease onset. In addition, we identified 13 loci (Table 3), which, despite their lack of significant association in this study, had independently replicated

before November 2022. Therefore, they represent associations with radiographic damage in RA as likely to be true positive associations as the 15 loci replicated in this study. Several factors could explain a lack of association in our study: (1) different ancestries and MAF between the original cohort and NOAR; (2) differences in demographic and clinical cohort characteristics (including differences in the proportion of seropositive patients and treatment regimens); (3) insufficient power in NOAR (several P -values are close to the significance threshold in Table 3); (4) differences in analytical techniques and methodologies. Various methodological factors can impact replication of genetic findings across cohorts. These could be differences in genetic models (e.g., additive vs. recessive), different methods used to measure radiographic damage, varying approaches to assessing progression over time or to adjust for the effect of treatment. This heterogeneity across studies highlights a key challenge in identification of genetic factors associated with prognosis.

One SNP (rs59902911) remained significant in this study after multiple testing. This SNP is located within the *CARD9* (caspase-recruitment domain 9) gene, which has not been associated with susceptibility to RA. This association was previously reported in a cohort of 424 European American patients with RA [45]. Specifically, the minor allele (T) was associated with increased Sharp score ($\beta = 0.86$; $P = 1.01 \times 10^{-6}$) [45]. In the NOAR cohort, this effect was replicated; the minor allele (T) was also associated with increased Larsen score ($\beta = 4.65$; 95% CI, 2.87–6.42; $P = 1.46 \times 10^{-7}$) and with increased odds of erosions (OR, 1.64; 95% CI, 1.04–2.60; $P = 0.015$). Notably, this effect was independent of anti-CCP status. Further analysis of this genetic locus revealed 30 SNPs in the 95% CSS, in which the minor allele of the lead SNP (rs78892335) reached genome-wide significance ($\beta \pm SE = 4.78 \pm 0.83$; $P = 9.01 \times 10^{-9}$). The minor allele of this intronic SNP located in the nearby *ENTR1* gene increases expression of *CARD9* in B, CD4⁺ and CD8⁺ T cells, monocytes and neutrophils. *CARD9* plays an integral role in the immune system through upregulation of inflammatory cytokines such as IL-6, TNF, and interferon γ , and processes such as maturation of antigen-presenting cells, activation of T cells, neutrophil recruitment, and polarisation of macrophages. This gene has been associated with susceptibility to multiple autoimmune diseases including inflammatory bowel disease and ankylosing spondylitis, despite its lack of association with RA susceptibility in a large multiethnic meta-analysis comprising over 276,000 individuals [134,136].

A further example found in this study was rs2243250, located in the *IL-4* gene. Hussein et al, in a small study of 172 patients of which the majority were positive for anti-anti-CCP (91%), found that the minor allele (T) was associated with a 2-fold increased risk of erosions (OR, 2.04; 95% CI, 1.26–3.30; $P = .024$). In NOAR, the minor allele was also associated with increased propensity to erosive change and increased Larsen score. Patients with the TT genotype were found to have lower levels of serum IL-4 compared with those who possessed the CC genotype. IL-4 has a pivotal role in immunoregulation through modulation of macrophage expression profile, reduction of interferon gamma from Th1 cell and polarisation towards a Th2 phenotype [137]. A *IL-4*^{−/−} mouse arthritis model demonstrated a greater severity of disease [138,139]. IL-4 is thought to have a dual antiarthritic effect [140], through both inhibition of cartilage degradation and osteoclastogenesis [141–145].

Anti-CCP positivity has been consistently associated with worse prognosis, including higher severity of radiographic damage [3,146]. There is also evidence that anti-CCP positivity is associated with improved response to tocilizumab, abatacept,

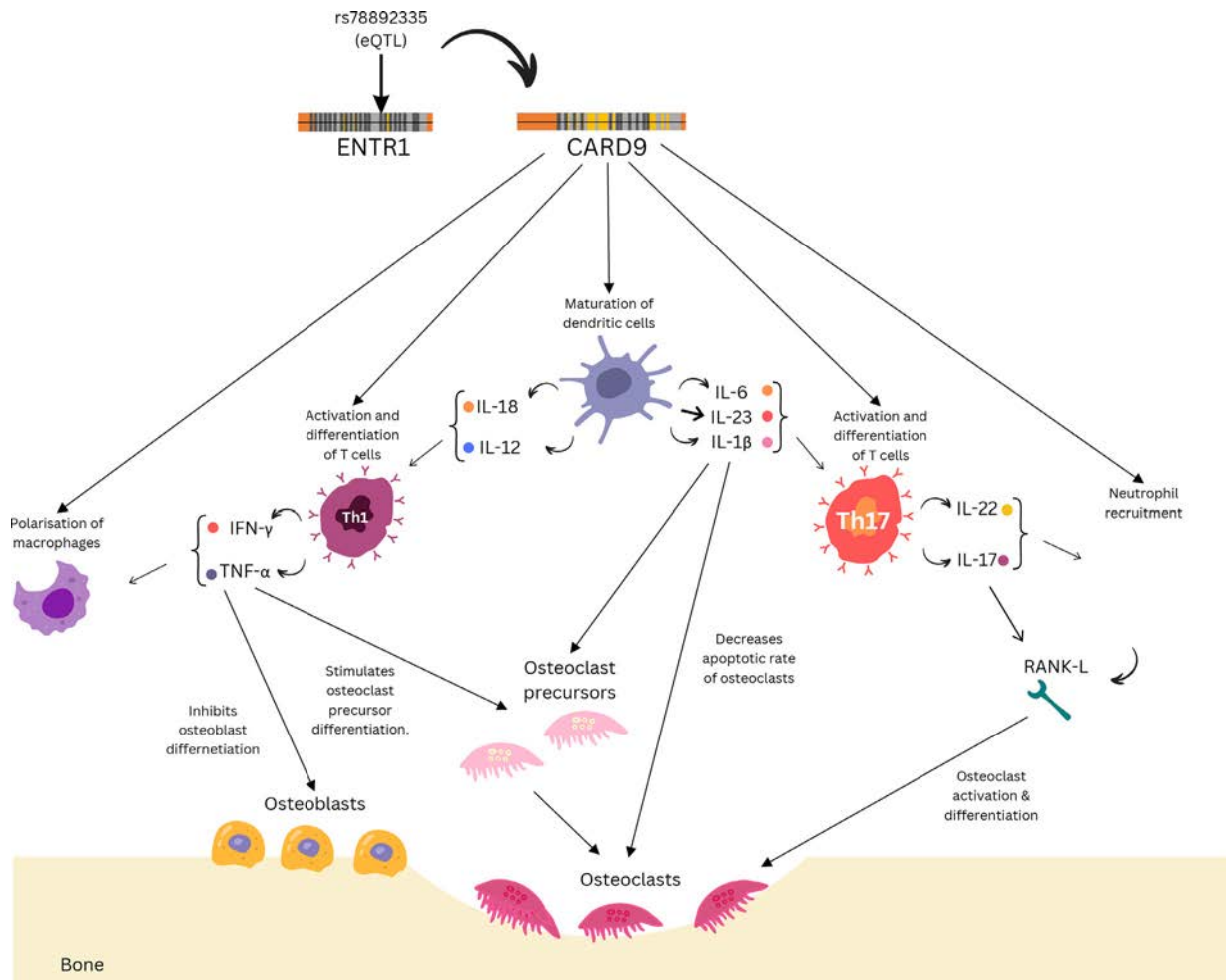


Figure 4. Potential role of rs78892335 (ENTR1) in rheumatoid arthritis (RA). The potential mechanisms by which rs78892335 located on the *ENTR1* gene may lead to increased disease activity and subsequent radiographic damage in RA. rs78892335, an intronic SNP located on the *ENTR1* gene, regulates expression of *CARD9* (minor allele increases expression). *CARD9* is involved in both the innate and adaptive immune response through mediation of cytokines as represented in the diagram [134]. Many of these cytokines are known to have potential effects on bone through acting on osteoblasts and osteoclasts. Tumour necrosis factor (TNF) α stimulates osteoclast precursor differentiation through a number of mechanisms including upregulating expression of c-FMS (colony-stimulating factor-1 receptor). It also inhibits osteoblast differentiation though inhibition of RUNX2 and increasing expression of RANKL and M-CSF in osteoblast lineage cells, as well as inducing osteoblast apoptosis. Interleukin (IL)-1 increases osteoclastogenesis and decreases rate of apoptosis. IL-6 stimulates prostaglandin (PG)E₂ synthesis and thereby increases RANKL, thus increasing osteoclastogenesis indirectly. IL-17 increases osteoclastogenesis through induction of RANKL and RANK and upregulates IL-6 through TNF α [135]. This heightened osteoclast activity and inhibition of osteoblast function results in net loss of bone and leads to increase erosive changes in RA.

Table 4
Predicted consequence of exonic SNPs in *CARD9* region

Chr	Position	SNP rsID	R ² with rs78892335	Major allele	Minor allele	MAF	β Coefficient	P	Gene	CADD	Synonymous?	Exon	PolyPhen2
9	139256495	rs3812553	0.87	G	T	0.04	4.54	$5.32 \times e^{-7}$	<i>DNLZ: CARD9</i>	21.3	No	Exon 3	Probably damaging (score, 0.996)
9	139265870	rs11145769	0.85	G	A	0.04	4.31	$1.2 \times e^{-6}$	<i>CARD9</i>	14.84	Yes	Exon 3	
9	139265088	rs59902911	0.90	C	T	0.04	4.65	$2.92 \times e^{-7}$	<i>CARD9</i>	14.43	Yes	Exon 5	
9	139270876	rs3812561	0.90	G	A	0.04	4.66	$2.4 \times e^{-7}$	<i>SNAPC4</i>	7.417	No	Exon 22	Benign (score, 0.021)
9	139316459	rs11145972	0.87	C	T	0.04	4.62	$2.69 \times e^{-7}$	<i>PMPA</i>	0.824	No	Exon 6	Score not available
9	139301960	rs3812580	0.95	G	A	0.04	4.58	$3.54 \times e^{-7}$	<i>SDCCAG3</i>	0.292	Yes	Exon 4	

Within the 95% credible SNP set (CSS) for rs59902911, there were 6 exonic SNPs, shown in this table. These were all in high LD with the lead SNP (SNP with the lowest *P*) rs78892335 and included the index SNP (original association) rs59902911. β Coefficients and *P* values for association with Larsen score in NOAR are displayed. Three of these variants were synonymous, meaning they do not cause a change in amino acid sequence, and 3 were nonsynonymous, leading to changes in the amino acid sequence of the encoded protein. Nonsynonymous variants may potentially impact protein function and thus contribute to the observed phenotypic differences. PolyPhen2 is a tool used to predict potential impact of nonsynonymous changes, and scores are displayed in the table [25]. Of note, rs3812553, located on *DNLZ: CARD9*, was predicted to be highly likely damaging. CADD scores, from FUMA, for each of these SNPs are also displayed (see Methods) [20,22,23]. CADD, combined annotation-dependent deletion; Chr, chromosome; LD, linkage disequilibrium; MAF, minor allele frequency; RDB, RegulomeDB.

and rituximab [147,148]. Establishing whether genetic associations of radiographic severity are related to anti-CCP status is crucial to inform functional mechanisms and whether genetic information is likely to contribute to prediction in clinical practice. For example, rs10760130 (*TRAF1*) appears to influence radiographic severity primarily through association with anti-CCP positivity.

Furthermore, it is increasingly recognised that seropositive RA may have distinct genetic mechanisms compared with seronegative RA. For instance, rs59902911 (*CARD9*) exhibited a greater effect in anti-CCP-positive patients, suggesting a potential interaction with anti-CCP-related pathways. This highlights the heterogeneity of genetic contributions to RA severity and underscores the importance of stratifying analyses by anti-CCP status in future research.

One main strength of the NOAR cohort is the recruitment of patients very early in the disease course, before they satisfy classification criteria for RA. Inception cohorts typically include a larger proportion of anti-citrullinated protein antibodies-negative patients [2,149] and patients with a wider range of radiographic damage than cohorts of patients with established disease from secondary or tertiary care settings. In addition, a large proportion of patients remained untreated in NOAR and a very low proportion were treated with modern strategies, known to diminish radiographic damage. Since we adjusted for the cumulative effect of various treatments, our study design would likely capture the natural evolution of the disease. However, the same setting can also be a limitation, as it favours misclassification – that is, the inclusion of seronegative arthritides other than RA. Historically, data analysis in NOAR, performed both in IP (which will capture seronegative RA better than classification criteria might) [150] and in RA, has led to similar conclusions [2,10]. Similarly, for this study, we performed our analysis in IP, in patients satisfying the ACR 1987 criteria for RA (Supplementary File S2, Tabs 6 and 7) and in patients satisfying the EULAR/ACR 2010 criteria (Supplementary File S2, Tabs 6 and 7) for all SNPs. As an improvement to previous genetic studies in NOAR, we excluded patients with an alternative diagnosis. We also tested the level of correlation of effect sizes between IP and RA and observed a very strong association (β coefficient > 0.8 between effect sizes for Larsen scores in IP and 2010 RA with $P < 7.82 \times 10^{-54}$). This increases our confidence that misclassification is not a major issue in this study.

In summary, we provide a comprehensive list of validated genetic associations with RA outcome and demonstrate that non-*HLA* severity polymorphisms, despite lack of association with disease susceptibility, can reach genome-wide significance and point towards genetic mechanisms of radiographic outcome. Our findings support the need for dedicated genetic studies looking specifically at genetic drivers of disease severity and outcomes. There is evidence for this across other autoimmune diseases such as inflammatory bowel disease and multiple sclerosis [13,151]. Further research could include severity specific GWAS and development of polygenic risk scores, which would require prospective testing in an inception cohort to determine whether these approaches have clinical utility and are cost-effective.

Competing interests

All authors declare they have no competing interests.

CRedit authorship contribution statement

Seema Devi Sharma: Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Lysette Marshall:** Methodology, Investigation, Formal analysis. **Alice Storrie:** Writing – original draft, Formal analysis. **John Bowes:** Writing – original draft, Visualization, Methodology, Conceptualization. **Alexander MacGregor:** Writing – review & editing, Funding acquisition, Data curation, Conceptualization. **Max Yates:** Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Andrew P Morris:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis. **Suzanne Verstappen:** Writing – review & editing, Methodology, Data curation. **Anne Barton:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Investigation, Conceptualization. **Hanna van Steenberg:** Methodology, Investigation. **Rachel Knevel:** Supervision, Methodology, Investigation. **Sebastien Viatte:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

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Patient consent for publication

The Norfolk Arthritis Register (NOAR) is an inception cohort of patients with inflammatory polyarthritis recruited since 1989 and followed prospectively for up to 20 years. All patients were recruited after informed consent.

Ethics approval

Ethical approval was granted by the Norwich Research Committee (NOAR – REC Ref 2003/075, December 18, 2003, Norwich Local Research Committee [NHS]).

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ard.2025.04.007.

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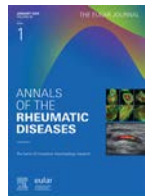
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Rheumatoid arthritis

Investigating endogenous immune-mediated monocyte memory in rheumatoid arthritis

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ABSTRACT

Objectives: Inflammation triggered by endogenous stimuli that signal cellular stress or tissue injury must be tightly controlled to balance robust protection from intrinsic danger while avoiding catastrophic destruction of healthy tissues. Here, we assess the contribution of innate memory to this balance. **Methods:** Memory evoked by the extracellular matrix protein tenascin-C, a damage-associated, toll-like receptor 4 (TLR4) agonist, was compared to that induced by the pathogenic TLR4 agonist lipopolysaccharide (LPS) by transcriptomic and epigenetic profiling of monocytes from healthy individuals or people with rheumatoid arthritis (RA), and tissue macrophages from the RA synovium. **Results:** Tenascin-C reprograms monocyte response to subsequent threats, inducing concomitantly suppressed and enhanced responses to rechallenge. Comparative analysis of tenascin-C and LPS revealed common and distinct gene expression signatures, effects controlled transcriptionally and associated with stimulus-specific epigenetic mediators. Altered responses following rechallenge after priming with tenascin-C were not limited to subsequent TLR4 activation but were evident in response to various pathogenic and endogenous stimuli detected by different receptors. In healthy monocytes primed with tenascin-C, rechallenge with stimuli found at high levels in the joints of people with RA resulted in trained responses that were not induced by LPS, including genes associated with chronic inflammation, tissue destruction, altered metabolism, and poor treatment response in RA. The expression of a large subset of these genes was elevated in monocytes from people with RA in the absence of any stimulation and in RA synovial macrophage populations associated with disease flare. Moreover,

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higher levels of permissive complexes within key epigenetic nodes and increased bivalent modification creating poised loci within endogenously trained genes were observed in RA cells. **Conclusions:** These data highlight how innate reprogramming during ‘sterile’ inflammatory diseases contributes to chronicity, uncovering pathways unique to endogenous immune triggers that could provide disease-specific points of intervention without engendering global immune suppression.

INTRODUCTION

Innate immune memory is a key regulator of defence against infection that evolved to potentiate protective immunity and limit collateral tissue damage. Inoculation with malaria or typhoid vaccine attenuated febrile responses to further pyrogenic agents, a method of treatment for neurological/psychiatric conditions that won the Nobel Prize in 1927 [1]. Reduced inflammation following repeated exposure to bacterial lipopolysaccharides (LPS) is commonplace in animal models, a macrophage-dependent phenomenon resulting from suppression of inflammatory cytokine synthesis [2,3]. Conversely, vaccination with bacille Calmette-Guérin (BCG) improves child mortality rates by protecting from infections with pathogens other than *Mycobacterium tuberculosis* [4], a lymphocyte-independent phenomenon conserved in mice [5,6]. Vaccines against influenza, measles, and yellow fever also produce broad protection from infection, which is not limited to the vaccine used [7]. Moreover, BCG vaccination linked to mitigated COVID-19 severity [8] prompted the proposal of early deployment of established vaccines as a way to secure protection from new pandemic threats [9]. At the molecular level, the best-characterised adaptation of myeloid behaviour based on past encounters comprises yeast-derived β -glucan, which potentiates monocyte/macrophage response to subsequent unrelated threats, and LPS, which inhibits the capacity to produce proinflammatory mediators following rechallenge; both β -glucan training and LPS tolerance result from complex transcriptional, epigenetic, and metabolic reprogramming of the myeloid compartment [10–13].

More recently, innate memory has been linked with the pathogenesis of immune-mediated diseases of noninfectious aetiology. In rheumatoid arthritis (RA), myeloid cells with features resembling a trained state have been described, including a distinct, proinflammatory macrophage in RA synovial fluid, with elevated CCR2, MMP12, and PHD3 expression, which promotes polarisation of healthy monocytes and macrophages towards a proinflammatory state [14]. In conjunction with hyperresponsiveness to LPS challenge *ex vivo*, circulating monocytes from RA patients displayed altered metabolism, including increased mitochondrial respiration and dependence on glycolysis. A similar RA-like monocyte phenotype was found in individuals at high risk of developing RA, predating clinical disease manifestation [15]. Finally, global DNA hypo-methylation, reversible following treatment with methotrexate, was described in RA monocytes [16], and tumor necrosis factor (TNF) biologics altered methylation and acetylation signatures at the promoter of the proinflammatory chemokine C-C motif chemokine ligand 2 (CCL2) [17], whereas inhibition of interleukin (IL)-6 promoter demethylation relieved the severity of experimental murine RA [18]. Despite evidence of myeloid reprogramming in RA, the immune stimuli that drive these altered states remain poorly characterised. Analogous to pathogen-associated molecular patterns, which signal microbial invasion, damage-associated molecular patterns (DAMPs) signal intrinsic threats such as cellular stress and tissue injury, activating inflammation designed

to drive tissue repair and restore homeostasis. Persistent accumulation of DAMPs is associated with a self-perpetuating cycle of tissue damage and inflammation in many diseases [19]. However, while we have a good understanding of myeloid adaptation following pathogen-related challenges, only a few studies show how noninfectious immune stimuli (ie, Western diet) train monocytes [20], and DAMPs (including HMGB1 and S100 proteins) produce an effect similar to LPS tolerance [21,22]. As such, little is known about DAMP-mediated memory and how these responses are linked to ‘sterile’ inflammatory diseases.

Here, we compare endogenously driven innate immune memory with memory resulting from infectious threats using LPS as an archetypal microbial toll-like receptor (TLR) 4 agonist and the TLR4-activating domain (the fibrinogen-like globe domain of tenascin-C [FBG-C]) of the extracellular matrix molecule tenascin-C as an exemplar DAMP [23]. Tenascin-C is not expressed or is expressed at low levels in healthy tissue and is transiently upregulated upon tissue injury. It is, however, persistently expressed in a number of inflammatory diseases, including RA, where high levels accumulate in the joints and circulation of people with erosive, hard-to-treat disease, and therapeutic antibodies targeting this molecule effectively ameliorate joint inflammation and destruction in preclinical disease models [24]. We show that tenascin-C generated a dynamic memory response in healthy monocytes, including suppression of canonically tolerised immune mediators, but that it also promoted potentiation of gene expression programmes linked to chronic inflammation, cell death/tissue destruction, altered metabolism, and poor treatment response in people with RA. These responses were not induced by LPS and were driven by the deployment of stimulus-specific epigenetic machinery. Modelling the accumulation of endogenous inflammatory triggers, including matrix molecules, nucleic acids, and cytokines, revealed novel training programmes distinct from LPS-induced rewiring. A large proportion of these genes exhibited elevated basal expression in monocytes from individuals with RA and/or permissive epigenetic modification, rendering them transcriptionally poised, a phenomenon conserved in inflammatory RA synovial tissue macrophage subsets associated with disease flare. These data demonstrate how myeloid cells adapt following challenges with RA-relevant immune stimuli, uncovering disease-specific pathways contributing to maladapted immune responses.

RESULTS

Tenascin-C induces memory states that are distinct from LPS

Induction of innate immune memory states involves the initial activation of cells by exposure to a primary stimulus (prime); primed cells are then presented with a rechallenge with a secondary stimulus (trigger); the response to this step can be potentiated (training) or diminished (tolerance). Consistent with classical memory protocols (Fig 1A) [10,11,13], monocytes primed with β -glucan demonstrated enhanced cytokine

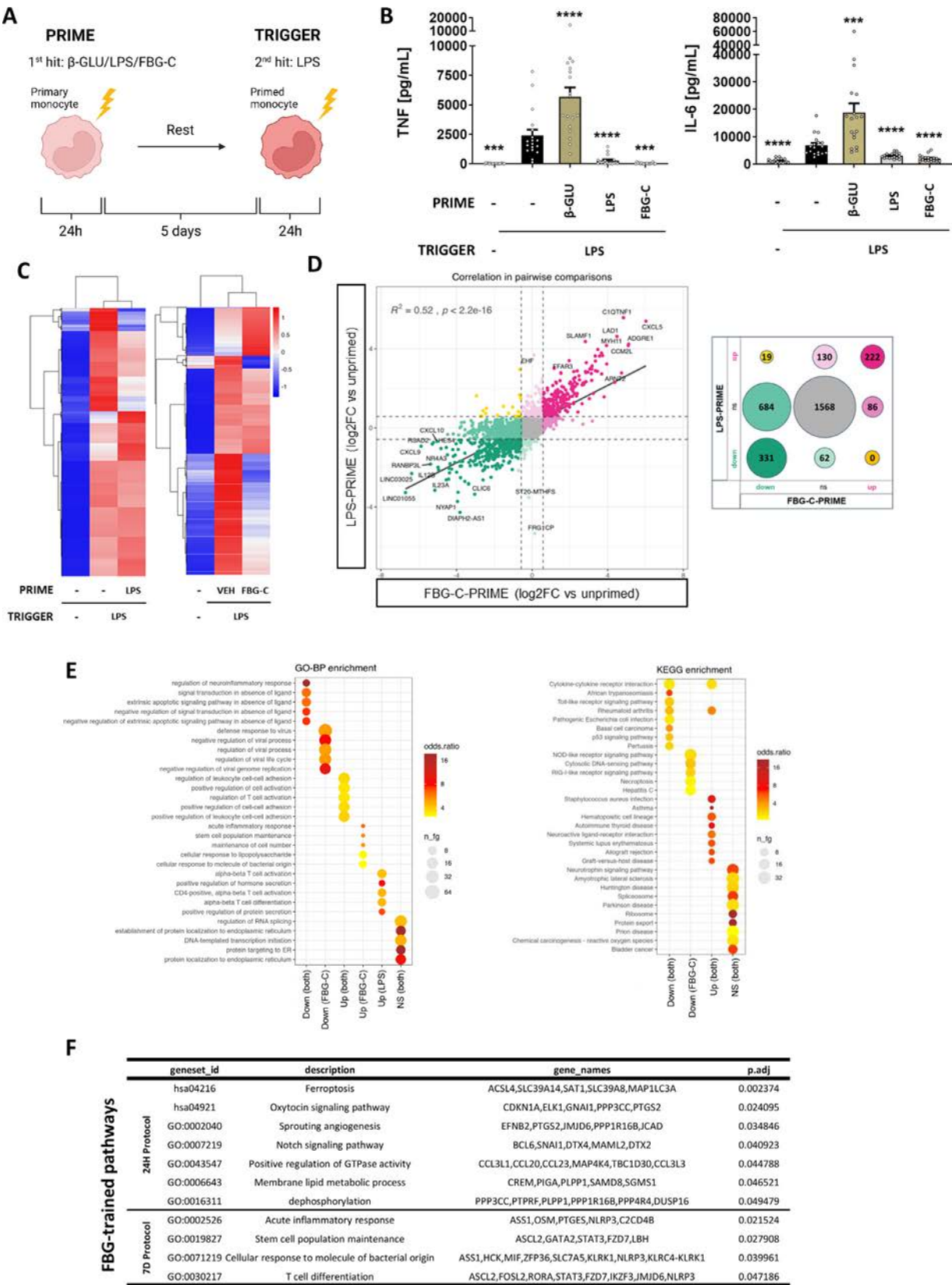


Figure 1. Tenascin-C induces innate immune memory responses. (A) Assay protocol: primary human monocytes were stimulated for 24 hours (24H) with lipopolysaccharide (LPS), β -glucan (β -GLU), or the fibrinogen-like globe domain of tenascin-C (FBG-C) (PRIME). Cells were then washed and rested for 5 days. On day 6, cells were restimulated with LPS (TRIGGER) for 24H, after which tumour necrosis factor (TNF) and interleukin (IL)-6 levels were quantified in the culture media by enzyme-linked immunosorbent assay. (B) Cytokine secretion in response to restimulation with 10 ng/mL LPS of monocytes primed with β -GLU (10 μ g/mL), LPS (100 ng/mL), or FBG-C (1 μ M). Data are shown as mean $\pm$ SEM from 18 independent donors. Data were analysed using multiple paired *t*-tests vs cells that were left unprimed and then restimulated with LPS ($\pm$ LPS); ****P* < .001, *****P* < .0001. (C–E) Primary human monocytes were primed and triggered as in (A) but lysed 4 hours after the trigger, and transcriptomic changes were analysed by bulk RNA sequencing. (C) Heatmap of significantly upregulated genes (fold change [FC] > 1.5, adjusted *P* [P.adj] < .05, N = 3148 genes) in triggered unprimed cells (–/LPS) compared with unstimulated cells (–/–), highlighting their expression in monocytes primed with LPS (LPS/LPS) or FBG-C (FBG-C/LPS). Data are mean VST-transformed values from 5 independent donors. VST, variance-stabilizing transformation. (D) Log₂ FC in gene

secretion following rechallenge with LPS, while LPS-primed cells demonstrated blunted responses (Fig 1B, [Supplementary Fig S1A–C](#)). Priming with FBG-C also caused a blunted response to the LPS rechallenge (Fig 1B). Absolute levels of baseline and induced cytokine production varied across donors; however, the degree of tolerance induced by LPS and FBG-C was very highly conserved ([Supplementary Fig S1D,E](#)). Priming monocytes with FBG-C induced dose-dependent cytokine release, confirming the activity of this domain ([Supplementary Fig S1F](#)). Following rest and rechallenge with LPS, FBG-C–primed cells demonstrated significantly blunted cytokine secretion, proportional to the concentration of FBG-C used during priming ([Supplementary Fig S1G](#)). Responses were unaffected by pretreatment of FBG-C preparations with polymyxin B, which blocked LPS-mediated TLR4 activation and were abolished by proteinase K (PK) cleavage of FBG-C protein. Cells primed with PK-treated FBG-C no longer demonstrated tolerance following LPS rechallenge, and cells incubated with PK in the absence of FBG-C priming did not exhibit tolerance upon LPS stimulation, while PK treatment in the absence of LPS rechallenge did not induce cytokine release ([Supplementary Fig S1H](#)).

RNA sequencing (RNAseq) of monocytes primed with LPS or FBG-C before LPS rechallenge confirmed that suppression of TNF and IL-6 secretion is transcriptionally mediated, and showed that LPS-primed monocytes are transcriptionally distinct from FBG-C-primed monocytes ([Supplementary Fig S1I–M](#), Fig 1C,D). Genes tolerised by both stimuli were associated with inflammatory cytokine signalling and cytokine/chemokine-receptor interactions. Gene expression was also enhanced following priming with both stimuli, including mediators of leucocyte adhesion and T-cell activation, with strong enrichment of human leukocyte antigen (HLA) genes. Strikingly, more genes were tolerised by FBG-C than LPS; 684 genes were specifically affected by FBG-C, including intracellular pattern sensing receptors such as NOD-like receptor, RIG-I–like receptor/cytosolic DNA sensing, and necroptosis. Eighty-six genes were enhanced by FBG-C only; these were associated with haematopoietic development (including transcription factor *GATA2* and human insufficiency, which causes monocytopenia) and cell proliferation, as well as arginine synthesis and positive regulators of immunity (eg, *STAT3*, a transcriptional activator of myriad inflammatory genes, and *MIF*, a proinflammatory cytokine that counteracts the anti-inflammatory activity of glucocorticoids [25]). Genes whose induction was not affected by priming were linked to gene transcription and splicing, as well as protein translation and export (Fig 1E,F).

Analysis of monocyte responses over time revealed that TNF secretion was blocked immediately following priming with LPS and FBG-C and maintained for up to 7 days ([Supplementary Fig S2A](#)), while IL-6 exhibited biphasic regulation, with a rapidly induced superinduction preceding the onset of tolerance ([Supplementary Fig S2B](#)). This was reflected at the messenger RNA (mRNA) level ([Supplementary Fig S2C–F](#)). Upon rechallenge of FBG-C- or LPS-primed monocytes with LPS, TNF levels did not increase in contrast to unprimed cells ([Supplementary Fig S2E](#)), while IL-6 was transiently elevated following rechallenge ([Supplementary Fig S2F](#)), consistent with higher residual IL-6 following primary activation compared with TNF, which returned to

baseline after 24 hours of priming ([Supplementary Fig S2C,D](#)). Transcriptomic analysis of early memory events confirmed enhanced IL-6 expression along with more than 300 genes that were also trained and revealed a number of pathways modulated in the opposite direction or not regulated compared with the long assay, within which both overlapping and distinct genes defined pathway enrichment ([Supplementary Fig S2G–O](#)). Stimulus-specific early responses were also observed, including genes associated with ferroptosis (programmed cell death that causes inflammation, oxidative stress, mitochondrial dysfunction, and lipid oxidative damage), angiogenesis, and Notch signalling that were only elevated in FBG-C–primed cells (Fig 1F, [Supplementary Fig S2N](#)).

These data confirm that monocyte activation with infectious or endogenous TLR4 stimuli does induce tolerance to further challenge, but also causes enhanced gene expression. Moreover, gene programmes are altered in a time- and stimulus-dependent manner. We identified a biphasic response to priming with both stimuli; early after activation, cell infiltration and selected proinflammatory mediators were augmented, and T-cell cytotoxicity was suppressed, while later stages were characterised by downregulation of leucocyte migration and innate signalling in favour of enhanced T-cell effector function. We also uncovered a specific role for endogenous priming in rapid upregulation of cell death/destructive tissue remodelling after monocyte activation, and downregulation of viral defence programs concomitant with altered monocyte metabolism and the induction of a select repertoire of immune mediators that does not occur with pathogenic primary challenge.

Tenascin-C drives challenge-specific cross-protection and training

Using LPS as the secondary stimulus in this model mimics subsequent infection following primary infection (LPS and β -glucan) or tissue injury (FBG-C). However, the immune microenvironment of the inflamed joint is more complex. To determine whether memory responses are limited to rechallenge with LPS, we primed cells with FBG-C, LPS, or β -glucan and then rechallenged with FBG-C (Fig 2A). Monocytes primed with FBG-C or LPS demonstrated blunted TNF secretion following rechallenge with FBG-C (Fig 2B, [Supplementary Fig S3A](#)). Interestingly, monocytes primed with β -glucan did not demonstrate enhanced TNF secretion following rechallenge with FBG-C, in contrast to those rechallenged with LPS ([Supplementary Fig S3B](#)). These data suggest that tolerance responses induced by FBG-C protect from both subsequent infection and repeated exposure to injury-associated stimuli, but training downstream of β -glucan exposure is specific to rechallenging with infectious stimuli.

LPS-induced tolerance is not limited to rechallenge by TLR4 agonists but drives cross-protection against many inflammatory stimuli recognised by a wide range of pattern recognition receptors (PRRs) [26]. To determine the specificity of FBG-C priming, we exposed FBG-C–activated monocytes to a variety of ligands derived from bacteria and yeast, as well as nucleic acid and viral mimics and noninfectious stimuli (Fig 2C). Here, we included IL-8 protein secretion as an additional readout alongside TNF and IL-6 to assess the activity of ligands that do not induce TNF or IL-6. Blunted cytokine release in monocytes primed with FBG-C was not

expression obtained from independent differential gene expression analyses (DEA) of monocytes primed with LPS (LPS/LPS, x-axis) or FBG-C (FBG-C/LPS, y-axis) compared with unprimed monocytes. Data are from $N = 3102$ genes that are upregulated at baseline ($-/LPS$ vs $-/-$ comparison) and expressed in both follow-up DEA (LPS/LPS vs $-/LPS$ and FBG-C/LPS vs VEH/LPS). (E) Top 5 Kyoto Encyclopedia of Genes and Genomes/Gene Ontology-Biological Process (KEGG/GO-BP) pathways enriched in upregulated, downregulated, and unaffected gene groups, as determined from overrepresentation analysis. (F) The table depicts a selection of pathways enriched among genes that are significantly upregulated in FBG-C–primed monocytes following stimulation for 24H and rechallenged with LPS after 24H or 7 days (7D). VEH, vehicle control.

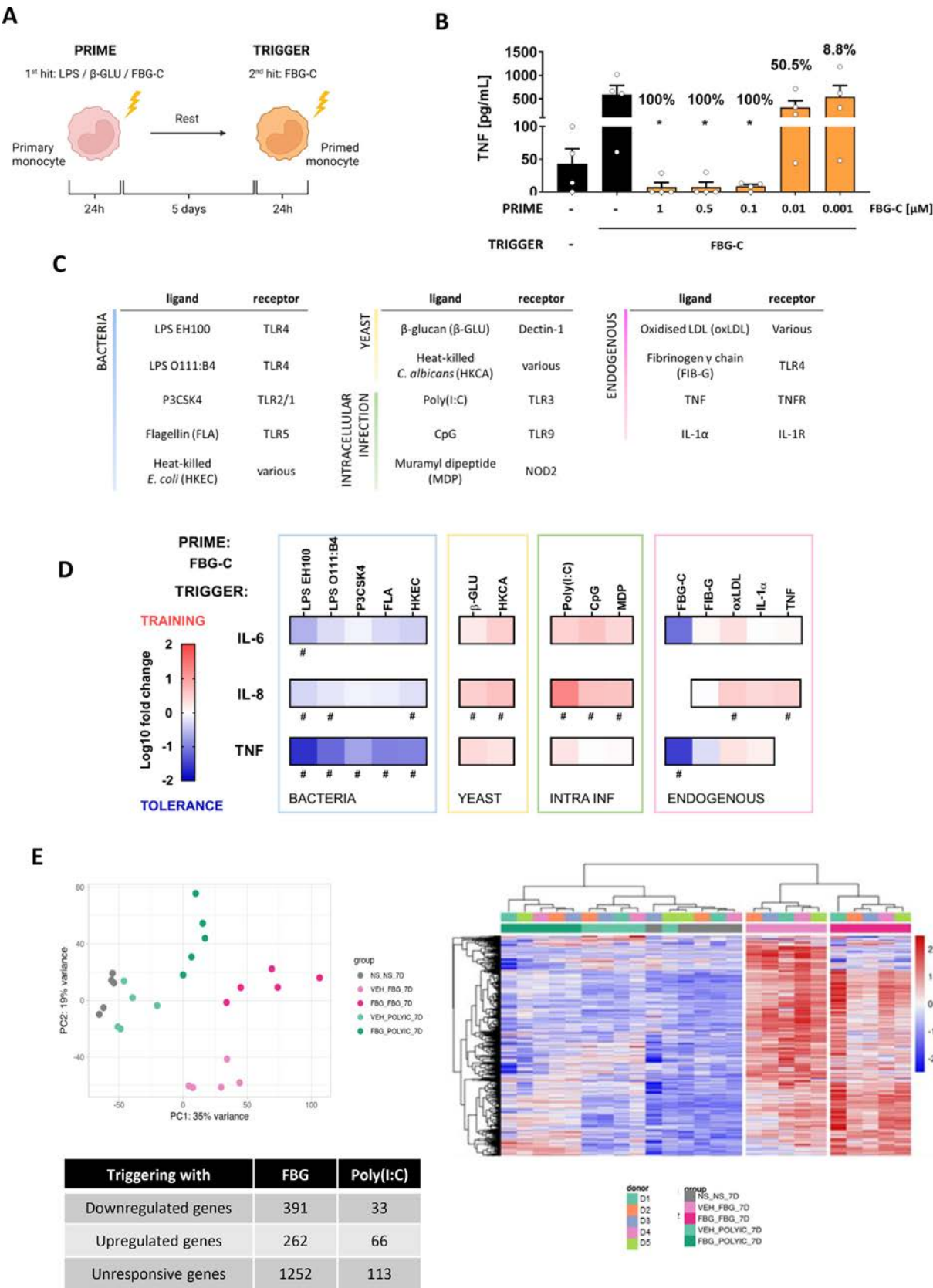


Figure 2. Tenascin-C reprograms monocyte responses to danger. (A) Assay protocol: primary human monocytes were stimulated for 24 hours with lipopolysaccharide (LPS), β -glucan (β -GLU), or the fibrinogen-like globe domain of tenascin-C (FBG-C) (PRIME). Cells were then washed and rested for 5 days. On day 6, cells were restimulated with FBG-C (TRIGGER) for 24 hours, and cytokine levels were quantified in the culture media by enzyme-linked immunosorbent assay. (B) Tumour necrosis factor (TNF) secretion in response to restimulation with 1 μ M FBG-C of monocytes that were primed with increasing doses of FBG-C. The numbers above the bars represent the percentage of TNF signal inhibition compared with a sample that was not primed but was triggered with FBG-C (–/–FBG-C), normalised to background quantified in unstimulated cells (–/–). Data are shown as mean $\pm$ SEM from 4 independent donors. (C) A selection of pathogen-derived (bacterial, yeast, and viral) and endogenous ligands sensed by various pattern recognition receptors was used to examine FBG-C–induced cross-memory. (D) TNF, interleukin (IL)-6, and IL-8 secretion in monocytes that were primed with 1 μ M FBG-C for 24 hours, rested for 5 days, and then restimulated with pathogenic or endogenous triggers for 24 hours. Data on the

restricted to rechallenge via TLR4 but was also observed in response to rechallenge with bacterial ligands of TLR2/1 and TLR5, Pam3CSK4, and flagella, as well as with heat-killed *Escherichia coli*, each of which significantly reduced TNF secretion. In contrast, rechallenge with ligands of intracellular PRRs, including TLR3 (polyinosinic:polycytidylic acid, Poly(I:C)), TLR9 (CpG), and NOD2 (muramyl dipeptide, MDP), or with yeast, had no impact on IL-6 or TNF release, but all elicited enhanced IL-8 secretion. Moreover, while TNF was significantly blunted in FBG-C–primed cells following rechallenge with FBG-C, rechallenge with fibrinogen, a structurally related endogenous TLR4 agonist [27], did not affect cytokine secretion. Finally, IL-8 release following rechallenge with oxidised low-density lipoprotein (oxLDL) or TNF was enhanced (Fig 2D, Supplementary Fig S3C,D). RNAseq of monocytes primed with FBG-C and then rechallenged with FBG-C or Poly(I:C) confirmed specific induction of *CXCL8*, the gene encoding IL-8, only by the latter, and revealed 262 trained genes upon FBG-C rechallenge and 66 trained upon Poly(I:C) rechallenge (Fig 2E, Supplementary Fig S4).

These data indicate that FBG-C–induced memory not only impacts TLR4 signalling capabilities but also modulates responses to other receptors. Moreover, while FBG-C priming caused tolerance across the cytokines examined here following rechallenge with itself and bacterial triggers (TLR4, TLR2/1, and TLR5), rechallenge with other classes of stimuli (TLR3, TLR9, NOD2, Dectin-1, TNF receptor, and oxLDL receptor) led to a broadly enhanced IL-8 response. Comparison of FBG-C and Poly(I:C) rechallenge uncovered activation of both common and distinct gene expression programmes. These results suggest that FBG-C priming exacerbates monocyte responses to rechallenge that may be experienced in the RA synovium, including high levels of TNF, accumulating tenascin-C deposition, and elevated TLR3 agonism caused by RNA released from necrotic synovial cells [28].

Altered intrinsic transcriptomic responses in RA

To determine whether training responses seen in healthy cells are reflected in disease, we examined RNAseq data from monocytes isolated from people with RA compared with healthy controls (HCs) in the absence of any stimulation (Supplementary Fig S5). We interrogated the expression of 4 gene signatures in this data set (Fig 3A); genes elevated after priming with FBG-C or LPS and rechallenged with LPS (from Fig 1C,D) and genes elevated after the FBG-C/FBG-C or FBG-C/Poly(I:C) challenge (from Fig 2E). Thirty-six percent to 41% of genes from the FBG-C/FBG-C, FBG-C/LPS, and LPS/LPS signatures, and nearly 60% of genes from the FBG-C/Poly(I:C) signature were significantly higher in RA monocytes than in HCs (Fig 3A–C). Among the 4 signatures were genes that were regulated in common by 1 or more challenge protocols, as well as genes unique to different stimuli; the overlap in genes upregulated by each challenge is shown in Figure 3D.

heatmap are shown as the mean of log10 fold change (FC) over cells that were not primed with FBG-C but were restimulated from $n = 4$ (FBG-C), $n = 5$ (LPS 0111:B4), $n = 6$ (FibG, IL-1a, and TNF), or $n = 7$ (all other stimuli) independent donors. Data were analysed using ratio paired *t*-tests vs unprimed restimulated samples, followed by multiple testing corrections using the Benjamini, Krieger, and Yekutieli methods; #corrected *P* value < .05. Mean FCs over unprimed restimulated samples are presented in the table. INTRA INF, intracellular infection. (E) Primary human monocytes were primed with 1 μ M FBG-C for 24 hours and rested for 5 days before restimulation with 1 μ M FBG-C or 10 μ g/mL Poly(I:C) for 4 hours. Cells were lysed, and transcriptomic changes were analysed by bulk RNA sequencing. Data are from 5 independent donors. The principal component (PC) analysis reveals transcriptomic changes in FBG-C- and Poly(I:C)-triggered monocytes upon priming with FBG-C. Stimulation of monocytes with FBG-C or Poly(I:C) in the absence of priming resulted in increased expression of 1905 or 212 genes, respectively. The table depicts how many of these genes were upregulated, downregulated, or not affected in cells primed with FBG-C and triggered with FBG-C or Poly(I:C). The heatmap shows significantly upregulated genes (FC > 1.5, adjusted *P* < .05, $N = 1932$) in triggered unprimed cells (vehicle control [VEH]/Poly(I:C) or VEH/FBG-C) compared with unstimulated cells (–/–), highlighting their expression in monocytes primed with FBG-C (FBG-C/FBG-C and FBG-C/Poly(I:C)). Data are VST-transformed values from 5 independent donors. VST, variance-stabilizing transformation.

Pathway analysis revealed genes specific to endogenous challenge (FBG-C/FBG-C) that were upregulated in RA monocytes, including pathways already linked to disease pathogenesis, granulocyte migration (*CCL8*, *CXCL6*, and *ADAM8*), and positive regulation of mitogen-activated protein kinase (MAPK) and transforming growth factor beta (TGF- β) signalling. The latter was associated with elevated expression of the matrix protein *THBS1*, which activates latent TGF- β and drives synovial hypertrophy in experimental arthritis [29]. Moreover, the regulation of ferroptosis was linked to elevated expression of *SLC2A3*, a glucose transporter required for glycolysis, and deletion, which is associated with protection from RA [30]. By contrast, while genes specific to FBG-C/Poly(I:C) (FBG-C/PIC) challenge were also linked to immune cell migration, a different subset was elevated, including *CCL3* and *CXCL8*, both of which influence neutrophil infiltration and activation but which also have well-documented roles in promoting osteoclastogenesis and osteolysis [31], indicating that distinct challenges modulate nonoverlapping aspects of chemokine biology. One pathway uniquely associated with FBG-C/PIC challenge comprised upregulation of negative regulators of canonical nuclear factor (NF)- κ B signalling (*NFKBIA/NFKBIZ/ZC3H12A/SOD2*), alongside induction of the noncanonical subunit *RELB*, implying a switch away from broadly proinflammatory p65/p50 subunit usage towards RelB/p50 usage, typically associated with lymphoid tissue development [32]. FBG-C/PIC challenge also upregulated *ADORA2A*, an adenosine receptor required for the anti-inflammatory effects of free adenosine created by methotrexate, polymorphisms in which genes are associated with RA patient response to glucocorticoid treatment [33]. Finally, a number of genes were common in every challenge protocol, including *ETS2*, a central regulator of human macrophage inflammation recently identified to be causative of inflammatory bowel disease [34], while only 1 gene was upregulated, which was common in any protocol involving FBG-C, but not LPS, priming: *NLPR3*, a central inflammasome component with long-established links to RA onset and progression [35] (Supplementary Fig S6, Fig 3E,F). Together, these data identify a subset of genes, enhanced in healthy monocytes following FBG-C priming, that are also significantly raised in circulating monocytes from people with RA, and uncover challenge-specific gene expression programmes associated with RA.

Distinct regulatory nodes control epigenetic reprogramming of cytokine synthesis

Monocyte memory induced by LPS is controlled via chromatin remodelling and histone modification. To better understand how FBG-C priming is mediated, we used a library of 58 small-molecule inhibitors targeting a range of epigenetic readers, writers, and erasers (<https://www.thesgc.org/chemical-probes>). Healthy monocytes were primed with LPS or FBG-C, treated with inhibitor or vehicle control (VEH), and then rechallenged with LPS after 24 hours to allow examination of early tolerance

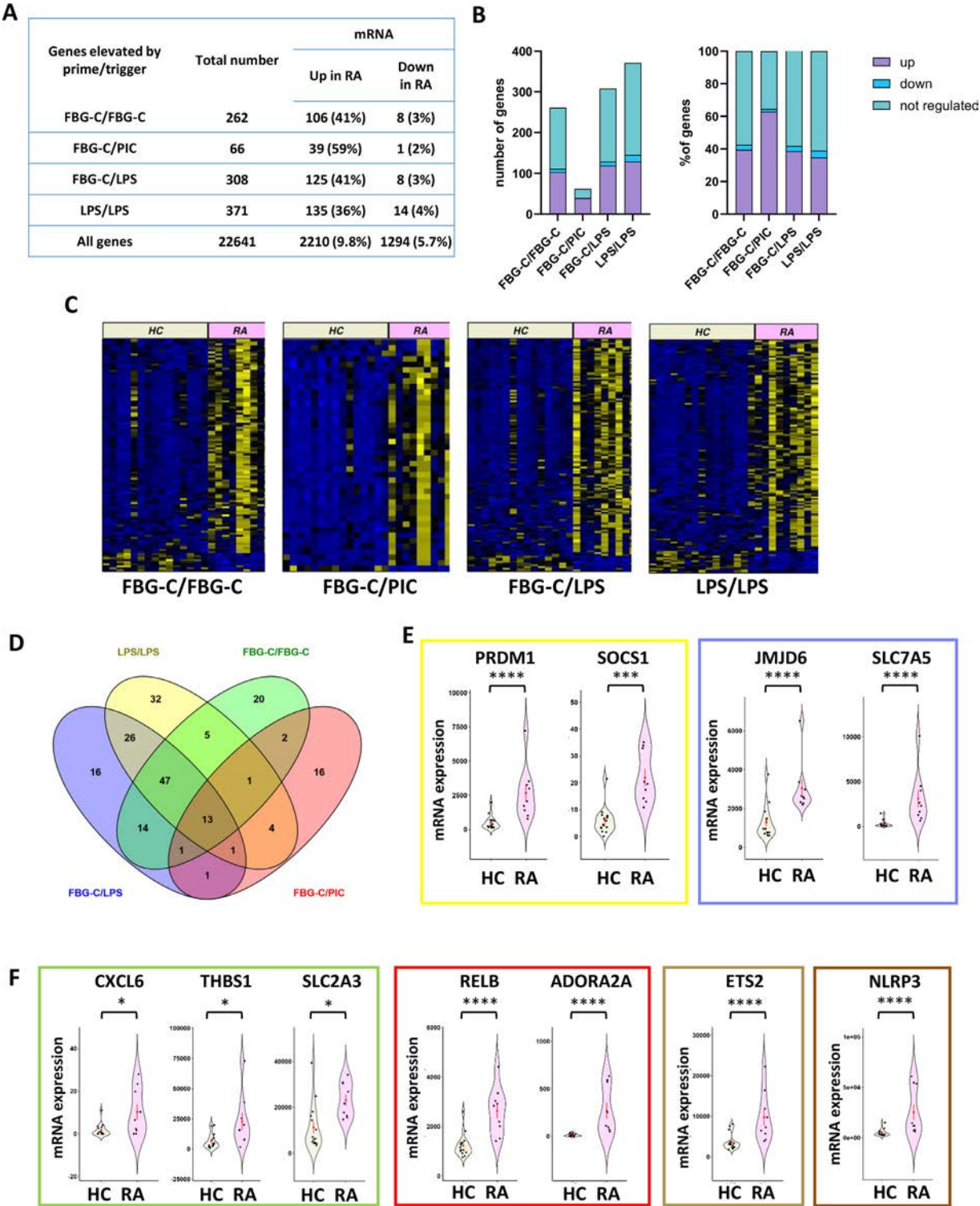


Figure 3. Innate immune memory is altered in rheumatoid arthritis (RA) monocytes at the level of gene transcription. (A) Primary human monocytes were primed with 1 μ M of the fibrinogen-like globe domain of tenascin-C (FBG-C) or 100 ng/mL lipopolysaccharide (LPS) for 24 hours, rested for 5 days before restimulation with 10 ng/mL LPS, 1 μ M FBG-C, or 10 μ g/mL Poly(I:C) (PIC) for 4 hours. Cells were lysed, and transcriptomic changes were analysed by bulk RNA sequencing. Data are from 5 independent donors. The table depicts the number of genes that are upregulated in triggered cells alone compared with nonstimulated cells and are further elevated upon priming (training signatures). In order to assess transcriptomic changes in monocytes from patients with RA, monocytes were isolated from whole blood collected from patients with active RA ($n = 9$) and healthy volunteers (HC) ($n = 15$). Immediately following isolation, with no further stimulation, RNA was isolated and sequenced. In total, 2210 genes were upregulated, and 1294 genes were downregulated (adjusted P [Padj] $< .05$, absolute $\log_2$ fold change > 0.58). The table represents the number of genes of each training signature that were also deregulated in RA monocytes. (B) Bar chart representing data from panel (A) showing the number (left bar) or percentage (right bar) of genes from each training signature (left bar) that are upregulated, downregulated, or not regulated (either not detected or not significantly regulated) in RA monocytes. (C) DESeq2-normalised gene expression heatmap highlighting the size and consistency of differences between HC and RA monocytes for genes from each training signature. Samples are given by column, and the significantly differential genes (Padj $< .05$ and absolute $\log_2$ fold change > 0.58) are given by row. The colour intensity indicates row-scaled (z-score) gene expression with blue as low and yellow as high. Rows have been hierarchically clustered using Spearman distances, while columns within each group are clustered randomly. (D) Venn diagram showing the overlap of genes in each training signature. (E,F) Normalised gene messenger RNA (mRNA) expression violin plots for statistically different genes in RA vs HC monocytes identified in the training signatures. *Padj $< .05$, **Padj $< .001$, ***Padj $< .0001$.

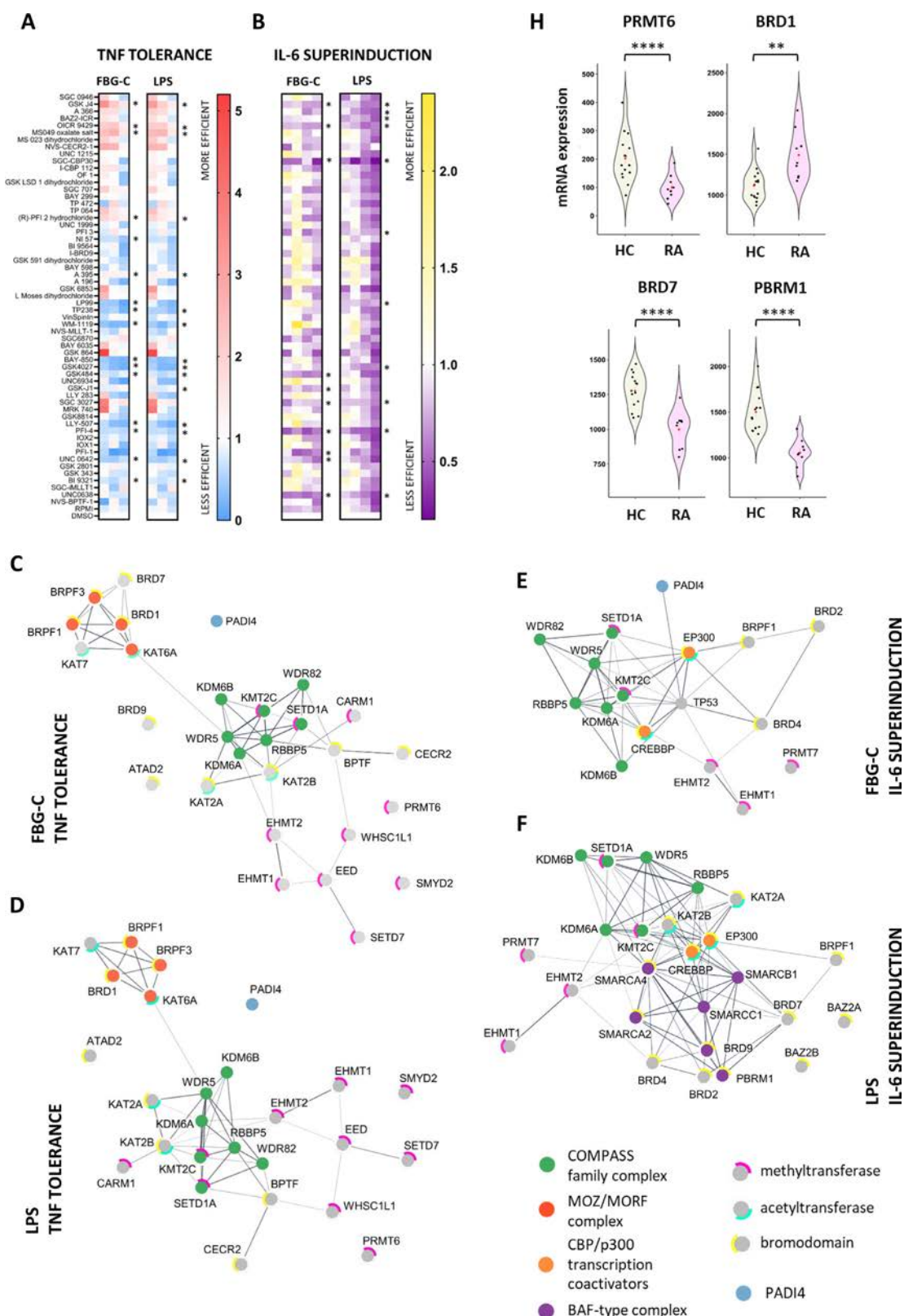


Figure 4. Epigenetic remodelling underpins innate immune memory. (A,B) Primary human monocytes were preincubated with a library of epigenetic probes for 24 hours and then primed with the fibrinogen-like globe domain of tenascin-C (FBG-C; 1 μ M) or lipopolysaccharide (LPS; 100 ng/mL) for 24 hours. Next, cells were washed and restimulated with LPS (10 ng/mL) for 24 hours; then, tumour necrosis factor (TNF) and interleukin (IL-6) release in the media was quantified by enzyme-linked immunosorbent assay. Data are shown as fold change of TNF tolerance (A) or fold change of IL-6 superinduction (B) over dimethyl sulfoxide (DMSO) preincubated control from 3 (TNF) or 4 (IL-6) independent donors. Data were analysed using a paired *t*-test vs DMSO preincubated control; **P* < .05. (C-F) STRING protein–protein interaction networks of the targets of epigenetic probes significantly alter the establishment of TNF tolerance and IL-6 superinduction and the 5 best-predicted interaction partners. Nodes are coloured according to their specific chromatin remodelling complexes and/or their catalytic functions. The thickness of the connecting lines indicates the degree of confidence in the predicted functional association between the molecules. (H) Monocytes were isolated from whole blood collected from patients with active RA (*n* = 9) and healthy volunteers (HC) (*n* = 15). Immediately following isolation, with no further stimulation, RNA sequencing was performed. Normalised gene messenger RNA (mRNA) expression violin plots for a selection of transcriptional regulators that were significantly changed in RA vs HC monocytes. **Adjusted *P* (Padj) < .01, ****Padj < .0001.

and superinduction events. Protein targets of inhibitors that significantly affected TNF tolerance (Fig 4A) and IL-6 superinduction (Fig 4B) were identified (Supplementary Table S1). Pathway analysis of proteins linked to TNF tolerance and IL-6 superinduction induced by both FBG-C and LPS was predominantly associated with chromatin structure and histone modification, as expected. Interestingly, biological processes overrepresented during IL-6 superinduction by FBG-C specifically required proteins involved in the upregulation of TGF- β signalling pathways, while those induced by LPS centred more around cellular metabolism (Supplementary Fig S7A,B). Interaction networks of target proteins required for TNF tolerance downstream of LPS and FBG-C priming were characterised by 2 major nodes: the COMPASS family of histone methyltransferases and MOZ/MORF histone acetyltransferase complexes. PAD4, a peptidyl arginine deiminase, was also required for efficient TNF blockade (Fig 4C,D). Interaction networks for IL-6 superinduction downstream of both FBG-C and LPS priming were also characterised by COMPASS family proteins, along with the transcriptional coactivators CREBBP/EP300. LPS-mediated superinduction specifically required the chromatin remodelling BAF complexes, and exhibited an overall dependence on bromodomain function, while FBG-C-driven superinduction required PAD4 (Fig 4E,F). Among the 33 inhibitor targets identified, the expression of 14 of these genes was significantly regulated in HC vs RA monocytes. Higher levels of the COMPASS complex lysine demethylase *KDM6b*, which removes repressive H3K27 gene marks, and lower levels of *EHMT2* and *PRMT6*, which add repressive histone marks, were found in RA cells. Similarly, bromodomains from the MOZ/MORF complex were differentially regulated, with higher levels of activating complex members (*BRPF3*, *BRD1*, and *BRD4*) and lower levels of the transcriptional repressor *BRD7* in people with RA. Finally, chromatin remodelling genes were also altered, including members of the BAF complex, *PBRM1*, *SMARCA2*, and *SMARCA4*, which were all downregulated in RA cells (Supplementary Fig S8, Fig 4H). These data highlight differences in how infectious or endogenous TLR4 agonists control chromatin accessibility and identify enhanced levels of specific mediators in RA monocytes, consistent with published data [36] and our observation that RA cells exhibit altered basal gene expression but also respond differently to further activation (Supplementary Fig S9).

Altered epigenetic responses in RA

To determine whether the altered expression of epigenetic modulators we observed in RA cells was accompanied by altered histone modification, we examined chromatin immunoprecipitation followed by sequencing (ChIP-seq) data generated from the same RA and HC donors as RNAseq data. For each gene signature from 4 challenge protocols (FBG-C/FBG-C, FBG-C/PIC, FBG-C/LPS, and LPS/LPS), we assessed: (1) H3K4me3 peaks (active), (2) H3K27me3 peaks (repressed), and (3) H3K4me3 peaks that also have H3K27me3 peaks present (bivalent; 'primed' or poised for rapid activation). There was no significant difference in H3K27me3 marks for any of the genes assessed, but a number had elevated active H3K4me peaks, among which a large proportion were also bivalent (Fig 5A-C). Specific to the FBG-C/FBG-C challenge, *PRDM8* bivalency was elevated in RA cells. This histone methyltransferase was previously shown to be required for monocyte training by oligomeric S100A4 and was associated with elevated plasma chemokine levels and therapy resistance in RA [36], suggesting common pathways are deployed across different endogenous TLR4 agonists. Unique to the FBG-C/PIC challenge was

myristoylated alanine-rich C-kinase substrate *MARCKSL1*, a membrane-associated protein that is a substrate for protein kinase C, which promotes macrophage migration, cytokine synthesis, and MAPK and JAK/STAT signalling [37]. Both FBG-C/FBG-C and FBG-C/PIC protocols were linked to elevated *MMP9* bivalency, which is a protease that degrades cartilage and remodels the joint in RA [38].

Genes whose bivalency increased following priming with FBG-C, but not LPS, included *ASS1* (an enzyme responsible for arginine biosynthesis required to meet the metabolic demands of proliferation, protein synthesis, and nitric oxide synthase (NOS) production in activated macrophages [39]), *CEMIP* (a hyaluronic acid [HA] catabolising enzyme that hydrolyses high-molecular-weight HA polymers to intermediate-sized products, creating TLR4 agonists [40] and is upregulated in serum, synovial, fluid, and synovial tissue from people with RA, where levels are associated with disease severity and cartilage loss [41]), *GATA2* (zinc-finger transcription factor, a master regulator of monocyte/macrophage differentiation, but is also emerging as a potent proinflammatory mediator enabling enhanced cellular response to cross-PRR stimulation [42]), and *RORA* (NR1 family nuclear hormone receptor regulating clock gene transcription is required for TNF-induced stimulation of *BMAL1* expression in RA synovial tissue, and dysregulation is linked to worse symptom severity in people with RA [43]). While the large majority of gene bivalency detected in our gene signature was increased in RA, a handful exhibited lower bivalency in RA cells (eg, *POU2F2*, a transcription factor necessary for escape from oncogene-induced senescence). Moreover, the bivalent modification does not always have a positive effect on gene expression but can also repress transcription (eg, *DACT3*, a negative epigenetic regulator of Wnt/ β -catenin signalling), suggesting that bivalency at this locus in RA monocytes may contribute to uncontrolled Wnt pathway activation. Together, these data suggest that RA monocytes contain basally higher levels of mRNA coding for permissive regulators of the COMPASS and MOZ/MORF epigenetic complexes and altered BAF complex chromatin remodellers than healthy monocytes and that RA monocytes exhibit a number of genes that are epigenetically poised.

Conservation of training signatures in synovial tissue macrophages

Data so far have been derived from circulating monocytes in healthy individuals and people with RA. To determine whether genes trained in these cells are expressed in synovial tissue myeloid cells, we examined single cell RNA sequencing data from biopsy tissue from people with RA and HCs [44,45]. Signatures comprising genes trained following each of the 4 challenge protocols (Fig 3A) and a combined signature comprising all genes that were poised following any challenge protocol (Fig 5A) were detected in synovial tissue myeloid cells. In general, across all signatures, genes were more enriched in tissue-infiltrating macrophage populations (M4-M8) and less abundant in MERTK + tissue-resident macrophages (M0-M3). A comparison of cells across disease status revealed that all 5 signatures were elevated in M6, M7, and M8 macrophages from people with RA, with the most significant enrichment in M7 IL-1 + macrophages (Fig 6A, Supplementary Fig S10). To corroborate these data, we examined the same signatures in synovial tissue macrophages from an independent scRNA sequencing dataset comprising cells isolated from biopsies from HCs, people with active RA, and people whose RA is in remission [46]. In this analysis, trained gene signatures were also enriched in the IL-1 + macrophage subset

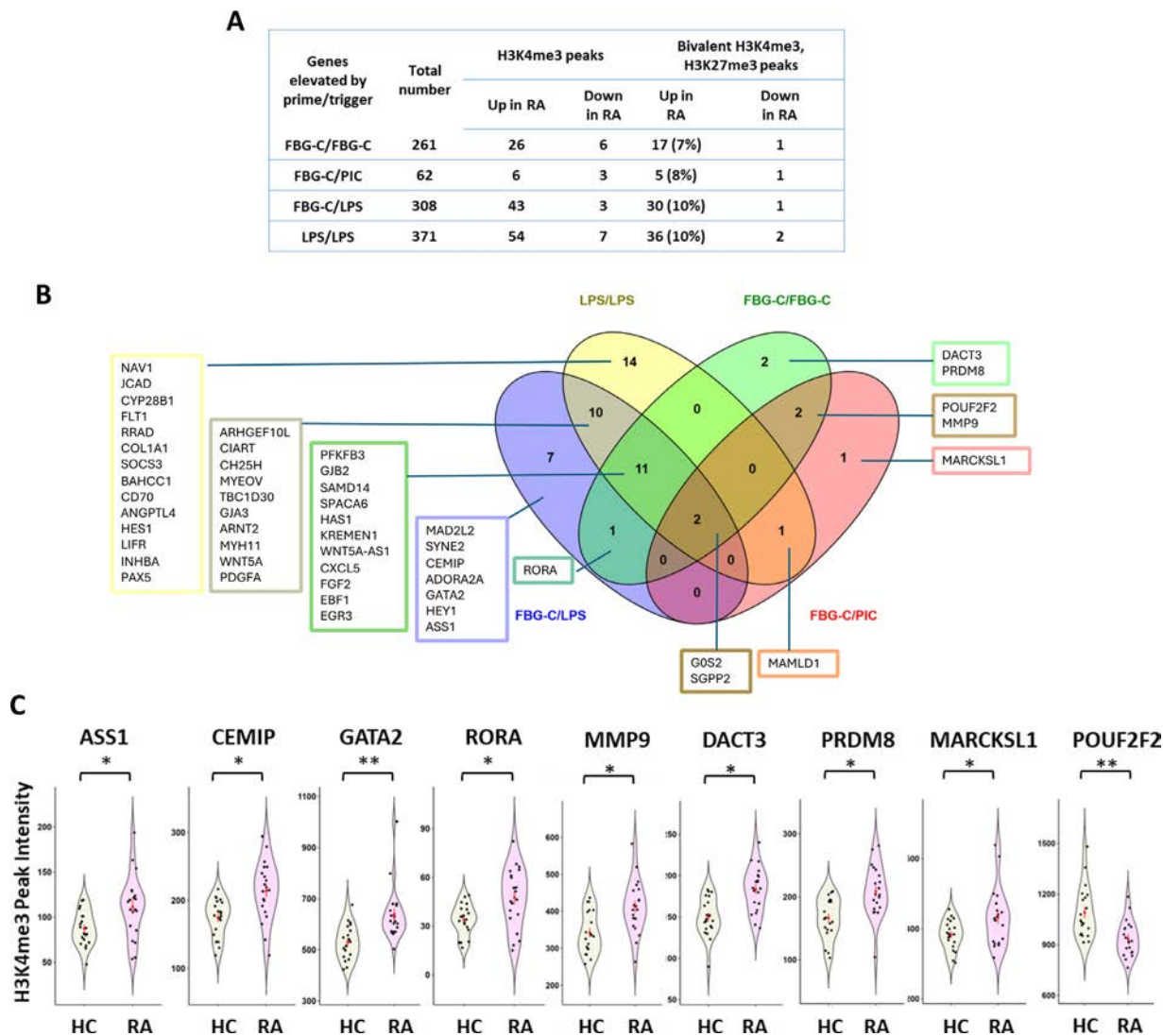


Figure 5. Innate immune memory is altered in rheumatoid arthritis (RA) monocytes at the epigenetic level. Monocytes were isolated from whole blood collected from patients with active RA ($n = 9$) and healthy volunteers (HC) ($n = 15$). Immediately following isolation, with no further stimulation, chromatin immunoprecipitation followed by sequencing (ChIP-seq) was performed. (A) Table depicting the number of genes that harbour H3K4me3 peaks alone or are bivalent in each training signature. (B) Venn diagram showing the overlap of bivalent genes between different training signatures. (C) Normalised H3K4me3 peak intensity violin plots for statistically different genes in RA vs HC monocytes identified in the training signatures. *Adjusted P (Padj) $< .05$, **Padj $< .01$. FBG-C, fibrinogen-like globe domain of tenascin-C; LPS, lipopolysaccharide; PIC, Poly(I:C).

analogous to M7 (CD48 + S100A12 + IL-1 + macrophages), and expression was higher in people with active RA compared with HCs. Interestingly, in IL-1 + synovial macrophages, a subset of trained genes remained elevated in people whose disease was in remission compared with active disease (including *VEGF*, *CCL3*, and *MARCKSL1*), while some were no longer elevated in remission (including *CCL2*, the ion channels *SLC7A5* and *SLC25A37*, and *SOCS3*) (Fig 6B, Supplementary Fig S11). These data indicate that genes trained following the challenge of circulating monocytes are also elevated in monocytes that have infiltrated the RA synovium and, in particular, the proinflammatory IL-1 + subset. This population uniquely persists in tissue from people whose disease is in remission and is associated with disease flare [47]. These data highlight new pathways that may help to more fully understand the complex pathogenesis of RA and the recurrence of disease during episodes of flare.

DISCUSSION

RA is an incurable condition affecting ~1% of the population. A major cause of chronic inflammation in this disease is

unrestrained monocyte influx and activation in the synovium, resulting in persistent synthesis of proinflammatory and tissue-destructive mediators. Current treatments demonstrate life-changing efficacy for some people, but not the majority, and prolonged suppression of global immune responses, including cytokines such as TNF and IL-6, or lymphocytic cell populations, frequently results in side effects and opportunistic infections [48]. Emerging data show that trained responses in monocytes from people with RA become unchecked early in disease development, contributing to exacerbated inflammatory signalling and potentiated cell activation. However, the mechanisms underpinning this loss of control remain poorly characterised. Here, we use healthy human monocytes and monocytes from people with RA to examine how endogenous immune stimuli programme myeloid memory, uncovering trained behaviour that is distinct from adaptation caused by exposure to infectious immune triggers, and highlighting novel potential points of intervention that could offer disease-specific treatment.

We found that the TLR4-activating FBG domain of tenascin-C induces innate immune memory, evoking both suppressive and potentiated cell adaptation. Comparative analysis with

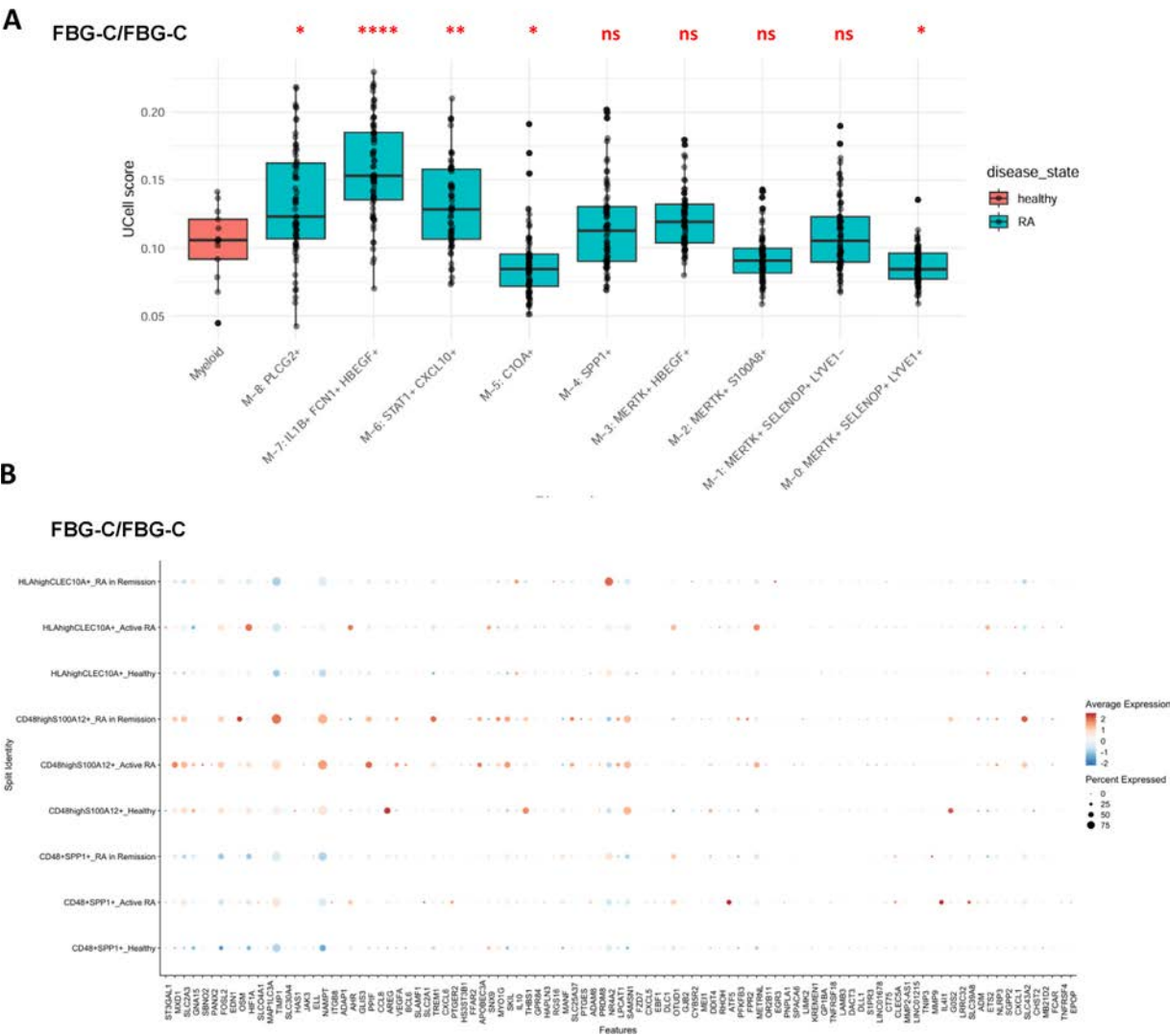


Figure 6. Trained gene expression is enriched in tissue-infiltrating macrophages in the rheumatoid arthritis (RA) synovium. (A) Box plot displays UCell gene scores for healthy and RA synovial myeloid populations from published single-cell datasets. Cells were pseudobulked by sample and cluster (RA, $n = 70$ patients and $n = 73$ samples) or by donor (healthy $n = 13$) and scored using genes associated with the FBG-C/FBG-C challenge protocol. The IQR contains 50% of the data, and the median values are displayed; each dot represents a sample (RA) or donor (healthy). Significance was calculated using a Wilcoxon test, with P values $< .05$ considered significant. * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$, ns, not significant. (B) Dot plot of genes trained by the FBG-C/FBG-C challenge protocol in tissue-infiltrating monocyte/macrophage subsets identified in the Alivernini single-cell RNA sequencing (scRNAseq) dataset. Dot size represents the percentage of cells in the cluster expressing the gene of interest, and dot colour represents its level of expression. Cell clusters are split by sample condition. FBG-C, fibrinogen-like globe domain of tenascin-C.

infectious TLR4 activation identified altered gene expression that was common to FBG-C and LPS, as well as unique to either challenge. In line with known mechanisms of LPS-induced tolerance [11], alterations in chromatin accessibility and histone modification were associated with FBG-C challenge, but these stimuli did not engage identical epigenetic mediators to achieve transcriptional suppression and potentiation. While these data highlight groups of chromatin remodellers contributing to altered cytokine expression in FBG-C-primed cells, we still do not have a full understanding of the mechanisms by which endogenous immune stimuli rewire gene expression programs, nor how these mechanisms differ depending on the nature of the priming challenge. ChIP-seq analysis would be interesting in revealing transcriptome-wide epigenetic modifications, mapping these to specific gene loci in LPS- and FBG-C-primed monocytes in order to better understand stimulus-specific mechanisms underpinning innate memory. Differences in cross-protective responses following priming with LPS and FBG-C were also observed. FBG-C-activated monocytes exhibited

adapted behaviour upon rechallenge with a range of secondary stimuli, including different triggers of the same receptor (LPS: TLR4) and agonists of different receptors (eg, TLR3, NOD2, and TNFR). However, chemokine synthesis, while suppressed following bacterial ligand activation of cell surface PRRs, was potentiated following rechallenge with agonists of intracellular PRRs and endogenous stimuli, including TNF and oxLDL. An in-depth analysis of monocyte adaptation following FBG-C priming and FBG-C or Poly(I:C) rechallenge revealed widespread gene training in pathways that were not affected by LPS priming. Distinct primary responses of myeloid cells to LPS and endogenous TLR4 triggers have been widely reported, with FBG-C and LPS inducing overlapping but distinct phenotypes in monocyte-derived macrophages [49]. Mechanistically, different responses are likely mediated by differences in receptor avidity, differential coreceptor usage, and/or distinct downstream ligand-receptor processing. MD-2 and CD14, while required for LPS signalling, are dispensable for FBG-C [23], and although LPS-TLR4 complexes are internalised, and can activate lysosomal

TRIF signalling before receptor recycling [50]; what happens downstream of endogenous TLR4 activation is unknown. Physiologically, stimulus-specific cross-protection may reflect the need to prevent excessive inflammation following tissue injury caused by subsequent opportunistic infection, but that also allows for functional nonredundancy among inflammatory signalling. In this case, signalling by cell surface TLRs may overlap sufficiently to warrant further activation, which is unnecessary and even detrimental in the presence of further threat. Alternatively, signalling by intracellular PRRs or cytokine receptors may benefit from the potentiation of selected effectors; for example, a 440-fold increase in the secretion of the neutrophil chemoattractant IL-8 following TLR3 rechallenge suggests that FBG-C-activated cells are primed to evoke a targeted granulocytic antimicrobial response to ensure rapid defence against microbial invasion or removal of necrotic tissue at sites of tissue damage, while tissue injury after yeast infection does not potentiate trained responses. Some reports document that low doses of LPS can induce potentiation of TNF in human monocytes [51]; however, examination of a wide range of concentrations of FBG-C, including quantities no longer capable of eliciting cytokine release in naïve cells, did not result in enhanced TNF synthesis (Supplementary Fig S12). Instead, we find that FBG-C priming results in trained monocyte behaviour in genes beyond these canonical proinflammatory cytokines, including those linked to disease pathogenesis in RA.

While the benefit of FBG-C-induced myeloid adaptation lies in robust response to tissue damage, examination of endogenously trained gene signatures in RA monocytes shows that this process becomes maladapted in disease. Inarguably, long-term culture of monocytes *ex vivo*, even in human growth factor-supplemented models designed to mimic tissue maturation of circulating cells, is liable to artefact. While healthy monocyte viability was not affected, we observed a decrease in monocyte density over time, independent of cell stimulation (Supplementary Fig S13), which was not apparent at days 1 to 3. Neither cellular nor murine models recapitulate events occurring in long-term autoimmune diseases that can develop over many years; therefore, we used circulating immune cells isolated from people with RA to directly assess memory states in disease. We asked whether genes trained by endogenous challenge protocols, using matrix-derived TLR4 agonists (FBG-C/FBG-C) and TLR3 agonists released from damaged RA synovium (FBG-C/PIC), are dysregulated in disease. RA monocytes displayed elevated basal mRNA levels of a large proportion of FBG-C/FBG-C and FBG-C/PIC trained genes and higher basal levels of permissive methylases and bromodomains, combined with lower levels of repressive methylases and bromodomains and/or higher levels of H3K4me at bivalent loci. These data confirm that RA monocytes are trained *in vivo* and that this preprogramming conspires towards exacerbated inflammation in these individuals while revealing for the first time that a large subset of genes trained only by endogenous challenge form part of this memory response. Reprogramming of myeloid precursors in the bone marrow by infectious (β -glucan) and endogenous (Western diet) triggers results in long-term trained phenotypes of mature myeloid cells [20,52]. We observed high serum tenascin-C levels in people with RA compared with HCs, suggesting a mechanism of extra-articular priming of circulating cells by tenascin-C, which, upon subsequent arrival in the inflamed synovium, are rechallenged by locally and persistently elevated tissue tenascin-C, self-nucleic acids released in areas of tissue damage, and/or high levels of TNF. Indeed, we find trained gene signatures are significantly enriched in tissue-infiltrating RA synovial macrophages, in particular in subsets linked to disease flare.

Examination of trained signatures specific to endogenous stimuli in RA monocytes identified genes known to contribute to disease pathogenesis. Genetic changes in *NLRP3*, *SLC2A3*, and *RELB* are linked with an increased risk of developing RA and *PDRM8*, *SLC7A5*, *CEMIP*, and *MARCKSL1* have been proposed as biomarkers of inflammation and joint tissue damage in RA. Moreover, elevated *PDRM8* correlates with poor response to multiple treatments, and *MIF* counteracts the anti-inflammatory activity of glucocorticoids. These data suggest mechanisms by which people with RA with high synovial tenascin-C and/or tissue damage might fare worse in response to existing therapies, while upregulation of *ADORA2*, an adenosine receptor required for effective methotrexate signalling, may identify a subset of patients that may instead do well with glucocorticoid treatment. New themes underpinning disease development driven by endogenous monocyte training also emerged. These include remodelling of the extracellular matrix via TGF- β -mediated fibrosis and altered HA catabolism, suggesting that endogenous monocyte challenge may promote a more fibrotic endotype with poorer outcomes than other cell-type abundance phenotypes (CTAPs) [45] and/or that it may contribute to a persistent cycle of inflammation where deposition of tenascin-C causes proteolytic generation of small HA fragment TLR4 agonists, perpetuating high synovial DAMP levels. Another theme centred on metabolic changes, including altered solute carrier expression controlling glucose transport (*SLC2A3*) and leucine influx (*SLC7A5*), as well as the sodium hydroxide exchanger *SLC9A8*, whose cellular role is unknown, and altered *ASS1*, a key checkpoint in arginine biosynthesis. Finally, elevated expression of a number of genes linked to ferroptosis, including *C2CD48*, *SLC39A14*, *SAT1*, *SLC39A8*, and *MAP1LC3A*, implicates endogenous monocyte training in exacerbated iron-dependent programmed cell death and associated inflammation, oxidative stress, and mitochondrial dysfunction, linked to the development and progression of RA [53]. Identification of pathways potentiated by endogenous, but not infectious, immune stimuli, which are elevated in RA monocytes and enriched in flare-associated synovial macrophages, moves us closer to the development of safer, more effective points of disease intervention.

METHODS

Cell isolation and stimulation

Primary human monocytes from healthy donors were isolated from leukocyte cones purchased from NHS BT at the John Radcliffe Hospital, Oxford (catalogue code NC24; LREC/NREC number 11/H0711/7). Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient separation over His-topaque (Sigma), followed by the removal of contaminating red blood cells with ammonium–chloride–potassium (ACK) Lysing Buffer (Gibco). Monocytes were isolated from the purified PBMC fraction by immunomagnetic negative selection using the EasySep Human Monocyte Enrichment Kit without CD16 Depletion (STEMCELL Technologies), according to the manufacturer's instructions. Cell purity was confirmed by flow cytometry, and the resulting monocytes had a purity of 85% or greater, containing 0.2% or fewer CD3+ T cells, 3% or fewer CD19+ B cells, and 9% or fewer CD56+ NK cells. Monocytes were plated in 96-well plates at 1×10^5 cells/well in RPMI 1640 (Dutch modified) supplemented with 2 mM GlutaMAX, 1 mM sodium pyruvate, and 50 μ g/mL gentamicin (all reagents from Gibco) and rested for 1 hour before stimulation. Primary stimulation (prime) was performed by the addition of stimuli to the serum-free medium.

After priming, the medium was collected, and cells were washed in PBS and left to rest in medium supplemented with 10% pooled human serum (type O, kindly provided by Dr Henk Schipper, Kennedy Institute, University of Oxford). After resting, the medium was collected before the addition of the secondary stimulus (trigger) in medium containing 10% human serum. Cell stimuli were used at the concentrations listed below, and for each experiment, the length of the prime, rest, and rechallenge steps is stipulated in the text and the figure legend. Cell viability following stimulation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Invitrogen) and CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) according to the manufacturer's instructions.

Stimuli

Ultrapure LPS from *E coli* serotype O55:B5 was purchased from Sigma (catalogue number L4524). β -glucan was kindly provided by Prof David L Williams (East Tennessee State University). The FBG-C and fibrinogen γ (FIB- γ) were synthesised, purified, and validated as described [23]. Preparation of recombinant proteins included on-column washing of bound proteins with Triton X-114 to remove endotoxins. Endotoxin levels in purified protein samples were tested using the Recombinant Factor C Assay (Lonza) according to the manufacturer's instructions. Protein preps containing less than 10 pg/mL of endotoxin were used in this study; 10 pg/mL of LPS *E coli* serotype O55:B5 neither induces primary cytokine secretion nor tolerance to rechallenge (Supplementary Fig S1B,C). All protein preps were preincubated for 30 minutes with 10 μ g/mL polymyxin B (Sigma) on ice before adding to cells. Where stated, protein preps were treated with PK (Sigma) for 1 hour at 37°C, followed by boiling for 15 minutes. FBG-C (5–10^{−6} μ M), LPS (100–10^{−4} ng/mL), and β -glucan (100–0.1 μ g/mL) were used to both prime and rechallenge cells at various concentrations, as stipulated in the text. Where stated, cells were rechallenged with FIB- γ (1 μ M), Pam3CSK4 (1 μ g/mL), Poly(I:C) HMW (10 μ g/mL), ultrapure flagellin from *Salmonella typhimurium* (1 μ g/mL), muramyl dipeptide (10 μ g/mL), CpG oligonucleotides (ODN 2006; 5 μ M), heat-killed *Candida albicans* (10⁶ cells/mL), heat-killed *E coli* (10⁶ cells/mL), and heat-killed *Staphylococcus aureus* (10⁶ cells/mL) (all purchased from Invivogen), LPS from *E coli* serotype EH100 (Ra) (TLR grade) (10 ng/mL, Enzo Life Sciences), LPS from *E coli* O111:B4 (100 ng/mL, Sigma), oxLDL from human plasma (10 μ g/mL, Invitrogen), recombinant human TNF- α (10 ng/mL, BioLegend), and recombinant human IL-1 α (10 ng/mL, STEMCELL Technologies).

Cytokine measurements

TNF, IL-6, and IL-8 secretion in media following stimulation was detected by enzyme-linked immunosorbent assay (ELISA; BD Pharmingen).

For transcriptional analysis, monocytes were plated in 96-well plates at 1 $\times$ 10⁵ cells/well in RPMI 1640 (Dutch modified) supplemented with 2 mM GlutaMAX, 1 mM sodium pyruvate, and 50 μ g/mL gentamicin (all reagents from Gibco) and stimulated as described above. RNA was isolated after stimulation using the RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. Next, 30 to 50 ng of the isolated RNA was reverse transcribed into complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). TNF and IL-6 mRNA were quantified by standard curve quantitative PCR (qPCR) using TaqMan Universal PCR Master Mix and FAM-conjugated TaqMan Gene Expression

Assays, with GAPDH as a control reference gene (Hs00174128_m1, Hs00174131_m1, and Hs02758991_g1) (all from Applied Biosystems). qPCR was performed using the ViiA7 instrument (Applied Biosystems) according to the manufacturer's recommendations. qPCR data were analysed using the delta Ct method; relative expression values are presented.

RNAseq of stimulated monocytes

Monocytes seeded in 12-well plates (1.09 $\times$ 10⁶ cells/well) were isolated and stimulated as described above, and RNA was isolated after stimulation using the RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. Quality controls and sequencing of RNA samples were performed by Novogene Europe. mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. After fragmentation, the first-strand cDNA was synthesised using random hexamer primers, followed by second-strand cDNA synthesis using dTTP for the nondirectional library. For the nondirectional library, it was ready after end repair, A-tailing, adapter ligation, size selection, amplification, and purification. The library was checked with a Qubit, real-time PCR was used for quantification, and a bioanalyser was used for size distribution detection. Sequencing was performed using the NovaSeq X Plus Series (PE150) with 20 million reads using >200 ng RNA. Raw data (raw reads) in fastq format were processed through Novogene's in-house Perl scripts for quality control purposes, which confirmed the high quality of the sequencing data.

Raw fastq files were aligned to the human genome (build GRCh38) and quantified by calling salmon (version 1.10.0) via the txseq pipeline (<https://txseq.readthedocs.io/en/latest/index.html>). Salmon counts were imported into R (version 4.3.1, R Foundation for Statistical Computing) and aggregated at the gene level using tximeta (version 1.20.3 [54]) and drawing from Ensembl version 110 for annotations and transcript-to-gene conversion. Lowly expressed genes (ie, which did not show at least 5 read counts in at least 2 distinct samples across the whole dataset) were removed at this stage, leaving a total of 21,528 genes for downstream analyses. Independent pairwise differential expression analyses (DEA) were performed comparing (1) 'nonstimulated/trigger' vs 'nonstimulated/nonstimulated' and (2) 'prime/trigger' vs 'nonstimulated/trigger' where 'prime' and 'trigger' may be 1 of FBG-C, LPS, or Poly(I:C), and 'nonstimulated' corresponds to no treatment or treatment with a VEH performed in R (version 4.4.0) using DESeq2 (version 1.44.0 [55]). For each comparison, the DESeq2 model controlled for the donor of the sample to enable paired testing. Log2 fold change (LFC) values estimated by DESeq2 were corrected using the ashR shrinkage estimator (version 2.2-63 [56]). Significance in differential expression between 2 conditions was defined as an adjusted *P* value (*Padj*) < .05 and |LFC| > 0.58. Follow-up analysis focused on genes that were significantly upregulated by the trigger stimulus in the absence of priming, ie, in the 'nonstimulated/trigger' vs 'nonstimulated/nonstimulated' comparison (trigger-induced genes). To analyse if trigger-induced genes are significantly further upregulated, downregulated (*Padj* < .05, |LFC| > 0.58), or not regulated (*P* value $\geq$.05) in primed cells, DEA was performed as described above by comparing 'prime/trigger' vs 'nonstimulated/trigger' conditions. Since all DEAs were performed independently, a small subset of genes that were tested by DESeq2 in the 'nonstimulated/trigger' vs 'nonstimulated/nonstimulated' comparison were not expressed in all conditions tested and thus were excluded from the analysis. The set of genes that was

upregulated by the trigger at baseline and further upregulated upon priming formed the training signature. Four different training signatures were generated for the different sets of prime/trigger pairs assessed in this study. To analyse how memory responses overlap between different prime/trigger pairs, it was assessed which genes are commonly or differently upregulated, downregulated, or not regulated. In the case of comparing memory responses between FBG-C/Poly(I:C) vs VEH/Poly(I:C) and FBG-C/FBG-C vs VEH/FBG-C, we focused on the intersection of genes, i.e., genes that were significantly upregulated by both triggers at baseline and, therefore, whose memory response could be assessed in both conditions. Heatmaps were generated with pheatmap (version 1.0.12) and display the mean Variance-Stabilizing Transformation (VST)-normalised counts, scaled by row (gene); the associated hierarchical clustering for both rows and columns was derived from Pearson's distances in vst-normalised values, calculated using WGCNA::cor (version 1.72-5). Overrepresentation analyses were run using gsFisher [57] and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Gene Ontology (Biological Processes subset) as resources, and the gene background was defined as all genes that could be tested in both the contrasted DEA(s) and that were significantly upregulated at baseline (as detailed above).

Epigenetic probes

A library of 58 epigenetic probes was obtained from the Structural Genomics Consortium (Supplementary Table S2). Monocytes were plated in 96-well plates at 1×10^5 cells/well in RPMI 1640 (Dutch modified) supplemented with 2 mM Glutamax, 1 mM sodium pyruvate, and 50 µg/mL gentamicin (all reagents from Gibco). Cells were rested for 1 hour and then preincubated with each probe at 1 µM in 0.01% dimethyl sulfoxide (DMSO) in PBS, 0.01% DMSO in PBS alone, or left untreated for 24 hours. At the end of the preincubation period, FBG-C at 1 µM or LPS O55:B5 at 100 ng/mL was added for 24 hours (prime). The medium was collected, cells were washed, and fresh medium supplemented with 10% human serum was added. Cells were restimulated with LPS O55:B5 at 10 ng/mL for 24 hours (trigger), medium was collected, and an MTT assay was performed. TNF and IL-6 secretion in the media was quantified by ELISA. Measured cytokine concentration was normalised to MTT, and TNF tolerance was calculated for each probe as a decrease in normalised TNF secretion between control cells that were not primed but received a trigger, and primed and triggered cells. IL-6 superinduction was calculated for each probe as an increase in IL-6 secretion between unprimed triggered cells and primed triggered cells. Data were presented as fold change of TNF tolerance and IL-6 superinduction over DMSO control. In this experiment, 1 out of the 4 donors did not make any detectable TNF in response to activation and was excluded from the analysis.

Memory assay in RA monocytes

Whole blood was obtained from 14 patients with RA, fulfilling the 2010 American College of Rheumatology (ACR)/The European Alliance of Associations for Rheumatology (EULAR) criteria for RA. Participants recruited were treatment-naïve or treated with hydroxychloroquine, methotrexate, or both. None of the subjects were receiving biologic disease-modifying drugs. Samples were obtained with written, informed consent and approval from the Black Country Research Ethics Committee (12/WM/0258) in compliance with the Declaration of Helsinki.

Control blood obtained from 6 healthy volunteers was purchased from Cambridge Bioscience (REC R73570/RE001) (Supplementary Table S3). All samples were collected into EDTA-coated tubes and transported in ambient conditions. RA and control monocytes were isolated and stimulated, and cytokine secretion in culture media was quantified by ELISA as described above.

RNAseq of unstimulated monocytes

Peripheral blood was collected from 10 patients with RA at the Gartnavel General Hospital Rheumatology Unit, Glasgow. The inclusion of RA samples required patients to have active disease, defined as a disease activity score (DAS) 28 score > 2.7. Five HCs were obtained from age-matched volunteers at the University of Glasgow. Informed consent was obtained prior to sample collection alongside appropriate ethical approval (West of Scotland REC 4 approval 19/WS/0111, project reference CG_2019_09_A_AM01), and all donor information was treated anonymously immediately after collection (Supplementary Table S3). PBMCs were separated from blood samples by Ficoll-Paque density centrifugation (Sigma Aldrich). CD14⁺ monocytes were magnetically isolated from the PBMCs using the EasySep Human CD14 Positive Selection Kit II (STEMCELL Technologies). Isolated CD14⁺ monocytes were lysed using an RNA lysis buffer containing β-mercaptoethanol (ThermoFisher), and RNA was extracted using the PureLink RNA Mini Kit (Invitrogen). Alongside this, 10 additional age-matched HC CD14⁺ monocyte RNA samples were collected at the University Medical Centre Utrecht, the Maasstad Medical Centre Rotterdam, by a collaborator at Utrecht University, the Netherlands. RNA samples consisting of 10 RA patients and 15 HCs were sequenced by GenomeScan BV, based in Leiden, the Netherlands. RNA sample libraries were prepared using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (New England BioLabs). RNA-seq was performed using the NovaSeq 6000 (Illumina), paired-end 150 bp, 20 million reads, using 1.1 nM of DNA. The reads generated by RNAseq were aligned to the reference genome (GRCh38.p13, Ensembl) using Spliced Transcripts Alignment to a Reference (STAR) (version 2.7.3) with default settings. DESeq2 was then used to normalise read counts and identify differentially expressed genes between sample groups.

Chromatin immunoprecipitation (ChIP) of unstimulated monocytes

Monocytes from the same 10 RA patients and 15 HCs used for RNAseq in the section above were fixed in 1% methanol-free formaldehyde (Thermo Fisher Scientific) for 10 minutes at room temperature and immediately quenched using 125 mM glycine for 5 minutes. Cells were collected by centrifugation at 4 °C. Pelleted cells were washed 3 times with ice-cold Dulbeccos PBS (Gibco, Thermo Fisher Scientific) and lysed in lysis buffer (20 mM Hepes pH 7.6, 1% sodium dodecyl sulfate [SDS], 1× protease inhibitor cocktail, and 10 mM sodium butyrate). Chromatin samples were sonicated for 12 ± 2 cycles of 30 seconds ON/30 seconds OFF with the Bioruptor Pico (Diagenode) until most of the DNA was sheared into 200- to 600-bp-long fragments. An aliquot was taken from each of the sonicated chromatin samples to use as a 10% input.

For each immunoprecipitation (IP), 20 µL of Dynabeads Protein A (Invitrogen, Thermo Fisher Scientific) was used. For antibody conjugation, beads were washed in chromatin immunoprecipitation (ChIP) dilution buffer (1% Triton X-100, 1.2 mM EDTA,

16.7 mM Tris-HCl pH 8, and 167 mM NaCl) containing 0.1% SDS and 0.1% BSA and incubated with either the H3K4me3 or H3K27me3 antibody (Merk Millipore, 1.5 µg/IP) in the same buffer for 1 hour at room temperature with rotation. After conjugation, beads were washed, and the sonicated chromatin added; conjugated beads and chromatin were incubated in ChIP dilution buffer on a rotator for 3 hours at 4 °C. After incubation, beads were washed once with ChIP washing solution 1 (2 mM EDTA, 20 mM Tris-HCl pH 8, 1% Triton X-100, 0.1% SDS, and 150 mM NaCl), twice with ChIP washing solution 2 (2 mM EDTA, 20 mM Tris-HCl pH 8, 1% Triton X-100, 0.1% SDS, and 500 mM NaCl), and twice with ChIP washing solution 3 (1 mM EDTA, 10 mM Tris-HCl pH 8). Finally, the beads were eluted in 100 µL elution buffer (0.5% SDS, 300 mM NaCl, 5 mM EDTA, and 10 mM Tris-HCl pH 8) containing 400 µg/mL recombinant PK (Thermo Fisher Scientific). Decrosslinking was done by incubating the ChIP or 10% input samples at 55 °C for 1 hour, followed by overnight at 65 °C. The supernatant containing the immunoprecipitated DNA or input DNA was purified using the Qia-gen MinElute PCR Purification Kit, following the manufacturer's instructions for subsequent ChIP-seq.

ChIP-seq library preparation and sequencing

DNA libraries were prepared for sequencing using the NEB-Next Ultra II DNA Library Prep Kit for Illumina (New England BioLabs), following the manufacturer's instructions. DNA libraries were barcoded using the NEBNext Multiplex Oligos for Illumina Dual Index Primers Set 1 or Set 2 (New England BioLabs). Quality control was performed on DNA libraries using a high-sensitivity DNA ChIP (Agilent) on the 2100 Bioanalyzer Instrument (Agilent). Samples were pooled and sequenced on the NextSeq 500 platform (Illumina). Each sample was sequenced to a depth of ~30 million reads, with fragments undergoing single-end sequencing of 75 bp read lengths.

ChIP-seq data analysis

For each histone modification, the fastq files were quality-controlled using FastQC (GitHub, v0.11.7) and then aligned to the reference genome and transcriptome GRCh38 (release 102) using Bowtie2 (v2.4.4). Peaks were called using MACS2 (v2.2.8), specifying the appropriate ChIP input for each sample. The usable genome length was calculated from the reference fasta file (2,945,849,067). The called peaks across all samples and conditions were merged (for each modification separately) using BEDTools merge (v2.3) [58], creating a master list of regions containing the modification. An input table of normalised read counts per sample at each region was generated using Diffbind (v3.1.7) [59]. An initial principal component analysis (data not shown) highlighted a batch effect between the Dutch and UK samples and was corrected using the SVA package ComBat-seq [60] with the batch correction function. The fraction of reads in peaks (FRiPs) was calculated using the bowtie align reads metric and the raw read counts table. The read counts were then additionally corrected for FRiPs using the ComBat-seq batch correction function. The corrected counts were used for differential binding analysis. Differential binding analysis was performed using DESeq2 (v1.24) [55], specifying pairwise models with no additional covariates and a significance threshold of $P_{adj} < .05$. To generate bivalent regions, the BEDTools intersect was used under default settings to find regions containing both H3K4me3 and H3K27me3 in HCs. To increase statistical power for H3K4me3, the newly generated data were

combined with our previously published HC and RA dataset (GSE15291) [61], which was reprocessed using the above pipeline. Both datasets were merged after the peak calling step.

Bioinformatic analysis

Protein–protein interaction networks were generated using the STRING Database (<https://string-db.org/>). Interactions were visualised using Cytoscape (<http://www.cytoscape.org>). Functional enrichment was performed using the Gene Ontology Consortium enrichment analysis tool (<http://www.geneontology.org>). Illustrations were created using BioRender (<https://biorender.com/>). Venn diagrams were generated using Venny 2.1 (<https://bioinfogp.cnb.csic.es/tools/venny/>) [62].

Gene expression in scRNA sequencing data from synovial cells was analysed in tissue from HCs and people with active RA [44,45]. The myeloid compartment of both datasets was subsetted, and counts were pseudobulked using the AggregateExpression function in Seurat (version 5.2.1)(Satija lab). For data from the Accelerating Medicines Programme II (AMPII) study, author-annotated dendritic cell (DC) populations were excluded prior to pseudobulking, and counts were aggregated by sample and annotated clusters. Owing to a smaller number of cells in the healthy data (183 cells), all cells were used and aggregated by the donor. Pseudobulked cells were scored according to their expression of challenge-associated genes, using the package Ucell (version 2.6.2) [63]. Statistical significance was investigated using the wilcoxon package in R (version 4.3.2)(Frank Wilcoxon) based on Ucell scores for pseudobulked cells.

Gene expression in scRNA sequencing data from synovial cells was analysed in tissue from HCs, people with active RA, and people with RA in remission; E-MTAB-8322 [46]. Analyses were performed using R version 4.0.3 (2020-10-10) and RStudio version 1.3.1093 (Posit) for MacOS. Plots were generated using ggplot2 version 3.3.5 (Hadley Wickham). The Seurat pipeline described in the relevant vignette (https://satijalab.org/seurat/articles/pbm3k_tutorial.html) was used to subset the monocyte and macrophage cell subsets identified in the original study of each dataset and used to plot individual gene expression through the DotPlot() function. Genes from the target gene list that were not captured in a dataset were excluded from plotting. For DEA, MAST was run directly on the log-normalised data using the Seurat FindMarkers() function, and P values were adjusted using the p.adjust() function and Benjamini-Hochberg adjustment [64,65]. LFC and P_{adj} values were plotted as volcano plots using the ggplot2 R package.

Statistical analysis

Data analysis was performed, and graphs were generated using GraphPad Prism software. Multiple group datasets were analysed by 1-way analysis of variance (ANOVA), followed by Dunnett's multiple comparisons tests. A two-tailed paired t-test, or ratio paired t-test, was used for comparisons involving only 2 groups. Multiple testing corrections were performed using the False Discovery Rate (FDR) approach and the Benjamini, Krieger, and Yekutieli methods during the analysis of the data from the experiment involving a variety of infectious and endogenous stimuli. Two-way ANOVA and Sidak multiple comparisons testing were used to analyse data from the citrullination experiment. Patient samples and HC data were analysed using a 2-tailed unpaired t-test. P values lower than .05 were considered significant.

Competing interests

The authors declare no competing interests.

CRediT authorship contribution statement

Anna M. Marzeda: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Anja Schwenzer:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis. **Bogdan S. Didov:** Writing – review & editing, Visualization, Investigation, Formal analysis. **Kieran Woolcock:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Jean-Baptiste Richard:** Writing – review & editing, Visualization, Investigation, Formal analysis. **Libby K. Jennings:** Writing – review & editing, Visualization, Investigation, Formal analysis. **Amélie M. Julé:** Writing – review & editing, Visualization, Methodology, Formal analysis, Data curation. **Nan Yang:** Writing – review & editing, Visualization, Investigation, Formal analysis. **Sarah Davidson:** Writing – review & editing, Formal analysis. **Steve Sansom:** Writing – review & editing, Supervision, Methodology, Data curation. **Adam P. Cribbs:** Writing – review & editing, Validation, Supervision, Methodology, Formal analysis. **Calliope A. Dendrou:** Writing – review & editing, Formal analysis. **Wyatt W. Yue:** Writing – review & editing, Visualization, Supervision, Project administration, Formal analysis. **Carl S. Goodyear:** Writing – review & editing, Validation, Supervision, Resources, Investigation. **Karim Raza:** Writing – review & editing, Resources, Conceptualization. **Kim S. Midwood:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization.

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Contributors

AMM, AS, APC, WWY, CSG, KR, and KSM designed aspects of the research; AMM, BSD, NY, AS, and KW performed the research; AS and KR contributed new reagents; AMM, AS, LKJ, KW, AMJ, SS, J-BR, CAD, SD, and KSM analysed data; AMM and KSM wrote the paper; all authors edited the paper.

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Patient consent for publication

Not applicable.

Provenance and peer review

Not commissioned, externally peer reviewed.

Data availability statement

Datasets generated in this study can be found in the NCBI Gene Expression Omnibus (GEO): healthy monocyte RNA seq (GSE273837), RA and healthy monocyte RNA seq (GSE294225) and CHIP seq (GSE294235).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.ard.2025.03.016](https://doi.org/10.1016/j.ard.2025.03.016).

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Systemic lupus erythematosus

Anti-TFAM antibodies link mitochondrial damage with antiphospholipid syndrome and thrombosis in SLE

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ABSTRACT

Objectives: Mitochondria are a source of autoantigens and damage-associated molecular patterns (DAMPs) in systemic lupus erythematosus (SLE). Nucleoids carrying TFAM (transcription factor A, mitochondrial) and mitochondrial DNA (mtDNA) are important DAMPs in SLE. While mtDNA has been associated with anti-double-stranded (ds)DNA antibodies and type I interferon (IFN-I), the immunogenic role of TFAM in SLE pathogenesis is unknown. Here, we characterised the clinical and transcriptional phenotypes linked to anti-TFAM antibodies in SLE.

Methods: Anti-TFAM antibodies were discovered in an exploratory sample of 22 SLE patients and 9 healthy controls. To define the prevalence, clinical significance, and associations with transcriptional profiles and IFN levels, anti-TFAM antibodies were detected using enzyme-linked immunosorbent assay (ELISA) in 98 healthy controls and 158 SLE patients. Sera from patients with dermatomyositis, rheumatoid arthritis, and primary antiphospholipid syndrome (PAPS) were also tested.

Results: Anti-TFAM antibodies were discovered in patients with SLE while analysing neutrophil autoantigens and confirmed by ELISA and immunoblotting. One-third of SLE patients (48/158) were positive for anti-TFAM antibodies. Unlike anti-dsDNA antibodies, anti-TFAM antibodies were not associated with disease activity or the IFN signature. Instead, anti-TFAM antibodies were associated with thrombosis, antiphospholipid syndrome (APS) (odds ratio [OR], 2.9 and 5.4, respectively), thrombosis-associated transcriptional profiles, and elevated IFN-III. Anti-TFAM antibodies were also found in PAPS, supporting their role in APS but not SLE pathogenesis. Lupus anticoagulant increased the risk of thrombosis associated with anti-TFAM antibodies (OR, 8.71), indicating they are markers of independent prothrombotic pathways.

Conclusions: Anti-TFAM antibodies identify a distinct clinical and transcriptional disease subset associated with mitochondrial damage, thrombosis, and APS in SLE.

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WHAT IS ALREADY KNOWN ON THIS TOPIC

- Mitochondrial damage has been implicated in the pathogenesis of systemic lupus erythematosus (SLE), particularly in relation to the production of anti-double-stranded DNA antibodies and the induction of type I interferon.
- Thrombotic events and antiphospholipid syndrome (APS) in SLE are immune-mediated conditions associated with the production of autoantibodies, particularly lupus anticoagulant.

WHAT THIS STUDY ADDS

- This is the first study to identify the mitochondrial nucleoprotein TFAM (transcription factor A, mitochondrial) as an autoantigen in SLE.
- Anti-TFAM antibodies are significantly associated with thrombotic events and APS in SLE patients, independent of traditional APS-associated antibodies. Notably, antibodies to TFAM increase the risk of thrombosis associated with lupus anticoagulant, indicating that they are markers of independent prothrombotic pathways.
- Gene expression analysis reveals unique transcriptional signatures associated with anti-TFAM antibodies, implying a mechanistic link with immune activation pathways associated with thrombosis in SLE.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- Anti-TFAM antibodies are potential biomarkers for identifying SLE patients at risk of thrombosis, independent of traditional APS-associated antibodies. These novel autoantibodies may aid in disease evaluation and treatment strategies for SLE, as well as provide novel insights into disease mechanisms, particularly those related to thrombotic events and APS.

INTRODUCTION

Mitochondria are evolutionary endosymbionts derived from bacteria [1] that contain potent immunological activators via damage-associated molecular patterns (DAMPs) [2]. TFAM (transcription factor A, mitochondrial) is a member of the high-mobility group (HMG) family of DNA-binding proteins and is essential for mitochondrial DNA (mtDNA) transcription and packaging into a nucleoprotein complex known as the nucleoid [3–5]. TFAM is also a DAMP. During mitochondrial stress, TFAM relocates from mitochondria to the cytosol, where it enhances cyclic GMP-AMP synthase (cGAS) activation by pre-structuring cytosolic DNA to nucleate or stabilise cGAS dimers [6]. TFAM also promotes NLRP3 inflammasome activation in macrophages by facilitating oxidised (Ox) mtDNA formation or release [7].

Extracellularly, TFAM released from necrotic cells binds RAGE (receptor for advanced glycation end-products) on plasmacytoid dendritic cells (pDCs) [8–10], thereby functioning as a nucleoprotein carrier to facilitate the delivery of mtDNA into toll-like receptor (TLR) 9 endosomes, which promotes the production of type I interferon (IFN-I) and tumour necrosis factor alpha [9,10]. Recombinant TFAM has also been shown to drive cytokine secretion by cultured human monocytes and primary microglial cells [11], and a TFAM polymorphism has been linked to the risk of venous thromboembolism recurrence [12]. In systemic lupus erythematosus (SLE), an autoimmune disease characterised by high-titre antibodies against nucleoprotein self-antigens [13,14], nucleoids containing Ox mtDNA and TFAM are extruded from

IFN-I-activated neutrophils upon stimulation with ribonucleoprotein (RNP) immune complexes (ICs), serving as potent interferogenic stimuli for pDCs.¹⁵ Moreover, mtDNA, particularly Ox mtDNA in nucleoids, is thought to be an immunogenic trigger for anti-double-stranded (ds)DNA antibodies in SLE [15,16]. Thus, as a carrier of Ox mtDNA, TFAM may play a dual role in SLE pathogenesis, inducing both IFN-I and anti-dsDNA antibodies.

Here, we determined TFAM is also a common autoantigen in SLE. However, unlike autoantibodies targeting dsDNA and nucleoprotein complexes [17–20], antibodies to TFAM were not associated with markers of disease activity or the IFN signature. Instead, these novel autoantibodies showed a striking association with thrombosis, antiphospholipid syndrome (APS), thrombosis-associated transcriptional profiles, and increased IFN-III activity levels. Interestingly, anti-TFAM antibodies are also found in patients with primary APS (PAPS), implying that these antibodies are related to APS but not to SLE pathogenesis. In addition, the risk of thrombosis associated with anti-TFAM antibodies is similar to and independent of lupus anticoagulant (LAC), indicating that these antibodies are markers of thrombotic pathways distinct from those associated with LAC.

METHODS

Sex as a biological variable

Male and female subjects were included in this study, but the majority were female (9:1 female:male ratio) due to the disease demographics.

Study design and patients

The study included an exploratory sample of 9 healthy controls and 22 SLE patients and a validation sample of 98 healthy controls (median age [IQR] 37 (30, 45) years; female sex 71/98, 72%) and 158 SLE patients from the ‘Study of biological Pathways, Disease Activity and Response markers in patients with Systemic Lupus Erythematosus’ (SPARE) cohort, which has been previously described [21,22]. Briefly, adult patients (aged 18–75 years) who met the definition of SLE per the revised American College of Rheumatology classification criteria [23] were enrolled into the study. Patients were seen as outpatients at the Johns Hopkins Lupus Center and treated according to standard clinical practice. Disease activity was assessed using the Safety of Estrogens in Lupus Erythematosus: National Assessment version of the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) [24] and physician global assessment [25]. C3, C4, anti-dsDNA (*Crithidia*), complete blood cell count, and urinalysis were performed at every visit. Study participants also underwent whole blood gene expression analysis using the Affymetrix GeneChip HT HG-U133+ [21,22] as well as quantification of circulating IFN-I, IFN-II, and IFN-III activity levels [26]. Antibodies to TFAM were also measured in 36 patients with rheumatoid arthritis (RA), 40 with dermatomyositis (DM), and 50 with PAPS.

Study approval

All samples were obtained with the approval from the institutional review boards at the Johns Hopkins University School of Medicine and Sapienza University of Rome.

TFAM cloning, protein expression, and structure

cDNA encoding mature human TFAM (amino acids 43–246) was synthesised using RNA from human peripheral blood mononuclear cells and cloned into pET-28a(+). Recombinant N-terminal His-tagged TFAM was expressed in *E. coli* BL21 (DE3) and purified by Ni-NTA affinity chromatography. TFAM structure was predicted using AlphaFold Protein Structure Database (<https://alphafold.com/>).

Detection of antibodies to TFAM

Anti-TFAM antibodies were measured in serum/plasma using an in-house developed enzyme-linked immunosorbent assay (ELISA). Briefly, Nunc Maxisorp plates were coated with 200 ng per well of recombinant TFAM. The plates were blocked for 1 hour in phosphate-buffered saline with 0.1% Tween-20 (PBST) and 3% nonfat milk. Serum/plasma was diluted 1:1000 in PBST with 1% nonfat milk and assayed in duplicate using antigen-conjugated plates and plates without antigen for background subtraction. For competition assays, diluted SLE serum/plasma was preincubated with 10 µg/mL of human recombinant high-mobility group box 1 (HMGB1; Sino Biological) or β2-glycoprotein I (β2GPI) purified from human plasma (Arotec Diagnostics) for 1 hour at room temperature. Horseradish peroxidase-conjugated goat anti-human IgG was used as a secondary antibody (1:10,000 in PBST with 1% nonfat milk). Anti-TFAM antibody arbitrary units (AU) were calculated using a standard curve made using serial dilutions of serum from a high-titre SLE patient. Anti-TFAM positivity cutoff was set as the mean + 2 SD of anti-TFAM antibodies (in AU) from a healthy control.

Indirect immunofluorescence

Peripheral blood human neutrophils were purified as previously described [27], attached to microscope slides using Histo-Grip (cat. 008050; ThermoFisher Scientific), fixed with 4% paraformaldehyde, and permeabilised with acetone. After blocking with 2% bovine serum albumin and incubation with SLE serum diluted at 1:100 in PBS or commercial anti-TFAM antibody (clone C9, cat. sc-376672; Santa Cruz Biotechnology) at 1:10, human IgG and commercial anti-TFAM antibodies were visualised using Alexa Fluor 488-conjugated goat anti-human IgG or Alexa Fluor 594-conjugated rabbit anti-mouse antibodies, respectively. DNA was detected using 4',6-diamidino-2-phenylindole (DAPI). In addition, TFAM detection using the commercial anti-TFAM antibody (clone C9) and anti-TFAM positive SLE sera was performed using commercially available HEp-2 cell slides (NOVA Lite HEp-2 ANA Kit with DAPI, cat. 708102; Wefen) according to manufacturer instructions. For HEp-2 testing, serum was diluted at 1:80 and the commercial antibody at 1:10. Human IgG and commercial anti-TFAM antibodies were visualised using Alexa Fluor 488-conjugated goat anti-human IgG or Alexa Fluor 594-conjugated rabbit anti-mouse antibodies, respectively. For competition assays, diluted SLE serum was preincubated with 40 µg/mL of mature human recombinant TFAM for 1 hour at room temperature.

Gene expression analyses

Gene expression analysis from the SPARE cohort was previously described [22]. CEL files were subjected to robust multiarray average background correction and quantile normalisation

using the *oligo* R package [28]. To select only expressed genes in whole blood, we filtered out transcripts that had a raw signal <100 in <10% of samples using the *genefilter* R package. All calculations and analyses were performed using R (ver 4.0.2) and Bioconductor (ver 3.13) [29].

Feature selection using the Fisher score algorithm and principal component analysis

To select the most relevant transcripts to distinguish between patients who were positive or negative for anti-TFAM antibodies, thrombotic events, or anti-dsDNA antibodies, we employed the Fisher score algorithm [30]. Briefly, a Fisher score is calculated for each individual transcript by considering the number of samples, mean, and SD for each category (eg, anti-TFAM positive or anti-TFAM negative), and each transcript is then ranked according to its score. For the principal component analysis (PCA), we selected the top 1000 genes because this number of transcripts is useful to identify biologically relevant pathways using the Fisher score algorithm [30]. Fisher scores were calculated using the *Rdimtools* R package [31]. PCA was performed using base R functions.

Gene set enrichment analyses

To perform gene set enrichment analyses, we determined the transcripts with the highest loading on the anti-TFAM antibody, thrombosis, and anti-dsDNA antibody PC1 by calculating Pearson's *r* correlation coefficient. *P* values were adjusted using the Benjamini–Hochberg method. Transcripts with an adjusted *P* value ≤ .01 were considered to be significantly correlated. To compare the anti-TFAM antibody-, thrombosis-, and anti-dsDNA antibody-related transcriptomes, gene set enrichment analysis was performed using the metanalysis function of Metascape [32].

Statistical analyses

Continuous variables between groups were compared using Student's *t* test and analysis of variance with Tukey's post hoc test as indicated. Mann–Whitney *U* and Kruskal–Wallis tests were used for groupwise comparisons of nonnormally distributed variables. Fisher's exact and χ^2 tests were used for univariate analysis on SPARE cohort variables, as appropriate. The *exact2x2* package in R version 3.5.1 was used for binary variables to obtain *P* value, odds ratio (OR), and 95% CI. Correlations between variables were calculated using Pearson's *r*. Comparisons of paired samples were performed using Wilcoxon signed-rank test. Multivariate analyses were performed using multivariate logistic regression. Statistical significance was set at *P* < .05. The statistical analyses were carried out using R software version 4.3.3.

RESULTS

TFAM is an autoantigen in SLE

Autoantibodies in SLE bind to different antigens in human neutrophils, generating distinct cellular patterns when analysed by indirect immunofluorescence, including nucleolar, nuclear, and cytoplasmic patterns (Supplementary Fig S1). Coincidentally, while performing parallel studies with neutrophils and TFAM, we noticed that commercial antibodies to TFAM generated a cellular pattern similar to that of SLE sera targeting

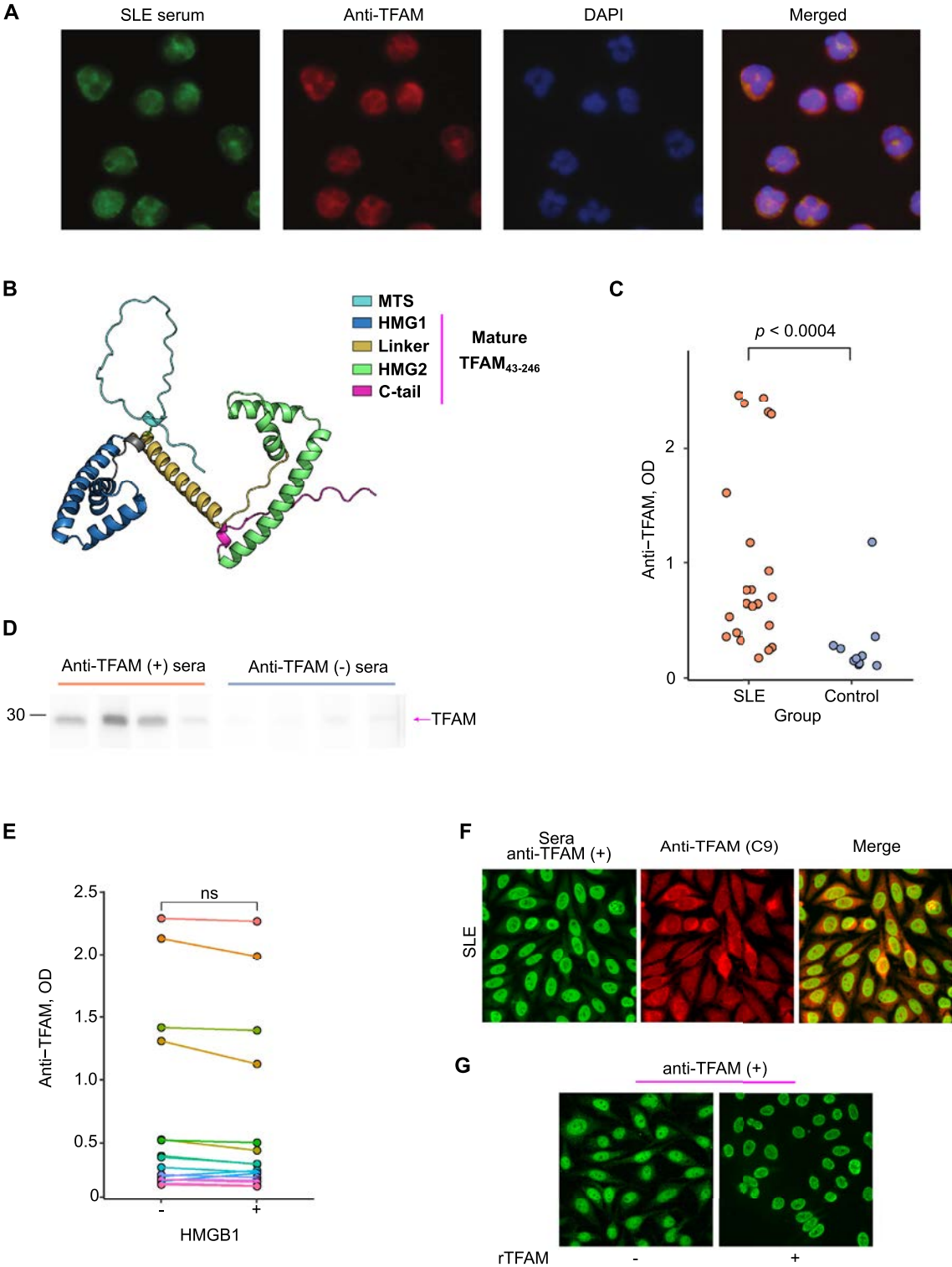


Figure 1. TFAM is a novel autoantigen in SLE. (A) Healthy control neutrophils were stained with SLE serum detecting cytoplasmic antigens (green), anti-TFAM monoclonal antibody C9 (red), and DAPI (blue) and viewed by immunofluorescence microscopy. Individual and merged images are shown. (B) TFAM structure predicted by AlphaFold. Amino acids (aa) 1-42 form the mitochondrial targeting sequence (MTS), while aa 43-246 comprise the mature form of TFAM, which includes 2 high-mobility group domains (HMG1 and HMG2), a linker, and a C-terminal tail (C-tail). (C) Serum levels of anti-TFAM antibodies in SLE (n = 22) and healthy controls (n = 10). The P value was obtained using the Wilcoxon rank sum test. (D) Representative immunoblots showing the reactivity of healthy control serum (n = 4) and anti-TFAM-positive SLE serum (n = 4) against human recombinant TFAM. (E) Serum levels of anti-TFAM antibodies before and after blocking with human recombinant HMG box 1 (HMGB1) protein (n = 9). Comparisons were done using a paired samples Wilcoxon signed-rank test. (F) HEp-2 cells were stained using anti-TFAM-positive SLE serum (green) and anti-TFAM monoclonal antibody C9 (red) and viewed by immunofluorescence microscopy. Individual and merged images are shown. (G) HEp-2 cells were stained with anti-TFAM-positive SLE serum (green) in the absence (left) and presence (right) of blocking with recombinant TFAM (rTFAM). Representative images of 12 (F) and 8 (G) anti-TFAM-positive SLE sera are shown. DAPI, 4',6-diamidino-2-phenylindole; SLE, systemic lupus erythematosus; TFAM, transcription factor A, mitochondrial.

cytoplasmic antigens (Fig 1A), leading to the hypothesis that TFAM is an autoantigen in SLE. TFAM is a nuclear-encoded protein of 204 amino acids (24 kDa) [4]. It is translated as a preprotein containing a mitochondrial targeting sequence (MTS; amino acids 1–42) that is cleaved upon mitochondrial import (Fig 1B). Mature TFAM (amino acids 43–246) consists of 2 HMG boxes separated by a linker and followed by a short tail at the C-terminus (Fig 1B) [4].

To address whether TFAM is a target of autoantibodies in SLE, we developed an ELISA using mature human recombinant TFAM as a substrate and screened sera from an exploratory cohort sample of patients with SLE ($n = 22$) and healthy controls ($n = 9$). Patients with SLE had significantly elevated serum levels of anti-TFAM antibodies compared with healthy controls ($P < .0004$) (Fig 1C). Notably, SLE sera associated with a cytoplasmic pattern in neutrophils (either alone or concomitant with a nuclear pattern) (Fig 1A and Supplementary Fig S1) were those with the highest levels of anti-TFAM antibodies detected by ELISA. We further confirmed the presence of antibodies to TFAM in SLE by immunoblotting (Fig 1D) and that these antibodies were not cross-reactive with HMGB1 (Fig 1E), a protein analogous to TFAM that is also an autoantigen in SLE [4,33]. In HEP-2 cells, anti-TFAM-positive SLE serum colocalised with TFAM staining, which showed a cytoplasmic reticular pattern consistent with the AC-21 antinuclear antibody pattern (Fig 1F). However, unlike in neutrophils (Fig 1A), TFAM also showed some nuclear localisation in HEP-2 cells, which might be explained by the fixation/permeabilisation method or the expression of a TFAM variant lacking the MTS [34]. The

antibody pattern that colocalised with TFAM was completely blocked by preincubating SLE serum with mature recombinant TFAM (Fig 1G).

Antibodies to TFAM are associated with APS and thrombotic events in SLE

To define clinical features and transcriptional pathways associated with anti-TFAM antibodies in SLE, we studied samples from the SPARE cohort for which extensive clinical and serologic variables are available, as well as whole blood gene expression data [21,22]. Demographic, clinical, and laboratory features of the SLE cohort are summarised in Supplementary Table S1. The presence of antibodies to TFAM were also measured in serum from patients with PAPS, RA, and DM. Among the different disease groups, only patients with SLE had significantly higher levels of anti-TFAM antibodies than did healthy controls (mean [SD], 126.1 [236.4] vs 32.2 [50.5], $P < .0001$) (Fig 2A). After determining the cutoff level of 2 SD above the mean anti-TFAM antibody level in healthy sera, 30.3% (48/158) of SLE patients tested positive for antibodies to TFAM compared to 4% (4/98) in healthy controls ($P < .0001$) (Fig 2B,C). In addition to SLE, PAPS was linked with an increased frequency of anti-TFAM antibodies, 16% (8/50) vs healthy controls ($P < .05$). Anti-TFAM antibodies were not significantly increased in patients with RA or DM compared to healthy controls (Fig 2A–C). In SLE, anti-TFAM antibodies were stable over time. Although anti-TFAM levels varied between visits (intraclass correlation 0.622, $P = .002$), none of the samples were negative

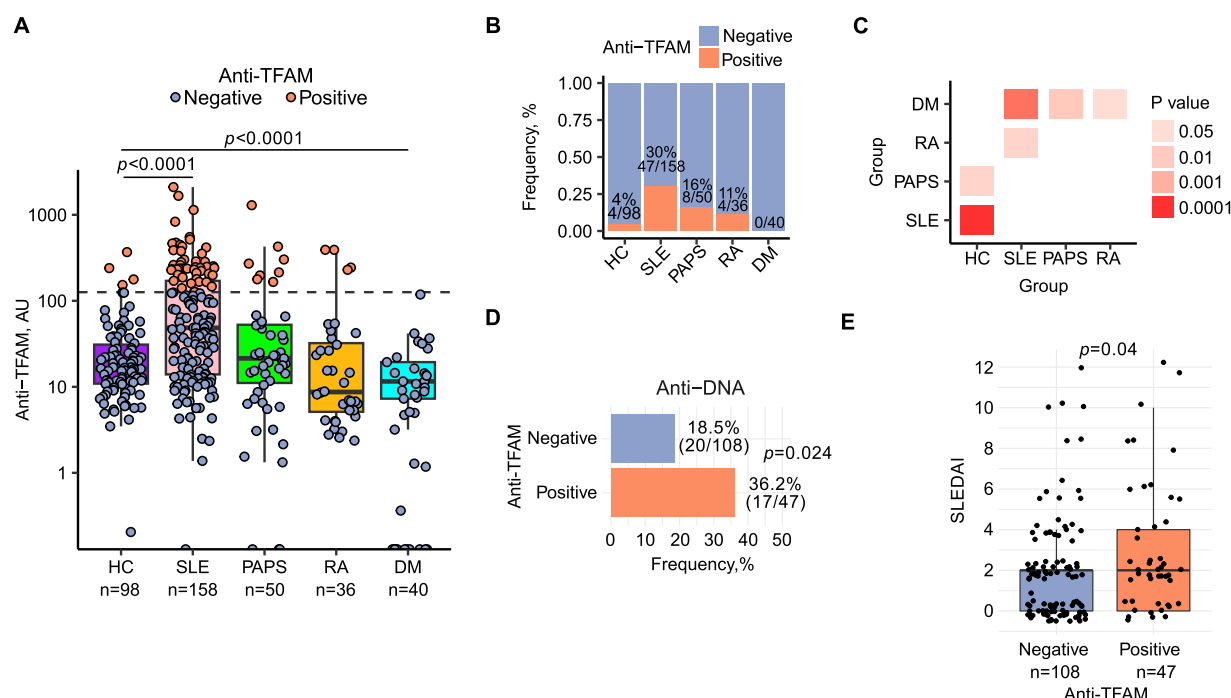


Figure 2. Anti-TFAM antibodies are prevalent in SLE. (A) Serum levels of anti-TFAM antibodies in SLE patients ($n = 158$) from the SPARE lupus cohort compared with those of healthy controls (HC, $n = 98$), primary antiphospholipid syndrome (PAPS, $n = 50$), rheumatoid arthritis (RA, $n = 36$), and dermatomyositis (DM, $n = 40$). Comparisons between groups were performed using one-way analysis of variance and Tukey's post hoc test. (B) Frequency of anti-TFAM antibodies in each group in (A). (C) P values indicating significant differences between the frequency in (B) of anti-TFAM in each group from (A). P values were obtained using Fisher's exact test. (D) Association between anti-dsDNA positivity and anti-TFAM antibodies. The association was estimated using Fisher's exact test. (E) SLEDAI score at the time of visit in SLE patients according to anti-TFAM positivity. The P value was obtained using a Student's t test. AU, arbitrary units; dsDNA, double-stranded DNA; SLE, systemic lupus erythematosus; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index; SPARE, Study of biological Pathways, Disease Activity and Response markers in patients with Systemic Lupus Erythematosus; TFAM, transcription factor A, mitochondrial.

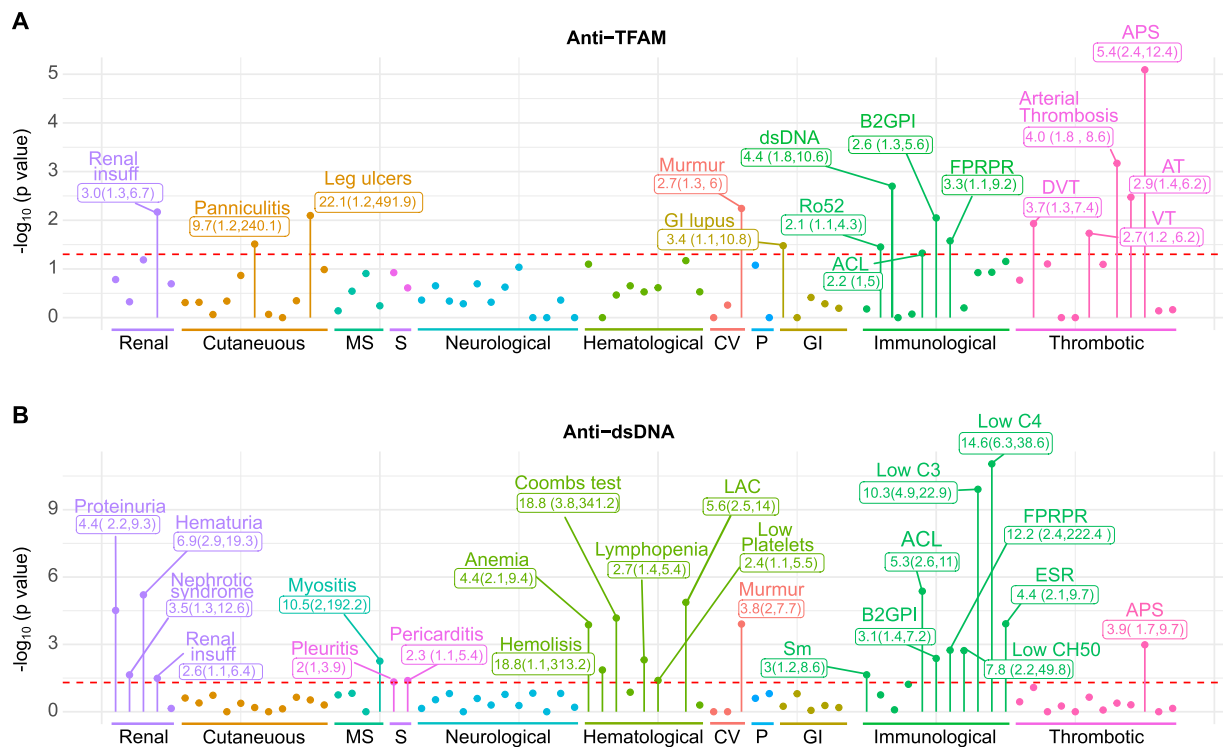


Figure 3. Anti-TFAM antibodies are associated with APS and thrombotic events in SLE. Clinical and immunological characteristics of SLE patients according to anti-TFAM (A) and anti-dsDNA antibody positivity (B). The graphs represent data for 157 SLE patients, including 48 who were positive and 109 negative for anti-TFAM antibodies. Each dot represents a clinical manifestation. Only clinical manifestations significantly associated ($P < .05$) with anti-TFAM and anti-dsDNA antibodies are shown. Quantities under each label correspond to the odds ratio (OR) and 95% CI of anti-TFAM (+) vs anti-TFAM (–) (A) and anti-dsDNA (+) vs anti-dsDNA (–) (B) calculated using a 2×2 table. P values were obtained using Fisher's exact test. ACL, anti-cardiolipin antibodies; APS, antiphospholipid syndrome; AT, any thrombotic event; B2GPI, anti- β 2-glycoprotein-I antibodies; CH50, 50% hemolytic complement test; CV, cardiovascular; dsDNA, anti-double-stranded DNA antibodies; DVT, deep vein thrombosis; ESR, erythrocyte sedimentation rate; FPRPR, false positive rapid plasma reagin test; GI, gastrointestinal; LAC, lupus anticoagulant; MS, musculoskeletal; P, pulmonary; Renal insuff, renal insufficiency; Ro52, anti-Ro52 antibodies; S, Serositis; SLE, systemic lupus erythematosus; Sm, anti-Sm antibodies; TFAM, transcription factor A, mitochondrial; VT, venous thrombosis.

for the autoantibody, and the mean anti-TFAM levels were not statistically different between visits (mean $\pm$ SD, 500 ± 282 AU/mL vs 432 ± 392 AU/mL, $P = .555$) (Supplementary Fig S2A).

Since mtDNA has been implicated in the production of anti-dsDNA antibodies in SLE [15,16], and TFAM forms a complex with mtDNA [3–5], we anticipated that antibodies to TFAM would be mechanistically linked to anti-dsDNA antibodies and disease activity in SLE. However, only 36.2% (17/47) of anti-TFAM-positive patients were also positive for anti-dsDNA antibodies (Fig 2D), and there was a mild correlation between anti-TFAM and anti-dsDNA antibody levels ($r = 0.311$, $P < .005$). Moreover, anti-TFAM antibodies only showed a mild association with SLEDAI score (Fig 2E and Supplementary Table S1). Instead, anti-TFAM antibodies were significantly associated with renal insufficiency (but not active nephritis) and features of APS (Fig 3A and Supplementary Tables S1 and S2), in contrast to anti-dsDNA antibodies, which showed prominent association with features linked to IC deposition, such as nephritis and low complement levels (Fig 3B). Notably, SLE patients positive for anti-TFAM antibodies were more likely to have a history of leg ulcers, cardiac murmur, or any thrombotic event and be classified with APS (OR [95% CI], 22.1 [1.2, 491.9], 2.7 [1.3, 6.0], 2.9 [1.4, 6.2], and 5.4 [2.5, 11.9], respectively) (Fig 3A and Supplementary Table S2). Anti-TFAM antibodies were also associated with increased frequency of antibodies to β 2GPI, cardiolipin, and dsDNA (OR [95% CI], 2.6 [1.3, 5.6], 2.2 [1.0,

5.0], and 4.4 [1.8, 10.6], respectively). Given the strong association between APS and anti-TFAM antibodies, we further discarded that anti-TFAM antibodies in SLE were cross-reactive with β 2GPI (Supplementary Fig 2B). Since patients with PAPS are defined by the presence of APS-associated antibodies, it not surprising that anti-TFAM antibodies were not associated with any particular antibody in patients with PAPS (Supplementary Table S3).

Anti-TFAM antibodies are predictive of thrombotic events in SLE

To analyse the predictive value of anti-TFAM antibodies and possible covariates, we classified SLE patients in 2 groups: (i) subjects with any thrombotic event ($n = 54$, 34%), and (ii) subjects without thrombosis ($n = 102$, 65.4%) (Supplementary Table S4). Univariate analysis revealed that patients who had thrombotic events had longer disease duration (23.7 vs 18.5 years, OR = 1.04 per year of disease duration, $P = .01$) (Fig 4A). Notably, only anti-TFAM antibodies and LAC (determined by increased dilute Russell viper venom test) were significantly associated with any thrombotic event (46% [25/54] vs 23% [23/102], OR = 3.74, $P = .003$; and 50% (27/54) vs 27% (28/102), OR = 3.46, $P = .007$) (Fig 4A and Supplementary Table S4). Similar to other studies in SLE [35], however, anti- β 2GPI or anti-cardiolipin (ACL) antibodies were not associated with thrombotic events in the SPARE cohort (Supplementary Table S4).

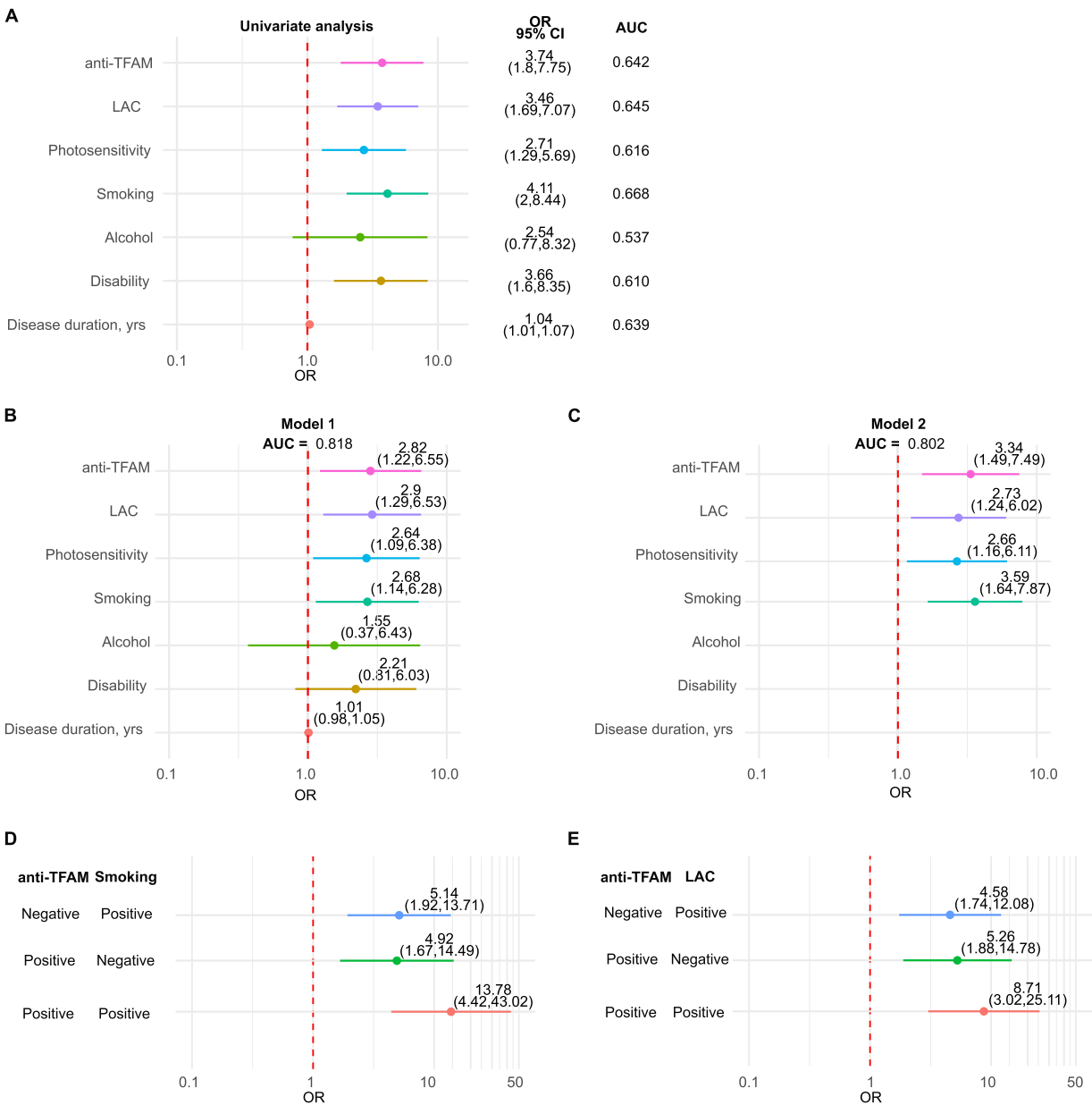


Figure 4. Anti-TFAM antibodies are predictive of thrombotic events in SLE. (A) Univariate logistic regression showing the predictive value of anti-TFAM antibodies and significantly associated factors from [Supplementary Table S3](#) for any thrombotic events in patients with SLE. (B,C) Multivariate logistic regression models analysing the predictive value of anti-TFAM antibodies after adjustment for disease duration, LAC, smoking, alcohol use, disability, and photosensitivity. (D,E) Logistic regression models showing the additive value of a history of smoking or LAC with anti-TFAM antibodies in SLE. The graphs represent data for 157 SLE patients, including 48 who were positive and 109 negative for anti-TFAM antibodies. AUC, area under the curve; LAC, lupus anticoagulant; OR, odds ratio; SLE, systemic lupus erythematosus; TFAM, transcription factor A, mitochondrial.

Among traditional cardiovascular risk factors, prior smoking was significantly associated with thrombotic events (59% [32/54] vs 26.5% [27/102], OR = 4.1, $P < .001$) (Fig 4A and [Supplementary Table S4](#)), and among the clinical manifestations not known to be related to thrombosis, photosensitivity was more frequent in patients with a history of thrombosis (72.2% [39/54] vs 48% [49/102], OR = 2.71, $P = .004$) (Fig 4A and [Supplementary Table S4](#)). Patients with thrombosis were also more likely to have some degree of disability (OR = 3.66) (Fig 4A). After adjustment for LAC, photosensitivity, and smoking, SLE patients positive for anti-TFAM antibodies were 2.82 to 3.34 times more likely to have a thrombotic event during their clinical course than anti-TFAM-negative patients (Fig 4B, C). Interestingly, whereas the likelihood of having a thrombotic event was similar among patients positive for anti-TFAM

antibodies or having a smoking history (OR = 4.92 and 5.14, respectively), we found an additive effect when both conditions were present (OR = 13.78) (Fig 4D). Similarly, the presence of LAC had an additive effect on the risk of thrombosis in SLE patients positive for anti-TFAM antibodies (OR = 8.71) (Fig 4E).

Antibodies to TFAM are associated to transcriptional fingerprints linked to thrombosis in SLE

Since the extracellular release of nucleoids from activated neutrophils has been associated with the induction of IFN-I in SLE [15], we addressed whether anti-TFAM antibodies are linked to unique transcriptional profiles—particularly the IFN signature—using gene expression data from blood collected in

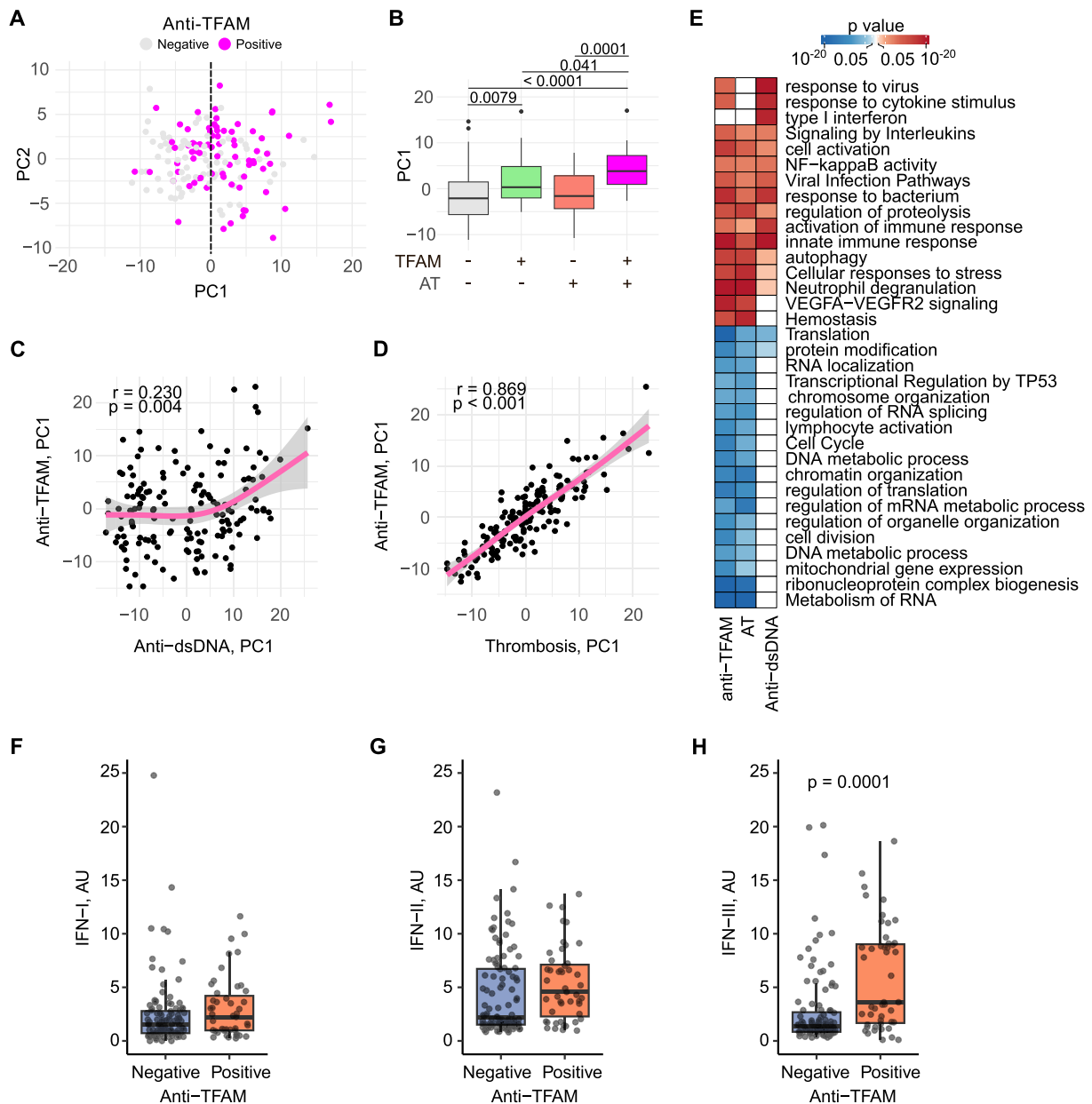


Figure 5. Anti-TFAM antibodies are linked to a thrombotic transcriptome in SLE. (A) Principal component (PC) analysis of the top 500 transcripts ranked by their Fisher score comparing anti-TFAM positive vs anti-TFAM negative SLE. (B) PC1 levels in patients with SLE according to the presence of both anti-TFAM antibodies and a history of any thrombotic event (AT). Comparisons were performed using analysis of variance and Tukey's post hoc test. (C,D) Correlation between anti-TFAM PC1 and anti-dsDNA PC1 (C) and anti-TFAM PC1 and thrombosis PC1 (D). Correlations were estimated using Pearson's r correlation coefficient. (E) Enrichment analysis of transcripts significantly correlated with anti-TFAM, thrombosis, and anti-dsDNA PC1. Significantly correlated genes were determined using Pearson's r correlation coefficients. Only genes with an adjusted $P < .01$ (Benjamini–Hochberg method) were considered in the analyses. The enrichment analysis was conducted using the Metanalysis function from Metascape.org. For this data set, we analysed 156 microarrays, of which 47 and 109 corresponded to anti-TFAM-positive and anti-TFAM-negative SLE, respectively. (F–H) Activity levels of IFN-I, IFN-II and IFN-III in patients with SLE according to anti-TFAM positivity. Anti-TFAM-positive, $n = 47$; anti-TFAM-negative, $n = 109$. Comparison of IFN levels was done using a Student's t test. AU, arbitrary units; dsDNA, double-stranded DNA; IFN, interferon; SLE, systemic lupus erythematosus; TFAM, transcription factor A, mitochondrial.

parallel with the samples used to measure anti-TFAM antibodies. To uncover gene expression signatures associated to anti-TFAM antibodies, we used PCA on the top 500 transcripts ranked by their Fisher score between SLE patients positive and negative for anti-TFAM antibodies (ie, the anti-TFAM transcriptome) (Supplementary File S1). The first principal component (PC1) stratified SLE patients on a gradient rather than into specific clusters (Fig 5A). PC1 was significantly higher in SLE patients who were positive for anti-TFAM antibodies and had a history of thrombotic events (Fig 5B).

To compare the transcriptome of anti-TFAM antibodies to those associated with thrombosis or activation by anti-dsDNA antibodies, we performed PCA analysis using the top 500 transcripts ranked by their Fisher score between patients with and without history of thrombosis (ie, thrombosis transcriptome) and between patients positive and negative for anti-dsDNA antibodies (ie, anti-dsDNA transcriptome) (Supplementary File S1). Interestingly, while the anti-TFAM antibody PC1 showed only mild correlation with the anti-dsDNA antibody PC1 ($r = 0.230$, $P = .004$), it had a strong correlation with thrombosis PC1

($r = 0.869$, $P = .001$) (Fig 5C, D, respectively). There was no correlation between anti-dsDNA antibody PC1 and thrombosis PC1 ($r = 0.01$, $P = .914$). Together, these data imply that PC1 represents transcripts and pathways associated with anti-TFAM antibody-related thrombosis rather than anti-dsDNA antibody-mediated immune activation.

To further determine the biological significance of the anti-TFAM antibody, anti-dsDNA antibody, and thrombosis PC1s, we performed enrichment analysis on the transcripts that were significantly correlated with each component. We found 1799 and 246 transcripts that were positively and negatively correlated with the anti-TFAM antibody PC1 (Supplementary File S1). Positively enriched transcripts were linked to pathways associated with immune-mediated activation and haemostasis, such as response to virus and cytokine stimuli, neutrophil degranulation, activation of the innate immune response, vascular endothelial growth factor A (VEGFA)-VEGFR2 signalling, responses to bacteria, cellular response to stress, haemostasis, and autophagy, among others. In contrast, transcripts that were negatively correlated were mainly associated with pathways linked to RNA and DNA metabolism, including protein translation, RNA localisation and splicing, chromatin organisation, RNP complex biogenesis, cell cycle, and mitochondrial gene expression, among others (Fig 5E).

As indicated by the strong correlation between anti-TFAM antibody and thrombosis PC1s (Fig 5C), the enrichment of both components was essentially identical, with the exception of 2 pathways related to response to virus and cytokine stimuli (Fig 5E). Notably, pathways involving haemostasis and VEGFA-VEGFR2 signalling, as well as RNA and DNA metabolism (except for RNA translation and protein modification), were exclusively linked to anti-TFAM antibodies and thrombosis, whereas type I IFN was only associated with anti-dsDNA antibodies (Fig 5E). Other immune-mediated activation pathways showed a significant similarity between anti-TFAM antibodies and thrombosis with anti-dsDNA antibodies (Fig 5E), which is most likely explained by the 30% overlap between anti-dsDNA and anti-TFAM antibodies. Collectively, the data support that anti-TFAM antibodies are mechanistically related to thrombosis in SLE but are independent of the hallmark features, such as disease activity, anti-dsDNA antibodies, and the IFN signature. Consistent with these findings, circulating activity levels of IFN-I or IFN-II were not associated with anti-TFAM antibodies (Fig 5F,G, respectively). Unexpectedly, however, IFN-III was significantly elevated in patients positive for antibodies to TFAM ($P = .0001$) (Fig 5H).

DISCUSSION

Mitochondria appear to play at least 2 immunogenic and potentially independent roles in SLE pathogenesis. First, nucleoids—particularly those containing Ox mtDNA—have been linked to the induction of IFN-I by pDCs, and mtDNA is considered an immunogenic candidate for the production of anti-dsDNA antibodies in SLE [15,16]. In this context, mitochondria may have a mechanistic role in clinical phenotypes associated with the IFN signature and disease activity features related to anti-dsDNA antibodies, such as complement activation and lupus nephritis. Second, antibodies to mitochondrial proteins (eg, 60-kDa heat-shock protein, HSP60) and cardiolipin-protein complexes have been associated with thrombosis [36,37], supporting a role for non-mtDNA-associated components in thrombotic events. Because TFAM is the main nucleoprotein involved in the packaging of mtDNA into nucleoids [3–5], and Ox

mtDNA nucleoids have been linked to the induction of IFN-I by pDCs in SLE [15], we were particularly interested in anti-TFAM antibodies and their relationship to antibodies to dsDNA and the IFN signature. Collectively, our clinical and transcriptional data show that anti-TFAM antibodies are not mechanistically linked to antibodies to dsDNA or IFN-I induced activation, but rather constitute a novel biomarker associated with APS and thrombosis in SLE. Indeed, the finding that antibodies to TFAM are also present in patients with primary APS supports the notion that these antibodies are generated in response to mechanisms of mitochondrial damage other than those potentially associated with anti-dsDNA and IFN-I induction.

While antibodies to TFAM and dsDNA overlap in about one-third of patients with SLE, they identify 2 distinct clinical and transcriptional subsets within SLE. Anti-dsDNA antibodies are associated with mechanisms related to IC formation, including complement activation, nephritis, TLR signalling, and IFN-I production. In contrast, anti-TFAM antibodies are linked to thrombotic events, APS, and thrombosis-associated transcriptional fingerprints but not to pathways activated by IFN-I. Indeed, unlike SLE-associated autoantibodies such as anti-dsDNA, anti-Sm, anti-RNP, anti-DNAse1L3, and anti-Ro52Ex4, which are strongly associated with high levels of circulating IFN-I [26,38], antibodies to TFAM showed no relationship with IFN-I levels. These data are consistent with the lack of correlation between APS-associated autoantibodies and IFN-I levels or the IFN-I signature [18,26,38]. Thus, although TFAM is the major carrier of mtDNA in nucleoids, our findings indicate that the immune response to TFAM in SLE is independent of any potential role of mtDNA in anti-dsDNA antibody production, and anti-TFAM antibodies appear to play no role in the induction of IFN-I by cell-free nucleoids. Nevertheless, it is worth noting that antibodies to TFAM were associated with increased IFN-III activity levels. Although it is unclear whether IFN-III promotes the production of anti-TFAM antibodies or these antibodies induce IFN-III expression, and whether IFN-III is relevant for thrombosis, an association between IFN- λ 1 and thrombotic events has been suggested in SLE, but it was not statistically significant [38]. The mechanistic relationship between anti-TFAM antibodies, IFN-III, and APS has yet to be determined.

Notably, the risk of thrombosis associated with anti-TFAM antibodies is similar to and independent of LAC, the stronger risk factor for thrombosis in SLE [35,39–41]. Moreover, a history of smoking or LAC has an additive effect on the risk of thrombosis in SLE patients positive for anti-TFAM antibodies, implying that these antibodies are markers of thrombotic pathways distinct from those associated with smoking and LAC. Indeed, while the prothrombotic effect of LAC is mediated by targeting the coagulation pathway [37,42], the presence of antibodies to TFAM indicates thrombotic mechanisms involving cellular and mitochondrial damage.

Although the study of neutrophils provided initial clues for the search of antibodies to TFAM, the cellular source of TFAM that drives the production of autoantibodies may have different origins in SLE, including neutrophils, erythrocytes, and platelets. For example, TFAM released from IFN-I-activated neutrophils in response to RNP IC stimulation [15], as well as by forms of cell death involving mitochondrial membrane permeabilisation, such as pyroptosis, apoptosis, and necrosis [43,44], are potential sources of immunogenic TFAM in SLE. Interestingly, although mtDNA is found in neutrophil extracellular traps (NETs) [45], TFAM has not been detected in NETs by mass spectrometry analysis [46,47]. The abnormal accumulation of erythrocytes carrying mitochondria in SLE and their clearance by macrophages

may also trigger the production of antibodies to TFAM [48]. Finally, extracellular mitochondria released from IC-activated platelets have been proposed as a key source of mitochondrial antigens in SLE [49].

Interestingly, mitochondria are essential for platelet function and therefore, mitochondrial damage or dysfunction can affect platelet survival and increase the risk of thrombosis [50]. In this case, the production of anti-TFAM antibodies may represent markers of mitochondrial dysfunction associated with thrombosis rather than direct drivers of thrombotic events in SLE. Alternatively, these antibodies may promote thrombosis by binding TFAM–DNA complexes released from activated neutrophils, degranulating platelets, apoptotic cells undergoing secondary necrosis, or from other forms of cell death. In either scenario, as markers of prothrombotic mitochondrial dysfunction or direct triggers of thrombosis, the discovery of antibodies to TFAM provides a novel tool independent of traditional APS-associated antibodies for identifying SLE patients at risk of thrombosis, in which mitochondrial damage is likely to play a pathogenic role.

Limitations of the study

Because the study only examined cross-sectional samples, it is unclear whether anti-TFAM seroconversion occurs synchronously or metachronously with vascular events in SLE. Similarly, it is important to further define the significance of anti-TFAM antibodies in other groups of patients with thrombotic events who do not have SLE or PAPS. Furthermore, it would be interesting to determine if anti-TFAM antibodies are prevalent among patients with anti-phosphatidylserine/prothrombin complex antibodies, which, similar to anti-TFAM antibodies, are associated with increased risk of thrombosis in subjects negative for ACL or anti- β 2GPI [51].

Competing interests

FA has received consulting fees and/or royalties from Celgene, Inova, Advise Connect Inspire, and Hillstar Bio Inc and is inventor on a licensed patent (US patent no. 14/617,412) and licensed provisional patent (US patent no. 62/481,158). EGB, AIC, MITZ, MeP, FC, CA, ET, DWG, and MiP have declared that no conflict of interest exists. There are no conflicts of interest with the work presented in this manuscript.

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Contributors

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Study conception and design were contributed by FA. Experimental work was contributed by EGB, AIC, MITZ, MeP, and FA. Acquisition of data was contributed by EGB, AIC, MITZ, JARDA, MeP, SC, FC, CA, ET, DWG, RAB, MiP, and FA. Data analysis was performed by EGB. Writing—original draft: EGB and FA. Writing—review and editing: EGB, AIC, JARDA, MITZ, MeP, SC, FC, CA, ET, DWG, RAB, MiP, and FA.

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Patient consent for publication

Not applicable.

Ethics approval

All samples were obtained under approval by the Institutional Review Board at the Johns Hopkins University School of Medicine and Sapienza University of Rome.

Provenance and peer review

Not commissioned; externally peer reviewed.

Data availability statement

Microarray data are available from the Gene Expression Omnibus with accession numbers GSE45291 and GSE121239. Any additional data presented in this work can be obtained from the authors upon request.

Patient and public involvement

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

Supplementary materials

Supplementary material associated with this article can be found in the online version at [doi:10.1016/j.ard.2025.04.015](https://doi.org/10.1016/j.ard.2025.04.015).

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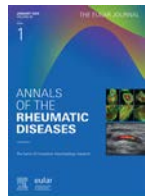
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Sjögren's syndrome

Variants in the *DDX6-CXCR5* autoimmune disease risk locus influence the regulatory network in immune cells and salivary gland

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ABSTRACT

Objectives: Sjögren's disease (SjD) and systemic lupus erythematosus (SLE) share genetic risk at the *DDX6-CXCR5* locus (11q23.3). Identifying and functionally characterising shared SNPs spanning this locus can provide new insights into common genetic mechanisms of autoimmunity.

Methods: Transdisease meta-analyses, fine-mapping, and bioinformatic analyses prioritised shared likely functional single nucleotide polymorphisms (SNPs) for allele-specific and cell type-specific functional interrogation using electromobility shift, luciferase reporter, and quantitative chromatin conformation capture assays and clustered regularly interspaced short palindromic repeat (CRISPR) gene regulation.

Results: Five shared SNPs were identified as likely functional in primary human immune cells, salivary gland and kidney tissues: rs57494551, rs4936443, rs4938572, rs7117261, and rs4938573. All 5 SNPs exhibited cell type-specific and allele-specific effects on nuclear protein binding affinity and enhancer/promoter regulatory activity in immune, salivary gland epithelial, and kidney epithelial cell models. Mapping of chromatin–chromatin interactions revealed a chromatin regulatory network that expanded beyond *DDX6* and *CXCR5* to include *PHLDB1*, *lnc-PHLDB1-1*, *BCL9L*, *TRAPPC4*, among others. Coalescence of functional assays and multiomic data analyses indicated that these SNPs likely modulate the activity of 3 regulatory regions: intronic rs57494551 regulatory region, intergenic SNP haplotype (rs4938572, rs4936443, and rs7117261) regulatory region, and rs4938573 regulatory region upstream of the *CXCR5* promoter.

Conclusions: Shared genetic susceptibility at the *DDX6-CXCR5* locus in SjD and SLE likely alters common mechanisms of autoimmunity, including interferon signalling (*DDX6*), autophagy (*TRAPPC4*), and lymphocytic infiltration of disease-target tissues (*CXCR5*). Further, using multiomic data from patients with SjD, combined with bioinformatic and *in vitro* functional studies, can provide mechanistic insights into how genetic risk influences the biological pathways that drive complex autoimmunity.

INTRODUCTION

Sjögren's disease (SjD) and systemic lupus erythematosus (SLE) are distinct yet related autoimmune diseases with heterogeneous clinical presentations with multifactorial aetiologies including both polygenic and environmental risk factors [1–3]. SjD and SLE share several serologic features of systemic disease including aberrant B cell activity, production of circulating auto-antibodies (eg, antinuclear antibody, and anti-Ro/SSA), a prominent interferon signature, and chronic secretion of proinflammatory cytokines in peripheral blood. In addition to systemic features, SjD exhibits organ/tissue-specific lymphocyte accumulation, proinflammatory cytokine secretion, and, ultimately, glandular dysfunction [4–6]. The dysregulated molecular mechanisms underlying the systemic and tissue-specific pathologies result from a complex interplay between incompletely defined polygenic and environmental risks.

Genome-wide association studies (GWASs) have established ~200 SLE risk loci exceeding genome-wide significance (GWS; $P < 5 \times 10^{-8}$) in populations of European and non-European ancestries [2]. In comparison, the genetic susceptibility of SjD is largely understudied. Only 22 GWS SjD risk loci have been identified in populations of European ancestry, many of which are common with SLE [7]. GWASs leverage linkage disequilibrium (LD) to identify statistical associations between a disease trait and a haplotype of co-inherited SNPs, the majority (80%–90%) of which are in noncoding regions of the genome [8–10]. Statistical genetics cannot distinguish functional causal SNPs from benign SNPs spanning risk haplotypes in strong LD that are co-inherited [8,9]. Thus, fine-mapping and functional genetic studies are necessary to identify and mechanistically define how non-coding variants spanning a risk locus alter cell type-specific and context-specific gene expression through the modulation of transcription factor binding, activity of regulatory elements, and/or

the organisation of chromatin regulatory networks [8,10,11]. Further, contrasting the functional effects of risk alleles between SjD and SLE is an important step towards understanding the dysregulation of common and disease-specific molecular mechanisms.

The *DDX6-CXCR5* locus spans ~200 kb of chromosome 11q23.3 and is a common risk association in SLE and SjD. In SLE, the *DDX6-CXCR5* locus was established as GWS in a GWAS of Asian populations (rs4639966: odds ratio [OR], 1.29 [1.22–1.37]; $P = 1 \times 10^{-16}$) [12] and a transancestral meta-analysis of European, African, and Hispanic Amerindian populations (rs10892286: OR, 1.36 [1.08–1.20]; $P = 2.0 \times 10^{-6}$) [13]. The SjD *DDX6-CXCR5* risk locus was established in a GWAS of European ancestry (rs7119038: OR, 0.74 [0.64–0.86]; $P = 1.10 \times 10^{-8}$) [7]. *DDX6* (DEAD-box RNA helicase 6) is a central regulator of the viral RNA recognition pathway of the innate immune system and modulator of type I interferon responses [14–16], but little is known about a potential role in autoimmunity. *CXCR5* (C-X-C chemokine receptor type 5) plays an important role in adaptive immune responses by modulating B and T follicular cell trafficking to the lymphoid germinal centres and germinal center-like structures that form in the salivary gland of patients with SjD and kidneys of patients with SLE [17–19]. Despite these common molecular mechanisms underlying the shared *DDX6-CXCR5* risk association, the allele-specific functional implications of shared risk alleles spanning the SjD and SLE *DDX6-CXCR5* risk haplotype in the periphery and disease-target tissues have not been extensively studied.

This study performed meta-analyses and genetic fine-mapping, coupled with bioinformatic interrogation of publicly available epigenetic data, to prioritise and functionally characterise shared SNPs spanning the SLE and SjD *DDX6-CXCR5* risk haplotypes in immune, salivary gland epithelial, and kidney epithelial cell models. Understanding how shared functional SNPs regulate

WHAT IS ALREADY KNOWN ON THIS TOPIC

- Sjögren's disease and systemic lupus erythematosus are related autoimmune diseases with common clinical manifestations and genetic risk loci associated with increased disease susceptibility, but how genetic risk alters the underlying molecular mechanisms that drive disease onset and/or progression remain unclear. The *DDX6-CXCR5* genetic risk locus is one of the genetic risk loci common between Sjögren's disease and lupus, but the functional implications of shared genetic variants spanning this locus have not been studied.

WHAT THIS STUDY ADDS

- This study performed a genetic meta-analysis and fine-mapping of the common single nucleotide polymorphisms (SNPs) spanning the *DDX6-CXCR5* association in Sjögren's disease and lupus. Subsequent deep bioinformatic interrogation of the common SNPs identified 5 SNPs that demonstrated allele-specific and cell type-specific effects on nuclear protein binding and activity of 3 regulatory regions in human immune, salivary gland epithelial, and kidney epithelial cell models. Mapping of the complex chromatin regulatory network spanning the *DDX6-CXCR5* locus and multiomic analyses suggested that the SNPs likely alter the expression of *DDX6* and *CXCR5* in the tested cell types, as well as other genes in the network including *PHLDB1*, *lnc-PHLDB1-1*, *BCL9L*, *TRAPPC4*, and *HINFP*.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- This study provides several new insights to the field of autoimmune disease genetics. The *DDX6-CXCR5* risk locus has a complex, expanded chromatin regulatory network that likely modulates the expression of multiple genes beyond the index genes, *DDX6* and *CXCR5*, defined based on nearest proximity to the top SNP association in previous genetic studies. Further, coalescence of the bioinformatic data and functional studies suggest that the 5 functionally characterised SNPs may contribute to common disease mechanisms, including dysregulated interferon signalling, lymphocyte infiltration, and autophagy, by dysregulating the expression of several genes spanning the locus. Therefore, this study provides foundational knowledge to support future studies aimed at understanding how the dysregulation of specific target genes influences autoimmune disease pathogenesis.

cell type-specific expression of *DDX6*, *CXCR5*, and/or other genes within the context of a complex chromatin regulatory network will provide new insights into how their genotypic effects modulate common molecular mechanisms of autoimmunity.

METHODS

Patient and public involvement

Transdisease meta-analyses were performed using pre-existing deidentified genotype, Immunochip (IC), and genomic summary results in accordance with Oklahoma Medical Research Foundation (OMRF) institutional review board (IRB) approval (see Data availability statement) [7,13,20,21]. Minor salivary gland biopsies used to perform RNA sequencing of single nuclei (snRNA-seq) were similarly received as deidentified samples in accordance with the OMRF IRB. Written informed consent was obtained in accordance with the IRB of each respective study [7,13,20,21]. All SjD cases fulfilled the America-European Consensus Group criteria for primary SjD [22,23] according to

clinical evaluations performed within their respective studies. All SLE cases fulfilled at least 4 of the 11 American College of Rheumatology revised criteria for SLE [24,25] according to clinical evaluations performed within their respective studies. Retrospective use of pre-existing, deidentified data prohibited patient involvement in this study. Refer to [Supplementary Methods](#) for a complete description of all methods and materials used in this study.

RESULTS

Meta-analysis and genetic fine-mapping of the *DDX6-CXCR5* risk locus show similar profiles in SjD and SLE

Meta-analysis of the *DDX6-CXCR5* locus in SjD was performed using previously published GWAS and IC data for 3851 SjD cases of European ancestry and 23,652 population controls (meta-analysis data set (DS)1 [DS1-SjD]) (Fig 1A) [7,21]. Similarly, meta-analysis was performed in SLE using a European IC DS and summary statistics from GWASs of European or Korean ancestries for 11,840 SLE cases and 28,869 populations controls (meta-analysis DS 2 [DS2-SLE]) (Fig 1A) [13,20]. Similar association signals were observed, but different peak SNP-trait associations (index SNPs) were identified: DS1-SjD – rs7481819 ($OR_{meta} = 0.813$; $P_{meta} = 2.11 \times 10^{-9}$) (Fig 1B; [Supplementary Table S1](#)) and DS2-SLE – rs11217045 ($OR_{meta} = 0.892$; $P_{meta} = 2.02 \times 10^{-10}$) (Fig 1C; [Supplementary Table S2](#)). A transdisease meta-analysis performed after merging DS1-SjD and DS2-SLE similarly identified index SNP rs11217045 ($OR_{meta} = 0.869$; $P_{meta} = 2.42 \times 10^{-17}$) (Fig 1D; [Supplementary Table S3](#)).

To further refine the *DDX6-CXCR5* association signals, Bayesian analyses were performed on DSs from which we had direct access to genotype data using Trinculo [26] and PICS2 [27]. Bayesian analysis of DS1-SjD identified 66 SNPs in the 95% credible set, with a maximum posterior probability of 0.087 (rs7123726) (Fig 1E; [Supplementary Table S4](#)). Since the only SLE genotype DS available was from IC, separate logistical regression and Bayesian analyses were performed using SjD or SLE IC data ([Supplementary Fig S1A](#)). Of note, this SjD IC DS consisted of 916 IC cases and a subset ($n = 1000$) of the genotyped cases from the DS1-SjD GWAS that were independently analysed by IC. The SLE DS included the IC data from DS2-SLE. The 6194 IC population controls from DS2-SLE were used as controls for both SjD and SLE IC analyses. Consistent with the meta-analysis, similar association signals were observed with different peak index SNPs ([Supplementary Fig S1B-G](#); [Supplementary Table S5-S7](#)).

RegulomeDB [28] was used to screen the top 100 SNPs from the DS1-SjD credible SNP set, ranked by highest posterior probability, to identify variants with potential biological relevance (Fig 2A; [Supplementary Table S4](#)). RegulomeDB is a database that uses a scoring system of 1 (best score) to 7 (worst score) to predict SNP functionality based on reported evidence of expression quantitative trait loci (eQTLs), transcription factor binding, transcription factor motifs, chromatin accessibility peaks, and chromatin states consistent with regulatory activity [28]. Thirteen candidate SNPs were identified as (1) having exhibited significant association ($P < 1 \times 10^{-7}$) in the meta-analyses and receiving a RegulomeDB score of ≤ 4 or (2) were index SNPs identified in the current meta-analyses or previously reported in SjD, SLE, or related autoimmune diseases (Fig 2A; [Table](#) [7,13,18,29]).

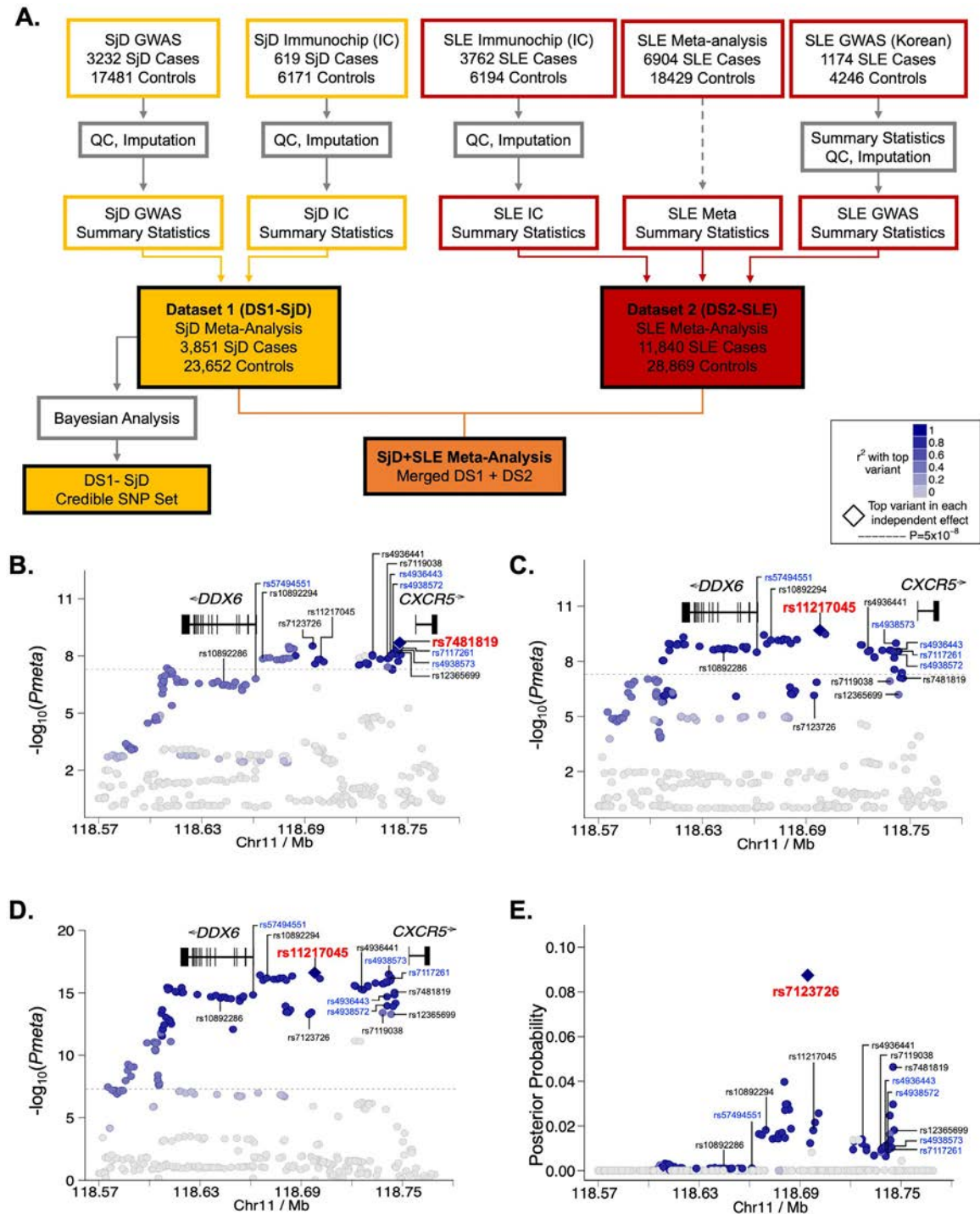


Figure 1. Meta-analysis and genetic fine-mapping of the shared *DDX6-CXCR5* locus associated with Sjögren's disease (SjD) and systemic lupus erythematosus (SLE). (A) The statistical genetic analysis workflow applied to data set (DS)1-SjD (yellow), DS2-SLE (red), and merged DS1-SjD and DS2-SLE (orange) data sets. (B-D) Logistic regression analysis of (B) DS1-SjD, (C) DS2-SLE, and (D) merged DS1 + DS2 after quality control and imputation. For (B-D), the index SNPs of the *DDX6-CXCR5* association are in red. Candidate single nucleotide polymorphisms (SNPs) selected from the credible SNP set for additional bioinformatic screening are labelled in black. The 5 SNPs prioritised for functional characterisation are labelled in blue. (E) Posterior probability distribution of SNPs in the *DDX6-CXCR5* region of DS1-SjD (top SNP: rs7123726 [red]).

We evaluated LD to ensure the 13 candidate functional variants were coinherited in European ancestry. LD analysis showed $D' \geq 0.85$ and $r^2 \geq 0.64$ (Supplementary Fig S2A,B). Specifically, index SNPs rs7481819 (DS1-SjD) and rs11217045 (DS2-SLE) exhibited moderately high LD ($r^2 = 0.84$) and strong coinheritance ($D' = 0.94$) on the *DDX6-CXCR5* risk haplotype of SjD in European populations (Supplementary Fig S2A,B). Strong coinheritance of SNPs spanning the risk locus may explain why the Bayesian analyses were unable to sufficiently refine the

association to predict likely functional SNPs from the 95% credible SNP set (threshold of >0.05).

Bioinformatic interrogation prioritises 5 common SNPs on the SjD and SLE DDX6-CXCR5 risk locus with evidence of functionality

To further evaluate the potential functional implications of the 13 candidate SNPs, a deep interrogation of multiple publicly available bioinformatic databases was performed (Fig 2A). In

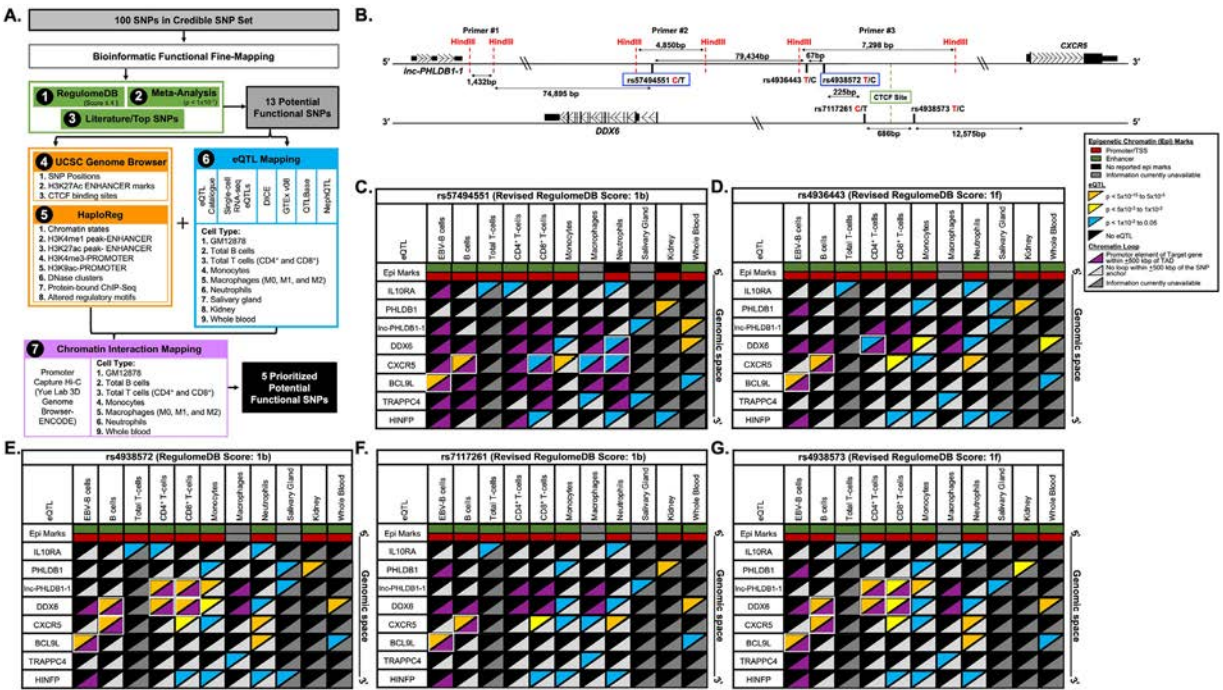


Figure 2. Bioinformatic interrogation and prioritisation of candidate functional single nucleotide polymorphisms (SNPs) shared on the Sjögren's disease (SjD) and systemic lupus erythematosus *DDX6-CXCR5* risk haplotypes. (A) The bioinformatic interrogation workflow used to identify candidate functional SNPs from the top 100 SNPs in the credible SNP set from the Bayesian analysis of data set (DS)1-SjD. (B) Schematic of the 5 SNPs prioritised for functional characterisation arranged in genomic space. Risk alleles are indicated by red font and nonrisk alleles by black. HindIII restriction sites (red dotted line), CTCF site (green box), and positions of 3C-qPCR primers 1-3 are indicated. SNPs rs57494551 and rs4938572 (blue boxes) tag the 3C-qPCR loop anchors. (C-G) Revised RegulomeDB scores and publicly reported cell type-specific epigenetic modifications indicative of promoter or enhancer function (horizontal rectangles), select expression quantitative trait loci (eQTLs) from the databases shown in A (top triangles), and Hi-C chromatin-chromatin interactions within ±500 kbp of each SNP (bottom triangles) are shown for rs57494551 (C), rs4936443 (D), rs4938572 (E), rs7117261 (F), and rs4938573 (G) across the 11 indicated human immune cell types or tissues. White boxes indicate eQTLs and chromatin interaction data coalescence.

addition to GTEx (v08), several additional eQTL databases were mined for reported eQTLs in disease-relevant immune cell types, salivary gland, and kidney tissues. All 13 candidate SNPs have a reported eQTL and chromatin accessibility peak in at least 1 reported cell type (Supplementary Fig S3). A revised RegulomeDB score was calculated to reflect additional reported eQTLs (Supplementary Fig S3). HaploReg [30] was then used to specifically assess the potential cell type-specific regulatory activity (ie,

chromatin states) of SNPs in immune cells and kidney tissues. Chromatin state data for the salivary gland were not publicly available. Despite revised RegulomeDB scores of 1d or 1f, 8 of the candidate SNPs exhibited minimal evidence of regulatory activity and were eliminated from further study (Supplementary Fig S3).

To evaluate the potential functional implications of the remaining 5 SNPs, publicly available cell type-specific

Table
Summary of the meta-analysis results for the 13 shared candidate SNPs on the SjD and SLE *DDX6-CXCR5* risk locus

SNP rsID	Chr 11 Base pair	Minor	Major	DS1-SjD		DS2-SLE		Merged SjD + SLE (DS1 + DS2)	
				Meta		Meta		Meta	
				OR	P	OR	P	OR	P
rs10892286	118642085	C	A	0.8441	2.978×10^{-7}	0.9056	2.224×10^{-9}	0.8842	2.64×10^{-15}
rs57494551	118661398	T	C	0.8404	1.377×10^{-7}	0.9068	3.159×10^{-9}	0.8836	1.45×10^{-15}
rs10892294	118667357	C	G	0.8266	1.093×10^{-8}	0.9009	1.09×10^{-9}	0.875	9.87×10^{-17}
rs7123726 ^a	118694297	C	T	0.8189	2.614×10^{-9}	0.9028	7.019×10^{-7}	0.8736	5.31×10^{-14}
rs11217045 ^b	118697865	A	C	0.8254	1.361×10^{-8}	0.8922	2.015×10^{-10}	0.869	2.42×10^{-17}
rs4936441	118725660	C	G	0.8252	1.982×10^{-8}	0.9017	2.936×10^{-9}	0.8751	5.24×10^{-16}
rs7119038	118738281	G	A	0.8369	3.362×10^{-8}	0.921	1.177×10^{-7}	0.8918	3.88×10^{-14}
rs4936443	118740864	C	T	0.8289	4.73×10^{-8}	0.9047	6.469×10^{-9}	0.8785	2.03×10^{-15}
rs4938572	118740931	C	T	0.8299	5.669×10^{-8}	0.9031	2.772×10^{-8}	0.8778	1.11×10^{-14}
rs7117261	118741157	T	C	0.8206	8.314×10^{-9}	0.9015	3.415×10^{-9}	0.8736	1.48×10^{-16}
rs4938573	118741842	C	T	0.82	6.945×10^{-9}	0.9004	1.009×10^{-9}	0.8728	3.26×10^{-17}
rs12365699	118743286	A	G	0.8086	7.308×10^{-9}	0.8952	6.493×10^{-7}	0.8656	5.17×10^{-14}
rs7481819 ^c	118745278	C	T	0.813	2.105×10^{-9}	0.8987	4.021×10^{-8}	0.869	9.37×10^{-16}

DS, data set; SjD, Sjögren's disease; SLE, systemic lupus erythematosus; SNP, single nucleotide polymorphism; OR, odds ratio.

^a Top SNP from the Bayesian analysis of DS1-SjD.
^b DS2-SLE and merged DS1 + DS2 meta-analysis index SNP.
^c DS1-SjD meta-analysis index SNP.

promoter-capture Hi-C data (hereafter simplified as Hi-C) were used to map chromatin-chromatin interactions (± 500 kbp of each SNP) between the regulatory region carrying the indicated SNP and the promoter of cell type-specific or tissue-specific eQTLs spanning the *DDX6-CXCR5* locus (Fig 2C–G). Coalescence of select reported eQTLs and Hi-C data suggest that the remaining 5 SNPs are positioned in regulatory elements that may modulate the expression of *DDX6*, *CXCR5*, and other genes spanning the *DDX6-CXCR5* risk locus (Fig 2C–G). For example, rs57494551 is an eQTL for several transcripts in immune cells and positioned in an intronic regulatory element that forms cell type-specific chromatin-chromatin interactions with the promoters of *DDX6*, *CXCR5*, and *BCL9L*, but not *IL10RA* (Fig 2B,C). These data suggest that rs57494551 may exhibit regulatory effects on *DDX6*, *CXCR5*, and *BCL9L* through modulating interactions between the intronic enhancer and the promoters of these genes. Coalescence of eQTLs (eg, *lnc-PHLDB1-1*, *DDX6*, *CXCR5*, *IL10RA*, and *TRAPPC4*) and Hi-C data were also observed for the 4 SNPs in the shared regulatory region (within the 7298-bp loop anchor defined by HindIII restriction enzyme cut sites) between *DDX6* and *CXCR5* (Fig 2B,D–G). Unfortunately, publicly available Hi-C data were unavailable for the salivary gland; therefore, any potential coalescence between the reported *PHLDB1*, *TRAPPC4*, *HINFP*, and *lnc-PHLDB1-1* eQTLs and chromatin-chromatin interactions with these regulatory elements could not be inferred.

Prioritised SNPs demonstrate allele-specific nuclear protein complex binding

Electromobility shift assays (EMSAs) were performed in Epstein-Barr virus (EBV) B cells, Daudi B cells, Jurkat T cells, THP1 monocytes, HEK293T immortalised human embryonic kidney cells, and/or A253 cells originating from a submandibular gland squamous cell carcinoma to assess the cell type-specific and allelic effects of the 5 prioritised SNPs on nuclear protein binding (Supplementary Tables S8 and S9). Binding specificity was confirmed by competition assay with unlabelled probes. All 5 SNPs demonstrated varying allele-specific and cell type-specific nuclear protein binding with varied band patterns (Fig 3; Supplementary Fig S4–S7).

The risk allele of rs57494551 impaired nuclear protein binding compared with the nonrisk allele in EBV B cells ($P_{\text{EMSA}} < 5.8 \times 10^{-3}$) and THP1 monocytes ($P_{\text{EMSA}} < 3.9 \times 10^{-2}$) but increased binding in A253 cells ($P_{\text{EMSA}} = 1.4 \times 10^{-2}$) (Fig 3A,B; Supplementary Fig S4A,B). The risk alleles of rs4938572 ($P_{\text{EMSA}} < 4.2 \times 10^{-2}$) (Fig 3C,D; Supplementary Fig S4C–E) and rs7117261 ($P_{\text{EMSA}} < 2.0 \times 10^{-3}$) (Supplementary Fig S5) increased protein binding compared with the nonrisk allele in all tested immune cell lines and A253 cells, but not in HEK293T cells. The risk allele of rs4936443 decreased nuclear protein binding compared with the nonrisk allele in all cell lines tested ($P_{\text{EMSA}} < 1.1 \times 10^{-2}$), except EBV B cells (Supplementary Fig S6). The risk allele of rs4938573 increased protein binding in B cell lines ($P_{\text{EMSA}} < 1.8 \times 10^{-2}$) but decreased binding in the Jurkat T cells ($P_{\text{EMSA}} < 3.7 \times 10^{-3}$) and exhibited opposing allelic effects in the THP1 monocytes and A253 cells depending on the band analysed (Supplementary Fig S7). As a negative control for the SNP prioritisation pipeline, 2 previously reported SNPs with less bioinformatic evidence of functionality and previously eliminated from this study, rs10892294 and rs12365699, exhibited no change in allele-specific binding in the cell types tested (Supplementary Fig S8) [7]. Collectively, the EMSA results demonstrated that the risk alleles of the 5 prioritised SNPs alter the

binding of cell type-specific nuclear proteins to the DNA sequences flanking each SNP.

Prioritised SNPs demonstrate allele-specific effects on regulatory activity

Luciferase expression assays were performed using a promoter-less vector or minimal-promoter vector to test the allele-specific and cell type-specific effects of the prioritised SNPs on promoter or enhancer activity, respectively (Supplementary Table S10). All 5 SNPs exhibited varying cell type-specific and allele-specific effects on promoter and/or enhancer activity in the tested cell models (Fig 4). A253 cells demonstrated the most allele-specific differences among all tested cell types (Fig 4F). THP1 cells demonstrated the most allele-specific effects among tested immune cell lines (Fig 4F). Observed variable allele-specific and cell type-specific effects of the individual SNPs on regulatory activity in the *in vitro* luciferase assay, which assessed activity independent of the native chromatin architecture, are suggestive of a potential complex and nuanced regulatory mechanism, whereby coinherited SNPs in close proximity may function synergistically in the context of the local chromatin regulatory network to modulate activity.

Three of the 4 SNPs in the regulatory region between *DDX6* and *CXCR5* (rs4936443, rs4938572, and rs7117261) span a 292-bp region separated from rs4938573 by a CTCF-binding site (Fig 2B) and exhibit similar epigenetic enrichment, reported eQTLs, and chromatin-chromatin interactions (Supplementary Fig S3). To test whether the 3 SNPs facilitate concordant or discordant allele-specific effects on regulatory activity, the gBlocks used to clone the SNPs into the luciferase constructs were redesigned such that the nonrisk or risk alleles of the 3 SNPs were cloned together. The risk alleles significantly increased promoter ($P_{\text{luc}} < 1.5 \times 10^{-2}$) and enhancer activity ($P_{\text{luc}} < 2.8 \times 10^{-2}$) in EBV B, Jurkat, and HEK293T cells relative to the nonrisk alleles (Fig 4G). In A253 cells, the 3 risk alleles also significantly increased promoter activity ($P_{\text{luc}} = 1.7.0 \times 10^{-3}$) but impaired enhancer activity ($P_{\text{luc}} = 9.0 \times 10^{-4}$) (Fig 4G). These findings suggest that the 3 SNPs, hereafter referred to as the three SNP haplotype, likely function synergistically to modulate the haplotype-specific and cell type-specific regulatory activity at this locus. The opposing allele-specific and cell type-specific effects observed between rs4938573 (Fig 4E) and the three SNP haplotype (Fig 4G) suggested that the ~978-bp region (within the 7298-bp loop anchor defined by HindIII restriction enzyme cut sites) between *DDX6* and *CXCR5* likely has more than 1 regulatory element, separated by a CTCF site, which are influenced by the *DDX6-CXCR5* SjD/SLE risk haplotype.

Chromosome architecture exhibits common and unique features across immune, salivary gland epithelial, and kidney epithelial cells at the *DDX6-CXCR5* risk locus

Coalescences of bioinformatic analyses, *in vitro* functional assays, and publicly available multiomic data (Fig 5; Supplementary Figs S9–S13) suggest that the 5 prioritised SNPs are positioned in regulatory elements that may modulate allele-dependent, cell type-specific expression of *DDX6*, *CXCR5*, and/or other genes within the chromatin regulatory network, resulting in high inference and modeling of phenotype-related active transcription (IMPACT) [31] scores (Supplementary Fig S14). To test this and address the limited availability of Hi-C data for the tested cell lines, 3C-quantitative polymerase chain reaction (qPCR) was performed in 4 patient-derived EBV B cell lines (2

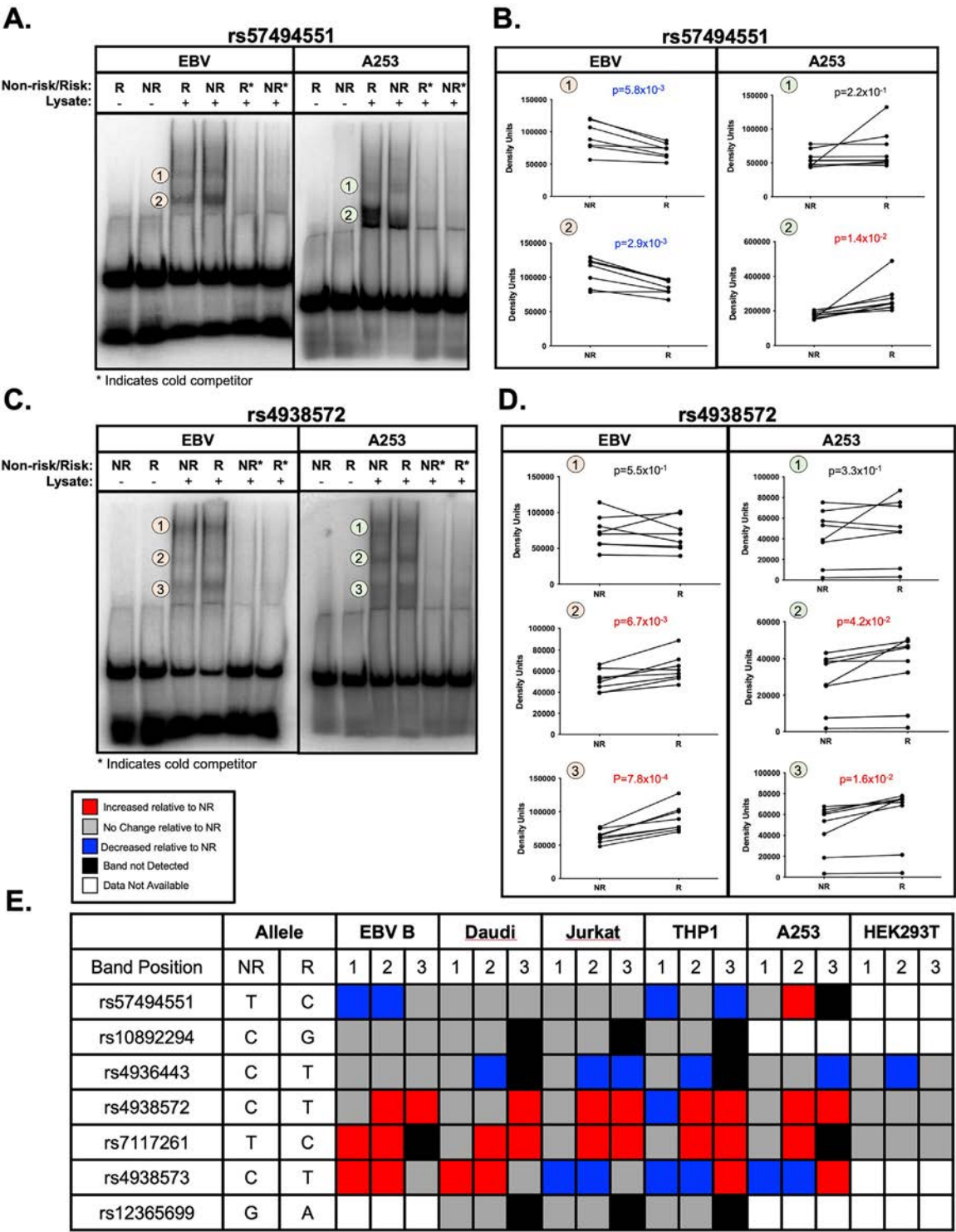


Figure 3. Allele-specific and cell type-specific differential nuclear protein affinities of the prioritised single nucleotide polymorphisms (SNPs) on the shared *DDX6-CXCR5* risk locus. (A–D) Radiolabelled electromobility shift assays were performed to assess the binding affinity of ribonucleoproteins isolated from Epstein-Barr virus (EBV) B or A253 cells to oligonucleotides containing the nonrisk (NR) or risk (R) allele of rs57494551 (A, B) or rs4938572 (C, D). Probes incubated in the absence of nuclear lysate were used as negative controls (lanes 1 and 2). Cold competitors were used to assess nonspecific binding (lanes 5 and 6). Images shown in (A) and (C) are representative of n>6 biological replicates. (B, D) Bands indicated in (A) and (C) by the peach or green circles were quantified by densitometry and analysed using paired *t* test (n>6); *P* values are indicated. (E) Summary analysis of the allele-specific nuclear protein affinities of the 5 prioritised SNPs in EBV B, Daudi, Jurkat, THP1, A253, and HEK293T cells shown in (A–D) and Supplementary Figures S4–S8. Increases in binding relative to NR are shown in red; decreases relative to NR in blue; no change relative to NR in grey; no detected band in black; and data not available in white.

homozygous for the *DDX6-CXCR5* nonrisk haplotype; 2 homozygous for the *DDX6-CXCR5* risk haplotype), as well as commercially available A253, Jurkat, THP1, and HEK293T cell lines, using rs574994551 or rs4938572 to tag the loop anchor fragments determined by HindIII digestion (Fig 2B, 6; Supplementary Table S11). The three SNP haplotype and the rs4938573 regulatory elements are positioned between the same HindIII sites used in the Hi-C and 3C-qPCR and, therefore,

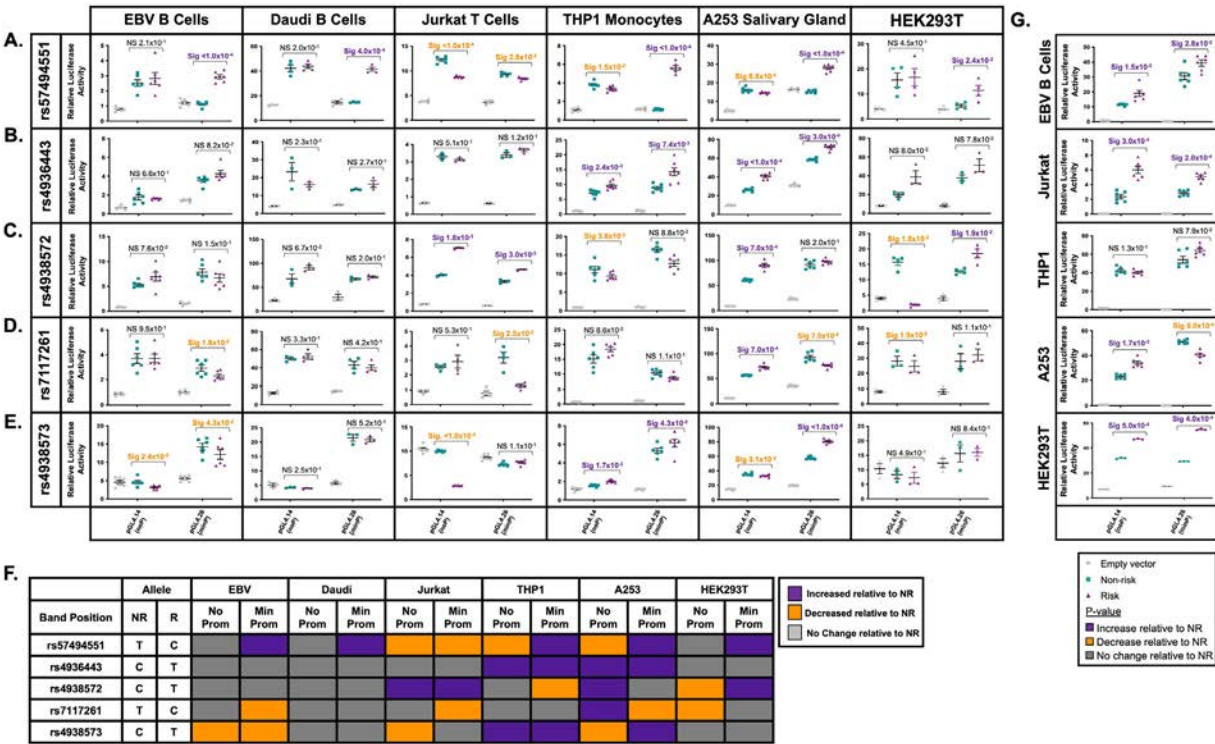


Figure 4. Allele-specific and cell-type specific promoter and enhancer activity of the prioritised single nucleotide polymorphisms (SNPs) on the shared *DDX6-CXCR5* risk region. (A–E) gBlocks carrying the nonrisk (NR) or risk (R) alleles of rs57494551 (A), rs4936443 (B), rs4938572 (C), rs7117261 (D), or rs4938573 (E) were cloned into a promoter-less (pGL4.14; noP) or minimal promoter (pGL4.26; minP) luciferase vector. Plasmids were transfected into Epstein-Barr virus (EBV) B, Daudi, Jurkat, THP1, A253, or HEK293T cells. Luciferase activity was measured after 24 hours and normalised to the Renilla transfection control, reported as relative luciferase activity. Statistical comparisons were performed using a paired *t* test ($n > 3$); *P* values are indicated. (F) Summary analysis of the allele-specific luciferase activity of the 5 prioritised SNPs in EBV B, Daudi, Jurkat, THP1, A253, and HEK293T cells. Increases in luciferase activity relative to NR are shown in purple; decreases relative to NR in orange; and no change relative to NR in grey. (G) gBlocks carrying all NR or all of the R alleles of the three SNP haplotype (rs4936443, rs4938572, and rs7117261) were cloned into the promoter-less or minimal promoter and transfected into EBV B, Jurkat, THP1, A253, or HEK293T cells, and luciferase activity was tested as described. Statistical comparisons were performed using a paired *t* test ($n > 3$); *P* values are indicated.

could not be evaluated independently. Publicly available multiomic data on salivary gland tissues are limited, and Hi-C or long-range chromatin interaction data are nonexistent. Therefore, 3C-qPCR data from A253 cells were plotted in the context of in-house CUT&RUN (H3K27me3 pull-down primer), ATAC-seq, and RNA-seq data (Fig 5B). To gain further insights into how the risk alleles of the 5 prioritised SNPs positioned in these regulatory elements likely modulate gene expression, we analysed the scaled effect sizes of the risk relative to that of nonrisk alleles of reported cell type-specific eQTLs from GTEx.

The rs57494551 regulatory region exhibited the highest relative interaction frequency with the upstream promoter region of *lnc-PHLDB1-1* in all tested cell types except THP1 and HEK293T (Fig 6A). Similar interactions were observed in Hi-C data from several examined cell types (Fig 5; Supplementary Figs S10–S12). The scaled effect size of reported eQTLs from GTEx suggest that the rs57494551 risk allele reduces *lnc-PHLDB1-1* expression in the minor salivary gland (Supplementary Fig S15A).

The rs57494551 regulatory region also interacted with the shared promoter region of *BCL9L* and *UPK2* across most cell types (Figs 5A,B and 6A; Supplementary Figs S9–S13). Hi-C revealed an additional cell type-specific (EBV B, B, and CD8⁺ T cells) interaction with the promoter of *TRAPPC4* (Fig 5A; Supplementary Figs S9 and S10B), but this was not observed in the 3C-qPCR of EBV B cells. The scaled effect sizes of the *BCL9L*

eQTL (blood and EBV B cells) and *TRAPPC4* eQTL (minor salivary gland) suggest that the rs57494551 risk allele may exhibit cell type-specific opposing effects on the long-range enhancer activity (Supplementary Fig S15A).

The SNP haplotype tagged by rs4938572 also revealed the highest relative interaction frequency with the promoter region of *lnc-PHLDB1-1* in all tested cell types (Fig 6B) and Hi-C data from T cells (Supplementary Fig S10). The scaled effect size of *lnc-PHLDB1-1* eQTL was reduced in minor salivary gland in the context of the rs4936443, rs4938572, and rs7117261 risk alleles (Supplementary Fig S15B–D).

An interaction was also observed between the SNP haplotype regulatory element and the shared promoter region of *BCL9L* and *UPK2* in all tested cell types (Fig 6B) and in Hi-C data from EBV B and CD4⁺ and CD8⁺ T cells (Fig 5A; Supplementary Fig S10). Risk alleles of the SNP haplotype were associated with decreased expression of *BCL9L* in blood and EBV B cells (not in rs7117261) (Supplementary Fig S15B–E). Hi-C data from B and T cells, macrophage, and neutrophils also demonstrated interactions between this regulatory region and the rs57494551 regulatory region, but not with the *BCL9L-UPK2* promoter (Supplementary Figs S9, S10, S12, and S13). The risk alleles of rs4936443, rs4938572, and/or rs4938573 also exhibited increased scaled effect size for *DDX6* in peripheral blood mononuclear cells (rs4936443 and rs4938572 only), T cells (rs4936443 and rs4938572 only), and monocyte populations

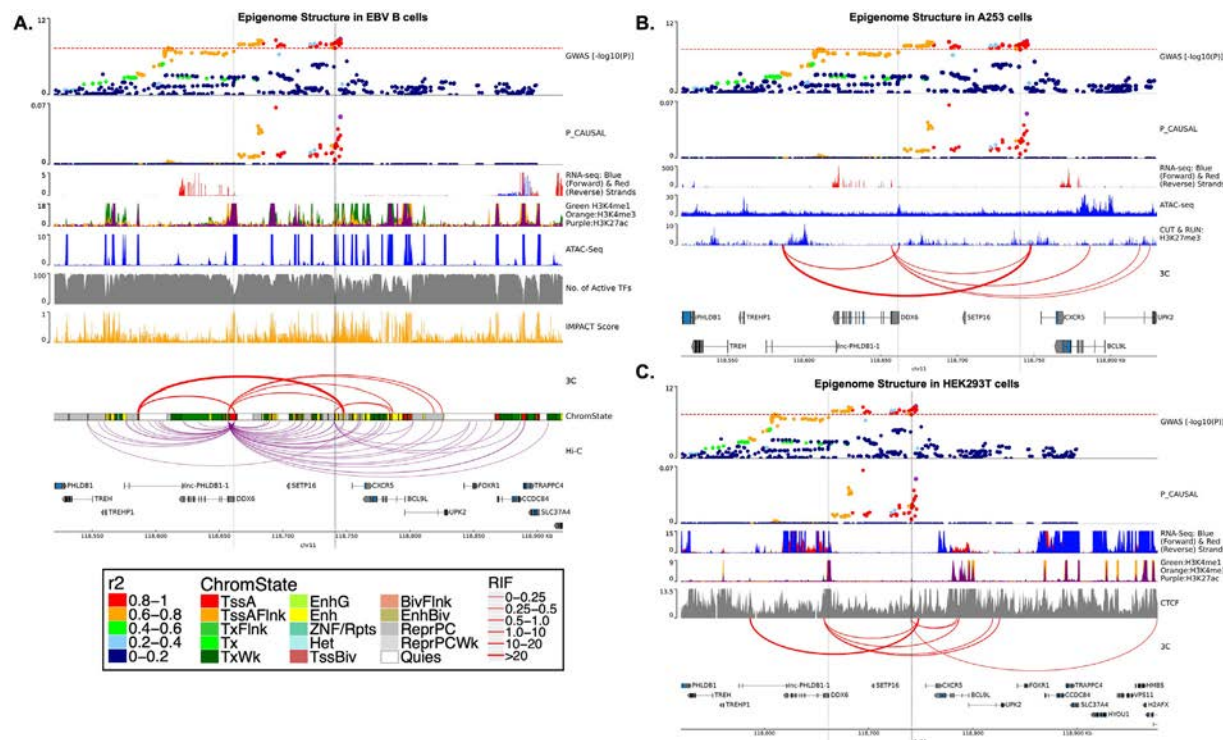


Figure 5. Complex chromatin architecture and epigenetic regulation revealed across the *DDX6-CXCR5* region in Epstein-Barr virus (EBV) B, A253, and HEK293T cell lines by Hi-C. (A) Sjögren’s disease (SjD) genome-wide association study (GWAS) association (top panel) and publicly available epigenomic enrichment across the *DDX6-CXCR5* region in GM12878 EBV B cells. Promoter-capture Hi-C looping (purple lines) contrasts the summary 3C-qPCR results (red lines); line thickness indicates relative interaction frequency (RIF) of the 3C data. For (A–C), vertical grey lines indicate the locations of rs57494551 or rs4938572. (B) SjD GWAS association (top panel) across the *DDX6-CXCR5* region in A253 cells. In-house ATAC-seq, CUT&RUN: H3K27me3 (Epicpyher), and RNA-seq data from A253 cells are shown because of limited publicly available epigenetic data on salivary gland. For (B) and (C), summary 3C-qPCR results are shown (red lines); line thickness indicates RIF. (C) SjD GWAS association (top panel) and publicly available epigenomic enrichment across the *DDX6-CXCR5* region in HEK293T cells.

(Supplementary Fig S15B,C,E). Together, the chromatin-chromatin interaction data and calculated eQTL effect sizes revealed a complex chromatin regulatory network, whereby the 5 prioritised SNPs were positioned in regulatory elements that likely exhibited cell type-specific and allele-specific effects of target gene expression.

Clustered regularly interspaced short palindromic repeat (CRISPR)-targeted modulation of the rs57494551 and three SNP haplotype regulatory elements alters gene expression

To validate the predicted functional effects of the rs57494551 and three SNP haplotype (rs4936443, rs4938572, and rs7117261) regulatory elements, CRISPR inhibition (CRISPRi) and CRISPR activation (CRISPRa) assays were performed using A253 salivary gland epithelial cell lines selected for stable expression of dCas9-KRAB-MeCP2 or dCas9-VP64, respectively, and transduced to express gRNAs targeting rs57494551 or the three SNP haplotype (Supplementary Table S12). qPCR was used to assess the expression of target genes that (1) showed enhancer-promoter interactions by Hi-C and/or 3C-qPCR in the A253 cells (eg, *DDX6* and *BCL9L*) or (2) were reported eQTLs in publicly available salivary gland data (eg, *PHLDB1*, *TRAPPC4*, and *HINFP*) (Fig 2C–F) (Supplementary Table S13). The expression of *lnc-PHLDB1-1* and *CXCR5* were not detected by qPCR in the parental or CRISPR-modified A253 cell lines, and therefore, the suggestive regulatory effects of the SNPs could not be assessed.

CRISPRi of rs57494551 in the A253 cells significantly reduced expression of *DDX6* ($P = 2.1 \times 10^{-5}$), *TRAPPC4*

($P = 1.3 \times 10^{-2}$), and *HINFP* ($P = 3.0 \times 10^{-4}$) (Fig 7A). In contrast, CRISPRa significantly increased the expression of *PHLDB1* ($P = 1.3 \times 10^{-2}$), *DDX6* ($P = 3.0 \times 10^{-3}$), and *HINFP* ($P = 1.3 \times 10^{-2}$). *BCL9L* exhibited trending increased expression ($P = 5.8 \times 10^{-2}$). While CRISPRi of the three SNP haplotype in A253 cells did not reduce the expression of tested genes, CRISPRa significantly increased the expression of *PHLDB1* ($P = 3.0 \times 10^{-4}$) and *DDX6* ($P = 6.0 \times 10^{-4}$) (Fig 7B). Interestingly, CRISPRi of the three SNP haplotype significantly increased the expression of *DDX6* ($P = 3.0 \times 10^{-2}$) and *BCL9L* ($P = 3.6 \times 10^{-2}$). These data collectively suggested that, in A253 cells, the expression of *DDX6*, *PHLDB1*, and, possibly, *BCL9L* are likely modulated by both regulatory elements harbouring rs57494551 and three SNP haplotype, while *TRAPPC4* and *HINFP* are likely modulated by the rs57494551 element. Further, observed changes in the expression of *PHLDB1* and *HINFP*, which are positioned beyond the chromatin regulatory network observed by Hi-C, suggested that additional regulatory mechanisms beyond predicted enhancer-promoter interactions may also occur at the *DDX6-CXCR5* locus.

To further explore the biological implications of rs57494551 and the three SNP haplotype in the context of a SjD target tissue, snRNA-seq data from minor salivary gland biopsies collected from patients with SjD and healthy controls were used to perform eQTL analyses for rs57494551 and rs4938572 (data shown only for rs4938572 since all 3 SNPs showed identical results) (Fig 7C; Supplementary Fig S16). Although no homozygous non-risk subjects were present in our DS, these data showed that rs57494551 and the three SNP haplotype likely modulate allele-specific and cell type-specific expression of *DDX6*, *BCL9L*,

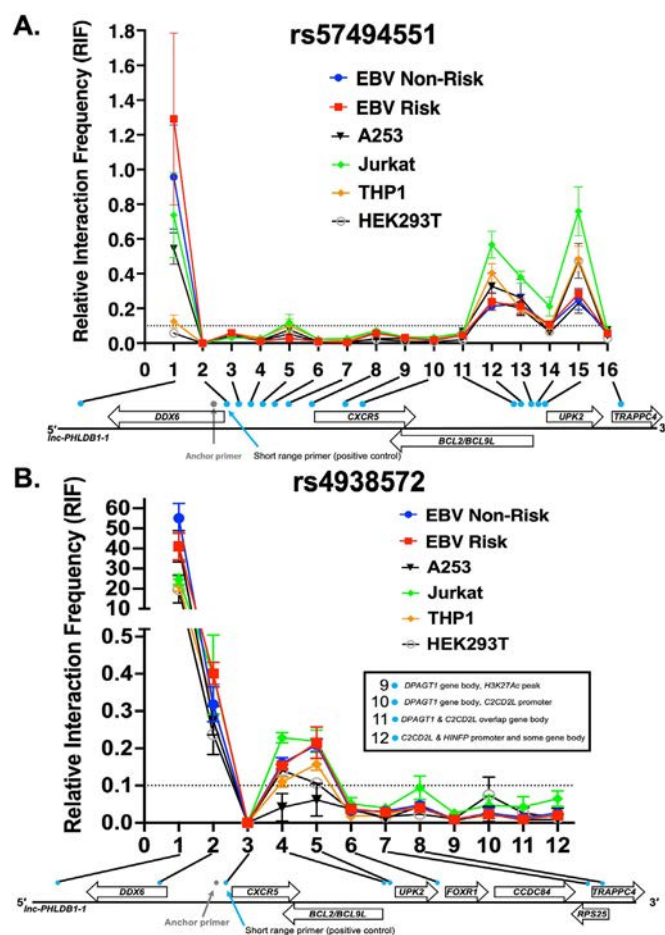


Figure 6. The rs57494551 or rs4938572 regulatory element form chromatin-chromatin interactions with promoters spanning the *DDX6*–*CXCR5* region in immune, A253, and HEK293T cell lines. Chromatin conformation capture with quantitative polymerase chain reaction (3C-qPCR) across the *DDX6*–*CXCR5* region was performed in Epstein-Barr virus (EBV) B cell lines homozygous for the nonrisk or risk haplotype, as well as A253, Jurkat, THP1, and HEK293T cell lines using either (A) rs57494551 or (B) rs4938572 as the anchor single nucleotide polymorphism (grey dot). Relative interaction frequency (RIF) is plotted relative to the primer number in 5'–3' genomic orientation (blue dots; see Fig 2B for additional detail). Primers 9–12 are shown in a text box for simplicity in (B). Each data point represents the mean of $n > 12$. Each cell line was normalised to the loop containing the anchor primer and the short-range primer (positive control) and then normalised to zero. The arbitrary cut-off line at RIF 0.1 was used to determine interaction frequency intensity.

TRAPPC4, and *HINFP* in nuclei from minor salivary gland biopsies (Fig 7C; Supplementary Fig S16). *CXCR5* eQTLs were not observed in the snRNA-seq data, but this was likely due to coarse cell typing and small sample size, leading to loss of specific *CXCR5*-expressing cell types in the homozygous nonrisk samples during analysis. *CXCR5* was a reported eQTL in several cell types in publicly available data (Fig 2C–G) and previously reported to exhibit allele-specific expression in circulating B cell subsets in SjD [18], emphasising the importance of *CXCR5* gene expression regulation in disease pathology.

DISCUSSION

This study leveraged a transdisease meta-analysis, genetic fine-mapping, integration of bioinformatic data to prioritise 5 putative functional SNPs spanning the *DDX6*–*CXCR5* risk locus for allele-specific functional studies in cell types and tissues

relevant to autoimmune disease, specifically immune cell types and the SjD target tissue, salivary gland. Collectively, the *in vitro* functional assays demonstrated that the 5 SNPs exhibited both allele-specific and cell type-specific effects on 1 of the 3 regulatory elements: the rs57494551 intronic, three SNP haplotype (rs4936443, rs4938572, and rs7117261), and rs4938573 regulatory elements (Supplementary Fig S17). Comparisons of cell type-specific eQTL effects sizes in the context of reported intra-chromosome interactions spanning the *DDX6*–*CXCR5* locus suggest that the risk alleles of these 5 SNPs likely modulate expression of several genes through altered enhancer and/or promoter elements (Supplementary Fig S17). Further, reported eQTLs were found lacking evidence of intrachromatin interactions, suggesting additional regulatory mechanisms may also be modulated in response to the risk alleles (Supplementary Fig S17). While *CXCR5* and *BCL9L* have previously defined functional implications in autoimmune disease, *PHLDB1*, *DDX6*, *TRAPPC4*, and *lnc-PHLDB1-1*, among others, remain incompletely understood or have yet to be reported in SjD or SLE.

The *CXCR5*–*CXCL13* axis is a critical regulator of adaptive immune responses. Patients with SjD carrying the *DDX6*–*CXCR5* risk haplotype (tagged by the risk allele [T] of rs4938573 [$r^2 = 0.87$]) reportedly exhibited lower *CXCR5* expression in circulating *CXCR5*⁺ cells [18]. Owing to strong LD and the location of the HindIII sites leveraged in the Hi-C and 3C-qPCR analyses, the effects of the rs4938573 risk allele on reported eQTL effect sizes and DNA looping could not be discerned from the observed effects of the three SNP haplotype. However, the *in vitro* functional studies reported in this study showed that the rs4938573 risk allele decreased nuclear protein binding and impaired promoter activity in Jurkat T cells. Given the position of rs4938573 proximal to the *CXCR5* promoter, this finding is consistent with the observed loss of *CXCR5* expression in T cell subsets in the context of the risk allele (Supplementary Fig S17). In contrast, the functional studies and eQTL effect sizes reported in this study suggest that the rs57494551 risk allele likely increases *CXCR5* expression, specifically in the context of CD19⁺ B cells, through a predicted enhancer-promoter looping interaction (Supplementary Figs S15 and S17). Collectively, these findings suggest that the rs57494551 and rs4938573 regulatory elements may modulate *CXCR5* expression in cell type-specific contexts and that presence of the *DDX6*–*CXCR5* risk haplotype may contribute to dysregulated *CXCR5*–*CXCL13* signaling in genetically susceptible individuals.

DDX6 is a central regulator of the innate immune system and is prominent regulator of interferon signalling in response to viral infection [14–16]. Interferon signalling dysregulation is well established in anti-Ro/SSA autoantibody-positive SjD and SLE [32–35]. Chronic upregulation of interferon-stimulated genes, known as the interferon signature, has been implicated in the pathogenesis of both diseases [35–39]. *DDX6* reportedly binds viral RNA and modulates interferon signalling by interacting with pathogen-sensing regulators, such as RIG-I and OAS1 [14–16]. Interestingly, *OAS1* is also a risk locus for SjD and is implicated in dysregulated interferon signalling [40]. Results from this study suggest that *DDX6* expression is also likely modulated by both the rs57494551 and three SNP haplotype regulatory elements (Supplementary Fig S17). Altered *DDX6* expression after CRISPRi and CRISPRa targeting to rs57494551 suggested that the rs57494551 regulatory region (positioned in the first intron of *DDX6*) likely exhibits proximal regulatory effects on expression. In contrast, the three SNP haplotype region forms a chromatin-chromatin interaction with the *DDX6* promoter in several immune cell types and the A253 cells and is

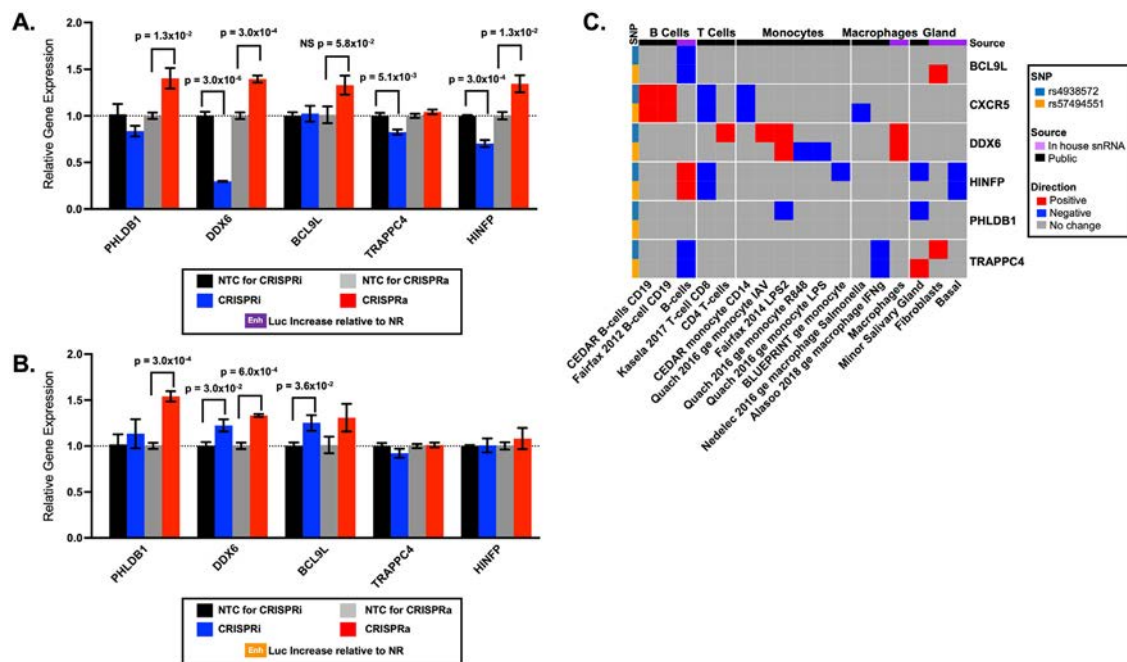


Figure 7. Clustered regularly interspaced short palindromic repeat (CRISPR)-targeted modulation of the rs57494551 and three SNP haplotype regulatory elements alter expression of target genes spanning the *DDX6*–*CXCR5* locus in A253 cell lines. (A, B) A253 cell lines stably expressing either dCas9-KRAB-MeCP2 (CRISPRi; A) or dCas9-VP64 (CRISPRa; B) were transduced with 3 guides targeting either (A) rs57494551 or (B) the three SNP haplotype spanning rs4938572, rs7117261, and rs4936443. Puromycin was used for gRNA selection, cDNA was collected, and relative gene expression of target genes *PHLDB1*, *DDX6*, *BCL9L*, *TRAPPC4*, and *HINFP* were assessed. CRISPRi (blue bar) and CRISPRa (red bar) was determined by comparing gene expression of the target SNP to nontarget control (NTC). Statistical comparisons were performed using a paired *t* test (*n* > 3); *P* values are indicated. Summarised enhancer luciferase data for rs57494551 (A) and the three SNP haplotype (B) are shown for A253 cells in the key, with the direction indicated relative to the nonrisk (NR) allele. (C) Heatmap of expression quantitative trait loci (eQTLs) for SNPs rs57494551 and rs4938572 in 6 genes spanning the *DDX6*–*CXCR5* locus. eQTLs shown are from in-house human salivary gland snRNA-seq (purple) and publicly available database data (black) in multiple different immune and gland cell types. Only publicly sourced eQTL data with directionality available were plotted. eQTLs that increase (red), decrease (blue), or show no change (grey) in relative gene expression are plotted.

a reported eQTL for rs4938572 with positive effect sizes for the risk allele in peripheral blood mononuclear cells, T cells, and activated monocytes in public databases and in resident macrophage from the salivary gland (Supplementary Fig S17). Given the implicated role of *DDX6* in interferon regulation, upregulated *DDX6* expression may contribute to the chronic interferon upregulation implicated in the pathogenesis of SjD, SLE, and many other autoimmune diseases.

Expanding beyond *DDX6* and *CXCR5*, this study revealed complex chromatin-chromatin interactions between the rs57494551 and three SNP haplotype regulatory elements and the promoters of *TRAPPC4* and/or *BCL9L*, as well as concordant eQTL effect sizes and observed allelic effects on luciferase activity (Supplementary Fig S17). Specifically, *TRAPPC4* and *BCL9L* were discovered eQTLs for rs57494551 and rs4938572, with the risk alleles exhibiting cell type/tissue-specific directional effects in peripheral B cells and monocytes, as well as fibroblasts and resident macrophages in the salivary gland. *TRAPPC4* is an important regulator of autophagy [41], while *BCL9L* is an oncogene that was shown to promote T cell infiltration of the lymphoid tissue in breast cancer [42,43]. Although incompletely understood, dysregulated autophagy and subsequent accumulation of autoantigens, as well as increased lymphocytic infiltration of target tissues, have been implicated in the loss of self-tolerance in specific disease-target tissues, such as salivary gland in SjD and kidney tissue in SLE [44]. The analyses reported in this study suggest the *DDX6*–*CXCR5* risk haplotype may facilitate autoimmune disease pathogenesis by modulating several mechanisms implicated in loss of self-tolerance.

Identification and characterisation of lncRNAs and their role in the pathogenesis of autoimmune disease is of growing interest but continues to be restricted by insufficient classification standards, omission from publicly available bioinformatic databases, and limited technologies for functional characterisation [45]. This study revealed that the previously uncharacterised *lnc-PHLDB1-1* is positioned within the expanded chromatin regulatory network of the *DDX6*–*CXCR5* locus. Further, results revealed chromatin interactions between the promoter region of *lnc-PHLDB1-1* and both the rs57494551 and three SNP haplotype regulatory elements. Although the functional significance of altered *lnc-PHLDB1-1* expression in autoimmunity remains unclear, *lnc-PHLDB1-1* is reportedly downregulated in whole blood of individuals with juvenile idiopathic arthritis, several primary and cultured immune cell types under varying inflammatory conditions, and in multiple types of cancer (Supplementary Fig S18) [46], suggesting that it may function as a negative regulator of inflammation.

Results from this study were limited by the availability of publicly accessible genetic and bioinformatic data and restricted largely to *in vitro* immortalised cell lines. While the use of immortalised cell lines may not translate directly to primary human cells or address the heterogeneity within salivary gland or kidney tissues, they provide an important foundational model to functionally test the allelic effects of predicted likely functional SNPs within the context of specific human cell types and regulatory landscapes. Limited or nonexistent single-cell multiomic data availability, specifically from salivary gland and kidney tissues, continues to limit the interpretations of allelic cell type-specific effects in heterogeneous tissue types. To bridge this gap,

the A253 salivary epithelial cell model and HEK293T kidney epithelial cell model were extensively analysed in this study. Inclusion of in-house CUT&RUN, ATAC-seq, and RNA-seq data from A253 cells, as well as minor salivary gland biopsies, is an important step towards filling this void. Although concordant findings from the *in vitro* functional assays, deep bioinformatic interrogations of multiomic data, eQTLs, Hi-C/3C-qPCR results, and CRISPRi/CRISPRa experiments support the functional implications suggested in this study, future use of prime-editing technologies in primary immune, salivary epithelial, and kidney epithelial cells will be necessary for validation and downstream functional studies. Finally, this study used bioinformatics as an effective method to prioritise SNPs for functional validation; however, there are likely additional functional SNPs spanning the *DDX6-CXCR5* risk locus.

Despite limitations, this study successfully identified 5 shared SNPs spanning the SjD/SLE *DDX6-CXCR5* risk haplotype that exhibited allele-specific and cell type-specific molecular functions. Of note, 4 of the 5 SNPs have been associated with rheumatoid arthritis (rs7117261, rs4938572, and rs4936443) [47], primary biliary cholangitis (rs7117261) [48], and follicular lymphoma (rs4938573) [49,50]. SNP rs4938573 is also a reported SLE-associated and SjD-associated biological sex-influenced eQTL [51]. Results reported in this study provide important new insights into how genetic susceptibility at the *DDX6-CXCR5* locus likely modulates cell type-specific and allele-specific expression of *DDX6*, *CXCR5*, and other genes within the regulatory network (*BCL9L*, *TRAPPC4*, *PHLDB1*, *Inc-PHLDB1-1*, and *HINFP*) and how altered target gene expression may contribute the dysregulation of common molecular mechanism in SjD and SLE.

Competing interests

AR received consulting fees from Immunovant and Clinical Outcomes Solutions. ND received a teaching honorarium from the British Association of Dermatologists. JLJ received travel support from the University of Oslo. MVJ received travel support from the International Symposium for Sjögren's Syndrome 2022 and serves as a members' representative for the *Journal of the Norwegian Dental Association (Tidende)*. SK is currently employed at Beckman Coulter Inc. TM is currently employed by UCB Pharma. GNoc received consulting fees from Abbvie, Novartis, Galapagos, and Amgen and travel support from Amgen and UCB and is a member of the Boehringer Ingelheim Advisory Board. PO received consulting fees from UCB and Lilly and a speaker honorarium from the Internetmedicin.se (website). CS is employed as Director, CAR-T Immunology European Medical Engagement Lead, at Bristol-Myers-Squibb (since April 2024); received speaker honorariums from AstraZeneca, Novartis, and Bristol-Myers Squibb; and is a member of the Fresenius Kabi Advisory Board. GET is employed by DeCODE genetics/Amgen Inc. SRV served on the American College of Rheumatology Board of Governors and has received research support from Navidea Biopharmaceuticals Inc, ARGENTX, Mallinckrodt, Janssen, and Kiniksa Pharmaceuticals. EMV received research support from AstraZeneca; consulting fees from AstraZeneca, UCB, Otsuka, Abbvie, Novartis, Aurinia, and Pfizer; and payment for expert testimony from the National Institute of Health and Clinical Excellence and serves as a member of the Aurinia Data Safety and Monitoring Board, General Secretary of SLEuro, and Chair of BILAG. MTB received research support and consulting fees from Meira GTx, Lipella, and Sun Pharmaceuticals and travel support from the North Carolina Dental Society. JAJ received research support from the National Institutes of Health and

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Phs000672.v1.p1: Genotype data from the Sjögren's International Collaborative Clinical Alliance (SICCA) Registry was obtained through dbGaP accession number phs000672.v1.p1. This study was supported by the NIDCR, the National Eye Institute, and the Office of Research on Women's Health [N01-DE-32636]. Genotyping services were provided by the Center for Inherited Disease Research (CIDR). CIDR is fully funded through a federal contract from the NIH to the Johns Hopkins University [HHSN268200782096C, HHSN268201100011I, and HHSN268201200008I]. Funds for genotyping were provided by the NIDCR through CIDR's NIH contract. Assistance with data cleaning and imputation was provided by the University of Washington.

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Contributors

CJL supervised the research and serves as senior author. MMW, MR, BK, MLJ, AMS, SY, and AAJ conceived and designed experiments under the supervision of CJL. CJL, BK, AR, J-MA, LAA, S-CB, EB, AB, JGB, SMB, ND, M-LE, FE, HF-d'E, CF, CG, J-EG, DH, JI-K, JLJ, SJAJ, MVJ, JAK, SK, KK, MK, TM, JM, DLM, GNoc, KBN, PO, ØP, J-OP, NLR, CS, KS, KET, PT, GET, SV, EMV,

DJW, KMG, LR, MTB, JAJ, RHS, PMG, LAC, RJ, SA, PE, SJB, RO, LR, BMW, MR, TW, ADF, XM, CHS, MW-H, MEA-R, W-FN, KLA, JMG, IA, TJV, BPT, and GNor collected, characterised, and/or genotyped the cases with SjD and/or SLE and population controls used in this study. SBG, JAK, and JMG managed the technology and/or data required for processing and analysis of large-scale sequencing approaches used. MMW, MR, BK, MLJ, AMS, SY, JL, and CP-F performed experiments. MMW, MR, BK, MLJ, AMS, and JL performed statistical analyses. MMW, MR, BK, MLJ, KLT, AMS, JL, BPT, GNor, and CJL analysed the data. CJL, MMW, MR, BK, KLT, AMS, JL, and CP-F wrote the paper. All authors critically reviewed the paper and accepted authorship responsibilities.

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Patient consent for publication

Written informed consent was obtained in accordance with the institutional review board of each respective study.

Ethics approval

Transdisease meta-analyses were performed using pre-existing deidentified genotype, ImmunoChip, and genomic summary results in accordance with Oklahoma Medical Research Foundation (OMRF) institutional review board approval.

Provenance and peer review

This manuscript was not commissioned. It was independently submitted to the journal by the authors and underwent external peer reviewer and editorial review.

Data availability statement

SjD genome-wide association summary statistics and individual-level data used in SjD-DS1 are previously reported [21] and available through the Databases of Genotypes and Phenotypes (dbGaP) under accession number: phs002723.v1.p1 (http://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs002723.v1.p1), phs000672.v1.p1 (n = 735) (https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs000672.v1.p1), phs000428.v2.p2 (n = 8,519) (https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs000428.v2.p2), phs000196.v3.p1 (n = 995) (https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs000196.v3.p1), and phs000187.v1.p1 (n = 602) (https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs000187.v1.p1). SLE ImmunoChip and GWAS data used in SLE-DS2, previously reported [13,20], are available from the corresponding authors of the original articles in accordance with respective institutional review board approval and subject consent. All remaining data are previously published and can be accessed by the URLs provided or generated by coauthors in this study and will be made available upon request to the corresponding author (chris-lessard@omrf.org) in accordance with an established material transfer agreement.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ard.2025.04.023.

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Vasculitis

Exploring the limit of image resolution for human expert classification of vascular ultrasound images in giant cell arteritis and healthy subjects: the GCA-US-AI project

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ABSTRACT

Objectives: Prompt diagnosis of giant cell arteritis (GCA) with ultrasound is crucial for preventing severe ocular and other complications, yet expertise in ultrasound performance is scarce. The development of an artificial intelligence (AI)-based assistant that facilitates ultrasound image classification and helps to diagnose GCA early promises to close the existing gap. In the projection of the planned AI, this study investigates the minimum image resolution required for human experts to reliably classify ultrasound images of arteries commonly affected by GCA for the presence or absence of GCA.

Methods: Thirty-one international experts in GCA ultrasonography participated in a web-based exercise. They were asked to classify 10 ultrasound images for each of 5 vascular segments as GCA, normal, or not able to classify. The following segments were assessed: (1) superficial common temporal artery, (2) its frontal and (3) parietal branches (all in transverse view), (4) axillary artery in transverse view, and (5) axillary artery in longitudinal view. Identical images were shown at different resolutions, namely 32×32 , 64×64 , 128×128 , 224×224 , and 512×512 pixels, thereby resulting in a total of 250 images to be classified by every study participant.

Results: Classification performance improved with increasing resolution up to a threshold, plateauing at 224×224 pixels. At 224×224 pixels, the overall classification sensitivity was 0.767 (95% CI, 0.737–0.796), and specificity was 0.862 (95% CI, 0.831–0.888).

Conclusions: A resolution of 224×224 pixels ensures reliable human expert classification and aligns with the input requirements of many common AI-based architectures. Thus, the results of this study substantially guide projected AI development.

INTRODUCTION

Giant cell arteritis (GCA) is the most common form of systemic vasculitis in adults and typically affects large and medium-sized arteries, such as the aorta and temporal and axillary arteries. Signs and symptoms of GCA demand urgent action to verify or exclude a diagnosis in order to prevent severe complications, such as permanent vision loss, which occurs in an average of 20% of all cases [1,2], ischaemic stroke [3–5], and arterial aneurysm and/or dissection [6]. Temporal artery biopsy (TAB) has historically been the diagnostic gold standard but is increasingly being replaced by ultrasound of the temporal and axillary artery as the first-line diagnostic modality in many parts

of the world, a practice supported by the latest European Alliance of Associations for Rheumatology (EULAR) recommendations on imaging for GCA [7]. Ultrasound offers widespread use and rapid availability, patient-friendliness, and cost-effectiveness [8], but also excellent image resolution of less than 0.1 mm for superficial arteries with modern ultrasound transducers and the ability to evaluate the entire temporal artery and its branches [9]. This is in contrast to TAB, which may miss GCA detection due to patchy involvement of the artery by vasculitis. The arrival of modern handheld ultrasound devices, characterised by high affordability (<€3000), could further expand future access to point-of-care diagnostic imaging, particularly in resource-limited settings [10]. While their image quality still

WHAT IS ALREADY KNOWN ON THIS TOPIC

- Vascular ultrasound is clinically considered the method of choice for the rapid detection of giant cell arteritis (GCA); however, expertise in ultrasound performance is scarce.
- Artificial intelligence (AI) could assist in image classification, thereby simplifying the examination process.
- However, to develop such an AI, as many AI architectures are constrained by an upper limit of processable image resolution, it is necessary to define the minimum resolution of ultrasound images required to ensure the detection of disease-specific abnormalities.

WHAT THIS STUDY ADDS

- A total of 7750 classification decisions made by 31 international experts were analysed, and 224×224 pixels were found to be the minimum image resolution required for the reliable classification of vascular ultrasound images by human experts.
- As this result aligns with the identical critical processing limit for many AI architectures, it allows a wide variety of AI architecture options to be considered for AI development.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- The findings of this study lay the essential foundation for developing an AI system to assist in ultrasound-based detection of GCA, an approach that holds great potential in allowing rapid diagnosis before ischaemic complications occur and improving examination accessibility even in resource-limited regions.

has some limitations that depend on the specific application, these constraints are expected to decrease with ongoing technological advancements. As a result, these advancements also have the potential to create new educational opportunities for training both specialists and nonspecialists in scanning and interpreting arterial findings in the context of GCA.

GCA ultrasound has proven its excellent reliability [11–16] among experts using appropriate ultrasound equipment, defined as a linear probe ≥ 7 MHz for axillary artery assessment and a linear probe ≥ 18 MHz for cranial artery evaluation [17]. However, the examination, especially the interpretation of findings, requires experience and expertise [14,18–20] that are rare to find and mostly limited to specialised centres. While there are initiatives to train rheumatologists all around the globe, these efforts will presumably be insufficient to achieve the desirable goal of a global network of fast-track clinics with sufficient expertise in GCA ultrasound. With modern technological advances, a suggested solution to close this gap is the development of an artificial intelligence (AI)-based assistant (eg, publicly accessible online) that can help classify submitted ultrasound images as abnormal or not, thereby assisting physicians globally in the early detection of GCA.

AI has been proclaimed to lead the fourth industrial revolution and is seen as having the potential to heavily impact our present and future [21]. Likewise, the introduction of AI in the healthcare sector has accelerated quickly over the last few years [22]. Previous attempts to apply AI in the classification of medical image data have proven feasible and have shown remarkable results when trained to label chest radiographs [23], classify skin cancer [24], or identify breast cancer in mammograms [25]. Recently, the first applications of AI in the field of imaging in rheumatology have been made: Andersen et al [26] developed a neural network for the automatic scoring of arthritis disease activity on joint ultrasound images, and Bressemer et al [27] trained neural networks for the accurate detection of specific

inflammatory joint abnormalities ('sacroiliitis') in pelvic x-rays. However, no attempt has yet been made to utilise AI in GCA for classifying B-mode ultrasound images—standard 2-dimensional greyscale ultrasound images without colour Doppler—as normal or abnormal.

The technological foundation of AI is marked by deep neural networks [28]. A variety of different deep neural network architectures exist, each with distinct characteristics, including limitations in image processing size, resolution, result accuracy, and resource efficiency [28]. In pursuing the outlined development of an AI-based system (see [Supplementary Material](#)), it is crucial to consider the technological constraints of diverse neural network architectures. Neural network architectures that are not able to handle images presented in a sufficiently high resolution, necessary for the adequate evaluation of disease-characteristic features, should be excluded in advance. In this context, the image resolution of 224×224 pixels represents a critical threshold, as it is the maximum limit for input in many common neural network architectures [27,29]. On the other hand, neural network architectures allowing for higher resolution image input are available, but the processing of higher image resolutions increases the computing power demand. Limitations in the available computing power require specifications that limit the maximum image resolution intended for processing. This ensures the selection of a suitable deep neural network architecture that balances resource efficiency with the ability to handle task-appropriate image sizes. In order to provide scientific evidence and determine the requirements a neural network must fulfil, this project aimed to define the minimum resolution required for human experts to reliably classify ultrasound images of arteries commonly affected by GCA for the presence or absence of the disease.

METHODS

Study design

We designed a web-based exercise in which physicians experienced in GCA ultrasonography were asked to classify 250 ultrasound images for the presence or absence of GCA typical morphology. Forty-one physicians from 15 countries were invited by email to participate. To qualify as an expert, all participants must be board-certified rheumatologists with extensive experience in large-vessel vasculitis ultrasonography, defined as a minimum of 4 years interpreting GCA ultrasound images and prior assessment of at least 100 patients with suspected GCA throughout their careers. Additionally, they were members of the Large Vessel Vasculitis subgroup within the Outcome Measures in Rheumatology (OMERACT) Ultrasound subgroup. The study followed the principles of the Declaration of Helsinki and the Guidelines of Good Clinical Practice and received ethical approval from the local ethics committee (Institutional Review Board number #101/22). Patients and the public were not actively involved in the design and conduct of this study.

Vascular ultrasound image source and selection

CJB selected 50 ultrasound images from an image collection provided by 5 international experts in GCA ultrasonography (CM, CP, MM, VSS, and WAS). The selection included 10 images of each of the following vessels: (1) superficial common temporal arteries, (2) their frontal branch, (3) their parietal branch (all in transverse view), (4) axillary arteries in transverse view, and (5) axillary arteries in longitudinal view. All ultrasound

images were obtained in B-mode. The sample included 6 images from patients with acute GCA and 4 images from healthy individuals for each vessel, except for the axillary artery in longitudinal view, which had 5 images from patients with acute GCA and 5 images from healthy individuals. The images were obtained from a total of 50 patients, of which 29 had GCA and 21 had no evidence of GCA.

Each image was derived from an individual patient or a healthy subject. Images were obtained from different ultrasound machine brands (Esaote, GE, and Philips) using 3 to 12 (Philips EPIQ), 4 to 12 (GE LOGIQ e), 6 to 15 (GE LOGIQ S8), 5 to 18 (Philips EPIQ), 6 to 18 (Esaote MyLab 8), 8 to 18 (GE LOGIQ E9), and 6 to 24 MHz (GE LOGIQ E10) linear transducers for the investigation of axillary arteries and 5 to 18 (Philips EPIQ), 8 to 18 (GE LOGIQ S8 and GE LOGIQ E9), and 6 to 24 MHz (GE LOGIQ E10) hockey stick probes for the investigation of cranial arteries. Image acquisition at each site was performed according to a previously published scanning protocol [14]. Intima-media thickness (IMT) measurements were available for all images. All GCA patients had a diagnosis confirmed by a board-certified rheumatologist and met either the extension of the 2016 American College of Rheumatology classification criteria for GCA [30] or the 2022 American College of Rheumatology/EULAR classification criteria for GCA [31] (depending on the date of diagnosis). Diagnosis was confirmed either by TAB or imaging, including ultrasound. None of the patients had a change in

clinical diagnosis from GCA to a non-GCA diagnosis during the follow-up period.

Vascular ultrasound image processing

All ultrasound images were individually loaded into an image editing software (GNU Image Manipulation Program version 2.10.8) to apply the necessary image preparation for this study. IMT measurements were removed if present. From each ultrasound image, a square 512×512 -pixel image section focusing on the appropriate artery was selected and cut out. This section was subsequently downsized (without interpolation) to all study-specific resolution variants, namely 32×32 , 64×64 , 128×128 , and 224×224 pixels. Figure 1 reflects the visual perception of all 5 image resolution variants applied in the study. All 4 downsized variants, in addition to the initial 512×512 -pixel variant, resulted in a total of 250 vascular ultrasound images representing 50 distinct vessel images, each at 5 different resolutions.

Conduct of the web-based classification exercise

The web-based image classification exercise was performed via Research Electronic Data Capture (REDCap, provided by Vanderbilt University [32]) version 13.2.5. It included all vascular ultrasound images without any information about the

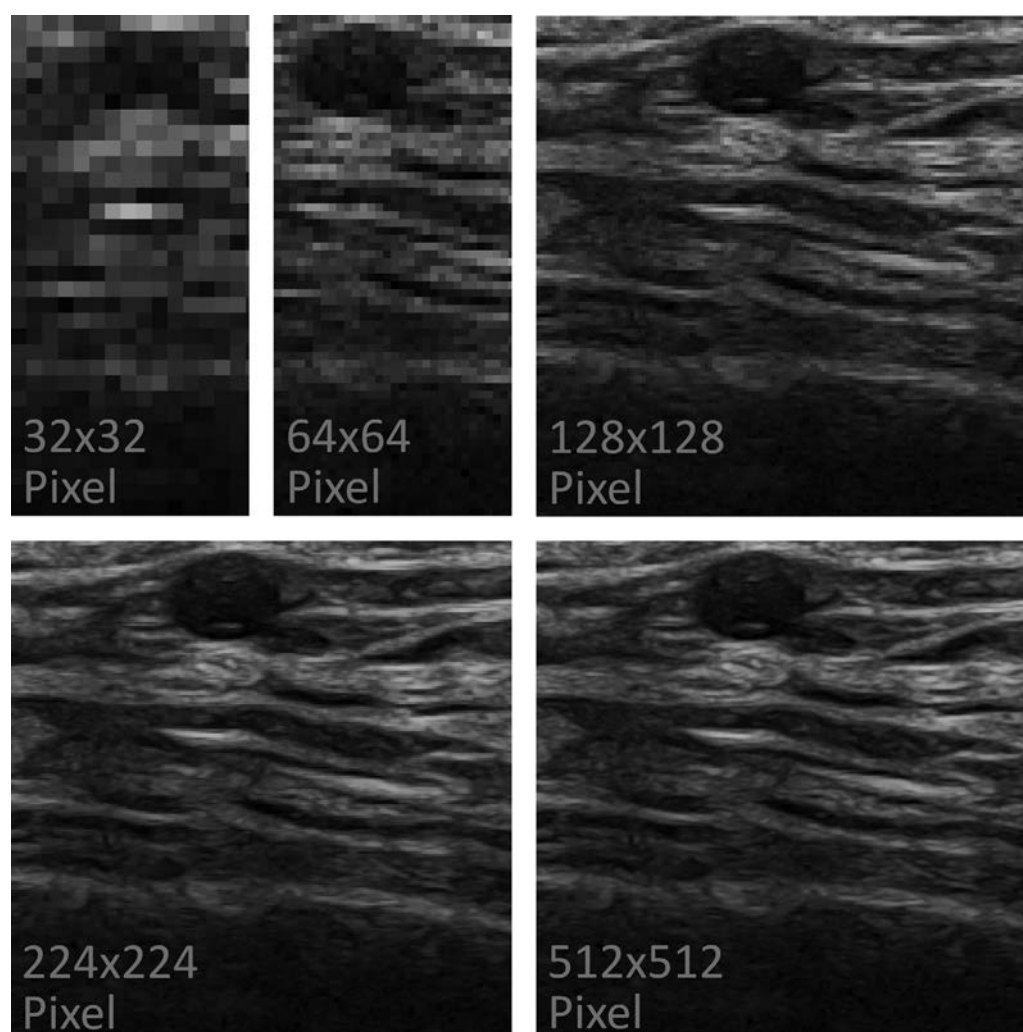


Figure 1. Comparison between the original square 512×512 -pixel cut out from a native B-mode ultrasound image of the superficial common temporal artery in transversal view and its downsized study-specific resolution variants, namely 32×32 , 64×64 , 128×128 , and 224×224 pixels.

corresponding IMT measurements, final ultrasound results, or clinical patient details. Intentionally, participants of the exercise were not told that they were evaluating identical images at different resolutions. To prevent image recall, images were displayed sequentially by artery and section, starting with the lowest resolution, with images presented in random order at each resolution level. During the classification exercise, each image was shown individually (1 per page), with each vascular ultrasound image representing a separate patient case (see [Supplementary Fig S1](#)). Participants were given 3 response options to the question, “I classify this ultrasound image as belonging to a ...”: (1) “GCA patient,” (2) “Healthy individual,” and (3) “Meaningful classification is not possible.” Whenever the latter option was selected, a follow-up dialogue box appeared, prompting the participant to choose either “GCA patient” or “Healthy individual” in response to the question, “If only one of the above-mentioned answers was accepted, I would pick...” This approach to gathering ‘forced classifications’ aimed at a deeper understanding and recognition of patterns in the participants’ decision-making process. Personal data regarding professional experience were also collected from all participants.

Statistical analysis

Statistical analyses were conducted using SPSS Statistics (version 26.0.0.0; IBM Corporation) and R (version 4.3.0; R Foundation for Statistical Computing) [33]. Descriptive statistics and exploratory data analysis were used. For metric parameters, mean, SD, median (in the case of the monthly average image classification figures, which contained individual outliers), and ranges were calculated. Categorical data were summarised by absolute and relative frequencies. Further analysis was performed to determine which change in image resolution is linked to a significant effect on correct image classification using logistic mixed-effects models. An increased chance of correct classification, indicated by an estimated odds ratio (OR) larger than 1, is interpreted as an improvement in classification performance. An estimated OR smaller than 1 is interpreted as a deterioration in image classification performance. To account for repeated measurements per rater and image, these were included as random intercept terms. Further factors impacting the classification decision, such as image resolution, working experience, and whether a study participant had provided study images, were investigated and, therefore, included as fixed effects. The image resolution and arteries were included as categorical independent variables. When the analyses were adjusted for the number of years experts had already been reading GCA ultrasound images or the number of patients that experts estimated to have seen, these were included as metric independent variables. Sensitivity and specificity were calculated on the basis of correct and incorrect classification numbers. In addition to that, 95% CIs were calculated. Due to a technical error in REDCap, detected only after study completion, participants could bypass the ‘forced classification’ within the follow-up question after the initial response had indicated an unclassifiable image. This affected all participants equally. Missing classifications from this issue were excluded from the analysis. The threshold for statistical significance was set at $p = .05$.

RESULTS

Study participant characteristics and expertise

Out of 41 international experts in GCA ultrasonography invited to participate in the web-based exercise, 31 responded,

resulting in a response rate of 75.6%. The cohort comprised 5 board-certified rheumatologists from Denmark, 4 from the United States of America, 4 from Germany, 3 from Italy, 3 from Austria, 2 from the United Kingdom, 2 from the Netherlands, and 1 each from Bulgaria, Norway, Poland, Portugal, Slovenia, Spain, Switzerland, and Turkey. Study participants had an average of 21.2 years of medical experience, ranging from 9 to 40 years. They had been interpreting GCA ultrasound images for an average of 10.9 years, with experience ranging from 4 to 30 years. Participants reported evaluating a median of 16.0 (mean, 70.1; range, 2–500) images from GCA patients per month.

Image classification results

Overall, 7750 classification decisions were obtained (31 participants conducting a total of 310 decisions per artery, view and resolution combined).

Overall, the frequency of participants selecting the response option ‘meaningful classification is not possible’ decreased with increasing image resolution (constantly with every sequential stage of raised image resolution) from 90.5% (at the image resolution stage of 32×32 pixels) to 25.9% (at 512×512 pixels). [Figure 2](#) illustrates how frequently the response option was selected during vascular ultrasound image classification, depending on the respective artery (and view) and image resolution. It was selected least frequently for images of the axillary artery in longitudinal view (19.0%), the axillary artery in transverse view (22.3%), and the temporal artery in transverse view (26.1%) at the 512×512 -pixel resolution.

When experts initially selected ‘meaningful classification is not possible,’ they were subsequently asked to make a forced decision on whether the ultrasound image belonged to a GCA patient or a healthy individual (referred to as ‘forced classification’). [Table 1](#) presents detailed results of ‘confident classification’ provided in the first instance, as well as the ‘forced classification’ outcomes for all arteries, views, and image resolutions. Generally, increasing image resolution from 32×32 to 224×224 pixels led to a higher number and percentage of correct classifications across all arteries and views. This improvement was consistent whether considering only correct confident classifications (32×32 pixels: 4.8%; 64×64 pixels: 13.8%; 128×128 pixels: 37.7%; 224×224 pixels: 57.6%) or both confident and forced classifications (32×32 pixels: 37.8%; 64×64 pixels: 54.8%; 128×128 pixels: 68.6%; 224×224 pixels: 74.4%). No relevant increment was observed in classification results once image resolution increased further from 224×224 pixels to 512×512 pixels (overall correct confident classification at 512×512 pixels: 61.1%; overall correct confident and forced classification at 512×512 pixels: 75.3%).

A detailed view showed that the highest percentage of correct classifications was achieved in ultrasound images of the axillary artery in longitudinal view (total percentage of correct classifications at an image resolution of 512×512 pixels: 83.9%). Images of the axillary artery in transverse view yielded the lowest percentage of correct classifications at any image resolution stage compared with other combinations of arteries and views.

Further analysis of the complete dataset across all arteries and views revealed that any increase in image resolution significantly improved classification performance compared with the reference category of 32×32 -pixel images. Specifically, image resolutions of 64×64 pixels (OR, 2.19; 95% CI, 1.84–2.61; $p < .001$), 128×128 pixels (OR, 3.49; 95% CI, 2.91–4.19; $p < .001$),

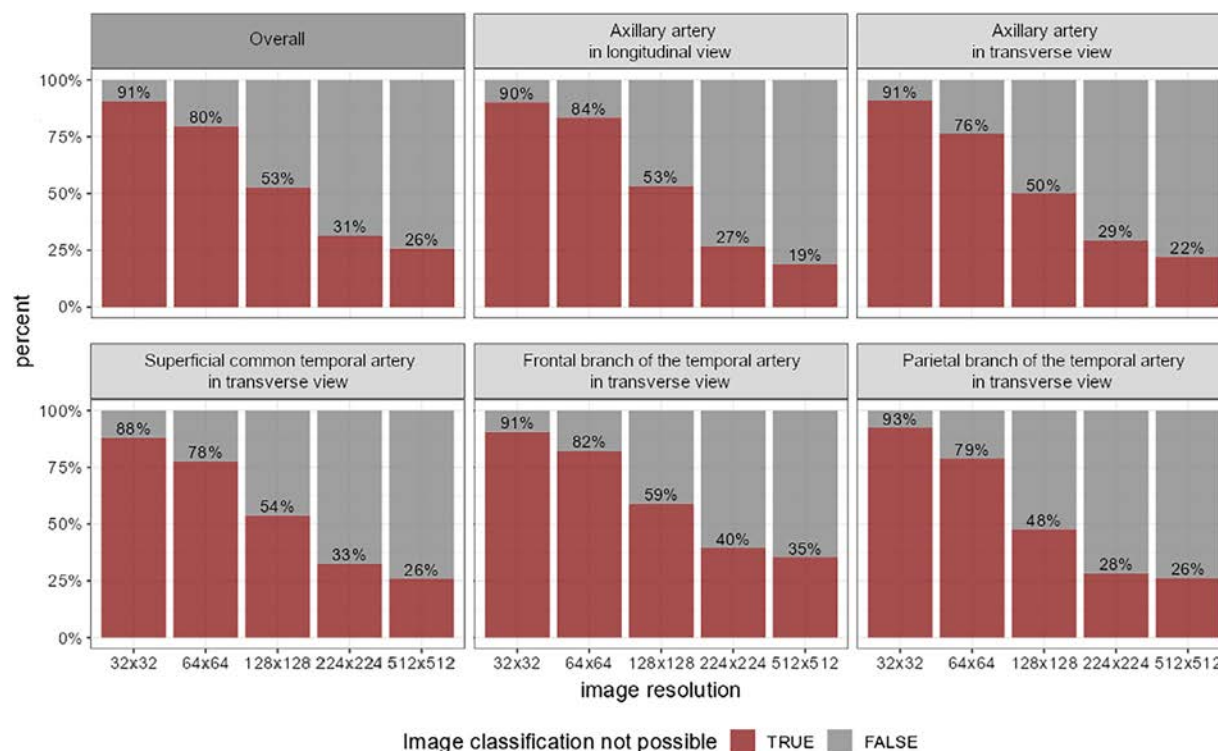


Figure 2. Frequency of the response option ‘Meaningful classification is not possible’ being selected during vascular ultrasound image classification, depending on the respective artery (and view) and image resolution. For all arteries, the frequency of this response option being selected decreased with increasing image resolution (consistent with every sequential stage of increased image resolution).

224 × 224 pixels (OR, 4.11; 95% CI, 3.42–4.94; $p < .001$), and 512 × 512 pixels (OR, 3.90; 95% CI, 3.25–4.69; $p < .001$) were all associated with significant improvements in image classification performance. In comparison with 128 × 128-pixel images as the reference category, increasing the image resolution further improved classification performance numerically, although the improvement did not reach statistical significance (224 × 224 pixels: OR, 1.18; 95% CI, 0.97–1.43; $p = .094$; 512 × 512 pixels: OR, 1.12; 95% CI, 0.93–1.35; $p = .248$). A sub-analysis selectively considering images of the axillary artery in longitudinal view or the temporal artery in transverse view did not reveal any relevant differences in the overall trend compared with the main analysis.

Heatmap plots illustrating the frequency with which each individual image included in this web-based exercise was classified correctly at a certain image resolution level are enclosed in [Supplementary Figure S2](#).

Sensitivity and specificity

The sensitivity and specificity of classifying vascular ultrasound images for the presence or absence of GCA indicative abnormalities based on single static B-mode images were assessed for any vessel/view and image resolution. Classification of 512 × 512-pixel longitudinal view images of the axillary artery achieved the highest specificity (specificity: 0.948, sensitivity: 0.734), while the lowest specificity was observed in 64 × 64-pixel transverse view images of the axillary artery (specificity: 0.596). Classification of 512 × 512-pixel transverse view images of the temporal artery’s frontal branch achieved the highest sensitivity (sensitivity: 0.851, specificity: 0.793), while the lowest sensitivity was observed in 32 × 32-pixel transverse view images of the parietal branch of the temporal artery

(sensitivity: 0.153). In general, sensitivity and specificity metrics constantly improved with rising image resolution between 32 × 32 and 128 × 128 pixels. In further increasing the image resolution beyond 128 × 128 pixels, sensitivity and specificity metrics continued to rise or remained constant, depending on the considered artery and view. Detailed data are listed in [Table 2](#).

Factors impacting the classification decision

A logistic mixed-effects model, which was performed in order to detect influential factors in classification decision-making and potential confounders (study participants’ contribution to study image provision, image resolution, study participants’ level of experience in GCA image interpretation as quantified by temporal duration, and quantity of scans interpreted throughout their career), identified image resolution as the only significant determining factor. A lower image resolution had a higher chance that the image was marked as ‘meaningful classification is not possible.’ Compared with a resolution of 512 × 512 pixels, the chance of receiving the label ‘Meaningful classification is not possible’ (1: yes, 0: no) was increased by a factor of 1.43 ($p < .001$) for images of size 224 × 224 pixels, a factor of 5.05 ($p < .001$) for images of size 128 × 128 pixels, a factor of 40.97 ($p < .001$) for images of size 64 × 64 pixels, and a factor of 198.84 ($p < .001$) for images of size 32 × 32 pixels. Likewise, compared with a resolution of 512 × 512 pixels, lower image resolutions led to significantly lower rates of correct image classification (eg, for an image size of 32 × 32 pixels: OR, 0.25; $p < .001$; for an image size of 64 × 64 pixels: OR, 0.56; $p < .001$). The number of years experts had already been reading GCA ultrasound images had no significant effect on the selection of the response category ‘Meaningful classification is not possible’ (OR, 1.02;

Table 1
Image classification results across all arteries, views, and image resolutions (in pixels)

Location	Pixel	Confident classification			Forced classification			% confident correct	% total correct*	% confident incorrect	% total incorrect**
		Correct	Incorrect	Not possible	Correct	Incorrect	Not answered				
Axillary artery - Longitudinal view	32x32	18	12	280	92	99	89	5.81	35.48	3.87	35.81
	64x64	38	13	259	146	51	62	12.26	59.35	4.19	20.65
	128x128	124	21	165	112	26	27	40.00	76.13	6.77	15.16
	224x224	201	26	83	49	27	7	64.84	80.65	8.39	17.10
	512x512	227	24	59	33	25	1	73.23	83.87	7.74	15.81
Axillary artery - Transverse view	32x32	12	16	282	98	106	78	3.87	35.48	5.16	39.35
	64x64	49	24	237	97	78	62	15.81	47.10	7.74	32.90
	128x128	113	42	155	70	42	43	36.45	59.03	13.55	27.10
	224x224	172	47	91	35	28	28	55.48	66.77	15.16	24.19
	512x512	176	65	69	29	19	21	56.77	66.13	20.97	27.10
Superficial common temporal artery - Transverse view	32x32	19	18	273	105	85	83	6.13	40.00	5.81	33.23
	64x64	42	27	241	117	64	60	13.55	51.29	8.71	29.35
	128x128	115	28	167	87	41	39	37.10	65.16	9.03	22.26
	224x224	166	43	101	52	18	31	53.55	70.32	13.87	19.68
	512x512	180	49	81	43	16	22	58.06	71.94	15.81	20.97
Frontal branch of the temporal artery - Transverse view	32x32	14	15	281	112	92	77	4.52	40.65	4.84	34.52
	64x64	38	17	255	142	39	74	12.26	58.06	5.48	18.06
	128x128	99	28	183	128	20	35	31.94	73.23	9.03	15.48
	224x224	159	28	123	70	23	30	51.29	73.87	9.03	16.45
	512x512	168	32	110	68	17	25	54.19	76.13	10.32	15.81
Parietal branch of the temporal artery - Transverse view	32x32	11	12	287	105	112	70	3.55	37.42	3.87	40.00
	64x64	47	18	245	134	56	55	15.16	58.39	5.81	23.87
	128x128	133	29	148	82	24	42	42.90	69.35	9.35	17.10
	224x224	194	28	88	55	8	25	62.58	80.32	9.03	11.61
	512x512	196	32	82	47	11	24	63.23	78.39	10.32	13.87

During the image classification exercise, experts first chose 1 of 3 options to answer the question, “I classify this ultrasound image as belonging to a . . .”: (1) “GCA patient,” (2) “Healthy individual,” or (3) “Meaningful classification is not possible.” This initial decision is termed “Confident classification” (results in the first 3 columns). If option (3) was selected, a follow-up dialogue prompted a forced choice between “GCA patient” and “Healthy individual” (termed “Forced classification,” results in the next 3 columns). Percentage figures can be found in the 4 columns on the right. In total, 310 classifications were submitted for each artery, view, and image resolution (10 images classified by 31 experts).

*Percent total correct: the number of correct confident classifications plus the number of correct forced classifications, divided by the total number of classifications (n = 310).

**Percent total incorrect: the number of incorrect confident classifications plus the number of incorrect forced classifications, divided by the total number of classifications (n = 310).

$p = .725$). Neither did the number of patients that experts estimated to have seen and evaluated for suspected GCA throughout their career (OR, 1.00; $p = .422$). The presented statistical data were derived from a model adjusted for differences across artery locations and views.

DISCUSSION

In this first and international study exploring the minimum image resolution mandatory for reliable human expert

classification of vascular ultrasound images in GCA and healthy subjects, we observed ascending classification performance with increasing image resolution up to an image resolution of 224×224 pixels, where decision confidence and image classification performance plateaued without further significant gains despite a continual increase in image resolution.

In fact, an in-depth analysis of the classification exercise performed in this study by human experts revealed that, compared with images of 128×128 pixels set as the reference category, higher image resolutions still showed further numerical

Table 2
Sensitivity and specificity of vascular ultrasound image classification by human experts at various image resolutions, arteries, and views

Artery	Metric	Image resolution (pixels)				
		32 × 32	64 × 64	128 × 128	224 × 224	512 × 512
Overall	Sensitivity	0.299	0.585	0.755	0.767	0.761
	Specificity	0.814	0.832	0.814	0.862	0.856
Axillary artery—longitudinal view	Sensitivity	0.328	0.549	0.727	0.733	0.734
	Specificity	0.719	0.929	0.943	0.915	0.948
Axillary artery—transverse view	Sensitivity	0.350	0.584	0.707	0.702	0.678
	Specificity	0.663	0.596	0.65	0.784	0.757
Superficial common temporal artery—transverse view	Sensitivity	0.346	0.549	0.759	0.734	0.709
	Specificity	0.846	0.773	0.724	0.855	0.871
Frontal branch ^a —transverse view	Sensitivity	0.329	0.690	0.837	0.834	0.851
	Specificity	0.860	0.872	0.807	0.793	0.793
Parietal branch ^a —transverse view	Sensitivity	0.153	0.555	0.738	0.829	0.830
	Specificity	0.979	0.950	0.898	0.939	0.878

^a Branch of the temporal artery.

improvement in classification performance. However, classification performance improvement with increasing image resolution largely plateaued at 224 × 224 pixels, in line with sensitivity and specificity metrics, which also accentuated a ‘sweet spot’ at 224 × 224 pixels. In a combined conservative consideration of these results, an image resolution of 224 × 224 pixels crystallised as the optimum between preserved information content and data size efficiency, thereby defining the minimum requirement for image resolution a neural network architecture in this scenario of application will be supposed to handle. These findings resonate with the applied image resolution in other application scenarios of AI-driven medical imaging classifications [34,35] in general, but more specifically, vessel ultrasound image classification within the scope of disorders other than GCA [36,37]. Particularly noteworthy is a study conducted by Li et al [37], which similarly focused on the detection of disease-specific vascular wall changes—in this case, the automatic detection of atherosclerotic plaques and calcification from intravascular ultrasound images. Despite contextual differences, parallels to our study’s conclusions can be drawn from the fact that Li et al [37] also successfully trained their neural network with images at a resolution of 224 × 224 pixels. Our study provides evidence that adequate GCA ultrasound image classification by human experts does not necessarily require the highest resolution level of 512 × 512 pixels, but can be appropriately achieved at a resolution level of 224 × 224 pixels. This finding broadens the range of neural network architectures suitable for AI development, including the widely chosen and resource-efficient neural network architectures such as ResNet [27] or DenseNet [29], which are limited to a maximum default image input size of 224 × 224 pixels.

Moreover, the reported results on human expert image classification performance serve as an essential benchmark for the classification performance of the projected neural network, which aims to match or exceed human expert classification performance, as achieved in other medical fields before [35]. The data presented in this study are representative, particularly considering that experts were only provided with 1 single static B-mode image per patient without any additional IMT measurement, halo, compression sign, insight into other anatomical segments, or clinical information. While participants in this study classified each image individually, it is important to note that there are typically 8 or more images available per patient, which may lead to higher sensitivity and lower specificity on a patient level when combined in clinical practice.

Also, sensitivity and specificity results, combined with heatmap visualisation (Supplementary Fig S2) and data from both confident and forced classifications, indicate that participants tended to classify an image as belonging to a GCA patient only when they were relatively confident. Among all cases in which the initial response option had been ‘meaningful classification is not possible,’ subsequent forced classifications found more than twice as many of these images to be attributed to GCA patients vs healthy individuals. Thus, images ultimately classified as belonging to GCA patients were clearly overrepresented within the category of ‘meaningful classification is not possible’ initial responses. This superordinate dynamic likely contributed to missed GCA classifications, explaining the persistent rate of misclassifications even at higher image resolutions. At the same time, this caused assessment specificity to remain consistently high across all resolution stages. Despite all these factors, the image classification performance yielded remarkable sensitivity and specificity results. Our reported sensitivity of 0.833 and specificity of 0.915 for 224 × 224-pixel longitudinal view images of the axillary artery, as well as 0.734 and 0.855, respectively, for 224 × 224-pixel transverse view images of the superficial common temporal artery, align with data from a systematic review and meta-analysis investigating the diagnostic performance of temporal artery ultrasound (considering full resolution images) for the diagnosis of GCA [38]. This meta-analysis, based on 11 studies with available data, reported a sensitivity of 0.78 and a specificity of 0.79 for detecting any sono-morphologic abnormalities (hypoechoic halo, stenosis, and/or occlusion) in GCA ultrasound. Likewise, this study’s results are in line with the results of the OMERACT Patient-based Reliability Exercise [14] that yielded an interreader reliability of around 0.8 (light κ, 0.76–0.86) for the overall diagnosis of GCA.

Limitations included the natural restrictions in expert availability to participate in this exercise. Indeed, 5 experts participated in this study who had previously granted access to their image libraries, out of which a fraction of images was randomly selected by an independent individual (CJB) not involved as a study participant. In weighing whether these study participants may have contributed to a bias in the results, we performed sensitivity analyses for all reported metrics, which did not lead to a significant change in results after the exclusion of these participants. Image selection may have been subject to selection bias and holds the potential to have impaired representativeness. Furthermore, we decided

not to actively include or exclude ultrasound images containing aspects of atherosclerosis, where an OMERACT ultrasound definition is available and validated [15]. Instead, our dataset represents a random sampling from real-world clinical cases, not stratified based on comorbidities. While in fact, fibromuscular dysplasia or many other vasculopathies are rarely encountered in temporal and axillary arteries, and atherosclerosis is also uncommon in temporal arteries, atherosclerotic changes can occur in the axillary arteries, though they are typically distinguishable by ultrasound based on their characteristic morphology [15]. Nonetheless, future research should explore whether higher image resolution is needed to differentiate GCA from these specific mimics, particularly in borderline cases where classification might be more challenging. Intentionally and with the operating principle of the projected AI-based GCA ultrasound image classification assistant in mind (see [Supplementary Material](#)), this study made use of a single static B-mode image per patient case without any additional IMT measurement, halo, compression sign, or clinical information. This represents a striking difference in the way human experts are used to approach a classification decision in clinical practice when performing GCA ultrasound in a dynamic manner, with appraisal of multiple anatomical segments. We regard this as the primary reason for the overall high rate of initially ‘non-classifiable’ images. An intrarater exercise could also have been conducted, but it was felt to be beyond the scope of this project. A technical error in REDCap allowed the omission of forced classifications, resulting in missing values within the dataset. While potential bias cannot be entirely excluded, removing these missing classifications was deemed the most appropriate strategy to minimise bias. For transparency, [Table 1](#) presents a detailed breakdown of classification results across all arteries, views, and image resolutions for both confident and forced classifications.

In conclusion, this international online study evaluated the minimum image resolution mandatory for reliable human expert classification of vascular ultrasound images in GCA and healthy subjects and found that 224×224 -pixel images provide the optimum between preserved information content and data size efficiency. This opens up a broad variety of applicable deep neural network architectures for the development of an AI-based assistant.

Future directions

Building on the results of the present study as the technical foundation, the realisation of an AI-based GCA ultrasound image classification assistant, as ultimately envisioned, follows a modularly structured project roadmap, specific intermediate milestones, and developmental steps that can be briefly outlined as follows:

1. Training and validating a neural network model utilising a task-appropriate dataset.
2. Expert-led clinical validation to prove reliability.
3. Staged rollout into clinical routine care.

While the primary developed neural network focuses on vessel morphology and pathological IMT in B-mode ultrasound images, it will subsequently be accompanied by 2 additional complementary neural networks that are to be developed for the evaluation of halo and compression signs. Each of these models will independently undergo the same steps of (1) dataset

development, training, and validation, as well as (2) expert-led clinical evaluation before undergoing (3) a staged rollout into routine care. To date, a 3800-ultrasound image library has already been provided for deep neural network training. Once development and clinical validation of the AI model have been completed, a publicly accessible website hosting the AI-based assistant that facilitates the classification of uploaded ultrasound images and helps to diagnose GCA early is envisioned. This approach holds great potential for the rapid and accurate diagnosis of GCA, including in resource-disadvantaged regions, a factor that could critically impact individuals by allowing diagnosis before ischaemic complications occur.

Competing interests

All authors declare they have no competing interests.

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Any dataset parts generated and analysed for this study that are not already included in this article are available upon

reasonable request. Further inquiries can be directed to the corresponding author.

Supplementary materials

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Still's Disease

Pulmonary arterial hypertension in adults with Still's disease: another pulmonary manifestation associated with HLA-DRB1*15

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ABSTRACT

Objectives: Inflammatory lung disease in Still's disease (SD) has recently been described. Among its manifestations, pulmonary arterial hypertension (PAH) is a rare and life-threatening event, with only a few case reports published. The objective was to report the largest adult cohort of PAH occurring in the context of SD.

Methods: We identified 16 adult SD patients with PAH (PAH+) by a call for observations from the CRI-IMIDIATE (Club Rhumatismes & Inflammation - Immune-Mediated Inflammatory Disease Alliance for Translational and Clinical Research) network (<https://cri-net.com/recherche/>)

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reseau-CRI-Imidiate/) and a search of the French pulmonary hypertension network registry. Patient characteristics and disease evolution were retrospectively compared with those of 111 SD controls without PAH (PAH-) followed in a reference centre.

Results: The profile of patients in the PAH+ and PAH- groups differed: 100% versus 69.4% female ($P = .006$), 75% versus 17.1% Black ($P < .0001$), more active SD both at diagnosis and throughout the disease course, and more likely to present macrophage activation syndrome (62.5% vs 14.4%, $P < .0001$) and exhibit eosinophilia during the disease course (68.7% vs 7.2%, $P < .0001$). For the 84 out of 127 patients with genetic typing, the HLA-DRB1*15 allele was more prevalent in PAH+ than PAH- patients (8/11 [72.7%] vs 22/73 [30.1%], $P = .014$). PAH+ patients more frequently received canakinumab and immunosuppressants than did PAH- patients and had higher frequency of drug reactions to interleukin 1 (IL-1) and/or IL-6 inhibitors (37.5% vs 7.2%, $P = .002$). Mortality was higher in PAH+ than PAH- patients (37.5% vs 0.9%, $P < .0001$), and all deaths were related to SD flare.

Conclusions: These results reinforce the association between the HLA-DRB1*15 allele and severe forms of SD and raise the question of therapeutic optimisation in such patients.

WHAT IS ALREADY KNOWN ON THIS TOPIC

- Inflammatory lung disease (ILD) in Still's disease (SD) has recently been described and is characterised by interstitial inflammatory infiltrates of CD4 and CD8 lymphocytes and intra-alveolar deposition of proteinaceous material. ILD seems associated with a history of recurrent macrophage activation syndrome (MAS), eosinophilia, high serum interleukin 18 (IL-18) levels, and presence of the HLA-DRB1*15 allele.
- Pulmonary arterial hypertension (PAH), a rare subtype of pulmonary hypertension (PH), is a potentially life-threatening complication of SD. It is crucial to differentiate PAH (group 1 PH), characterised by progressive remodelling of small pulmonary arteries, from PH associated with interstitial lung disease (PH-ILD), which falls under group 3 PH and results primarily from parenchymal lung involvement and hypoxic vasoconstriction. Although in the context of SD both mechanisms, PH-ILD and PAH, may be involved, PAH has rarely been reported.

What this study adds

- This work reports a large cohort of PAH in adults with SD. PAH develops on the same background as other manifestations of SD-related ILD (ie, in patients with MAS, especially when recurrent; eosinophilia; and carrying the HLA-DRB1*15 allele). Female sex and Black ethnicity seem to be risk factors.

How this study might affect research, practice or policy

- This work reinforces the association of the HLA-DRB1*15 allele with severe forms of SD and raises the question of treatment optimisations to better control both SD and its complications.
- The fact that PAH can be isolated in SD, ie, it can occur in the absence of major parenchymal involvement, should encourage physicians to screen for it using echocardiography in cases of unexplained dyspnoea, before confirmation by catheterisation in the case of intermediate or high probability of PAH.
- The existence of PAH has major therapeutic implications since it requires the addition of vasodilating agents to targeted and nontargeted immunomodulating therapies.

INTRODUCTION

Still's disease (SD) is a rare sporadic systemic condition driven by innate immunity, especially in the initial systemic phase of the disease [1]. In the event of life-threatening

complications, which can sometimes occur from the onset of the disease, adaptive immunity is also involved, leading to a hyper-inflammatory condition [2]. It is usually characterised by 4 clinical and biological features: spiking fever, evanescent salmon-pink maculopapular rash, arthralgia or arthritis, and white blood cell count $\geq 10,000/\text{mm}^3$, mainly neutrophil cells [3]; proinflammatory cytokines, mainly interleukin 1β (IL- 1β), IL-18, and IL-6, seem to play a key role [1]. Serious and potentially life-threatening complications may occur, including macrophage activation syndrome (MAS) and inflammatory lung disease (ILD) [1,4,5]. Recently, the European Alliance of Associations for Rheumatology (EULAR) and the Paediatric Rheumatology European Society (PREs) defined strategies for the diagnosis and management of these complicated forms, with particular focus on ILD [5]. Indeed, in the last decades, with progressively increasing frequency, physicians have encountered patients, particularly children, with this complication [6,7].

SD-related ILD was initially described in North America; however, cases have now been reported in Europe and other regions of the world [8]. SD-related ILD is characterised by interstitial inflammatory infiltrates of CD4 and CD8 lymphocytes and intra-alveolar deposition of proteinaceous material. ILD seems associated with young age at onset, Down's syndrome, more active forms of SD, the occurrence of MAS and particularly recurrent MAS, eosinophilia, and high serum IL-18 levels [7]. Evidence is accumulating for an association between HLA-DRB1*15 carriage and ILD [8,9]. Although the exact pathogenesis of SD-related LD is not yet understood, there is increasing evidence of a central role for the IL-18/interferon gamma (IFN- γ) pathway and some activated T-cell subsets [10], as for MAS [11,12]. EULAR/PReS have set the research agenda for a better understanding of these mechanisms [5]. However, the EULAR/PReS Task Force was unable to issue recommendations on pulmonary arterial hypertension (PAH) associated with SD because of the paucity of literature on the subject.

Pulmonary arterial hypertension (PAH), a rare subtype of pulmonary hypertension (PH), is a potentially life-threatening complication of SD [4]. Although transthoracic echocardiography is a useful non-invasive screening tool, it cannot establish the diagnosis of PH. Right heart catheterisation is essential for the definitive diagnosis of PH, as it provides direct haemodynamic measurements and allows classification of PH into distinct groups based on pathophysiological mechanisms [13,14]. Due to important therapeutic implications, it is crucial to differentiate PAH (group 1 PH), characterised by progressive remodelling of small pulmonary arteries, from PH associated with interstitial lung disease (PH-ILD), which

falls under group 3 PH and results primarily from parenchymal lung involvement and hypoxic vasoconstriction. The vascular involvement in PAH results in precapillary pulmonary hypertension, defined by elevated mean pulmonary arterial pressure (mPAP) and pulmonary vascular resistance (PVR) [13,14]. PAH may be associated with different conditions including inflammatory conditions such as connective tissue disease or HIV infection [13–15]. The link between inflammation and PAH pathogenesis can be supported by histological evidence of infiltration of perivascular inflammatory cells in remodelled arteries and plexiform lesions and the presence of elevated serum levels of proinflammatory cytokines (IL-1 β , IL-6) in PAH [16–18]. Of note, these cytokines also play a key role in SD pathogenesis [1,19].

In the context of SD, both mechanisms, PH-ILD and PAH, may be involved, although it is mainly PH-ILD that has been reported so far [6,7,9,20]. Rare cases of PAH have been reported in both paediatric and adult patients [4,7,20,21]. Kimura et al [20] described 16 paediatric patients with PAH (4 of whom also had advanced parenchymal involvement) in their cohort of 25 patients with SD-related LD. Schulert et al [7] described PAH in 3 out of 18 patients, ie, without major parenchymal involvement in their series. Other paediatric cohorts did not clearly distinguish PAH from other forms of PH, although clinical PH was estimated at 30% at the time of ILD diagnosis [6]. In adults, only a few isolated case reports have been published [4,21]. The present article represents the largest series of PAH reported to date in adults with SD. This is all the more important given that the concept of SD-related lung disease in previous publications and in the EULAR/PreS recommendations was mainly limited to inflammatory lung disease combining septa thickening and pulmonary alveolar proteinosis and in some cases PH-ILD, but did not point out a specific vascular involvement of the pulmonary arteries [5,7,8].

The objective of this study was first to survey the literature of SD-associated PAH as a background. Then, we aimed to establish a comprehensive nation-wide case series of SD-associated PAH and describe the clinical, functional, haemodynamic, and radiologic characteristics as well as pathologic assessment of these patients identified from 2 French clinical investigation networks. We analysed the response to SD and PAH therapies and long-term outcomes and compared the characteristics of SD patients with and without PAH.

METHODS

Systematic review of the literature on SD-associated PAH

We performed a systematic review (SR) of the literature according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines. The following databases were screened: Medline (PubMed), Embase and Cochrane Library up to February 8, 2024. The search strategy, selection process, data collection, outcomes and statistical analysis are in [Supplementary Methods](#) and [Supplementary Tables S1 to S3](#).

Identification of SD patients with PAH and controls

Adult SD cases with PAH were identified by a call for observations from the CRI-IMIDATE (Club Rhumatismes & Inflammation - Immune-Mediated Inflammatory Disease Alliance for Translational and Clinical Research) network (<https://cri-net.com/recherche/reseau-CRI-Imidiate/>) and a search of the French PAH Network Registry (see [Supplementary Materials](#)). All patients fulfilled the Yamaguchi or Fautrel criteria for SD [3,22]. The PAH was diagnosed according to the presence of a

precapillary PAH confirmed by right heart catheterisation (RHC) and after excluding other causes of PAH. The date of PAH diagnosis was defined as the date of the first RHC establishing the PAH diagnosis. Controls were recruited from the cohort of 111 adults with SD followed at the CEREMAIA Reference Centre (www.ceremaia.fr) in the Rheumatology Department of Pitié-Salpêtrière Hospital, Paris, France.

Ethics

This study was conducted in accordance with the Declaration of Helsinki. The methods used to collect and analyse data on PAH was approved by the ethics committee on May 24, 2003 (approval no. 842063). Regarding the data for SD, the French law does not require formal ethics committee approval or informed consent for retrospective data collection (<https://www.legifrance.gouv.fr/loda/id/JORFTEXT000000886460/>); however, all patients expressed nonopposition to the use of the data for research purposes.

Data collection

Data were retrospectively collected by using a structured and standardised form and included demographic information, age at diagnosis of SD and PAH, and the time between SD onset and PAH diagnosis.

SD characteristics

Manifestations of SD (fever, skin rash, pharyngitis, lymphadenopathy, hepatosplenomegaly, serositis, arthralgia and arthritis, MAS) and laboratory variables (haematologic profile, erythrocyte sedimentation rate in the first hour, C-reactive protein [CRP] level, serum ferritin level, and percentage of glycosylated ferritin when available) were recorded at the time of diagnosis of both SD and PAH. The modified Pouchot score (mPouchot score) was calculated [23]. Number of SD flares, treatments (corticosteroids, conventional synthetic or biological disease-modifying antirheumatic drugs), and therapeutic response assessed qualitatively (clinically inactive disease [CID] and remission [5], partial response or failure) were collected during follow-up. Eosinophilia was defined as an absolute eosinophil count $\geq 700/\text{mm}^3$ or eosinophils $\geq 10\%$ of the total white blood cell count measured on at least 2 occasions [24]. Definitions of CID, remission, partial response, and failure; high or low disease activity according to the mPouchot score (≥ 4 and ≤ 2) [5,25]; as well as drug reactions to IL-1 and/or IL-6 inhibitors [24] are available in [Supplementary Methods](#). Drug reaction with eosinophilia and systemic symptoms (DRESS) was defined with RegiSCAR score ≥ 4 [9,26]. When genetic material was available, HLA typing for a DRB1 was performed with the patient's signed consent ([Supplementary Materials](#)).

PAH characteristics

Patients in the PAH group were confirmed to have precapillary pulmonary hypertension, in line with the definition current at the time of PAH diagnosis, specifically, mPAP ≥ 25 mm Hg, pulmonary artery wedge pressure (PAWP) ≤ 15 mm Hg, and PVR > 3 Wood units (WU) on baseline RHC [27]. We collected clinic, functional, biologic, and haemodynamic data at diagnosis and during follow-up (3 to 4 months after treatment initiation or modification) and then once a year. Serial assessments included New York Heart Association (NYHA) functional class, 6-min walk distance (6MWD), haemodynamic parameters, and brain natriuretic peptide (BNP)/N-terminal prohormone of

brain natriuretic peptide (NT-proBNP) measurement. For patients at NYHA functional class IV unable to perform a 6-min walk test, the distance was imputed as 0 m. Haemodynamic measurements included right atrial pressure, mPAP, and PAWP. Cardiac output (CO) was measured by thermodilution. CI was calculated as CO divided by body surface area. PVR was calculated as mPAP-PAWP/CO. A pulmonary artery blood sample was collected to measure mixed venous oxygen saturation. We collected data on PAH-approved drugs including endothelin receptor antagonists, phosphodiesterase type-5 inhibitors, and prostanoids as well as inotropic support. Pathological assessment from postmortem examination was available for 1 patient.

Statistical analysis

Statistical analysis involved using SPSS Statistics 26 (SPSS Inc., Chicago, IL) or R Core Team (2021, <https://www.R-project.org/>). Continuous variables are expressed as median (interquartile range [IQR]). Comparisons between variables accessed at baseline and first follow-up as well as between patients with and without PAH (PAH+ and PAH-) involved the Wilcoxon matched-pair signed rank test and chi-squared test for qualitative binary data when all expected counts were > 5 and Fisher's exact test otherwise. Differences were considered significant at $P < .05$. The Kaplan–Meir method was used for survival analysis. The date of PAH diagnosis by RHC was used as the starting point to determine length of survival. The cut-off date was October 6, 2024.

RESULTS

SR of published SD-related PAH cases

The selection and flow of articles in the SR are provided in [Supplementary Material and Supplementary Figure S1](#). In total, 33 of 275 articles were assessed for detailed eligibility, and 20 were finally included (21 patients, 18 adults and 3 children). The results are detailed in [Supplementary Material and Supplementary Tables S4 to S12](#). In total, 17 articles (85%) were published after 2010, which approximately corresponds to the period of widespread use of IL-1 and IL-6 inhibitors. Twenty patients (95.2%) were female. Among the 15 (71.4%) patients with reported ethnicity, most were Asian (23.8%), followed by Black or Hispanic (19% for each ethnic group), and Caucasian and Middle-Eastern (4.8% for each). Serositis (pericarditis and/or pleuritis) and MAS occurred in 6 (28.5%) patients each during the disease course. Associated pulmonary parenchymal involvement was only reported in 2 out of 21 patients. Moreover, none of the cases reported pulmonary embolism. No articles reported eosinophilia or drug reactions to IL-1 or IL-6R inhibitors or HLA-DRB1 typing results. Of the 21 patients identified by the SR, 13 (61.9%) had never received IL-1i or IL-6i treatment before the PAH diagnosis; their characteristics, as well as those of the 8 patients who had received an IL-1i and/or an IL-6i prior to PAH onset, are detailed in [Supplementary Tables S10 and S11](#). Before PAH diagnosis, methotrexate had been used in 8 (38.1%) patients, at least one IL-1 inhibitor in 8 (38.1%; all received anakinra, 4 canakinumab, and some both) and IL-6R inhibitors in 4 (19%). Information about follow-up was available for 19 out of 21 patients. Overall, 31.6% (6/19) of patients had a poor outcome despite therapy: 4 patients died several days or months after PAH diagnosis, one required cardiopulmonary transplantation, and PAH continued to worsen despite treatment in one.

Characteristics of SD patients with and without PAH

We identified 16 SD patients with PAH from our call for observations and compared their characteristics to those of 111 SD patients without PAH ([Table 1](#), [Supplementary Tables S13 and S14](#)) [24,26]. In both cases, most SD diagnoses (approximately 80%) occurred after 2010. The PAH+ and PAH- groups differed in sex (100% vs 69.4% female, $P = .006$) and ethnicity (75% vs 17.1% Black, $P < .0001$). That SD patients with PAH were exclusively women in our series raised the hypothesis of a potential hormonal influence ([Supplementary Table S15](#)). Patients in whom PAH developed during SD disease had more active SD at diagnosis, as evidenced by the higher mPouchot score at PAH onset as compared with the PAH group (median 7 [IQR 6–8] vs 6 [5–7], $P = .03$). They also had more active disease during the disease course. Twelve patients in the PAH+ group presented mild radiological parenchymal features on the computed tomography (CT) scan (interlobular septal thickening in all cases, ground-glass opacities of nonspecific nature in half of the cases); in the PAH- group, only 3 (2.7%) patients presented mild radiological parenchymal involvement ([Supplementary Tables S16 and S17](#)). Importantly, no patient in either group presented with radiological aspect of pulmonary alveolar proteinosis and endogenous lipid pneumonia.

MAS was more frequent in PAH+ than PAH- patients (62.5% vs 14.4%, $P < .0001$), including 7 patients with multiple (≥ 2) episodes and 1 with a refractory MAS episode associated with uncontrolled PAH that led to death. More patients in the PAH+ than PAH- group exhibited eosinophilia during the disease course (68.7% vs 7.2%, $P < .0001$), with significantly higher maximum eosinophil count (median 2616 [1765–3460] and 937 [850–1100]/mm³, $P = .002$). HLA-DRB1*15 was typed in 84 patients (11 and 73 in the PAH+ and PAH- groups, respectively) and was significantly more frequent in PAH+ than PAH- patients (72.7% and 30.1%, respectively, $P = .014$). Interestingly, 2 of the 3 patients with pulmonary parenchymal involvement in the PAH- group were HLA-DRB1*15 positive ([Supplementary Table S16](#)).

The 2 groups did not differ in number of patients who received glucocorticoids (GCs), anakinra, or IL-6R inhibitors during the disease course. However, because of more active disease, PAH+ patients more frequently received canakinumab than did PAH- patients (50% vs 21.6%, $P = .026$) as well as methotrexate or immunosuppressants (mainly ciclosporin, azathioprine, cyclophosphamide, and etoposide). In addition, PAH+ patients more frequently experienced drug reactions to biologics than PAH- patients (37.5% vs 7.2%, $P = .002$), with 2 patients meeting the criteria for DRESS syndrome.

There were more deaths attributed directly to a SD complication in the PAH+ than PAH- group (6 [37.5%] vs 1 [0.9%], $P < .0001$). Causes of death are detailed below.

SD and PAH characteristics at PAH diagnosis

SD clinical and laboratory features at PAH onset are summarised in [Table 2](#) and [Supplementary Tables S13, S14, and S18](#). The median age at diagnosis of PAH was 31 (IQR 24–36) years. The diagnosis of PAH occurred after the diagnosis of SD in all cases, with a median delay of 37 (17–59) months between the SD and PAH diagnoses. Although underlying SD was not fully controlled in all patients at PAH onset, fever was absent in 25% of cases and joint involvement was active in only 37.5% of patients. The median CRP level at PAH diagnosis was 46 (17–101) mg/L and ferritin level 2690 (2300–9314) µg/L. The

Table 1
Characteristics of Still's disease patients with and without pulmonary arterial hypertension (PAH)

	Still's disease with PAH (n = 16)	Still's disease without PAH (n = 111)	P value (PAH vs no PAH)
Decade of Still's disease onset ^a , n (%)			
Before 2010	3 (18.7)	23 (20.7)	1
≥ 2010	13 (81.3)	88 (79.3)	1
Female, n (%)	16 (100)	77 (69.4)	.006
Ethnicity, n (%)			
Caucasian	3 (18.7)	64 (57.7)	< .0001
Black	12 (75.0)	19 (17.1)	
Asian	1 (6.3)	7 (6.3)	
North Africa	0 (0)	19 (17.1)	
Middle East	0 (0)	2 (1.8)	
Age at Still's disease onset ^a , median (IQR)	25 (21–36)	27 (20–37)	.982
Modified Pouchot score at Still's disease diagnosis Median [IQR]	(data available for 15 patients) 7 [6–8]	(data available for 67 patients) 6 [5–7]	.031
Still's disease manifestations (ever during disease course), n (%)			
Fever	16 (100)	110 (99.1)	1
Rash	16 (100)	99 (89.2)	1
Arthralgia/arthritis	15 (93.7)	107 (96.4)	.495
Odynophagia	14 (87.5)	77 (69.4)	.233
Myalgia	13 (81.2)	47 (42.3)	.005
Lymphadenopathy	16 (100)	49 (44.1)	< .0001
Hepatomegaly and/or abnormal liver tests	16 (100)	54 (48.7)	< .0001
Acute hepatitis with transaminases ≥10 N	7 (43.7)	20 (18.0)	.043
Splenomegaly	11 (68.7)	15 (13.5)	< .0001
Pericarditis	12 (75.0)	16 (14.4)	< .0001
Pleuritis	5 (31.2)	9 (8.1)	.017
Parenchymal involvement ^b	12 (75.0)	3 (2.7)	< .0001
Myocarditis ^c	4 (25.0)	10 (9.0)	.063
MAS ^d	10 (62.5)	16 (14.4)	< .0001
Eosinophilia ever during disease course, n (%) ^e	11 (68.7)	8 (7.2)	< .0001
Maximum eosinophil count, median [IQR], /mm ³	2616 [1765–3460]	937 [850–1100]	.002
HLA-DRB1 *15-positive, n/N available (%)	8/11 (72.7)	22/73 (30.1)	.014
History of treatment (ever during disease course) ^f			
Glucocorticoids, n (%)	16 (100)	108 (97.3)	1
csDMARDs, n (%)			
Methotrexate	13 (81.2)	56 (50.4)	.029
Leflunomide	1 (6.2)	2 (1.8)	.334
Sulfasalazine	1 (6.2)	1 (0.9)	.236
Intravenous immunoglobulins	2 (12.5)	2 (1.8)	.077
IL-1 and/or IL-6 blockade, n (%)	16 (100)	83 (74.7)	.021
IL-1 inhibitor, at least one	14 (87.5)	74 (66.7)	.145
Anakinra	14 (87.5)	71 (63.9)	.087
Canakinumab	8 (50.0)	24 (21.6)	.026
IL-6 inhibitor	8 (50.0)	45 (40.5)	.589
TNF inhibitors, n (%)	3 (18.7)	20 (18.0)	1
Immunosuppressants, n (%)			
Ciclosporin	5 (31.2)	4 (3.6)	.001
Mycophenolate mofetil	1 (6.2)	2 (1.8)	.334
Tacrolimus	1 (6.2)	2 (1.8)	.334
Sirolimus	0 (0)	1 (0.9)	1
Azathioprine	3 (18.7)	2 (1.8)	.014
Cyclophosphamide	2 (12.5)	0 (0)	.014
Etoposide	2 (12.5)	1 (0.9)	.041
JAK inhibitors, n (%)	3 (18.7)	6 (5.4)	.086
History of drug reactions to IL-1 or IL-6 inhibitors ^g , n (%)	6 (37.5)	8 (7.2)	.002
Deaths directly attributed to Still's disease complications, n (%)	6 (37.5)	1 (0.9)	< .0001

In bold, statistically significant values, i.e. $p \leq 0.05$.

^a In some patients, the disease developed in childhood (systemic juvenile idiopathic arthritis), but symptoms persisted and/or recurred in adulthood.

^b The radiological parenchymal involvement was mild in the 12 patients of the PAH + group: interlobular septal thickening in all cases, ground-glass opacities of non-specific nature in half of the cases (please see main text and [figure 1](#) for details). The radiological parenchymal involvement was mild to moderate on the CT scan for the 3 patients of the PAH- group who had parenchymal involvement (please see [supplementary tables 16 and 17](#) for details). Importantly, no patient in either group presented with pulmonary alveolar proteinosis and endogenous lipid pneumonia.

^c Including one patient with PAH in whom myocarditis developed in the context of major eosinophilia up to 8,000/mm³.

^d Among the population with PAH, 7 patients had multiple (≥2) episodes of MAS, and 1 had a refractory episode of MAS over several months associated with uncontrolled PAH that led to death.

^e Eosinophilia was defined as absolute eosinophil count ≥ 700/mm³ or eosinophils ≥ 10% of the total white blood cell count, measured on at least 2 occasions [24].

^f Some patients had several lines of treatment.

^g Drug reactions to IL-1 and/or IL-6 inhibitors were all confirmed by a dermatologist and/or an allergologist, and included rashes atypical for SD (eg, nonevanescent, pruritic, urticarial, a fortiori in the presence of blood eosinophilia), or acute reactions (respiratory distress, swelling or Quincke oedema during or near the time of medication administration). Two patients in the PAH + group met the criteria for DRESS syndrome (RegiSCAR for DRESS ≥ 4) [26]. Local reactions at the injection site were not included in this category, as they are common with subcutaneous biotherapies.

10N, 10 times above normal, csDMARDs, conventional synthetic disease-modifying antirheumatic drugs; IL, interleukin; IQR, interquartile range; JAK, Janus kinase; MAS, macrophage activation syndrome; PAH, pulmonary arterial hypertension.

Table 2
Clinical and laboratory features of patients with Still’s disease at PAH diagnosis and general evolution of the 16 patients with PAH

Age at PAH diagnosis, years, median (IQR)	31 (24-36)
Temporality between diagnosis of Still’s disease and PAH	
Still’s disease preceded PAH, n (%)	16 (100)
Time from Still’s disease diagnosis to PAH diagnosis, months, median (IQR)	37 (17-59)
Still’s disease clinical features at pulmonary diagnosis, n (%)	
Fever	12 (75.0)
Arthralgia/arthritis	6 (37.5)
Inflammatory laboratory parameters at PAH diagnosis, median (IQR)	
CRP level at PAH diagnosis, mg/L, n = 14	46 (17-101)
Ferritin level at PAH diagnosis, µg/L, n = 13	2690 (2300-9314)
Modified Pouchot score at PAH diagnosis, median (IQR)	5.5 (2.0-7.2)
High disease activity (modified Pouchot score ≥ 4), n (%)	11 (68.7)
Low disease activity (modified Pouchot score ≤ 2), n (%)	5 (31.3)
Glucocorticoids at time of PAH diagnosis, n (%)	16 (16)
Current glucocorticoid dose at PAH diagnosis, median (IQR)	11.7 (7.6-28.7)
No history of IL-1 and/or IL-6 blockade at PAH diagnosis, n (%)	4 (25)
Still’s disease complications at PAH onset or immediately after, n (%) ^a	
Macrophage activation syndrome	8 (50.0)
Pericarditis	11 (68.7)
Thrombotic thrombocytopenic purpura	2 (12.5)
Follow-up after PAH diagnosis, months, median (IQR)	48 (24-79)
Deaths, n (%)	6 (37.5)
Time from PAH diagnosis to death (months), median (IQR)	11 (3-34)

^a Within 6 months of PAH diagnosis.
CRP, C-reactive protein; IL, interleukin; IQR, interquartile range; PAH, pulmonary arterial hypertension.

median mPouchot score was 5.5 (2.0-7.2), but the range was 1 to 11, reflecting heterogeneous levels of activity of the underlying disease among patients. Indeed, 5 (31.3%) patients had low disease activity (mPouchot score ≤ 2) at PAH diagnosis. The period following PAH onset was particularly at risk of complications because 8 (50%) patients exhibited MAS, 11 (68.7%) pericarditis, and 2 (12.5%) thrombotic thrombocytopenic purpura at PAH diagnosis or within 6 months of diagnosis. All patients were receiving GCs at PAH diagnosis, with a median dose of 11.7 (7.6-28.7) mg/d prednisone (Table 2 and Supplementary Table S18). Although, most patients had a history of IL-1 and/or IL-6 blockade at the time of PAH diagnosis, 4 patients had never received these treatments. There was no statistically significant difference between patients who had never been treated with IL-1i or IL-6i and those who had been treated at the time of PAH diagnosis in terms of demographics, clinical, or paraclinical manifestations, treatment and disease evolution, except for MAS, which occurred in 10/12 patients who had been treated with IL-1i or IL-6i at the time of PAH diagnosis and in 0/4 patients in the untreated group ($P = .08$, Supplementary Table S19).

Initial PAH characteristics are provided in Table 3. Most patients were in NYHA functional class III and IV (81%), with severe haemodynamic impairment: mPAP 39 (35-46) mm Hg, CI 2.6 (2.0-3.3) L/min/m², and PVR 6.4 (5.6-8.9) WU. Twelve patients underwent high-resolution CT, revealing interlobular septal thickening in all cases (100%), ground-glass opacities of

Table 3
Initial PAH characteristics and therapeutic management at PAH diagnosis, n = 16

PAH characteristics	
BMI, kg/m ² , median (IQR)	21.2 (20.3-24.7)
Smoker or exsmoker, n (%)	3 (19)
NYHA functional class II/III/IV, n (%)	3 (19)/ 9 (56) / 4 (25)
6-min walk distance, m, median (IQR)	289 (0-441)
Haemodynamic parameters, median (IQR)	
Right atrial pressure, mm Hg	7 (3-13)
Mean pulmonary artery pressure, mm Hg	39 (35-46)
Pulmonary artery wedge pressure, mm Hg	7 (5-10)
Cardiac output, L/min	4.2 (3.3-5.3)
Cardiac index, L/min/m ²	2.6 (2.0-3.3)
Pulmonary vascular resistance, Wood units	6.4 (5.6-8.9)
Mixed venous oxygen saturation, %	59 (45-64)
BNP >800 ng/L or NT-proBNP > 1100 ng/L, n = 14, n (%)	11 (79)
Initial management	
ICU management, n (%)	6 (37.5)
Inotropic therapy, n (%)	6 (37.5)
Extracorporeal life support, n (%)	1 (6)
PAH specific treatment, n (%)	13 (81)
Monotherapy	3
Initial oral dual combination therapy	6
Dual combination therapy including IV prostacyclin	1
Triple combination therapy including IV prostacyclin	3
Still’s disease-specific-treatment (1 st line) ^a , n (%)	
High-dose glucocorticoids (PDN ≥ 0.5 mg/kg/d), n (%)	16 (100)
Preceded by IV MP pulses	11 (68.7)
IL-1 inhibitors, n (%)	8 (50)
Initiation	3
Maintenance	1
Reintroduction	2
Dose doubling	1
Switch from anakinra to canakinumab	1
IL-6 inhibitors, n (%)	6 (37.5)
Initiation	4
Maintenance	1
Reintroduction	1
Dose doubling	0
Methotrexate (maintenance), n (%)	4 (25)
Maintenance	3
reintroduction	1
Cyclosporin (introduction), n (%)	1 (6.2)
Cyclophosphamide (introduction), n (%)	1 (6.2)

^a Some patients had several treatments.
BMI, body mass index; BNP, brain natriuretic peptide; ICU, intensive care unit; IL, interleukin; IV, intravenous; MP, methylprednisolone; NT-proBNP, N-terminal prohormone of brain natriuretic peptide; NYHA, New York Heart Association; PAH, pulmonary arterial hypertension; PDN, prednisone.

nonspecific nature in half of the cases, and lymph node enlargement in 8 cases (67%) (Fig 1).

Initial management after PAH diagnosis, response to therapies, and outcomes

Therapies initially prescribed after PAH diagnosis are summarised in Table 3. Six (38%) patients were initially cared for in the intensive care unit because of severe right heart failure, all requiring inotropic support. Extracorporeal life support was initiated in one patient. Approved PAH drugs were initiated at the time of PAH diagnosis in 13 patients (81%), 4 months later in 1 patient, and after 12 months in the 2 remaining patients. Overall, all patients received PAH therapy during follow-up.

All patients received treatment intensification for SD immediately after PAH diagnosis. All received high-dose GCs (≥ 0.5 mg/kg/day prednisone); 11 patients received prior intravenous methylprednisolone pulses before transitioning to oral prednisone. Additionally, IL-1 or IL-6 inhibitor biological therapy was used in 14 (87.5%) patients. Eight (50%) received an IL-1 inhibitor and 6 (37.5%) an IL-6 inhibitor. Methotrexate was initiated

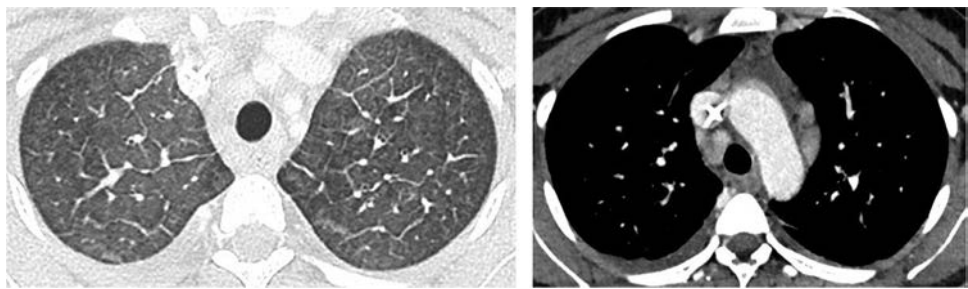


Figure 1. Illustrative high-resolution CT of a patient with Still’s disease at diagnosis of pulmonary arterial hypertension. High-resolution CT showing nonspecific ground-glass opacities, interlobular septal thickening, and lymph node enlargement.

Table 4
Change in clinical and haemodynamic variables between diagnosis and first reassessment (n = 15)

	Baseline	First reassessment Median 4.2 (3.4–5.0) months	P value
6-min walk distance, m, median (IQR)	318 (0–440)	445 (390–512)	.055
Right heart catheterization, n = 13, median (IQR)			
Right atrial pressure, mm Hg	7 (3–13)	5 (3–6)	.08
Mean pulmonary artery pressure, mm Hg	39 (35–40)	28 (25–31)	.006
Pulmonary artery wedge pressure, mm Hg	7 (5–10)	9 (7–10)	.26
Cardiac output, L/min	4.2 (3.3–5.3)	5.2 (4.6–5.6)	.16
Cardiac index, L/min/m ²	2.6 (2.0–3.3)	3.3 (2.8–3.8)	.15
Pulmonary vascular resistance, Wood units	6.4 (5.6–8.9)	3.5 (2.9–4.77)	.002
Mixed venous oxygen saturation, %, n = 6	58 (48–64)	69 (67–76)	.06
BNP >800 ng/L or NT-proBNP > 1100 ng/L, n = 12, n (%)	9 (75)	5 (42)	.10

In bold, statistically significant values, i.e. $p \leq 0.05$.
Note: One patient was not reassessed because she died within a month of PAH diagnosis.
BNP, brain natriuretic peptide; IQR, interquartile range; NYHA, New York Heart Association; PAH, pulmonary arterial hypertension; NT-proBNP, N-terminal prohormone of brain natriuretic peptide.

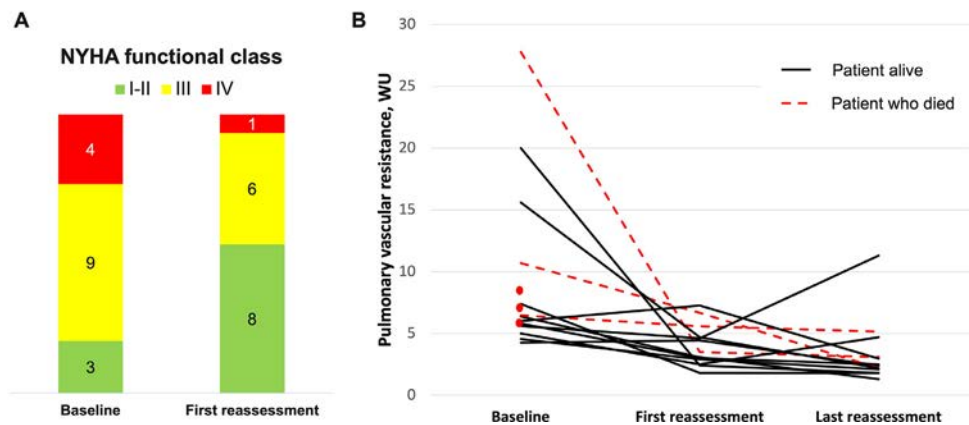


Figure 2. Change in New York Heart Association (NYHA) functional class (A) and pulmonary vascular resistance (B). Patients still alive are depicted in black. Patients who died are depicted in red. First reassessment visit median 4.2 (IQR 3.4–5.0) months and last reassessment visit median 39 (26–80) months.

in 4 (25%) patients, and ciclosporin and cyclophosphamide were each used in 1 patient. Details of treatments per patient received in the first and subsequent lines are summarised in [Supplementary Table S18](#).
Fifteen of the 16 patients were reassessed by RHC after a median follow-up of 4.2 (3.4–5.0) months ([Table 4](#)). One patient was not reassessed because she died within a month of the PAH diagnosis. Among the 15 reassessed patients, haemodynamic parameters significantly improved, including a 45% reduction in PVR ([Table 4](#) and [Fig 2](#)).
Over a median follow-up of 48 (24–79) months, 6 patients died ([Table 2](#), [Supplementary Table S20](#)); details of the causes of death are provided below. The 1- and 3-year overall survival rates were 81% and 75%, respectively ([Fig 3](#)). Of the 10 surviving patients, 9 were in SD remission (8 still on treatment and 1

off treatment) and one showed partial response on treatment at the last follow-up ([Supplementary Table S20](#)). Eight patients were at low risk of death, and 2 were at intermediate to high risk [[13,14,28,29](#)].
Deaths and pathologic assessment
Five deaths occurred during an SD flare with multivisceral failure, including septic shock to pseudomonas aeruginosa in 1 patient and blood coagulation disorders in 4 patients (disseminated intravascular coagulation in 2 patients, thrombotic microangiopathy in 1 patient, cerebral hematomas in the context of MAS-induced bicytopenias in 1 patient; [Supplementary Table S20](#), [Supplementary Fig S2](#)); one patient died suddenly. Of note,

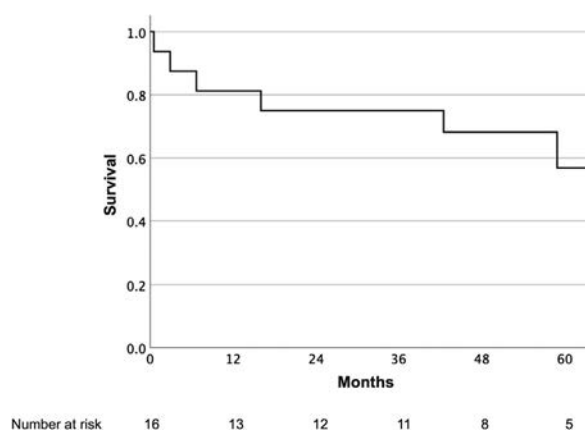


Figure 3. Overall survival after pulmonary arterial hypertension diagnosis in patients with Still's disease (n = 16). Overall survival at 1 and 3 years was 81% and 75%, respectively.

3 patients who died exhibited major haemodynamic improvement after initial therapy, which led to a reduction in immunomodulatory therapy in the months preceding the fatal disease flare ([Supplementary Fig S2](#)). The attending physician described another patient on anakinra as noncompliant in the months preceding the fatal disease flare. All patients who died had uncontrolled underlying SD; severe right ventricular failure developed and progressed rapidly ([Supplementary Table S20](#)). In comparison, the single death in the PAH- group was related to acute liver failure, which also occurred in the setting of underlying uncontrolled SD, which highlights the importance of controlling the disease.

Postmortem examination was available for 1 patient who died from SD flare complicated by PAH decompensation with multivisceral failure and diffuse intravascular coagulation (patient 16, [Supplementary Tables S20 and S21](#)). Lung tissue examination showed thickening of the pulmonary artery wall due to concentric intimal remodelling, with no evidence of thrombosis or plexiform lesions. The vasculopathy affected both pulmonary arteries and veins. In addition, there was significant

macrophage alveolitis with no thickening of the alveolar septa or destruction of the pulmonary parenchyma ([Fig 4](#)). Importantly, MAS was excluded ([Supplementary Table S21](#)).

Cytokine assays results available

Cytokine assays were available for 3 patients in the PAH+ group at different time points relative to the PAH diagnosis ([Supplementary Table S22](#)). However, the assays were all performed during an uncontrolled phase of SD and PAH. Plasma IL-18 levels were high, ranging from more than 50,000 pg/mL to more than 150,000 pg/mL. In 1 patient, the plasmatic IFN- γ levels were available during to flares of SD and PAH; they were high at 7.0 and 1507.0 pg/mL (N: 0.0–3.0), respectively.

DISCUSSION

This the largest series of SD patients with PAH (ie, group 1 PH related to a vascular dysfunction occurring independently of any significant parenchymal involvement) reported to date, and the comparison to cohort controls allowed for identifying key features differentiating PAH+ from PAH- patients. SD patients in whom PAH developed were all women, predominantly Black, and had more active disease at diagnosis and throughout the disease evolution. They had a high prevalence of recurrent MAS and eosinophilia. In most cases, they were positive for HLA-DRB1*15. These features were also associated with other manifestations of SD-related ILD [6,7,9]. However, in our clinical series (and in most patients retrieved by of our SR), there were no cases of patients with both PAH and parenchymal ILD, which suggests that PAH can occur independently of other manifestations of ILD in SD.

SD-related PAH and other manifestations of ILD occurring on the same background suggests a common pathophysiology and argues in favour of a similar group of complicated forms of SD: the 'hyperinflammatory' forms. The concept of hyperinflammation, addressed by the EULAR/PreS recommendations [5], can be defined as an activation of innate and adaptive immunity in

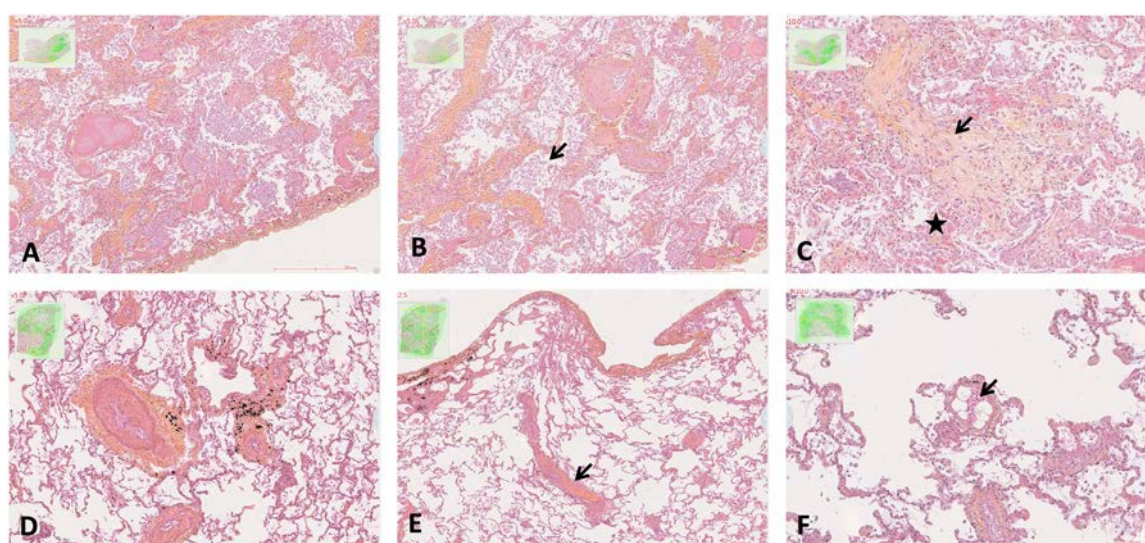


Figure 4. Pathologic assessment on explanted lungs. (A) Preserved pulmonary architecture. No interstitial fibrosis. Significant macrophage alveolitis with no thickening of the alveolar septa or destruction of the pulmonary parenchyma. (B) Significant shedding of macrophages within the alveoli (arrow). (C) Interstitial inflammatory load with endoalveolar fibroblastic budding (arrow); no granuloma. Pronounced capillary congestion (star). (D) Thickening of the pulmonary artery wall due to concentric intimal remodelling. No evidence of thrombosis or plexiform lesions. (E) Vasculopathy affecting both pulmonary arteries and veins. Fibrosis in the wall of a septal vein indicated by an arrow. (F) Bone marrow embolism (arrow).

response to a reasonable stimulus (eg, viral infection) that becomes excessive and leads to damaging the host. This concept of hyperinflammation has mainly been put forward in MAS [30], and of note, we found a statistical association with MAS (particularly recurrent) in our SD patients with PAH, as was described in those with other manifestations of ILD [6–9]. Although the pathophysiology is not yet fully understood, there is increasing evidence of a central role for the IL-18/IFN- γ pathway and some activated T-cell subsets in SD-related ILD [10]. Our observations in SD-related PAH point in this direction.

Indeed, 7 patients in our series showed improvement precisely by maintaining the IL-1 or IL-6R biotherapy and increasing the treatment (ie, by adding an immunosuppressive treatment targeting T cells, such as ciclosporin, tacrolimus, or mycophenolate mofetil), an immunomodulator (leflunomide), or a Janus kinase (JAK) inhibitor (Supplementary Tables S18 and S20). This observation is consistent with EULAR/PRoS recommendations, which suggest the initiation of T cell-directed immunosuppression (in addition to IL-1 or IL-6R inhibition) in patients at high risk for ILD or with ILD [5]. The data from our patients showing improvement after the addition of a T-cell-directed immunosuppression argues for a similar strategy in SD-related PAH. The benefits of JAK inhibitors in patients with SD-related LD has been reported in 2 cases [31,32] and observed in 3 of our patients with PAH. This improvement after optimisation of the biologic by adding a T cell-targeted therapy and the fact that PAH occurred in 4/16 patients (25%) in our series and 13/21 (61.9%) in our SR who had not previously received IL-1 or IL-6R inhibitors indicate that PAH as a drug reaction to these therapies is highly unlikely [9] but supports the Cytokine Plasticity Hypothesis described by Binstadt and Nigrovic [10]. In addition, the eosinophilia observed in 11 out of 16 patients during the SD course could suggest a possible role for IL-5 and T helper 2 cell subsets.

In all our patients with PAH, the underlying SD was not controlled, as assessed by the mPouchot score [23]. This observation reinforces the importance of the treat-to-target approach, which has been defined by the EULAR/PRoS [5]. The target should be remission, defined as CID for > 6 months. CID is defined as absence of SD-related symptoms and normal CRP level. None of our patients were in CID when PAH developed. Second, PAH can occur at any time during the disease course, including in cases in which the disease is only slightly active according to the mPouchot score.

Therefore, our results highlight the importance of identifying patients at risk of difficult-to-treat and complicated forms of SD. The EULAR/PRoS recommendations define a screening strategy for high-risk forms of SD–ILD [5] but do not address the specific case of PAH (ie, occurring without significant parenchymal involvement). This screening strategy for PAH could involve several elements. Our results argue in favour of systematic screening for HLA-DRB1*15 at SD onset. In the event of a positive result, especially in a female Black patient with recurrent MAS and eosinophilia, the physician should be particularly vigilant and focus on early detection of symptoms suggesting PAH, such as dyspnoea, chest pain, or signs of right heart failure. Eosinophilia is not a classical sign of SD in adults [1]. Although it can be found in children without ILD, in a recent series, it was numerically more frequent in children with SD with ILD than in those without any lung involvement and associated with MAS and drug reactions to IL-1 or IL-6R inhibitors [24]. The screening strategy, particularly the target population and follow-up frequency, has yet to be clearly defined. However, in cases of unexplained dyspnoea in SD patients, echocardiography

remains the recommended screening tool to assess the probability of PAH, in line with the 2022 European Society of Cardiology and the European Respiratory Society (ESC/ERS) guidelines [13,14]. If an intermediate or high probability of PAH is found, RHC is recommended because it is the only definitive test for confirming the diagnosis and determining both the underlying mechanisms and the severity of PAH. This strategy has been developed and validated in other immunological diseases at risk of PAH, such as scleroderma [13,14,33].

Our work has several strengths but also some limitations inherent to its retrospective nature. This study is the largest series reporting well-phenotype SD-related PAH (all confirmed by RHC) in adults to date, with a large control group of 111 PAH-SD patients. Although follow-up data and data in general are limited for the cases in our SR, the SR allowed for confirming certain important points highlighted in our series, such as the predominance of the female sex, the possibility of occurrence in the absence of prior treatment with IL-1 or IL-6R inhibitors (ruling out the hypothesis of a drug-related cause for PAH), and the need to control the inflammation of the underlying SD. HLA typing was not possible in all patients, mainly because some had died and genetic material was not available. We were also unable to perform cytokine assays in all our patients to confirm our pathophysiological hypotheses, ie, that patients with PAH have higher levels of IL-18 and IFN- γ than those without. However, in patients in whom they could be measured, the plasmatic IL-18 levels were very high. In one patient, the IFN- γ levels were also available and high, although this cytokine is difficult to measure in practice. Measuring CXCL9 (an IFN- γ induced chemokine) is indeed often technically easier [5,34], but these tests had not been carried out on our patients. For the first time, we describe a pathological assessment of SD-associated PAH, but it was limited to one patient. We did not include any paediatric patients; however, on the basis of numerous elements in the literature [34], EULAR/PRoS recently concluded that the paediatric and adult forms of SD are the same diseases [5].

In conclusion, the association of PAH with HLA-DRB1*15, similarly to other manifestations of ILD in SD, reinforces the association of the HLA-DRB1*15 alleles with severe forms of SD and raises the question of treatment optimisations to better control both SD and its complications. Physicians should contact reference centres with SD expertise (in Europe through the European Reference Network on Rare Immunodeficiency, Autoinflammatory and Autoimmune Diseases (ERN-RITA); <https://ern-rita.org/>) to discuss treatment escalation and in general the care of patients with inadequate response to standard care, as recommended by the EULAR/PRoS [5].

Competing interests

Stéphane Mitrovic has no permanent financial links, but has received consulting fees from BMS, Lilly, Pfizer, and SOBI. Bruno Fautrel has no permanent financial links, but received research grants from AbbVie, Lilly, MSD, and Pfizer and consultancy fees from AbbVie, Amgen, Biogen, BMS, Celltrion, Fresenius Kabi, Galapagos, Gilead, Janssen, Lilly, Medac, MSD, Nordic Pharma, Novartis, Pfizer, Roche, Sandoz, Sanofi-Genzyme, Sobi, UCB, and Viartis. The other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Contributors

The work was initiated by BF, SM, OS, AB, and DM. SM collected data for SD in cases and controls and performed the systematic review. AB collected data for PAH. Both SM and AB performed the descriptive statistics. MC performed and coordinated the HLA typing for patients. MRG performed the anatomopathological examination. SM, AB, OS, DM, and BF wrote the manuscript. All the other coauthors were either directly involved in patient management and/or reported a case and took part in the scientific reflection. All authors reviewed and amended the manuscript and formally approved the submitted version.

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Patient consent for publication

Not applicable.

Ethics approval

This study was conducted in accordance with the Declaration of Helsinki. The methods used to collect and analyse data on PAH was approved by the ethics committee on 24 May 2003 (approval no. 842063). Regarding the data for SD, the French law does not require formal ethics committee approval or informed consent for retrospective data collection (<https://www.legifrance.gouv.fr/loda/id/JORFTEXT000000886460/>); however, all patients expressed non-opposition to the use of the data for research purposes.

Provenance and peer review

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Supplementary materials

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Treatment

Janus kinase inhibitors enhance prostanoid biosynthesis in human whole blood *in vitro*: implications for cardiovascular side effects and prevention strategies

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ABSTRACT

Objectives: Janus kinase inhibitors (JAKis) effectively treat chronic inflammatory diseases but are associated with cardiovascular side effects through unknown mechanisms. This study aimed to investigate the impact of JAKis on prothrombotic thromboxane (TX)₂ production in human whole blood (WB) as a possible mechanism.

Methods: We evaluated the effects of 4 JAKis— tofacitinib, baricitinib, filgotinib, and upadacitinib (0.04–20.0 μM)—on TXB₂ biosynthesis in clotting WB from healthy subjects, serving as a marker for platelet TXA₂ generation. Additionally, we assessed the impact of these JAKis on TXB₂ production in WB from healthy subjects, patients with systemic lupus erythematosus (SLE), and treatment-naïve patients with axial spondyloarthritis (axSpA) after 24-hour lipopolysaccharide (LPS) stimulation, as a marker of platelet and leukocyte prostanoid biosynthesis.

Results: All JAKis increased serum TXB₂ production in clotting WB, although not in a concentration-dependent manner. In LPS-stimulated WB, tofacitinib (1 μM) significantly increased TXB₂ production in healthy subjects (HSs) (42% ± 33%, n = 17), patients with SLE (57% ± 39%, n = 12), and patients with axSpA (31% ± 23%, n = 15). Baricitinib (1 μM) also increased TXB₂ in HSs (30% ± 22%, n = 10). Upadacitinib showed a trend towards increased TXB₂ (46% ±

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40%, $n = 7$), while filgotinib did not ($21\% \pm 19\%$, $n = 7$). Aspirin ($100\ \mu\text{M}$) almost completely reduced serum TXB_2 in the presence of all JAKis.

Conclusions: The enhanced biosynthesis of TXA_2 in platelets, with a minor contribution from leukocytes, may contribute to the increased cardiovascular risk associated with JAKis. Low-dose aspirin may offer a protective effect, warranting further investigations.

WHAT IS ALREADY KNOWN ON THIS TOPIC

- Tofacitinib, a Janus kinase inhibitor (JAKi), is associated with increased cardiovascular risk.
- Due to concerns about cardiovascular safety, the European Medicines Agency has restricted the use of JAKis in patients older than 65 years or those with other cardiovascular risk factors.
- Thromboxane (TXA_2) is a vasoconstrictor and a lipid mediator with prothrombotic properties.

WHAT THIS STUDY ADDS

- This study demonstrates that JAKis increase the biosynthesis of prothrombotic TXA_2 in platelets, as well as TXA_2 and prostaglandin E_2 in leukocytes *in vitro*.
- Dysregulated TXA_2 biosynthesis induced by JAKis may contribute to an increased risk of cardiovascular side effects.
- Aspirin normalised tofacitinib-induced TXA_2 production *in vitro*.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- Combined treatment with JAKis and low-dose aspirin might counteract thrombotic side effects.
- Reducing the cardiovascular risk profile may enable more patients to safely benefit from treatment with JAKis.

INTRODUCTION

Janus kinase inhibitors (JAKis) have immunosuppressive effects and are used to treat rheumatoid arthritis (RA) and other chronic inflammatory, immune-mediated diseases [1]. These agents belong to a new class of targeted synthetic disease modifying antirheumatic drugs [2]. JAKis are often well tolerated by patients with RA and provide significant symptom relief, joint preservation, and improved quality of life [2].

Tofacitinib is a first-generation oral drug that inhibits JAK1 and JAK3 with minor inhibition of JAK2 and tyrosine-protein kinase 2 (TYK2) [3], developed for the treatment of RA [4]. Other indications for tofacitinib include ankylosing spondylitis and psoriatic arthritis [5]. In a randomised, open-label safety-endpoint trial, patients with RA treated with tofacitinib were found to have an increased risk of major adverse cardiovascular events and cancer compared with patients treated with tumour necrosis factor inhibitors [6]. As a result, the European Medicines Agency [7] endorsed safety measures to minimise the risk of serious side effects that apply to all JAK inhibitors [8].

Baricitinib is a JAK1 and JAK2 inhibitor with moderate activity against TYK2 but significantly less inhibition of JAK3. It is approved for the treatment of RA [9]. Filgotinib and upadacitinib are both JAK1-selective inhibitors [10] and were developed as second-generation JAKis. They are approved in Europe for the treatment of patients with moderate to severe active RA who are intolerant to methotrexate [10,11]. Upadacitinib is also used for the treatment of psoriatic arthritis and axial spondylitis [12]. In systemic lupus erythematosus (SLE), JAKis, including

tofacitinib and baricitinib, have shown beneficial effects [13] but have not been registered for this indication. Selectivity and pharmacologic properties of the JAKis have been summarised elsewhere [14], and an overview can be found in [Supplementary Table S1](#).

In a recent screening, we unexpectedly observed that tofacitinib can increase the biosynthesis of prostaglandin (PGE_2) and prothrombotic thromboxane (TXB_2) in human whole blood (WB) stimulated with the bacterial endotoxin lipopolysaccharide (LPS) *in vitro* [15]. These prostanoids are generated by the release of arachidonic acid (AA) from the plasma membrane phospholipids in platelets and leukocytes. AA is first converted to PGH_2 by cyclooxygenase (COX)-1 and/or COX-2 and subsequently converted into the principal prostanoids, for example, PGE_2 and TXB_2 , through the action of specific terminal synthases [16]. Platelets constitutively express COX-1 but not COX-2 [17]. They generate TXA_2 , a major product of AA metabolism, which is rapidly converted to the stable but almost biologically inactive TXB_2 through a nonenzymatic hydrolysis reaction [18]. TXB_2 can be determined by using a validated immunoassay [19] or by liquid chromatography-tandem mass spectrometry (LC-MS/MS) [15].

PGE_2 mediates signs of inflammation and is generated via the coordinated induction of the enzymes COX-2 and microsomal prostaglandin E synthase (mPGES)-1 [20]. The traditional non-steroidal anti-inflammatory drugs (NSAIDs) affect both COX isozymes, whereas coxibs act via the selective inhibition of COX-2 [16]. The inhibition of COX-dependent PGE_2 by these drugs translates into analgesic and anti-inflammatory effects [21]. During inflammation, enhanced biosynthesis of PGE_2 occurs via the induction of COX-2 and mPGES-1 in response to various proinflammatory stimuli, including cytokines and LPS (a major component of the outer cell membrane of gram-negative bacteria) [21].

TXA_2 is an unstable lipid mediator that plays a critical role in various pathophysiologic processes such as primary hemostasis, atherothrombosis, inflammation, and cancer [18]. TXA_2 is the primary AA derivative produced via the COX-1 pathway, mainly in human platelets [22], where its generation mediates different platelet responses, including platelet aggregation and granule release. Inhibition by the NSAID aspirin (acetylsalicylic acid [ASA]), when given at low doses, prevents cardiovascular disease and is commonly used worldwide as secondary prophylaxis against myocardial infarction or stroke [23].

We hypothesise that the altered production of TXA_2 , primarily from platelets, and PGE_2 from leukocytes may contribute to an increased risk of thromboembolic events and diminished anti-inflammatory effects, respectively, associated with the administration of JAKis. In this study, we aimed to investigate the effects of tofacitinib on LPS-stimulated WB prostanoid biosynthesis to validate our previous preliminary findings and included 3 additional JAKis (baricitinib, filgotinib, and upadacitinib). In LPS-stimulated WB assay (24 hours of incubation), the biosynthesis of PGE_2 depends on the time-dependent induction of COX-2 and mPGES-1 in leukocytes [24]. TXB_2 can be derived from mainly platelet COX-1 with a minor contribution from

leukocyte COX-2. Prostanoid biosynthesis in LPS-stimulated WB was investigated in healthy subjects (HSs) and patients with SLE or treatment-naïve axial spondyloarthritis (axSpA). We also evaluated the impact of JAKis on platelet TXA₂ biosynthesis by measuring TXB₂ generated during WB clotting (serum TXB₂) of HSs [25] (the study's primary end point). Several lines of evidence suggest that serum TXB₂ is generated in platelets via COX-1 during clotting and in response to the endogenously generated thrombin in humans [19,25]. Moreover, the importance of COX-1 has been demonstrated *in vivo* in mice lacking platelet COX-1 [26]. Finally, we investigated the effect of ASA to verify whether it can counteract the increased prostanoid formation in WB by JAKis *in vitro*.

METHODS

Materials

Tofacitinib citrate (PZ0017-5MG) was purchased from Merck (Darmstadt). Baricitinib (16707-25MG), filgotinib (17669-5MG), and upadacitinib (29706-5MG) were purchased from Cayman Chemical. All inhibitors were reconstituted in 10 mM dimethyl sulfoxide (DMSO, D2650-5 × 5 mL; Sigma Aldrich). Further aliquots of JAKi were made at concentrations 0.1, 1, and 10 μ M and stored at -80°C . ASA (A2093-100G; Sigma Aldrich) was reconstituted in DMSO to 1 M, and aliquots were prepared at concentrations of 100 μ M, 500 μ M, and 1 mM and stored at -20°C . LPS (L6529-1MG) was reconstituted in 1 mL of phosphate-buffered saline (PBS; D8537-500ML; Sigma Aldrich). These samples were aliquoted in Eppendorf tubes and stored at -20°C . AA (Sigma Aldrich) was dissolved in saline solution (0.9% NaCl), and aliquots were stored at -80°C .

Study population

Study population for WB clotting assay

Twelve HSs (8 females and 4 males) aged 31.10 ± 7.71 years (mean $\pm$ SD) were enrolled at the Center for Advanced Studies and Technology of G. d'Annunzio University, Chieti, Italy (Supplementary Table S2). The study was conducted in Italy after obtaining ethical approval (Approval no. 16, 2019). These subjects did not use NSAIDs, including ASA, for at least 2 weeks before donating blood for the WB clotting assay.

Study population for LPS-activated WB assay

The study enrolled 3 groups of individuals: 17 HSs; 12 patients with SLE; and 15 treatment-naïve patients with axSpA. Patients had stopped taking NSAIDs at least 1 week before blood donation (Supplementary Table S2). HSs were recruited from the Center for Blood Donors in Stockholm, Sweden, and had not experienced fever in the two weeks prior to donation. Individuals with rheumatic disease or major inflammation were also not allowed to donate blood. Patients with SLE were diagnosed according to the ACR SLE criteria [27] and recruited from the Rheumatology Clinic at Karolinska Hospital, Sweden. The median age was 37 years, ranging from 26 to 74 years. Ethical approval was granted by the Regional Ethical Review Board in Stockholm (Dnr: u2015/2001–32/2). Additionally, 6 female and 6 male treatment-naïve patients with axSpA with a median age of 31.5 years (ranging from 21 to 51 years) were recruited from the Rheumatology Clinic at Karolinska Hospital in Huddinge

and the Center for Rheumatology (CFR) in Solna, Sweden, with ethical approval (Dnr: 2022-01789-01). All patients provided written informed consent.

Bioassays

WB clotting assay

Tofacitinib, baricitinib, filgotinib, and upadacitinib (0.002–1.0 mM) were dissolved in PBS and 10% DMSO; 20 μ L of the solutions containing the compounds or vehicle were pipetted directly into glass tubes. The experiments were performed by a blinded researcher incubating 1 mL of WB aliquots drawn from healthy volunteers in glass tubes in the presence of vehicle or AA (Sigma Aldrich, Italy)—dissolved in saline solution (0.9% NaCl) at a final concentration of 20 μ M—alone or with 4 JAKis (4 μ M) and allowed to clot for 1 hour at 37°C [25]. In addition, WB was incubated with vehicle or ASA (dissolved in DMSO to a final concentration of 100 μ M) in the absence or presence of the 4 JAKis (4 μ M) and allowed to clot for 1 hour at 37°C . For all experiments, serum was separated via centrifugation (10 minutes at $1560 \times g$ at 4°C) and stored at -80°C until it was assayed for TXB₂ by a validated enzyme immunoassay [19]. In serum, 8-iso-PGF_{2 α} was also measured, as an index of nonenzymatic lipid peroxidation by a specific radioimmunoassay [28,29].

LPS-activated WB assay

WB *in vitro* stimulation

Fresh human blood was collected in heparinised tubes from male and female volunteers and patients. JAKis and the vehicle control (0.01% DMSO) were dissolved in PBS at room temperature. An aliquot of 200 μ L of freshly drawn heparin blood (within 4 hours after sampling) was added to the wells of U-shaped 96-well plates (83.3925-TC Platte; Thermo Fisher Scientific) in triplicates; 25 μ L aliquots of JAKis or vehicle control were added. The plate of 1 donor was incubated at 37°C for 30 minutes, and then, 25 μ L of 0.1 mg/mL LPS in PBS was added with a final concentration of LPS of 10 μ g/mL, and the mixture was mixed with a pipette 3 times. The plate was incubated for 24 hours at 37°C and centrifuged at $3000 \times g$ for 10 minutes at 4°C . An aliquot of 80 μ L plasma was collected in a new deep well plate (2-mL Square Collection plate; Waters). The plate was stored at -80°C until protein precipitation. The researcher performing the WB *in vitro* stimulation was not blinded to the different treatments. However, controls were investigated at the beginning and end of the plate to evaluate the risk of bias due to the small differences in time between treatments added to the plate at the beginning or end of the experiment.

Tofacitinib, baricitinib, upadacitinib, and filgotinib were investigated at different concentrations (0.01–1.0 μ M). Moreover, an mPGES-1 inhibitor (118), the COX1/2 inhibitor diclofenac (Diclo), and a COX-2 inhibitor (NS-398) were also investigated at 1 μ M to study the inhibition of the prostanoid pathway. ASA was studied at 3 different concentrations, with final concentrations in the wells of 10, 50, and 100 μ M. The combination of 1 μ M tofacitinib and ASA (10–100 μ M) was also studied.

Protein precipitation

Plasma samples were spiked with 50 μ L of the internal standard (IS) mixture of 100 μ g/mL (PGE₂-d₄, P/N 314010; PGD₂-d₄, P/N 312010; TXB₂-d₄, P/N 319030; 6-keto PGF_{1 α} -d₄, P/N 315210; PGF_{2 α} -d₄, P/N 316010; and 15d-PGJ₂-d₄, P/N

318570; Cayman Chemical), before protein precipitation; 800 μ L of 100% methanol (MeOH) was added to the samples in the deep well plate and mixed. The plate was subsequently centrifuged at 4 °C for 10 minutes at 3000 $\times$ g, after which the supernatants were collected in a new deep well plate. Using a speed vacuum (SPD120; ThermoScientific), the samples were evaporated for approximately 4 hours, and the plate was stored at –20 °C until solid-phase extraction.

Solid-phase extraction

The evaporated samples were redissolved in 1 mL of 0.05% formic acid (FA) in water. A solid-phase extraction plate (Oasis HLB 96-well plate; Waters) was preconditioned with 1 mL of pure MeOH and 1 mL of 0.05% FA in water. After the samples were added to the cartridges, the plate was washed with a solution consisting of 1 mL of 10% MeOH in acidic water (0.05% FA). Subsequently, 1 mL of 100% MeOH was used for elution into new plates (2-mL Square Collection plate; Waters). The plates were set in SpeedVac apparatus overnight evaporation. On the following day, the plate was retrieved and preserved at –20 °C.

Prostanoid profiling by LC-MS/MS

Ultraperformance LC-MS/MS (UPLC-MS/MS (Waters) was used for the analysis of prostanoids. The samples and standards were reconstituted in 50 μ L of 20% acetonitrile (MeCN) in water. The mobile phase consisted of a mixture of water (Milli-Q; Millipore) and MeCN, with 0.05% FA. A UPLC instrument (Acquity; Waters) connected to a triple quadrupole mass spectrometer (Waters) was used to analyse the samples in negative mode via multiple reaction monitoring as previously described (Supplementary Table S3) [15]. An ACQUITY UPLC BEH C18 analytical column measuring 50.0 mm $\times$ 2.1 mm $\times$ 1.7 μ m was used, and the column temperature was maintained at 40 °C. Mobile phase A consisted of 0.05% FA in water, while mobile phase B contained 0.05% FA in MeCN. The analytes were retained in the stationary phase and eluted by increasing the linear gradient (20%-95%) of organic solvent in the mobile phase (Supplementary Table S4). The flow rate was set to 0.6 mL/min with an injection volume of 5 μ L, and the samples were stored in the autosampler at 10 °C. The data quantification relied on the IS and standard curve. The external standards were injected at the beginning, middle, and end of the sample table from a 96-well plate, and quality control samples were injected before each batch. Peaks were integrated via MassLynx software, version 4.1. Although the WB *in vitro* stimulations were not blinded, the LC-MS/MS data analysis was conducted using automated software to integrate peaks, minimising human intervention. This automated approach ensured a high degree of consistency and objectivity in data handling, effectively reducing subjective influence, as the compound's locations were not known during the analysis. Additionally, for the analysis of 2 patients with axSpA, a second blinded researcher performed the data analysis in parallel with the other researcher, and the same results were obtained.

Statistical analysis

Data are presented as mean $\pm$ SD. The data were normalised to each individual's vehicle control to minimise intersubject variability. A *P* value <.05 was considered to indicate statistical significance (GraphPad Prism software, version Prism 10 for Windows or macOS). One-way analysis of variance (ANOVA) was used to investigate whether any JAKi at any concentration increased TXB₂ and PGE₂ compared with vehicle control, followed by the Dunnett's post hoc test. Reporting the actual

values of TXB₂ generation in serum (nanograms per millilitre) is appropriate since some studies have found that it correlates with clinical outcomes and the platelet response to ASA [18,30–32]. In the case of LPS-stimulated WB, reporting the data as a percentage of the control value (vehicle) is appropriate because no evidence suggests that absolute prostanoid concentrations in this model are markers related to clinical conditions. The levels of prostanoids produced depend on the concentration of LPS used, which is arbitrary. The LPS-stimulated WB data reported as % of control were analysed by one-way ANOVA and Dunnett post hoc test. The absolute levels in nanograms per millilitre of PGE₂ and TXB₂ in HSs, SLE, and axSpA groups can be found in Supplementary Tables S5 and S6. Since our goal was to compare each JAKi (at different concentrations) with the vehicle control, that is, study a relative increase/decrease, we performed pairwise comparisons (*t* test) with Bonferroni correction for multiple testing, and the results are reported in Tables 1 and 2.

Sample size calculation

The impact of the JAKis on serum TXB₂ was the study's primary end point. Assuming a SD of serum TXB₂ in HS of 160 ng/mL as previously [19], the study would require a sample size of 12 individuals for each treatment to achieve a power of 80% and a level of significance of 5% (2-sided), for detecting a true difference in means between the baseline (vehicle) and treated samples of 210 ng/mL (ie, an increase of 50% from the vehicle). For the LPS-activated WB assay in HSs (secondary end point), based on the effect size of 1.35 (calculated by dividing the mean difference [1.9 ng/mL] by the SD of the difference [1.4 ng/mL]), according to Cohen *d* for paired samples *t* test) for TXB₂, 8 individuals were required to achieve a statistical power of 80% at a significance level of 0.05 [33].

In the ASA experiments, serum TXB₂ (primary end point) inhibition was anticipated to exceed 95% [19]. Therefore, a sample size of at least 7 individuals was suitable to detect an inhibition percentage of 70 or greater to achieve a power of 80% and a significance level of 5% (2-sided).

RESULTS

JAKis increase prostanoids *in vitro*

Effects of JAKis on prostanoids generation in WB clotting assay

The generation of serum TXB₂ (a marker of TXA₂ biosynthesis from platelets in response to thrombin, which is produced endogenously during blood clotting) [24] increased significantly in the presence of tofacitinib, baricitinib, filgotinib, and upadacitinib compared with the levels detected in the presence of vehicle (Fig 1). The response was not clearly concentration dependent. At 0.4 mM, a statistically significant increase of serum TXB₂ was detected for baricitinib and filgotinib. At 4 mM, the increase in serum TXB₂ was significant versus vehicle for tofacitinib and baricitinib (Fig 1). At higher concentrations, tofacitinib and upadacitinib resulted in a significant increase in serum TXB₂. We also studied whether exogenous AA could further enhance serum TXB₂ levels. As shown in Figure 2, serum TXB₂ increased significantly in the presence of exogenous AA but the 4 JAKis did not further increase serum TXB₂ in the presence of exogenous AA (Fig 2).

Table 1
The effect of JAKis on PGE₂ biosynthesis in LPS-stimulated WB from HS, SLE, and axSpA

JAKis	Concentration	HS ¹			SLE			AxSpA		
		n	Increase ²	P ³	n	Increase ²	P ³	n	Increase ²	P ³
Tofacitinib	1 μM	n=17	48% ± 36%	<0.001	n=12	89% ± 127%	<0.01	n=15	45% ± 53%	<0.01
	0.1 μM	n=15	34% ± 28%	<0.001	n=12	47% ± 66%	<0.01	n=14	18% ± 31%	ns
	0.01 μM	n=15	16% ± 28%	<0.05	n=12	14% ± 16%	<0.05	n=11	-5% ± 11%	ns
Baricitinib	1 μM	n=10	39% ± 52%	<0.05	n=7	5% ± 26%	ns	n=14	27% ± 46%	ns
	0.1 μM	n=9	33% ± 31%	0.01	n=7	13% ± 21%	ns	n=11	7% ± 20%	ns
	0.01 μM	n=9	21% ± 23%	<0.05	n=7	7% ± 12%	ns	n=11	-2% ± 19%	ns
Filgotinib	1 μM	n=7	33% ± 18%	<0.05	n=7	24% ± 18%	ns	n=7	10% ± 12%	ns
	0.1 μM	n=7	21% ± 20%	ns	n=6	6% ± 13%	ns	n=6	-6% ± 13%	ns
	0.01 μM	n=7	18% ± 13%	ns	n=7	-1.6% ± 10%	ns	n=5	-13% ± 17%	ns
Upadacitinib	1 μM	n=7	59% ± 84%	ns	n=7	25% ± 36%	ns	n=8	24% ± 28%	ns
	0.1 μM	n=7	62% ± 44%	<0.05	n=7	21% ± 28%	ns	n=7	6% ± 23%	ns
	0.01 μM	n=7	37% ± 27%	<0.05	n=7	5% ± 15%	ns	n=6	-9% ± 20%	ns

axSpA, axial spondyloarthritis; ANOVA, analysis of variance; HS, healthy subject; JAKi, Janus kinase inhibitor; LPS, lipopolysaccharide; ns, nonsignificant; PG, prostaglandin; SLE, systemic lupus erythematosus; TX, thromboxane; WB, whole blood.

^aThe data for HSs have already been visualised in Figure 3 (using ANOVA) but are shown here for comparison with patient data, using paired *t* test to assess significance.

^bIncreased level of PGE₂ compared with the vehicle control. All vehicle solutions were 0.01% dimethyl sulfoxide. An increase>20% is highlighted in gray.

^cPaired *t* test. *P* < .05 is highlighted in bold. *P* < .01 is shaded in light orange and *P* < .001 is underlined. Compensating for multiple testing (36 comparisons) using Bonferroni correction corresponds to a cutoff of the nonadjusted *P* value at .0013 to maintain a significance level of .05. However, it should be noted that the Bonferroni correction was highly stringent, and the comparisons were not fully independent due to the inclusion of multiple concentrations of the same JAKi and the investigation of the same JAKi in 3 different cohorts. This suggests that unadjusted *P* values at thresholds of .01 and .05 could also be considered not to be too conservative, depending on the level of certainty required for interpretation. Furthermore, it is important to emphasise that the number of individuals analysed was higher in the tofacitinib and HS groups, which may increase the likelihood of achieving statistical significance in these groups.

Effects of JAKis on prostanoids generation in LPS-stimulated WB of HS

Tofacitinib (1 μM) significantly increased both PGE₂ (48% ± 36%, *n* = 17) (Fig 3) and TXB₂ (42% ± 33%, *n* = 17) (Fig 4). Non-normalised data can be found in Supplementary Table S5 and S6 and Supplementary Figure S1. In addition, tofacitinib at 0.1 μM also significantly (*P*_{adj} < .05) increased PGE₂ (34% ± 28%, *n* = 15) (Fig 3) and TXB₂ (23% ± 15%, *n* = 15) (Fig 4). These concentrations can be obtained after dosing with the therapeutic dose of tofacitinib of 10 mg (Supplementary Table S1). Baricitinib significantly increased TXB₂ at 1 μM (30% ± 22%, *n* = 10) (Fig 4). Filgotinib at 1 μM significantly increased PGE₂ (33% ± 18%, *n* = 7) but not TXB₂ (Figs 3 and 4). Upadacitinib at 0.01 μM significantly enhanced PGE₂ but did not reach significance at higher concentrations. Upadacitinib at 1 μM showed a trend towards an increase in TXB₂ (46% ± 40%, *n* = 7) but did not reach significance using very stringent Bonferroni correction for multiple tests.

Effects of JAKis on prostanoid generation in LPS-stimulated WB of patients

The effect of JAKis on WB of patients stimulated with LPS was investigated. In patients with SLE, tofacitinib (1 mM) significantly increased both PGE₂ (89% ± 127%, *n* = 12) (Table 1) and TXB₂ (57% ± 39%, *n* = 12) (Table 2). In patients with axSpA, tofacitinib (1 mM) significantly increased PGE₂ (45% ± 53%, *n* = 15) (Table 1) and TXB₂ (31% ± 23%, *n* = 15) (Table 2). Furthermore, tofacitinib at 0.1 μM also significantly

increased PGE₂ (47% ± 66%, *n* = 12) (Table 1) and TXB₂ (28% ± 35%, *n* = 12) (Table 2) in SLE, but not in axSpA.

In SLE and axSpA groups, baricitinib significantly increased TXB₂ at 1 μM (24% ± 16%, *n* = 7, and 43% ± 31%, *n* = 14, respectively), but not PGE₂ (Tables 1 and 2). Upadacitinib at 1 μM showed a trend towards increased TXB₂ (43% ± 41%, *n* = 7). Filgotinib showed no significant effect on PGE₂ and TXB₂ in either patients with SLE or patients with axSpA.

Investigation of prevention strategies

Effects of ASA on serum TXB₂ of HS in the presence of JAKis in the WB clotting assay

We verified whether the antiplatelet agent ASA can inhibit serum TXB₂ generation in the presence of JAKis. As shown in Figure 5, ASA almost completely inhibited the production of serum TXB₂ (96% ± 2%) and was not significantly affected by the presence of JAKis.

Effects of NSAIDs and a selective mPGES-1 inhibitor on prostanoids generated by LPS-activated WB assay of HS in the absence or presence of JAKis

Inhibitors of the AA cascade were investigated. Figures 3 and 4 show that production of PGE₂ and TXB₂ was inhibited by the addition of diclofenac (Diclo), a nonselective NSAID, or NS-398, a selective COX-2 inhibitor, supporting that prostanoids originate from COX-2. A selective mPGES-1 inhibitor, compound 118 [34], reduced PGE₂ but not TXB₂ as expected (Figs. 3 and 4).

Table 2
The effect of JAKis on TXB₂ biosynthesis in LPS-stimulated WB from HS, SLE, and axSpA

JAKis	Concentration	HS ¹			SLE			AxSpA		
		n	Increase ²	P ³	n	Increase ²	P ³	n	Increase ²	P ³
Tofacitinib	1µM	n=17	42% ± 33%	<0.001	n=12	57% ± 39%	<0.01	n=15	31% ± 23%	<0.001
	0.1µM	n=15	23% ± 15%	<0.001	n=12	28% ± 35%	<0.01	n=14	13% ± 19%	ns
	0.01µM	n=15	5% ± 19%	ns	n=12	14% ± 21%	ns	n=11	5% ± 21%	ns
Baricitinib	1µM	n=10	30% ± 22%	<0.001	n=7	24% ± 16%	<0.01	n=14	43% ± 31%	<0.001
	0.1µM	n=9	19% ± 19%	ns	n=7	7% ± 12%	ns	n=13	12% ± 23%	ns
	0.01µM	n=9	17% ± 15%	<0.05	n=7	12% ± 15%	ns	n=11	13% ± 30%	ns
Filgotinib	1µM	n=7	21% ± 19%	ns	n=7	15% ± 19%	ns	n=7	39% ± 47%	ns
	0.1µM	n=7	21% ± 21%	ns	n=7	0.5% ± 23%	ns	n=6	7% ± 24%	ns
	0.01µM	n=7	19% ± 16%	<0.05	n=6	0.5% ± 31%	ns	n=6	9% ± 53%	ns
Upadacitinib	1µM	n=7	46% ± 40%	<0.05	n=7	43% ± 41%	<0.05	n=8	20% ± 31%	ns
	0.1µM	n=7	33% ± 32%	ns	n=7	18% ± 29%	ns	n=7	15% ± 21%	ns
	0.01µM	n=7	41% ± 65%	ns	n=7	15% ± 20%	ns	n=6	12% ± 24%	ns

axSpA, axial spondyloarthritis; ANOVA, analysis of variance; HS, healthy subject; JAKi, Janus kinase inhibitor; LPS, lipopolysaccharide; ns, nonsignificant; PG, prostaglandin; SLE, systemic lupus erythematosus; TX, thromboxane; WB, whole blood.

^aThe data for HSs have already been visualised in Figure 4 (using ANOVA) but are shown here for comparison with patient data, using paired *t* test to assess significance.

^bIncreased level of PGE₂ compared with the vehicle control. All vehicle solutions were 0.01% dimethyl sulfoxide. An increase>20% is highlighted in gray.

^cPaired *t* test. *P* < .05 is highlighted in bold. JAKi vs vehicle control. *P* < .01 is shaded in light orange and *P* < .001 is underlined. Compensating for multiple testing (36 comparisons) using Bonferroni correction corresponds to a cutoff of the nonadjusted *P* value at .0013 to maintain a significance level of .05. However, it should be noted that the Bonferroni correction was highly stringent, and the comparisons were not fully independent due to the inclusion of multiple concentrations of the same JAKi and the investigation of the same JAKi in 3 different cohorts. This suggests that unadjusted *P* values at thresholds of .01 and .05 could also be considered not to be too conservative, depending on the level of certainty required for interpretation. Furthermore, it is important to emphasise that the number of individuals analysed was higher in the tofacitinib and HS groups, which may increase the likelihood of achieving statistical significance in these groups.

Part of the TXB₂ production can derive from platelet COX-1 activity, as shown by the finding that the NS-398 inhibited PGE₂ more profoundly than TXB₂. A combination of the mPGES-1 inhibitor and tofacitinib at 1 µM normalised the production of PGE₂ caused by JAKis (Fig. 3).

ASA inhibited TXB₂ in a concentration-dependent manner and reached 45% inhibition at 100 µM, while the inhibition of PGE₂ was only significant at the highest concentration (100 µM) (Fig 6). These data suggest that ASA reduced the contribution of platelets to TXB₂ generation in LPS-stimulated WB. Coincubation with ASA (100 µM) attenuated the enhanced generation of the 2 prostanoids induced by 1 µM tofacitinib.

DISCUSSION

We hypothesised that tofacitinib and other JAKis increase the biosynthesis of TXA₂ and that this may contribute to the increased risk of cardiovascular events associated with their use. To address this hypothesis, we examined the effects of 4 JAKis—tofacitinib, baricitinib, filgotinib, and upadacitinib—on the generation of TXB₂ in WB that was allowed to clot for 1 hour at 37 °C (serum TXB₂). Under these experimental conditions, thrombin is produced, which causes the release of AA from platelet membrane phospholipids [18,25]. AA is then converted to TXA₂ by platelet COX-1, and TXB₂ is generated through nonenzymatic hydrolysis of TXA₂ [18,25]. TXB₂ is a validated marker of TXA₂ formation; it is stable and can be readily measured [18]. We found that the 4 JAKis increased the production of serum TXB₂. Serum TXB₂ is an easily

measurable and highly reproducible capacity index primarily linked to platelet COX-1 and TXA-synthase activity. It can be used to monitor drug-induced or disease-induced changes in these enzyme activities [35]. For instance, it has been shown that serum TXB₂ production is significantly reduced by 60% in uremic patients compared with that in age-matched and sex-matched controls. This reduction contributes to the impairment of platelet function observed in uraemia [30]. Additionally, measuring serum TXB₂ levels a reduced platelet synthesis of proaggregatory TXA₂ was detected in cirrhotic patients, which may help explain the bleeding tendency seen in cirrhosis [31]. Serum TXB₂ is derived exclusively from platelet TXA₂, serves as a direct indicator of the platelets’ ability to produce TXA₂, and clearly reflects pharmacologic effects of ASA on platelets [19]. Furthermore, in a prospective study involving 700 ASA-treated patients who underwent angiographic evaluation for coronary artery disease, residual platelet COX-1 function, as measured by serum TXB₂, was found to correlate with subsequent major adverse cardiovascular events [32]. The evidence for a central role of TXA₂ in thrombosis and vascular occlusive events has come from the findings that TXA₂ biosynthesis is enhanced in cardiovascular diseases and that low-dose ASA, which reduces TXA₂ biosynthesis, is associated with a reduced risk [23]. The saturation of the clinical response at low doses of ASA correlates with the maximal inhibition of platelet TXA₂ biosynthesis (serum TXB₂) [19,36].

Although the effect of the JAKis was not clearly concentration dependent, we detected higher average levels of serum TXB₂ with all JAKis. However, upadacitinib was associated with

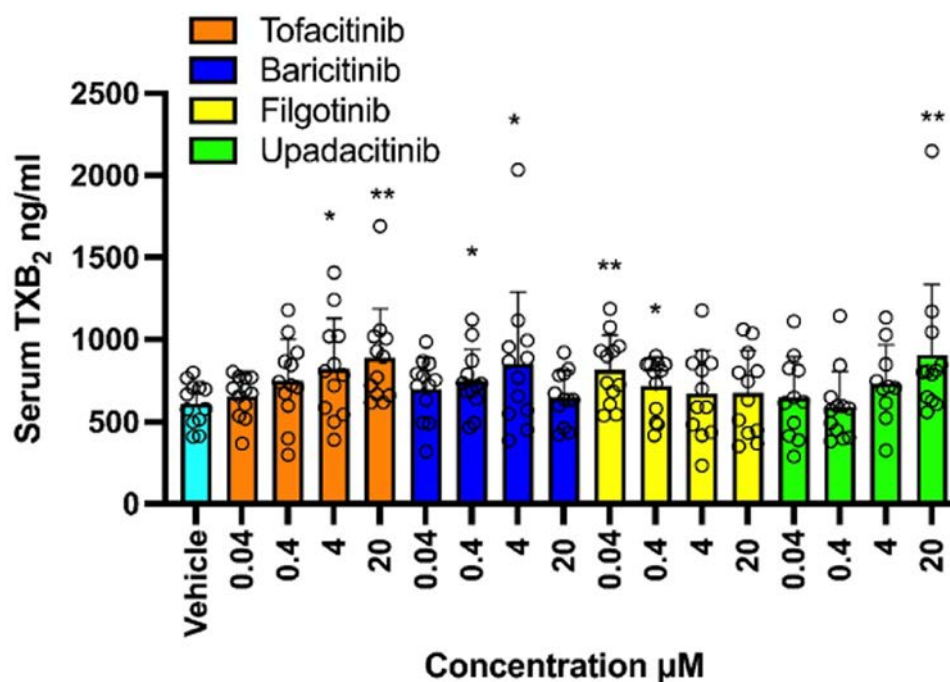


Figure 1. Effects of JAKis on the generation of TXB₂ using the WB clotting assay. Tofacitinib, baricitinib, filgotinib, and upadacitinib (dissolved in phosphate-buffered saline [10% dimethyl sulfoxide, final concentrations 0.04–20 mM]) or their vehicles were added directly into glass tubes. One-millilitre aliquots of WB drawn from HSs were immediately transferred into glass tubes and allowed to clot at 37 °C for 1 hour. Serum was separated by centrifugation (10 minutes at 1560g at 4 °C) and kept at –80 °C until assayed for TXB₂. Values (ng/mL of serum) are mean ± SD (n = 12). *P < .05, **P < .01 vs vehicle using one-way ANOVA, followed by the Dunnett post hoc test (GraphPad Prism software, version Prism 10 for macOS). ANOVA, analysis of variance; HS, healthy subject; JAKi, Janus kinase inhibitor; TX, thromboxane; WB, whole blood.

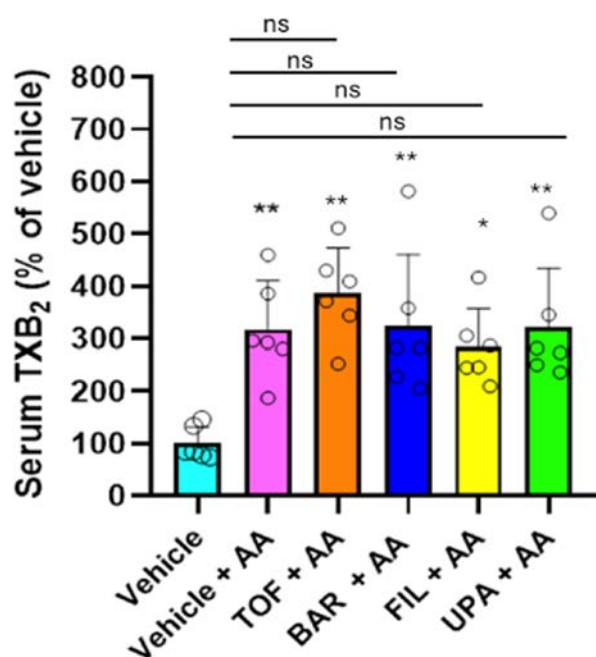


Figure 2. Effect of exogenous AA on serum TXB₂ generation in the absence and presence of JAKis. Tofacitinib (TOF), baricitinib (BAR), filgotinib (FIL), and upadacitinib (UPA; dissolved in phosphate-buffered saline, and 10% dimethyl sulfoxide, final concentration 4 µM) and AA (dissolved in saline solution at the final concentration of 20 µM) or their vehicles were added directly into glass tubes. One-millilitre aliquots of WB drawn from HSs were immediately transferred into glass tubes and allowed to clot at 37 °C for 1 hour. Serum was separated by centrifugation (10 minutes at 1560g at 4 °C) and stored at –80 °C until assayed for TXB₂. The results are depicted as serum TXB₂ (% of control, ie, vehicle), mean ± SD (n = 6), and individual values. Addition of AA resulted in a significant increase in serum TXB₂ compared with that in vehicle control (*P < .001 by one-way ANOVA and multiple comparisons with Tukey

a statistically significant increase in serum TXB₂ only at the supratherapeutic concentration of 20 µM. The most plausible explanation for the increase in TXB₂ is that the JAKis enhance the availability of free AA for the platelet COX-1 and thereby increase serum TXB₂ levels. As expected, the addition of exogenous AA increased serum TXB₂ levels, indicating the system's responsiveness to higher concentrations of the COX substrate. The 4 JAKis did not further increase serum TXB₂ levels in response to exogenous AA. This suggests that the effect of JAK inhibitors on TXB₂ production is probably not downstream of AA and supports the hypothesis that JAK inhibitors promote the release of AA from platelet phospholipids. JAKis may cause off-target effects via enhanced oxidative stress [37], thus contributing to AA release from platelet membrane phospholipids. To address this hypothesis, we verified whether the JAKis promoted the increased generation of 8-iso-PGF_{2α} in blood allowed to clot at 37 °C. Moreover, 8-iso-PGF_{2α} is an abundant F₂-isoprostane formed *in vivo* nonenzymatically through a free radical catalysed attack on esterified arachidonate and provides a reliable tool for detecting enhanced rates of lipid peroxidation [28]. However, in this study, we were only able to analyse a limited number of serum samples that remained after the assessment of TXB₂ levels. A comprehensive assessment of F₂-isoprostanes can be undertaken in a specific study focused on elucidating the molecular mechanisms driving the increased production of prostanoids by JAKis. The preliminary data show that JAKis enhanced serum 8-iso-PGF_{2α} levels, supporting our hypothesis that these drugs, as a class, caused an off-target effect by

test). The 4 JAKis did not further increase serum TXB₂ in response to exogenous AA (ns = no significant difference between vehicle + AA and TOF + AA, BAR + AA, FIL + AA and UPA + AA). AA, arachidonic acid; ANOVA, analysis of variance; HS, healthy subject; JAKi, Janus kinase inhibitor; TX, thromboxane; WB, whole blood.

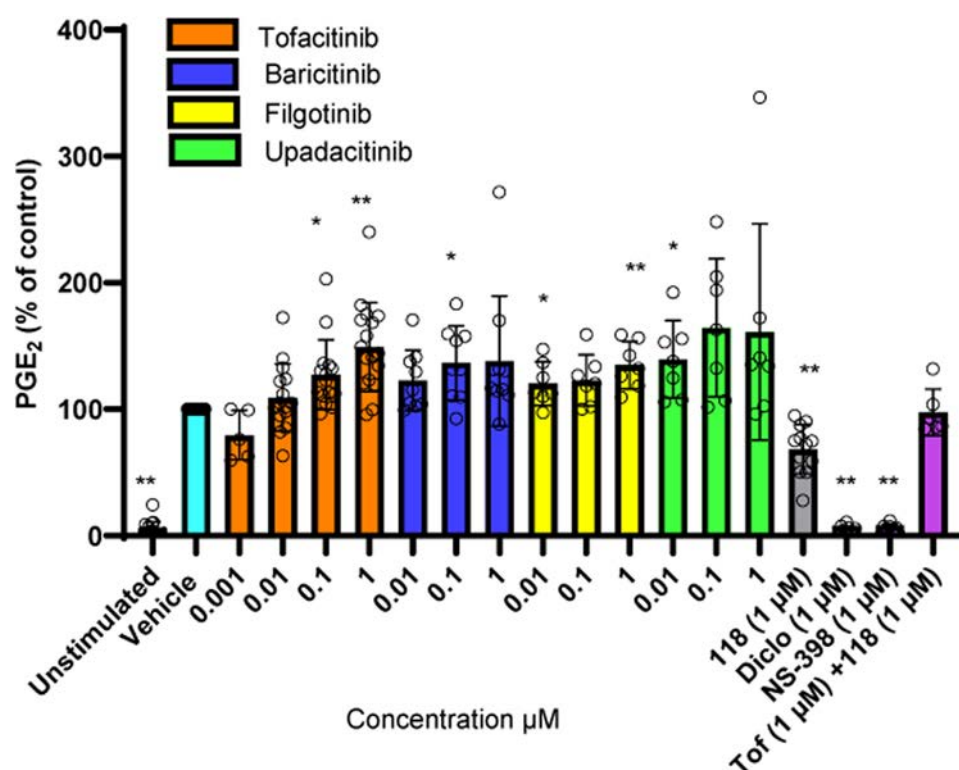


Figure 3. Effect of JAKis on PGE₂ biosynthesis in the LPS WB assay with blood from HS. Different concentrations of tofacitinib (Tof) (n = 15–17), baricitinib (Bar) (n = 9–10), filgotinib (Fil) (n = 7), upadacitinib (Upa) (n = 7) or vehicle (0.01% dimethyl sulfoxide in phosphate-buffered saline) were used. Unstimulated conditions were investigated without the addition of LPS. Diclofenac (Diclo), a nonselective nonsteroidal anti-inflammatory drug; NS-398, a selective COX-2 inhibitor; and 118, an mPGES-1 inhibitor. The data were normalised to the vehicle control. **P* < .05, ***P* < .01 vs vehicle, using one-way ANOVA on non-normalized data, followed by the Dunnett post hoc test (GraphPad Prism software, version Prism 10 for Windows). The nonnormalised data can be found in [Supplementary Table S5](#). ANOVA, analysis of variance; HS, healthy subject; JAKi, Janus kinase inhibitor; LPS, lipopolysaccharide; PG, prostaglandin; TX, thromboxane; WB, whole blood.

inducing oxidative stress ([Supplementary Fig S2](#)). It is proposed that the JAKis induce oxidative stress at low or clinical concentrations through an off-target effect and that, at higher concentrations, other signalling pathways can inhibit reactive oxygen species (ROS) formation. This could explain why the increased TXB₂ production by the JAKi did not follow a concentration-response relationship but instead exhibited a Gaussian model (a bell-shaped curve). In addition to triggering TXA₂ generation, the role of ROS in platelet function has recently emerged. They regulate platelet activation, aggregation, and recruitment, fine-tuning several cellular signalling pathways [38]. Excessive ROS can increase TXA₂ production by potentiating PLCγ2/PKC/p38α MAPK signalling, thus providing more AA substrate for COX-1 and in line more substrate (PGH₂) for the TXA synthase to produce more TXA₂, leading to platelet hyperactivity [39]. Oxidative stress can also lead to enhanced surface expression of the TXA₂ receptor, which contributes to enhanced platelet activation in response to TXA₂ [39].

It is noteworthy that JAKis did not affect the capacity of ASA (100 μM) to almost completely inhibit serum TXB₂ production in clotting WB, a necessary action to translate into an antiplatelet effect *in vivo* [18]. Our findings suggest that coadministration of low-dose ASA could counteract the increase of platelet TXA₂ and, therefore, mitigate the risk of thromboembolic events caused by JAKis.

LPS-stimulated WB was used to evaluate the effect of JAKis on the biosynthesis of both PGE₂ and TXB₂ during an inflammatory response triggered by LPS in WB from HSs and patients with SLE or axSpA. Under these experimental conditions, leucocytes generate mainly PGE₂ in response to the induction of COX-2/mPGES-1 by LPS stimulation [24]. However, a significant

amount of TXB₂ can also be produced by platelet COX-1/TXA₂ synthase [24]. Tofacitinib and baricitinib significantly increased both PGE₂ and TXB₂ in HSs. Filgotinib and upadacitinib significantly increased PGE₂, while only a trend towards increased TXB₂ was observed. This effect potentially mitigates these drugs' anti-inflammatory benefits, and it is crucial to note that the enhanced TXA₂ biosynthesis during inflammation is a hallmark of increased cardiovascular risk [40].

In patients with SLE, all JAKis, except for filgotinib, increased LPS-stimulated WB TXB₂ production at a concentration of 1 μM. The concentrations used in this study are within the range of the average maximum concentration (C_{max}) observed after therapeutic doses for all JAKis, except for filgotinib, for which a C_{max} of 5 μM is reported following a 200 mg/day dose ([Supplementary Table S1](#)).

Notably, filgotinib was associated with enhanced serum TXB₂ levels at 0.04 and 0.4 μM. A limitation of this study is that a smaller number of individuals were evaluated for filgotinib and upadacitinib in the LPS-activated WB assay compared with those for tofacitinib. However, assessing prostanoids generated in LPS-stimulated WB was a secondary end point. Serum TXB₂, which was a primary end point, was assessed with a comparable sample size for all JAKis. It is also important to note that this study was conducted *in vitro*, and its translational relevance requires validation through *in vivo* studies. Furthermore, after *in vivo* confirmation of the ability of JAKis to enhance platelet TXA₂ generation, their use should be approached with caution in patients carrying the JAK2V617F mutation.

Since we propose that the mechanism of enhanced platelet TXA₂ generation involves oxidative stress resulting from JAK inhibitors, we predict that the response might be greater in older

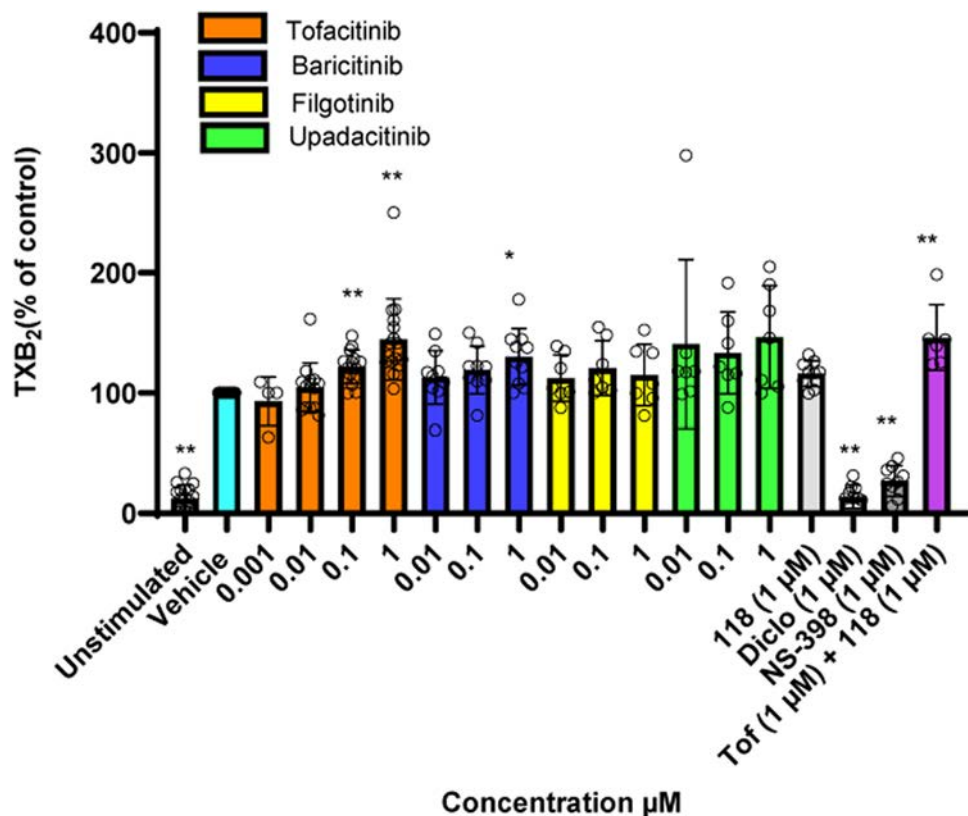


Figure 4. Effect of JAKis on TXB₂ biosynthesis in the LPS-activated WB assay. Different concentrations of tofacitinib (Tof) (n = 15-17), baricitinib (Bar) (n = 9-10), filgotinib (Fil) (n = 7), upadacitinib (Upa; n = 7), or vehicle (phosphate-buffered saline/dimethyl sulfoxide) were used. Unstimulated conditions were investigated without the addition of LPS. Diclofenac (Diclo), a nonselective NSAID, NS-398, a selective COX-2 inhibitor, and 118, an mPGES-1 inhibitor, were investigated at a concentration of 1 μM. The data were normalised to the vehicle control. **P* < .05, ***P* < .01 vs vehicle, using one-way ANOVA on non-normalized data, followed by the Dunnett post hoc test (GraphPad Prism software, version Prism 10 for Windows). The nonnormalised data can be found in [Supplementary Table S6](#) and [Supplementary Figure S2](#). ANOVA, analysis of variance; JAKi, Janus kinase inhibitor; LPS, lipopolysaccharide; TX, thromboxane; WB, whole blood.

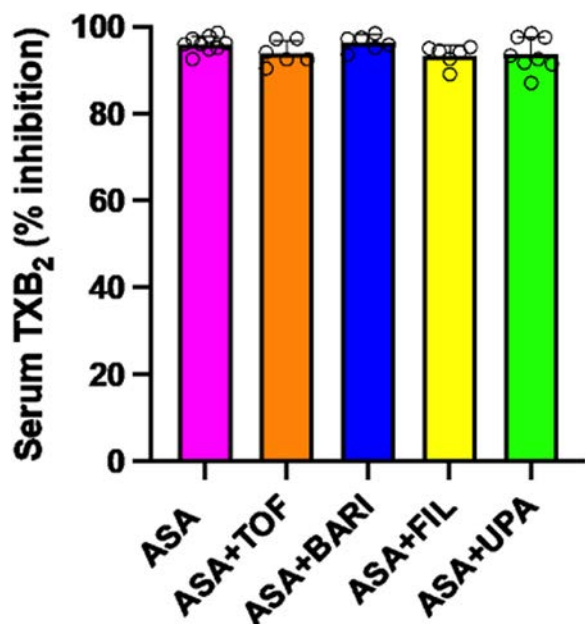


Figure 5. Aspirin profoundly affected serum TXB₂ generation in the absence and presence of JAKis. Tofacitinib (TOF), baricitinib (BARI), filgotinib (FIL), and upadacitinib (UPA) (dissolved in phosphate-buffered saline and 10% dimethyl sulfoxide [DMSO], final concentration 4 mM) and aspirin (acetylsalicylic acid [ASA] dissolved in DMSO at a final concentration of 100 μM) or vehicles were added directly into glass tubes. One-millilitre aliquots of WB drawn from HSs were immediately transferred into glass tubes and allowed to clot at 37 °C for 1 hour. Serum was separated by centrifugation (10 minutes at 1560g at 4 °C) and kept

populations or those with a history of cardiovascular risk. A specific study needs to be conducted to investigate this hypothesis. We show that enhanced platelet TXB₂ generation by JAKis can be prevented by the coinubation with ASA. The antiplatelet agent ASA is recommended for secondary prevention of atherothrombotic events in most patients with established atherosclerotic cardiovascular disease [41]. It exerts its antithrombotic effect by inhibiting platelet TXA₂ via the irreversible inhibition of the COX-1 enzyme [18,22,42]. ASA can raise the risk of bleeding. While its benefits for secondary prevention of cardiovascular disease outweigh these risks, the use of low-dose ASA for primary prevention is still controversial [43]. The challenge in assessing Asa's net benefits stems from the strong correlation between its benefits and risks, complicating the identification of individuals who are at high risk for vascular ischaemia but have a low risk of bleeding. New strategies, such as using coronary imaging to identify high-risk individuals and providing gastroprotective therapy, may help address this issue [43].

In conclusion, the enhanced biosynthesis of TXA₂ from platelets and leukocytes may increase the risk of atherothrombosis and venous thromboembolism associated with JAKis. This effect is

at –80 °C until assayed for TXB₂. Results are presented as serum TXB₂ (% inhibition of baseline vehicle values), mean ± SD (n = 6-9), and individual values are shown. There was no significant difference between ASA and ASA + JAKi (*P* > .05) using one-way ANOVA and multiple comparisons with the Tukey test (GraphPad Prism software version Prism 10 for macOS). ANOVA, analysis of variance; HS, healthy subject; JAKi, Janus kinase inhibitor; TX, thromboxane; WB, whole blood.

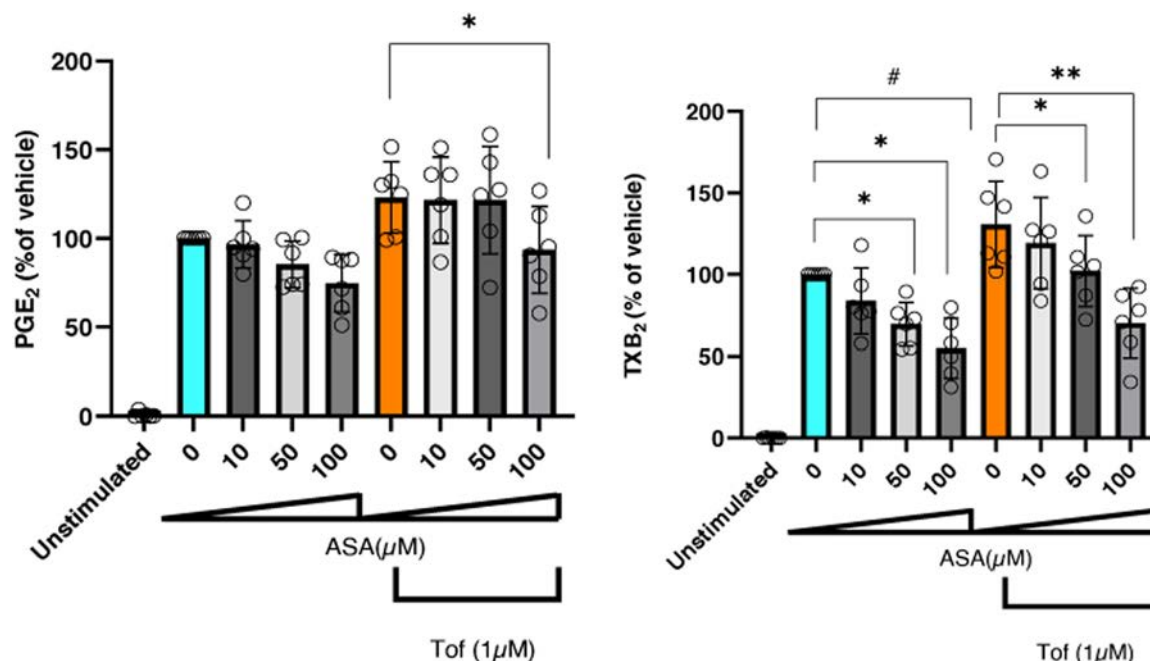


Figure 6. Aspirin inhibits the biosynthesis of PGE₂ and TXB₂ in LPS-stimulated WB *in vitro*. The effect of aspirin (acetylsalicylic acid [ASA]) was investigated in HSs (n = 6) to study its ability to inhibit the production of PGE₂ (left panel) and TXB₂ (right panel). ASA significantly reduced the production of prostanoids in a concentration-dependent manner in both the presence and the absence of tofacitinib 1 μM (**P* < .05, ***P* < .01; paired *t* test compared with 0 μM ASA). Tofacitinib 1 μM significantly increased TXB₂ (#*P* < .05, paired *t* test compared with vehicle). HS, healthy subject; JAKi, Janus kinase inhibitor; LPS, lipopolysaccharide; PG, prostaglandin; TX, thromboxane; WB, whole blood.

particularly pronounced with tofacitinib; however, all 4 JAKis examined influenced TXA₂ production. The findings suggest that combining JAKis with low-dose ASA may reduce cardiovascular side effects. To validate this hypothesis, it is crucial to conduct registry-based epidemiologic studies or randomised clinical trials involving patients with inflammatory diseases who need JAKis for treatment. If the risk of cardiovascular side effects can be effectively lowered, more patients may benefit from JAK inhibitor therapy.

Competing interests

Part of this work was conducted on behalf of the Aspirin for Cancer Prevention Group, Wolfson Institute of Preventive Medicine, Queen Mary School of Medicine and Dentistry, University of London (UK) to PP.

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Authors contributions

P-JJ, PP, and HI contributed to the ideas, formulation, or development. SA, HI, PP, and P-JJ drafted the manuscript. SA and HI set up the analysis of LPS-WB assay. HI was involved in the development of the methodology and the creation of models. SA performed experiments and analysed the data for HSs and patients in the LPS-activated WB. ST, ADM, and PDG performed WB experiments on clotting WB. PP performed the statistical analysis for the clotting WB. MV helped with patient samples with axial spondyloarthritis (axSpa). IV helped with patients with SLE at Karolinska University Hospital. VO and MK helped

to collect samples from patients with axSpa at CFR. All authors have revised and approved the manuscript.

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Patients consent for publication

Not applicable.

Ethics approval

The Regional Ethical Review Board in Stockholm: Dnr: u2015/2001–32/2 regarding healthy individuals and Dnr: 2022-01789-01 regarding patients with axSpA. The Regional Ethical Review Board in Italy: Approval no. 16, 2019, regarding samples from healthy individuals in Italy

Provenance and peer review

Not commissioned; externally peer reviewed.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used ChatGPT4 developed by OpenAI, in order to improve language and grammar. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Patients and public involvement

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

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ard.2025.03.014.

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Epidemiology

Antirheumatic drugs in reproduction, pregnancy, and lactation: a systematic literature review informing the 2024 update of the EULAR recommendations

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ARTICLE INFO

ABSTRACT

Objectives: This study aimed to summarise and update evidence to inform the 2024 update of the European Alliance of Associations for Rheumatology recommendations for the use of antirheumatic drugs in reproduction, pregnancy, and lactation.

Methods: A systematic literature review (SLR) was performed, including keywords on reproduction, adverse pregnancy outcomes (APOs), and lactation. Two appraised SLRs were the basis for the SLR on drug safety in men. If sufficient data were available, a meta-analysis was performed on maternal drug exposure and the risk of APOs.

Results: Of 6680 screened articles, 255 were included in the final analysis. In pregnancy, most evidence was available for biologic disease-modifying antirheumatic drugs (bDMARDs). Meta-analyses with adjusted risk estimates did not reveal APOs or serious infant infections to be associated with tumour necrosis factor inhibitor (TNFi) use. Data on non-TNFi bDMARDs did not raise concerns. In bDMARD-exposed infants, no serious adverse effects to rotavirus live

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vaccination were reported. Safety of Bacille Calmette–Guérin vaccination in TNFi-exposed infants could be a concern in the first 6 months of life. Regarding oral glucocorticoids, the SLR and meta-analysis using adjusted risk estimates found a dose-dependent association with an increased risk of preterm birth. Nonsteroidal anti-inflammatory drug use could reversibly reduce fecundability. Concerning lactation, available data on various bDMARDs was reassuring. In male patients, available evidence on methotrexate and most other drugs did not reveal adverse effects on sperm quality or birth outcomes. Cyclophosphamide remains the only drug that causes a dose-dependent irreversible infertility.

Conclusions: This SLR provides up-to-date evidence to guide the 2024 update of the European Alliance of Associations for Rheumatology recommendations for the use of antirheumatic drugs in reproduction, pregnancy, and lactation.

WHAT IS ALREADY KNOWN ON THIS TOPIC

- The safety of antirheumatic drugs (ARDs) is of important relevance for patients with rheumatic and musculoskeletal disorders who are planning to start a family. A reevaluation of the literature was necessary since new studies and treatment options are available since the publication of the 2016 guidelines.

WHAT THIS STUDY ADDS

- This systematic literature review (SLR) provides the following results:
 - An update of the 2016 SLR with regard to the effect of ARDs on miscarriage and congenital malformations.
 - An update of the 2016 SLR regarding lactating women and the effect of ARDs on adverse outcomes of the child.
 - A new search focusing on the effect of ARDs on adverse pregnancy outcomes (APOs) (eg, stillbirth, preterm birth, low birth weight, and small-for-gestational age born infants), maternal outcomes (eg, gestational diabetes), and infant outcomes (eg, serious infant infections after *in utero* exposure).
 - An additional search on the effect of ARDs on reproductive safety in male patients.
 - A new search on the effect of nonsteroidal anti-inflammatory drugs on fertility and fetal health issues.
 - Meta-analyses were performed to investigate the risk of different APOs related to tumour necrosis factor inhibitor treatment during pregnancy and the risk of preterm birth associated with glucocorticoid use during pregnancy.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- This SLR informed the European Alliance of Associations for Rheumatology task force of the 2024 update on ARDs in reproduction, pregnancy, and lactation including all available evidence.

INTRODUCTION

Rheumatic and musculoskeletal disorders (RMDs) encompass a diverse group of medical conditions, which often require the continuous use of antirheumatic drugs (ARDs) to manage symptoms and prevent disease progression. The safety of these medications for women planning a pregnancy and during pregnancy and breastfeeding, and for men, who want to father a child, remains a major concern for both clinicians and patients. Conversely, active inflammatory RMDs are known for being a relevant risk factor for fertility and adverse pregnancy outcomes (APOs) [1–4].

The first European Alliance of Associations for Rheumatology (EULAR) points to consider (PtC) for the use of ARDs before pregnancy, and during pregnancy and lactation published in 2016, gave guidance for clinicians and healthcare professionals [4]. Since then, a growing body of literature has emerged, and new treatment options have entered the market, necessitating a reevaluation of the available evidence.

Therefore, the objective of this systematic literature review (SLR) was to update the 2016 SLR on the safety of ARDs in pregnant and breastfeeding women with RMDs reflecting the most recent scientific literature. Moreover, this SLR includes additional relevant pregnancy outcomes, such as stillbirth, preterm birth (PTB), neonates born small-for-gestational age (SGA) or of low birth weight (LBW), and serious infant infections (SII). The review also investigated drug safety for male patients planning a family. A further objective was to investigate the association between maternal drug exposure and the risk of APOs in a meta-analysis if sufficient data were available. Results of this SLR served as a basis to inform the EULAR Task Force (TF) on ARDs in pregnancy and lactation. Of note, this SLR and the update of the 2016 EULAR PtC form an integral and should be read as such.

METHODS

For the update of the 2016 EULAR SLR, we followed the standardised operating procedures for EULAR-endorsed recommendations [5]. The Cochrane Handbook was used to conduct this SLR [6], and for the reporting, we adhered to the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) guidelines [7]. Review questions were developed by the TF steering group (FF, AF, and YM) and fellows (SH, AP, and LR) following the population–intervention–outcome (PIO) framework (details in the [Supplementary Material](#)):

- (1) In women planning a pregnancy or currently pregnant (P) with an indication for the given drug (I), what is the effect of the drug on congenital malformations and miscarriages (O)?
- (2) In lactating women (P) with an indication for the given drug (I), what is the effect of the drug on any adverse child's outcomes (O)?
- (3) In pregnant women with an indication for the given drug (P), what is the effect of the drug (I) on adverse pregnancy and maternal outcomes and adverse infant outcomes (O)?
- (4) In male patients with an indication for the given drug trying to conceive or with periconceptional drug exposure (P), what is the effect of the drug (I) on fertility issues and adverse pregnancy outcomes (O)?
- (5) In pregnant women using nonsteroidal anti-inflammatory

drugs (NSAIDs) (P), what is the effect of NSAIDs (I) on fetal health issues (O)?

- (6) In women on NSAIDs planning to conceive (P), what is the effect of continuing NSAIDs(I) on fertility issues (O)?

PIO 1 and 2 questions represent the update of the earlier EULAR SLR and encompass a search period starting from April 1, 2015, until April 30, 2023 (in the case of included abstracts, available published full-text articles were considered for data extraction). Additional searches were performed for research questions addressing new populations and outcomes (PIO 3–6). The search was not limited to RMDs, but to all indications for the given drug. Search starting point was from January 1, 2000 (era of broader treatment option), for PIO 3, 5, and 6. The search for PIO 4 started from September 1, 2019, as 2 SLRs were used as the base for PIO 4. Included SLRs were critically reviewed and appraised according to the AMSTAR2 guidelines [8] ([Supplementary Table S1](#)). At the first TF meeting, all members approved the review questions. The detailed SLR protocol was published in PROSPERO (CRD42022357689). No comparisons between ARDs on the review questions were requested to obtain broader search results.

Search strategy and eligibility criteria

An experienced librarian (TR) translated the main elements of the research questions into search terms by using controlled vocabulary and free text, and the initial search strategy was supported by analysing key studies. The search was conducted in the databases MEDLINE ALL (Ovid), Embase (Elsevier) and Cochrane Library (Wiley). The detailed search strategy for each database is provided in the supplement. As further information sources, abstracts from the EULAR and the American College of Rheumatology (ACR) congresses of the years 2022 and 2023 were screened, and reference lists of included studies and LactMed database were handsearched.

Studies were eligible for inclusion if they reported (I) the population of adult female or male patients with an indication for a drug defined by 1 of the PIO questions who planned to conceive (exposure time before conception < 5 half-lives) or with periconceptional drug exposure, or (II) the population of pregnant or lactating/breastfeeding women with an indication for a drug defined by PIO 2, and if they reported at least 1 outcome from the 6 PIO questions in at least 1 of the investigated treatment exposure groups. The SLR also included studies of newborns/infants with *in utero* exposure to 1 of the drugs listed for PIO 3 and reporting of the infant's outcome during the first year of life. Eligible study types comprised original prospective and retrospective studies including randomised or non-randomised studies, observational cohorts, case-control or cross-sectional studies, case series or reports, short reports, or letters including original data. Publications in English, Spanish, French, or German language were included. Exclusion criteria for the SLR search comprised animal studies, *in vitro* studies, editorials, literature reviews, non-peer-reviewed publications, publications describing cancer chemotherapy, and publications in other than the above-described languages.

Selection of studies, data extraction, and assessment of risk of bias

Screening and study selection of search results were performed by using the web-based SLR tool Rayyan (<https://www.rayyan.ai/>). Before the formal screening, a pilot was performed. Two

reviewers each (FF, SH, AP, LR, LFP-G, and IC) independently screened a subset (20%) of titles and abstracts by applying inclusion and exclusion criteria. Agreement between reviewers was sufficient (>80%), and consequently remaining results were screened by 1 reviewer. The same procedure was applied for full-text screening. Any discrepancies were resolved through discussions between reviewers or by involving the convenor and/or methodologists. Data were extracted into a standardised excel spreadsheet with a pilot extraction to ensure consistency across reviewers. Risk of bias and study quality of individual studies was assessed by the risk-of-bias tool for randomised trials (RoB2), the Newcastle–Ottawa Scale for observational studies, and JBI appraisal for case reports/case series [9–11]. The details on quality scoring of each study are provided in [Supplementary Table S2](#). For each drug, the level of evidence was assigned according to the evidence retrieved in the SLR of EULAR PtC 2016, the present SLR, and other SLRs when relevant [4,5,12].

Meta-analysis

A meta-analysis was conducted as part of this SLR, with sufficient data available to investigate 2 key topics: the risk of APOs (miscarriages, congenital malformations, stillbirth, PTB, LBW, SGA, and SII) associated with tumour necrosis factor inhibitor (TNFi) treatment versus no TNFi treatment during pregnancy, and the risk of PTB associated with glucocorticoid (GC) use versus no GC use during pregnancy. The meta-analysis used 2 different approaches to estimate the risks: model (a) calculated pooled unadjusted odds ratios (ORs) for exposed versus unexposed groups per outcome, while model (b) pooled risk estimates adjusted for confounding factors for exposed versus unexposed groups. Methodologic details are provided in the [Supplementary Material](#).

RESULTS

The combined search yielded 6680 results, of which 255 were eligible for inclusion ([Supplementary Fig S1](#)). The study quality overall was classified as good for 30% of studies, fair for 8%, and poor for 62%. Information on study quality for individual studies and by drug are provided in [Supplementary Figs S2 and S3](#) and [Supplementary Table S2](#). Notably, the number of published studies on drug exposure during pregnancy and associated APOs has increased over the past 2 decades, with a growing number of prospective cohort and national registry studies ([Supplementary Fig S4](#)). Of the included studies, 37 were incorporated in at least 1 of the meta-analyses ([Supplementary Tables S3 and S4](#)).

Effects of ARDs on congenital malformation, miscarriage, and other APOs

An overview of the crude results of the PIO questions 1 and 3 (only maternal/pregnancy outcomes) are provided in [Table 1](#).

Glucocorticoids

Previously, EULAR recommended that GC could be considered for use in pregnancy if needed to control active disease symptoms. This recommendation was based on 24 studies (including 17 case reports/series) [4]. Since then, we identified additional evidence from 19 studies (including 8439 pregnancies). No increased risk of congenital malformation was found in adjusted analyses [13,14]. Rates of LBW and PTB were notably high at 22.2% and 17.9%, respectively. Our meta-analysis based on adjusted risk estimates from 11 cohorts confirmed an association between oral GC use and PTB (pooled adjusted odds ratio

Table 1

Outcomes of pregnancy exposure to antirheumatic drugs: summary of results stratified by drug

Drug/EULAR SLR	No. of studies per study type [Ref] ^a	Total exposed pregnancies (per time points); total live births ^b	Pregnancy loss (per total pregnancies): MC, ^c SB, ND, ET	Pregnancy outcomes (per total pregnancies): PTB, LBW, SGA	CM ^c (per total live birth) ^d	Maternal outcomes: SMI, GH, GD	Comments ^e	Evidence: LoE ^f and study quality ^g
Glucocorticoids		11,717 ^h						
EULAR 2016	24 [s216]	3500	MC: 70/331		CM: 34/3180			
EULAR update 2024:	17 CS [s1–s17]	8439 Preg	MC: 103/1094	PTB: 931/5212	CM: 89/1457	SMI: NA	No excess risk of CM, MC, and SGA compared with unexposed controls in studies adjusted for confounders.	2b
prednisone/prednisolone,	1 CCS [s18]	(PRE ≥349; T1 ≥1200;	SB: 13/569	LBW: 152/685		GH: no increased prevalence		8 good
methylprednisolone pulse (IV)	1 CSE [s19]	T2 ≥1301; T3 ≥774)	ND: 2/258	SGA: 106/2049		GD: NA	Higher risk of PTB and LBW compared with nonexposed controls was confirmed by several studies with adjustment for disease severity/activity. Higher risk of PTB for both, early and late, glucocorticoid exposures. Lowest risk of PTB for low prednisolone doses (average daily dose < 5.0 mg/d)	7 fair 4 poor
		4454 LB	ET: 25/380					
NSAIDs		157,561 ^h						
Nonselective NSAIDs		17,992	MC: 530/5609		CM: 457/12,354			
EULAR 2016	6 [s216]	145,889 Preg	MC: 152/1740	PTB: 1504/21,234	CM: 4754/118,954	SMI: NA	No excess risk of CM and MC compared with nonexposed controls in studies with adjustment for confounders	2a
EULAR update 2024	11 CS [s4,s8,s20–s28] 1 CSE [s29]	(PRE ≥133; T1 ≥130,627; T2 ≥3317; T3 ≥1564)	SB: 61/12,551 ND: 27/11,053 ET: 132/1631	LBW: 7581/141,333 SGA: 7/125		GH: no increased prevalence GD: no increased prevalence		7 good 5 poor
		14,663 LB						
Selective COX-2 inhibitors		1038	MC: 11/71		CM: 9/114			
EULAR 2016	3 [s216]	215	MC: 16/174	PTB: 15/139	CM: 40/726	SMI: NA	No excess risk of CM and MC compared with unexposed controls in the prospective cohort study with adjustment for confounders	2b
EULAR update 2024	2 CS [s25,s30]	823 Preg (PRE NA; T1 823; T2 ≥7; T3 ≥3)	SB: 0/174 ND: NA ET: 19/174	LBW: 47/649 SGA: NA		GH: NA GD: NA		1 good 1 poor
		139 LB						
csDMARDs, immunosuppressives, and other drugs		5823 ^h						
Antimalarials (HCQ, CQ)		492	MC: 20/170		CM: 23/492			
EULAR 2016	6 [s216]	5331 Preg	MC: 99/889	PTB: 437/3023 ⁱ	CM: 204/4009	SMI: NA	Most data available for HCQ. No study reported a significantly higher risk of APO, except for 1 study based on claims data, which reported aRR of 1.33 (95% CI: 1.08, 1.65) for CM with HCQ ≥ 400.0 mg/d. No CM pattern was observed. A subsequent prospective cohort study did not confirm this risk (OR: 1.08, 95% CI: 0.51, 2.29)	2a (HCQ)
EULAR update 2024	20 CS [s10,s31–s49]	(exposure: NA)	SB: 15/858 ND: 1/161 ET: 7/549	LBW: 110/771 ⁱ SGA: 226/1709		GH: no increased prevalence GD: no increased prevalence		2c (CQ) 16 good 3 fair 1 poor
		854 LB						
Azathioprine/6-mercaptopurine		5619 ^h						
EULAR 2016	18 [s216]	1327	MC: 40/559		CM: 65/1327			
EULAR update 2024	14 CS [s7,s10,s18,s33,s48,s50–s58] 4 CCS [s59–s62] 1 CSS [s63]	4985 Preg (exposure NA) 3597 LB	MC: 69/698 SB: 6/313 ND: 2/308 ET: 3/197	PTB: 781/4957 ⁱ LBW: 137/1099 ^j SGA: 360/4416	CM: 69/1453	SMI: NA GH: no increased prevalence GD: no increased prevalence	Most studies reported no increased risk of APO, except for a slight increase in prematurity in a single nationwide cohort study of children born to women with Crohn disease, confounded by disease severity.	2a 10 good 2 fair 7 poor
Bosentan		1						
EULAR 2016	—	1 Preg	MC: 0/1	PTB: 1/1	CM: 0/1	SMI: NA	Too limited data to draw conclusions	4
EULAR update 2024	1 CR [s64]	(PRE NA; T1 1; T2 1; T3 0) 1 LB	SB: 0/1 ND: 0/1 ET: 0/1	LBW: 1/1 SGA: NA		GH: NA GD: NA		1 poor

(continued on next page)

Table 1 (Continued)

Drug/EULAR SLR	No. of studies per study type [Ref] ^a	Total exposed pregnancies (per time points); total live births ^b	Pregnancy loss (per total pregnancies): MC, ^c SB, ND, ET	Pregnancy outcomes (per total pregnancies): PTB, LBW, SGA	CM ^c (per total live birth) ^d	Maternal outcomes: SMI, GH, GD	Comments ^e	Evidence: LoE ^f and study quality ^g
Colchicine		564 ^h						
EULAR 2016	3 [s216]	460	MC: 30/417		CM: 11/460			
EULAR update 2024	2 CS [s65,s66] 1 CSS [s67]	342 Preg (exposure NA) 217 LB	MC: 25/300 SB: 0/300 ND: NA ET: NA	PTB: 44/276 ⁱ LBW: NA SGA: NA	CM: 12/321	SMI: NA GH: NA GD: NA	No adverse drug-related effect on pregnancy outcomes compared with unexposed controls.	2b 1 good 2 fair
Cyclophosphamide		433						
EULAR 2016	30 [s216]	276	MC: —		CM: 23/86			
EULAR update 2024	4 CS [s10,s14,s18, s68] 3 CR/CSE [s9,s69,s70]	157 Preg (PRE ≥146; T1 ≥1; T2 ≥3; T3 ≥2) 103 LB	MC: 35/151 SB: 7/114 ND: 1/94 ET: 8/61	PTB: 38/136 LBW: 4/8 SGA: 2/8	CM: 0/28	SMI: NA GH: NA GD: NA	Known teratogenic and embryotoxic risk if exposure occurs in the first trimester, which explains the high rate of MC. Few data of exposed patients vs unexposed controls, no studies with adjusted risk analyses available.	2b 2 fair 5 poor
Cyclosporine		1163 ^h						
EULAR 2016	14 [s216]	1126	MC: 137/953		CM: 9/261			
EULAR update 2024	1 CS [s71] 1 CSE [s72]	37 Preg (exposure NA) 33 LB	MC: 3/34 SB: 1/8 ⁱ ND: 0/7 ET: 1/29	PTB: 4/7 ⁱ LBW: 1/7 ⁱ SGA: 2/26 ⁱ	CM: 0/33	SMI: NA GH: no increased prevalence GD: NA	No adverse drug-related effects on pregnancy outcomes compared with unexposed controls.	2a 2 poor
IV immunoglobulin		100 ^h						
EULAR 2016	3 [s216]	96	MC: 24/93		CM: 0/96			
EULAR update 2024	1 CSE [s73] 1 CR [s74]	4 Preg (exposure NA) 4 LB	MC: 0/4 SB: 0/4 ND: 0/4 ET: NA	PTB: 1/3 ^j LBW: NA SGA: NA	CM: 0/4	SMI: NA GH: NA GD: NA	No adverse drug-related effects on pregnancy outcomes compared with unexposed controls.	3b 2 poor
Leflunomide/teriflunomide		1285 ^h						
EULAR 2016	7 [s216]	129	MC: 12/122		CM: 5/129			
EULAR update 2024	9 CS [s75–s83] 1 CCS [s84] 1 CR [s85]	1219 Preg (PRE ≥299; T1 ≥321; T2 ≥35; T3 ≥49) 653 LB	MC: 182/1160 SB: 7/1043 ND: 0/290 ET: 312/1160	PTB: 37/235 LBW: 10/53 SGA: 19/125	CM: 20/655	SMI: NA GH: no increased prevalence GD: no increased prevalence	No excess risk of CM, MC, LBW and PTB in studies with controls. The average rate of drug washout was 49%. Data on leflunomide/teriflunomide exposure in pregnancy without drug washout procedure remains insufficient.	2b 2 good 9 poor
Methotrexate		595						
EULAR 2016	12 [s216]	372	MC: 140/329		CM: 15/143			
EULAR update 2024	1 CS [s86]	223 Preg (exposure NA) 153 LB	MC: 70/223 SB: 49/223 ND: NA ET: 21/223	PTB: NA LBW: NA SGA: NA	CM: 1/153	SMI: NA GH: NA GD: NA	Known teratogenic and embryotoxic risk if exposure occurs in the first trimester, which explains the high rate of MC and stillbirth.	2b 1 poor
Mycophenolate mofetil		337						
EULAR 2016	23 [s216]	333	MC: 119/318		CM: 48/174			
EULAR update 2024	1 CSE [s9]	4 Preg (exposure NA) 4 LB	MC: 0/4 SB: 0/4 ND: 0/4 ET: 0/4	PTB: 2/4 LBW: 3/4 SGA: 1/4	CM: 0/4	SMI: NA GH: no increased prevalence GD: NA	Known teratogenic and embryotoxic risk if exposure occurs in first trimester, which explains the high rate of MC.	2b 1 poor
Sildenafil		166						
EULAR 2016	—							
EULAR update 2024	SLR [s251]	165 Preg	MC: NA SB: 3/69 ND: 5/129 ET: NA	PTB: NA LBW: NA SGA: NA	CM: 0/35	SMI: NA GH: NA GD: NA	Limited evidence available in patients with RMD. Most evidence was available from studies with control groups investigating whether sildenafil improves perinatal outcome in pregnancies with severe placental dysfunction.	4 1 poor
		1 CR [s64]	1 Preg (exposure NA) 1 LB	MC: NA SB: NA ND: NA ET: NA	PTB: 1/1 LBW: NA SGA: NA	CM: NA		

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Table 1 (Continued)

Drug/EULAR SLR	No. of studies per study type [Ref] ^a	Total exposed pregnancies (per time points); total live births ^b	Pregnancy loss (per total pregnancies): MC, ^c SB, ND, ET	Pregnancy outcomes (per total pregnancies): PTB, LBW, SGA	CM ^c (per total live birth) ^d	Maternal outcomes: SMI, GH, GD	Comments ^e	Evidence: LoE ^f and study quality ^g
Sulfasalazine/mesalazine (5-ASA)		623 ^b						
EULAR 2016	4 [s216]	525	MC: 12/186		CM: 16/339			
EULAR update 2024	5 CS [s4,s5,s48,s87,s88]	98 Preg (exposure NA) 3618 LB	MC: 10/54 SB: NA ND: NA ET: NA	PTB: 296/3849 LBW: NA SGA: 108/3599	CM: 145/3618	SMI: NA GH: NA GD: NA	No adverse drug-related effects on pregnancy outcomes compared with unexposed controls, including studies with adjustment for confounders.	2a 4 good 1 poor
Tacrolimus		540 ^b						
EULAR 2016	12 [s216]	505	MC: 91/344		CM: 3/107			
EULAR update 2024	2 CS [s89,s90]	35 Preg (exposure NA) 32 LB	MC: 3/35 SB: 0/35 ND: NA ET: NA	PTB: 3/20 ⁱ LBW: NA SGA: 3/20 ⁱ	CM: 1/35	SMI: NA GH: NA GD: NA	No adverse drug-related effects on pregnancy outcomes compared with unexposed controls, including 1 study with adjustment for confounders.	2b 1 good 1 fair
Avacopan, mepacrin, voclosporin, and iloprost/ilomedin: No studies available								
tsDMARDs								
Tofacitinib		85						
EULAR 2016	1 [s216]	27	MC: 7/27		CM: 1/15			
EULAR update 2024	1 CS [s91]	58 Preg (PRE NA; T1 ≥47; T2 NA; T3 NA) 33 LB	MC: 14/58 SB: 0/58 ND: 0/58 ET: 9/58	PTB: 7/33 LBW: NA SGA: NA	CM: 2/58	SMI: NA GH: NA GD: NA	No comparison with a control group. High rate of MC (but no data on comedication). Limited data indicate no increased risk of CM. No studies with control group available.	2b 1 poor
Apremilast, baricitinib, filgotinib, and upadacitinib: No studies available								
bDMARDs								
TNFi bDMARDs								
TNFi total^l		12,817 ^b						
EULAR 2016	49 [s216]	2492	MC: 265/2258		CM: 75/2110			
EULAR update 2024	50 CS [s2,s22,s23,s53,s55,s57,s58,s61,s62,s92–s132] 3 CSS [s63,s133,s134] 2 CCS [s15,s135] 3 CR [s136–s138] 5 CSE [s139–s143]	11,194 Preg (PRE ≥1471; T1 ≥5009; T2 ≥1801; T3 ≥2190) 11,826 LB	MC: 900/7540 SB: 53/7135 ND: 6/448 ET: 215/5683	PTB: 1488/14,343 LBW: 533/7330 SGA: 729/9250	CM: 277/7479	SMI: data insufficient GH: no increased prevalence GD: no increased prevalence	No adverse drug-related effects on pregnancy outcomes based on studies with control groups and adjustment for confounders.	2a 26 good 4 fair 33 poor
Adalimumab		1148						
EULAR 2016	23 [s216]	524	MC: 23/191		CM: 24/350			
EULAR update 2024	3 CS [s94–s96]	624 Preg (PRE ≥12; T1 ≥138; T2 ≥52; T3 ≥20) 502 LB 1798	MC: 60/610 SB: 2/610 ND: NA ET: 17/610	PTB: 58/558 LBW: 7/45 SGA: 17/206	CM: 23/502	SMI: NA GH: no increased prevalence GD: NA	No adverse drug-related effects on pregnancy outcomes compared with unexposed controls. Analyses with adjustment for confounders available.	2b 1 good 2 poor
Certolizumab								
EULAR 2016	6 [s216]	362	MC: 52/339		CM: 12/267			
EULAR update 2024	2 CS [s98,s99] 1 CR [s138] 2 CSE [s139,s140]	1436 Preg (PRE ≥13; T1 ≥1035; T2 ≥23; T3 ≥44) 1304 LB	MC: 111/1468 SB: 11/1454 ND: 0/29 ET: 39/1454	PTB: 125/1304 LBW: 103/1287 SGA: NA	CM: 44/1304	SMI: data insufficient GH: no increased prevalence GD: no increased prevalence	No adverse drug-related effects on pregnancy outcomes. No studies with control group available.	2b 1 good 4 poor
Etanercept		588						
EULAR 2016	19 [s216]	332	MC: 12/74		CM: 9/251			
EULAR update 2024	1 CS [s97]	256 Preg (PRE ≥233; T1 NA; T2 NA; T3 NA) 177 LB	MC: 56/256 SB: 2/256 ND: NA ET: 23/256	PTB: 32/166 LBW: 25/166 SGA: NA	CM: 10/165	SMI: NA GH: NA GD: NA	No adverse drug-related effects on pregnancy outcomes compared with unexposed controls.	2b 1 good

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Table 1 (Continued)

Drug/EULAR SLR	No. of studies per study type [Ref] ^a	Total exposed pregnancies (per time points); total live births ^b	Pregnancy loss (per total pregnancies): MC, ^c SB, ND, ET	Pregnancy outcomes (per total pregnancies): PTB, LBW, SGA	CM ^c (per total live birth) ^d	Maternal outcomes: SMI, GH, GD	Comments ^e	Evidence: LoE ^f and study quality ^g
Golimumab								
EULAR 2016	2 [s216]	50	MC: 13/47		CM: 0/26			
EULAR update 2024	No studies available ^k	≥39 Preg ^k					No adverse drug-related effects on pregnancy outcomes. No studies with control group available.	4
Infliximab		3127						
EULAR 2016	31 [s216]	1161	MC: 64/676		CM: 20/756			
EULAR update 2024	2 CS [s92,s93] 2 CR [s136,s137] 2 CSE [s141,s142]	1966 Preg (PRE ≥91; T1 ≥1315; T2 ≥109; T3 ≥554) 1640 LB 6912	MC: 242/1989 SB: 2/1883 ND: 0/8 ET: 83/1989	PTB: 149/1990 LBW: 72/1879 SGA: 10/1875	CM: 27/1640	SMI: NA GH: NA GD: NA	No adverse drug-related effects on pregnancy outcomes compared with unexposed controls.	2b 4 poor
Mixed TNFi^l								
EULAR 2016	—							
EULAR update 2024	42 CS [s2,s22,s23,s53,s55,s57,s58,s61,s62,s100–s132] 3 CSS [s63,s133,s134] 2 CCS [s15,s135] 1 CSE [s143]	6912 Preg (PRE ≥1122; T1 ≥2521; T2 ≥1617; T3 ≥1572) 8203 LB	MC: 431/3217 SB: 36/2932 ND: 6/411 ET: 53/1374	PTB: 1124/10,325 LBW: 326/3953 SGA: 702/7169	CM: 173/3868	SMI: data insufficient GH: no increased prevalence GD: no increased prevalence	No adverse drug-related effects on pregnancy outcomes compared with unexposed controls with adjustment for confounders	2a 23 good 4 fair 21 poor
Non-TNFi bDMARD								
B cell-targeted therapy								
Belimumab		410 ^b						
EULAR 2016	2 [s216]	153	MC: 41/153	CM: 7/71				
EULAR update 2024	2 CS (1 abstract) [s144,s145] 6 CR [s146–s151] 2 CSE [s152,s153]	410 Preg (PRE ≥24; T1 ≥26; T2 ≥14; T3 ≥18) 255 LB	MC: 82/409 ^j SB: 4/408 ND: 1/40 ET: 74/388	PTB: 14/410 LBW: 9/18 ⁱ SGA: 8/16 ⁱ	CM: 12/247	SMI: data insufficient GH: no increased prevalence GD: no increased prevalence	No adverse drug-related effects on pregnancy outcomes. No studies with control group available.	4 10 poor
Rituximab		484 ^b						
EULAR 2016	21 [s216]	256	MC: 48/210	CM: 6/172				
EULAR update 2024	4 CS [s54,s154–s156] 5 CR [s157–s164] 6 CSE [s165–s170]	393 Preg (PRE ≥216 T1 ≥19; T2 ≥24; T3 ≥11) 255 LB	MC: 43/296 SB: 3/242 ND: 2/162 ET: 35/277	PTB: 35/232 ⁱ LBW: 6/53 ⁱ SGA: 4/60	CM: 8/235	SMI: data insufficient GH: no increased prevalence GD: no increased prevalence	No adverse drug-related effects on pregnancy outcomes. No studies with control group available.	4 15 poor
T cell costimulation inhibitor								
Abatacept		170 ^b						
EULAR 2016	2 [s216]	152	MC: 40/151	CM: 7/87				
EULAR update 2024	2 CS (one abstract) [s171,s172] 2 CR [s161,s173]	169 Preg (PRE ≥2 T1 ≥2; T2 ≥0; T3 ≥0) 101 LB	MC: 42/169 ^j SB: 2/169 ND: 2/104 ET: 19/168	PTB: 2/15 ⁱ LBW: 0/2 SGA: 1/13	CM: 8/100 (not increased compared with overall rates for CM: in the thoroughly collected OTIS data)	SMI: NA GH: NA GD: no increased prevalence	Overall, 2 cohort studies with prospective data indicated no adverse drug-related effect on pregnancy outcomes. No studies with control group available.	4 4 poor
Interleukin inhibitors								
Anakinra		70 ^b						
EULAR 2016	4 [s216]	40	MC: 4/40	CM: 2/34				
EULAR update 2024	3 CS [s174–s176] 1 CR [s177] 1 CSE [s178]	39 Preg (PRE ≥19; T1 ≥6; T2 ≥5; T3 ≥5) 37 LB	MC: 3/39 SB: 0/29 ND: 0/29 ET: 0/4	PTB: 2/32 LBW: 0/14 SGA: 1/18	CM: 2/36	SMI: no increased prevalence GH: no increased prevalence GD: no increased prevalence	No increased risk for adverse pregnancy outcomes compared with unexposed controls. No studies with control group available.	4 5 poor

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Table 1 (Continued)

Drug/EULAR SLR	No. of studies per study type [Ref] ^a	Total exposed pregnancies (per time points); total live births ^b	Pregnancy loss (per total pregnancies): MC, ^c SB, ND, ET	Pregnancy outcomes (per total pregnancies): PTB, LBW, SGA	CM ^c (per total live birth) ^d	Maternal outcomes: SMI, GH, GD	Comments ^e	Evidence: LoE ^f and study quality ^g
Canakinumab		16						
EULAR 2016	—							
EULAR update 2024	2 CS [s174,s175] 1 CR [s179] 1 CSE [s175]	16 Preg (PRE ≥14 T1 ≥7; T2 ≥2; T3 ≥3) 13 LB 3	MC: 2/16 SB: 1/13 ND: 0/13 ET: 0/1	PTB: 0/7 LBW: 0/7 SGA: 0/7	CM: 0/10	SMI: no increased prevalence GH: NA GD: NA	Limited evidence showed no increased risk for adverse pregnancy outcomes. No studies with control group available.	4 poor
Mepolizumab								
EULAR 2016	—							
EULAR update 2024	2 CR [s180,s181]	3 Preg (exposure NA) 2 LB	MC: 0/3 SB: NA ND: NA ET: 1/3	PTB: NA LBW: NA SGA: NA	CM: 0/2	SMI: NA GH: NA GD: NA	Limited evidence showed no increased risk for adverse pregnancy outcomes. No studies with control group available.	4 2 poor
Sarilumab		1						
EULAR 2016	—							
EULAR update 2024	1 CR [s182]	1 Preg (exposure NA) 1 LB	MC: 0/1 SB: 0/1 ND: 0/1 ET: 0/1	PTB: 0/1 LBW: 0/1 SGA: 0/1	CM: 0/1	SMI: NA GH: NA GD: NA	Limited evidence from 1 case report showed no adverse pregnancy outcome. No studies with control group available.	4 1 poor
Tocilizumab		401 ^h						
EULAR 2016	3 [s216]	218	MC: 47/218		CM: 5/128			
EULAR update 2024	2 CS [s183,s184] 1 CSS [s14] 4 CR [s185–s188] 2 CSE [s189,s190]	363 Preg (PRE ≥84; T1 ≥216; T2 ≥10; T3 ≥6) 221 LB	MC: 84/360 SB: 1/294 ND: 2/294 ET: 59/359	PTB: 33/190 ⁱ LBW: 20/75 ⁱ SGA: 0/3	CM: 6/219	SMI: no increased prevalence GH: no increased prevalence GD: no increased prevalence	No adverse drug-related effects on pregnancy outcomes. No studies with control group available.	4 9 poor
Ixekizumab		99						
EULAR 2016	—							
EULAR update 2024	1 CS [s191]	99 Preg (PRE NA; T1 77; T2 NA; T3 NA) 39 LB 119	MC: 14/99 SB: 0/99 ND: 0/39 ET: 17/99	PTB: 6/39 LBW: NA SGA: NA	CM: 1/39	SMI: NA GH: NA GD: NA	No adverse drug-related effects on pregnancy outcomes. No studies with control group available.	4 1 poor
Secukinumab								
EULAR 2016	—							
EULAR update 2024	1 CS [s192]	119 Preg (exposure NA) 54 LB	MC: 26/119 ^j SB: NA ND: NA ET: 39/119	PTB: 6/119 LBW: NA SGA: NA	CM: 2/54	SMI: NA GH: NA GD: NA	No adverse drug-related effects on pregnancy outcomes. No studies with control group available.	4 1 poor
Ustekinumab		598 ^h						
EULAR 2016	5 [s216]	108	MC: 15/108		CM: 1/58			
EULAR update 2024	7 CS [s105,s106,s193–s197] 6 CR [s141,s198–s202] 1 CSE [s203]	571 Preg (PRE ≥487; T1 ≥125; T2 ≥73; T3 ≥153) 473 LB	MC: 71/576 SB: 3/487 ND: 0/28 ET: 31/574	PTB: 40/438 LBW: 21/429 SGA: 2/18 ⁱ	CM: 12/468	SMI: NA GH: no increased prevalence GD: no increased prevalence	No adverse drug-related effects on pregnancy outcomes compared with unexposed controls.	2b 1 good 13 poor

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Table 1 (Continued)

Drug/EULAR SLR	No. of studies per study type [Ref] ^a	Total exposed pregnancies (per time points); total live births ^b	Pregnancy loss (per total pregnancies): MC, ^c SB, ND, ET	Pregnancy outcomes (per total pregnancies): PTB, LBW, SGA	CM ^c (per total live birth) ^d	Maternal outcomes: SMI, GH, GD	Comments ^e	Evidence: LoE ^f and study quality ^g
Anifrolumab, guselkumab, risankizumab: No studies available							Due to the molecular structure analogous to most other biologics, a similar risk profile is expected.	5
Others		14						
Eculizumab								
EULAR 2016	—							
EULAR update 2024	4 CR [s204–s207] 2 CSE [s208,s209]	14 Preg (exposure NA) 13 LB	MC: 0/14 SB: NA ND: NA ET: 1/14	PTB: 7/13 ⁱ LBW: NA SGA: NA	CM: 0/13	SMI: NA GH: NA GD: NA	Limited evidence showed no increased risk for the pregnancy outcomes MC and CM. No studies with control group available.	4 6 poor

APO, adverse pregnancy outcome; bDMARD, biologic disease-modifying antirheumatic drug; C5i, complement protein C5 inhibitor; CCS, case-control study; CM, congenital malformation; COX, cyclooxygenase; CQ, chloroquine; CR, case report; CS, cohort study; csDMARD, conventional synthetic disease-modifying antirheumatic drug; CSE, case series; CSS, cross-sectional study; ET, elective termination; GD, gestational diabetes; GH, gestational hypertension; HCQ, hydroxychloroquine; IFNAR1, interferon α/β receptor subunit 1 inhibitor; ILi, interleukin inhibitor; IV, intravenous; IVIG, intravenous immunoglobulin; LB, live birth; LBW, low birth weight; LoE, level of evidence; MC, miscarriage; NA, not available; ND, neonatal death; NSAID, nonsteroidal anti-inflammatory drug; OTIS, Organization of Teratology Information Specialists; PDE4i, phosphodiesterase-4 inhibitor; PRE, exposure prepregnancy; Preg, pregnancies; PTB, preterm birth; RMD, rheumatic and musculoskeletal disorder; SB, stillbirth; SGA, small-for-gestational age; SLR, systematic literature review; SMI, serious maternal infection; SSZ, sulfasalazine; T1, exposure in first trimester; T2, exposure in second trimester; T3, exposure in third trimester; TNFi, tumour necrosis factor inhibitor; tsDMARD, targeted synthetic disease-modifying antirheumatic drug.

^a The complete list of references is available in the Supplementary Material.

^b The number of pregnancies and live births may vary due to different data reporting in the different studies. The same applies to the number of reported outcomes, where the number of pregnancies or live births may differ compared with the reported denominator. The same applies to the number of exposed pregnancies during the various exposure periods, which may differ from the total number of reported pregnancies. As the exposure period was not recorded in all studies, the number of pregnancies that were exposed during at least this period is used.

^c Due to the methodology of the SLR for the outcome MC and CM, some additional studies before 2015 may be included.

^d If number of live birth was not available as denominator, the number of pregnancies was used as denominator.

^e Summary on pregnancy exposure related to antirheumatic drugs including evidence of both previous and present SLRs. Where available, only high-quality studies were considered.

^f Level of evidence according to studies retrieved in the SLR of EULAR points to consider 2016 and the 2024 SLR Update as well as additional studies (including additional SLRs and MA).

^g Study quality rated as good, fair, and poor based on the Newcastle–Ottawa Scale; CR and CSE were rated as poor.

^h Total number of exposed cases including present EULAR SLR and previous EULAR SLR without data overlap.

ⁱ Increased rate (considering all studies regardless of quality) due to potential confounding by disease severity, disease activity, or concomitant medication.

^j All studies with different TNFi (monotherapy) and studies not differentiating between the different TNFi.

^k No separate studies with golimumab. In studies with different TNFi, >39 pregnancies with golimumab described.

^l Studies not differentiating between the different TNFi (if different bDMARD including >80%TNFi).

Table 2
Meta-analysis estimates of the risk of preterm birth with glucocorticoid use vs no use

Exposure	Unadjusted meta-analysis results					Adjusted meta-analysis results				
	No. of studies	Pooled odds ratio (95% CI)	Type of CI	I ²	τ ²	No. of studies	Pooled adjusted risk estimates (95% CI)	Type of CI	I ²	τ ²
GC vs no GC	8	2.02 (1.56, 2.62)	HKSJ	58.01	0.05	1	aHR: 1.12 (0.45, 2.78)	—	—	—
						5	aOR: 2.24 (1.84, 2.71)	HKSJ	0	0
						6	aRR: 1.78 (1.06, 3.00)	HKSJ	64.48	0.14
GC high dose vs no GC	3	^a	—	—	—	3	^a	—	—	—
GC low dose vs no GC	3	^a	—	—	—	3	aRR: 1.03 (0.56, 1.87)	HKSJ	41.4	0.02

aHR, adjusted hazard ratio; aOR, adjusted odds ratio; aRR, adjusted risk ratio; GC, glucocorticoid; HKSJ, CIs based on Hartung–Knapp–Sidik–Jonkman.

^a Estimate is not informative (ie, lower and/or upper confidence bounds fall outside the range of combined study estimates) and is therefore not reported.

[aOR]: 2.24; 95% CI: 1.84, 2.71 and pooled adjusted risk ratio [aRR]: 1.78; 95% CI: 1.06, 3.00) (Table 2 and Supplementary Fig S5). In the subanalysis stratified by dose, the association with PTB was particularly found for higher GC doses (≥5.0–10.0 mg daily or a cumulative dose of >300.0 mg within the first 20 weeks of gestation), but not for low doses (lowest risk with daily doses <5.0 mg) (Table 1 and Supplementary Table S5).

Nonsteroidal anti-inflammatory drugs

Based on the earlier EULAR SLR, which included 17,992 pregnancies exposed to nonselective NSAIDs, NSAIDs were considered compatible with the first and second trimesters of pregnancy. Regarding selective cyclooxygenase (COX) 2 inhibitors, it was recommended to avoid them during pregnancy, given the insufficient evidence (215 pregnancies from 3 studies) [4]. In this SLR update, we found an additional evidence from 12 studies of 145,889 pregnancy exposures to nonselective NSAIDs, most of which (58%) were of good quality. No increased risk of congenital malformation or miscarriage was reported in confounder-adjusted studies [15–18]. For selective COX-2 inhibitors, exposure in the first trimester was not associated with congenital malformations or miscarriages in 823 additional pregnancies [19]. Data on other outcomes were insufficient to draw conclusions.

Conventional synthetic disease-modifying ARDs, immunosuppressives, and other drugs

Antimalarials (hydroxychloroquine/chloroquine). The antimalarials hydroxychloroquine and chloroquine were previously considered safe for use during pregnancy according to the 2016 EULAR PtC [4], based on data from 492 pregnancy exposures. Our SLR identified 5331 new cases of antimalarial exposure (mainly hydroxychloroquine in systemic lupus erythematosus [SLE]). The majority of the studies were of good quality (80%). No congenital malformation risk was reported for ≤400.0 mg hydroxychloroquine per day, and no association with miscarriage, PTB, or SGA. One large study using claims data showed a slight increase of congenital malformation with hydroxychloroquine of ≥400.0 mg/d, without a specific pattern (aRR: 1.33; 95% CI: 1.08, 1.65) [20]. This finding was not confirmed by a subsequent prospective cohort study, neither in comparison with disease-matched nor with healthy subjects (OR: 1.08; 95% CI: 0.51, 2.29; and aOR: 0.76; 95% CI: 0.28, 2.05, respectively) [21]. Data remain insufficient for mepacrine.

Azathioprine/6-mercaptopurine (thiopurines). Based on the earlier EULAR SLR [4] with 1327 pregnancies exposures, azathioprine was considered compatible with pregnancy. In our updated SLR, thiopurine exposure was reported for 4985 pregnancies (mostly SLE

and inflammatory bowel disease [IBD]) by 19 studies (quality 53% good, 11% fair, and 37% poor). No significant increased risk of congenital malformation or miscarriage was identified. An increased rate of prematurity was observed by 1 study (aRR: 4.2; 95% CI: 1.4, 12.5) [22], which was not confirmed by others [23–25].

Bosentan. One case of bosentan exposure during the first and second trimester of pregnancy was reported, resulting in a live premature birth without congenital malformation [26].

Colchicine. Based on previous consensus and evidence [4] from 460 exposed pregnancies, colchicine was considered compatible with pregnancy. This SLR included 342 pregnancies with colchicine exposure, primarily patients with familial Mediterranean fever, with 1 study of good and 2 of fair quality. No increased rates of miscarriage and congenital malformation were observed, but there was an increase in PTB, which may have been confounded by disease severity [27].

Cyclophosphamide. Owing to its teratogenic properties, the previous EULAR PtC recommended that cyclophosphamide should be discontinued before pregnancy; however, it may be considered during the second or third trimester in case of severe, refractory maternal disease [4]. This recommendation was based on data from 276 pregnancies. Seven additional studies involving 157 pregnancies (in women with SLE and vasculitis) were reviewed in this SLR update. Study quality was low, with 29% being fair and 71% being poor. High overall rates of miscarriage (23.2%) and PTB (27.9%) were observed. Comedication was reported in only 3 studies (case series/reports): all patients were exposed to high doses of GC. No congenital malformations were reported in the more recent studies; however, of the 5 studies reporting the timing of exposure during pregnancy, only 1 pregnancy was exposed during the first trimester.

Cyclosporine. In addition to the 1126 exposed pregnancies described in the previous EULAR SLR [4], we identified 37 new cases of cyclosporine exposure during gestation (mostly SLE and IBD patients) from 2 studies of poor quality. The data did not raise concerns about miscarriages and congenital malformations. Increased rates of stillbirth, PTB, and LBW were likely confounded by elevated disease activity and GC use [28,29].

Intravenous immunoglobulins. The previous EULAR SLR identified 96 exposed pregnancies and considered intravenous immunoglobulins (IVIg) compatible with pregnancy [4]. Four additional pregnancies with IVIG exposure were included in this SLR (patients with adult-onset Still disease and vasculitis). Although limited, evidence did not raise concerns about miscarriages or congenital

malformations. The high rate of PTB cannot be interpreted due to the small sample size, the severity of the underlying disease, and comedication [30].

Leflunomide/teriflunomide. The previous EULAR 2016 consensus stated that leflunomide should be avoided in pregnancy given the limited data available [4]. A total of 1219 additional pregnancies exposed to leflunomide or teriflunomide (the active metabolite of leflunomide) were reviewed in this SLR. However, the quality of the studies was mostly poor (82%). A drug washout procedure was performed in 49% of cases where information was available (307/627 pregnancies) [31–37]. In 4 comparative studies, no increased risk of miscarriage, congenital malformations, LBW, or PTB was found, yet the drug washout rate ranged from unknown to 95% [36,38–40].

Methotrexate. The EULAR 2016 PtC concluded that methotrexate is teratogenic and should therefore be discontinued before pregnancy, based on data from 372 pregnancies [4]. Since then, 1 single retrospective cohort of poor quality investigated 223 pregnancies in patients with rheumatoid arthritis (RA) and reported an increased risk of miscarriage, congenital malformations, and stillbirth compared with controls [41].

Mycophenolate. The EULAR 2016 PtC included 333 pregnancies, leading to the conclusion that mycophenolate is teratogenic and should be withdrawn before pregnancy. In this review, we found an additional case series of 4 SLE pregnancies resulting in live births without malformations [42], providing little new evidence.

Sildenafil. Sildenafil was not included in the earlier EULAR SLR [4]. Three SLRs analysed the safety of sildenafil use for treatment of foetoplacental insufficiency, recurrent miscarriage, or maternal pulmonary hypertension, finding mild maternal side effects, no teratogenicity, and no increased rate of stillbirths or perinatal deaths [43–45]. In addition, we identified 1 case report [26] of sildenafil use for pulmonary hypertension in a patient with mixed connective tissue disease, which resulted in PTB. Of note, 1 SLR (10 studies, 1090 participants) concluded that a prolonged use of sildenafil for the treatment of severe fetal growth restriction may increase the risk of persistent pulmonary hypertension of the neonate [43].

Sulfasalazine/5-aminosalicylates. The earlier EULAR SLR considered sulfasalazine compatible with pregnancy based on 525 pregnancy exposures [4]. We identified additional 98 pregnancy exposures in patients with IBD and RA. Data quality was 67% good, 17% fair, and 17% poor. In line with the previous SLR, no adverse drug-related effects on miscarriage, congenital malformations, PTB, or SGA were described [46].

Tacrolimus. Tacrolimus was considered compatible with pregnancy in the previous consensus based on 505 exposures [4]. We found additional evidence from 2 studies of 35 pregnancies in women with SLE exposed to tacrolimus (1 good and 1 fair quality). Consistent with the earlier SLR, rates of miscarriage, and congenital malformations were not elevated. In addition, tacrolimus was not associated with PTB in 1 adjusted analysis (aOR: 0.76; 95% CI: 0.10, 4.99) [47].

Targeted synthetic disease-modifying ARDs. The previous consensus was based on a case series of 27 pregnancies exposed to tofacitinib and therefore advised avoiding tofacitinib in pregnancy due to insufficient data. We found additional evidence from 58 pregnancies exposed to tofacitinib. One cohort study of poor quality reported a 24.1% miscarriage and a 21.2% PTB rate, but no increased rate of congenital malformations [48]. Data on other outcomes were lacking.

Biologic disease-modifying ARDs

TNFi biologic disease-modifying ARDs. The previous EULAR consensus was that TNFi biologic disease-modifying ARDs (bDMARDs) are compatible during the first part of pregnancy, and etanercept and certolizumab may be used throughout pregnancy due to low transplacental passage [4]. This consensus was based on data of 2492 pregnancy exposures to TNFi bDMARDs. We found additional evidence from 63 studies of 11,194 pregnancy exposures to TNFi bDMARDs (Table 1). The underlying diagnoses were mixed (inflammatory rheumatic diseases, psoriasis, and IBD) [49–51], with more than half of the studies including only patients with IBD [24,51–53]. Study quality was diverse, with 50% rated as good and 50% rated as poor. Overall, no increased risk for any APO was apparent. A total of 28 studies including 11,282 pregnancy exposures to TNFi were eligible for meta-analyses (Supplementary Table S3). The pooled unadjusted OR showed increased risks for TNFi-exposed vs TNFi-unexposed pregnancies for the outcomes congenital malformations (1.37; 95% CI: 1.02, 1.84) and SGA (1.16; 95% CI: 1.01, 1.32) (Supplementary Figs S6A and S7A, Table 3). No significant increased risk was observed for miscarriage, stillbirth, PTB, and LBW (Supplementary Figs 8A, 9A, 10A, and 11A, Table 3). However, the meta-analysis of adjusted pooled estimates did no longer show an increased risk for TNFi-exposed vs TNFi-unexposed pregnancies for congenital malformations and SGA, and all other outcomes (Supplementary Figs 6B–11B, Table 3). Overall, the available evidence for the use of TNFi during pregnancy is reassuring.

Table 3
Meta-analysis estimates of the risk of adverse pregnancy and infant outcomes with tumour necrosis factor inhibitor use vs no use

Outcome	Unadjusted meta-analysis results						Adjusted meta-analysis results				
	No. of studies	Pooled odds ratio (95% CI)	Type of CI	<i>I</i> ²	τ^2		No. of studies	Pooled adjusted risk estimates (95% CI)	Type of CI	<i>I</i> ²	τ^2
Miscarriage	8	1.32 (0.99, 1.76)	HKSJ	0	0	1	1	aHR 2.22 (0.67, 7.29) aOR 1.30 (0.50, 3.30)	—	—	—
Stillbirth	8	0.92 (0.37, 2.25)	mKH	0	0	—	—	—	—	—	—
Congenital malformation	13	1.37 (1.02, 1.84)	mKH	0	0	4	4	aOR 1.24 (0.71, 2.16)	mKH	0	0
Preterm birth	25	1.23 (0.98, 1.54)	HKSJ	63.49	0.13	1	1	aHR 0.82 (0.66, 7.20)	—	—	—
						6	6	aOR 1.18 (0.83, 1.68)	HKSJ	29.6	0.03
Small-for-gestational age	12	1.16 (1.01, 1.32)	mKH	6.58	0	3	3	aOR 1.06 (0.60, 1.87)	HKSJ	0	0
Low birth weight	12	0.89 (0.45, 1.77)	HKSJ	84.14	0.86	2	2	aOR 1.16 (0.67, 2.00)	Z	—	—

aHR, adjusted hazard ratio; aOR, adjusted odds ratio; HKSJ, CIs based on Hartung–Knapp–Sidik–Jonkman; mKH, CIs based on modified Knapp–Hartung; Z, CI based on fixed-effects normal-normal model.

Non-TNFi bDMARDs. The earlier EULAR PtC recommended replacing rituximab, anakinra, tocilizumab, abatacept, belimumab, and ustekinumab with other ARDs before conception, mainly due to the limited data available, although there was no evidence of an increased rate of congenital malformations [4].

For the B cell-targeted therapies using belimumab and rituximab, the previous consensus was based on 153 and 256 exposed pregnancies; we identified further 410 and 393 pregnancy exposures from 6 cohort studies with poor quality (SLE and other immune-mediated inflammatory diseases). The studies did not report higher rates for miscarriage or congenital malformation. However, 1 cohort study investigating rituximab showed an increased miscarriage rate of 21.6%, which was confounded by potentially teratogenic comedication (eg, methotrexate and mycophenolate) in more than half of the exposed pregnancies [54]. Crude rates showed an increase of PTB in pregnancies exposed to anti-B cell agents, which may have been confounded by disease severity [55]. For belimumab, rates of LBW and SGA were high, but sample size was small (Table 1).

Regarding abatacept, 152 pregnancy exposures were reported in the previous EULAR SLR [4]. We identified additional 169 pregnancies in RA with exposure to abatacept, but study quality was poor. The studies mainly investigated miscarriages and congenital malformations, and no increased miscarriage rates were reported. The 8% rate of congenital malformations is higher than the typically reported malformation rate, which was due to methodological aspects of the Organization of Teratology Information Specialists (OTIS) register with thorough analysis by dysmorphology specialists and long follow-up period, but was in line with the rates of unexposed controls [49,56]. Other APOs were only investigated in a limited number of pregnancies.

For interleukin (IL) inhibitors (ILi), most new evidence was available for ustekinumab (IL12/23i) with 571 exposed pregnancies that were added to the previously reviewed 152 cases [4]. The study quality was 14% good and 86% poor. Regarding crude rates, no increase in APOs was apparent. This finding is consistent with the results of a prospective cohort, which found no increase in APOs in 27 ustekinumab-exposed pregnancies compared with TNFi- or non-bDMARD-exposed pregnancies [57].

Previously, evidence on IL1i was limited to 40 pregnancy exposures to anakinra and no data on canakinumab [4]. In our SLR update, we found additional evidence of 39 pregnancy exposures to anakinra and 16 to canakinumab (all studies of poor quality). Data did not show higher rates of any APO.

In the previous EULAR SLR, evidence on tocilizumab (IL6i) exposure in pregnancy was based on 218 cases [4]. We identified 363 additional pregnancies exposed to tocilizumab (IL6i). All studies were of poor quality and did not show increased rates of pregnancy loss or congenital malformations. One cohort study of RA pregnancies reported increased rates for PTB and LBW [58] but did not account for disease activity, which is a risk factor for PTB and LBW. Only 1 pregnancy exposed to sarilumab was included (IL6i) in this SLR [59].

The SLR identified 119 pregnancies exposed to the IL17i secukinumab and 99 to ixekizumab, all from studies of poor quality. No increased risk of miscarriage or congenital malformation was reported. One study analysed a pharmaceutical safety database of secukinumab-exposed pregnancies, showing a miscarriage rate of 21.8%. However, the number of unknown pregnancy outcomes due to lost to follow-up and ongoing pregnancies was high [60].

For mepolizumab and eculizumab, only case reports/series were available including 3 mepolizumab-exposed and 14

eculizumab-exposed pregnancies. No events of miscarriage or congenital malformation were reported.

Effect of ARDs on adverse infant outcomes

bDMARDs

A total of 4521 TNFi-exposed pregnancies and 7665 live births with *in utero* exposure to TNFi were investigated for the different infant outcomes within the first year of life. Less information was available for non-TNFi-exposed pregnancies. An overview of the results is given in Tables 2–4.

SII

In our meta-analysis (Table 3), the pooled unadjusted OR of SII in TNFi-exposed pregnancies showed an increased risk of SII (OR: 1.26; 95% CI: 1.14, 1.40); however, the association was attenuated when adjusted estimates were used (Fig A, B). Restricting the meta-analysis to studies that reported exposure in the third trimester did not show an increased risk for SII associated with maternal TNFi treatment (adjusted incidence rate ratio: 1.02; 95% CI: 0.79, 1.33; aOR: 0.88; 95% CI: 0.67, 1.16) (Fig C). The evidence regarding SII after *in utero* exposure to non-TNFi bDMARDs is scarce, but overall reassuring for rituximab, belimumab, ustekinumab, anakinra, and canakinumab. Limited data are available for sarilumab, tocilizumab, and abatacept, and no data for other non-TNFi bDMARDs. For rituximab, treatment in the second part of pregnancy resulted in haematologic alterations in up to 12% of the newborns without evidence of an increased risk of SII, and B cell counts normalised within 6 months [61]. In a belimumab cohort of 13 pregnancies, no haematologic changes in the infants were reported [62].

Vaccinations

In infants with *in utero* exposure to bDMARDs, no serious adverse events were reported after the application of nonlive vaccines according to standard vaccination schedules (Table 4). Most studies on this outcome addressed infants born at term, data on premature infants are sparse or missing. An adequate serologic response after *in utero* exposure to TNFi compared with that to non-TNFi was reported even if TNFi drug levels were detectable in the newborn at birth [53,63,64].

In our SLR, we found evidence for the safety of live rotavirus vaccination in 72 infants with *in utero* exposure to TNFi. In addition, no adverse events were observed after rotavirus vaccination in 1295 infants with *in utero* exposure to any bDMARD (60%–70% TNFi), with 28% to 93% exposed during the third trimester (1 SLR article and 1 study published after our search date) [65–68]. For the vaccination with *Bacillus Calmette–Guérin* (BCG), 1 case report described a fatal outcome of an infant who was exposed to infliximab *in utero* and vaccinated with BCG at 3 months of age [69]. No other serious adverse events related to BCG vaccination occurred in 192 infants with exposure to TNFi (>50% infliximab) (Table 4), with more than half exposed in the third trimester [68]. For non-TNFi bDMARDs, case reports described no serious adverse events after BCG vaccination, with most vaccines administered in children older than 6 months of age [70].

Effect of NSAID use on adverse fetal health outcomes

This update of the 2016 SLR encompassed a new PIO question about fetal health issues related to NSAID use during pregnancy. Two recent studies (1 good and 1 poor quality) evaluated 127,203 pregnancies exposed to NSAIDs (657 to COX-2

Table 4

Data on adverse infant outcomes following *in utero* exposure to bDMARDs: summary of results stratified by drug

Drug	No. of studies per study type [Ref] ^a	Total exposed pregnancies (n) (per time points); total live births (n) ^b	Serious infant infections with hospitalisation during first year of life (per total pregnancies)	Serious adverse events after nonlive vaccination within first year of life (per total infants vaccinated)	Serious adverse events after live vaccination within first year of life (per total infants vaccinated)	Comment ^c	Evidence: LoE ^d and study quality ^e
bDMARDs							
TNFi bDMARD							
TNFi total^f	22 CS [s53,s57,s58,s61,s93,s94,s100,s101,s103,s106,s108–s110,s112–s115,s119,s127,s130–s132] 2 CSS [s133,s134] 2 CR [s136,s137] 3 CSE [s139,s141,s142]	4521 Preg (PRE ≥461; T1 ≥2638; T2 ≥1183; T3 ≥1549) 7665 LB	SII: 813/8506	SAE: 0/144	RV: 0/72 BCG: 1/193	No increased risk of SII compared with controls. No increased risk of SII in the subanalysis with T3 exposure. Adequate serologic response for nonlive vaccines according to normal immunisation schedule is demonstrated in infants after <i>in utero</i> exposure to TNFi. No increased risk for SAEs in infants in the first year of life after vaccination with nonlive vaccines and with live RV vaccines. Regarding BCG vaccination during the first year of life in infants after <i>in utero</i> exposure to TNFi, there was 1 case (<i>in utero</i> infliximab exposure) of fatal disseminated BCGitis after BCG vaccinated at 3 mo of age, but no other report of SAEs in the majority of cases (n = 192). For TNFi with transplacental transfer, there was no evidence of an increased risk of SAEs in infants with <i>in utero</i> exposure and vaccination with BCG after the age of 6 mo.	2b 13 good 2 fair 14 poor
Adalimumab	1 CS [s94]	257 Preg (PRE NA; T1 ≥55; T2 ≥27; T3 ≥3) 221 LB	SII: 10/229	SAE: NA	RV: NA BCG: NA	No increased risk for SII.	2b 1 good
Certolizumab	1 CSE [s139]	14 Preg (PRE ≥10; T1 ≥10; T2 ≥14; T3 ≥14) 15 LB	SII: 0/15	SAE: 0/15	RV: 0/1 BCG: NA	Limited evidence showed no increased risk for SII or SAEs to nonlive vaccines. Regarding live vaccines 1 case with RV vaccination showed no SAE.	4 1 poor
Infliximab	1 CS [s93] 2 CR [s136,s137] 2 CSE [s141,s142]	1860 Preg (PRE ≥91; T1 ≥1216; T2 ≥10; T3 ≥515) 1559 LB	SII: 16/1558	SAE: 0/4	RV: NA BCG: 1/3	No increased risk for SII. No increased risk for SAEs to nonlive vaccines. Regarding live vaccines, 1 fatal event after BCG vaccination at 3 mo of age.	4 5 poor
Etanercept and golimumab: No studies available							4
Mixed TNFi^g	20 CS [s53,s57,s58,s61,s100,s101,s103,s106,s108–s110,s112–s115,s119,s127,s130–s132] 2 CSS [s133,s134]	2390 Preg (PRE ≥360; T1 ≥1357; T2 ≥1132; T3 ≥1017) 5870 LB	SII: 787/6704	SAE: 0/125	RV: 0/71 BCG: 0/190	No increased risk of SII compared with controls. No increased risk of SII in the subanalysis with T3 exposure. Regarding nonlive and live vaccines with RV and BCG, no SAEs after vaccination reported. Adequate serologic response for nonlive vaccines according to normal immunisation schedule is shown.	2b 12 good 2 fair 8 poor

(continued on next page)

Table 4 (Continued)

Drug	No. of studies per study type [Ref] ^a	Total exposed pregnancies (n) (per time points); total live births (n) ^b	Serious infant infections with hospitalisation during first year of life (per total pregnancies)	Serious adverse events after nonlive vaccination within first year of life (per total infants vaccinated)	Serious adverse events after live vaccination within first year of life (per total infants vaccinated)	Comment ^c	Evidence: LoE ^d and study quality ^e
Non-TNFi bDMARD							
B cell–targeted therapy							
Belimumab	1 CS (abstract) [s152] 1 CR [s148]	14 Preg (exposure NA) 14 LB	SII: 1/14	SAE: 0/1	RV: 0/1 BCG: NA	The limited evidence did not show increased risk of SII. Most pregnancies were exposed during T3. No studies with control group available. One case report without any adverse event to vaccination with RV and with adequate serologic vaccination response. Very limited evidence showed possible B cell depletion in neonates exposed to belimumab <i>in utero</i> (exposure time late T2). No SII in 1 infant with B cell depletion occurred.	4 2 poor
Rituximab	3 CS [s54,s154,s156] 4 CR [s158,s162–s164] 2 CSE [s165,s166]	280 Preg (PRE ≥111 T1 ≥9; T2 ≥21; T3 ≥10) 187 LB	SII: 4/194	SAE: 0/2	RV: NA BCG: NA	Three cohort studies showed that most <i>in utero</i> exposed infants did not develop SII. Regarding the exposure time, more than half of the pregnancies were exposed to rituximab before conception. No studies with control group available. Very limited evidence did not show a SAE to nonlive vaccines. One cohort study without controls reports haematologic alterations in 12% of the neonates. Most haematologic changes occurred with treatment in the second half of pregnancy. The haematologic changes normalised within weeks to 4 mo. No increased evidence of SII in neonates with B cell depletion or other haematologic changes.	4 9 poor
T cell costimulation inhibitor							
Abatacept	1 CR [s188]	1 Preg (exposure NA) 1 LB	SII: 0/1	SAE: 0/1	RV: 0/1 BCG: 0/1	One case report with <i>in utero</i> exposure in T1 without SII. Very limited evidence without any adverse events to nonlive or live vaccines (RV and BCG after the age of 6 mo).	4 1 poor
Interleukin inhibitors							
Anakinra	1 CS [s174] 1 CR [s177]	24 Preg (exposure NA) 23 LB	SII: 0/23	SAE: 0/1	RV: NA BCG: NA	Limited evidence did not show increased rates for SII. One case report without any adverse events to nonlive vaccines.	4 2 poor
Canakinumab	1 CS [s174] 1 CSE (abstract) [s210]	11 Preg (exposure NA) 9 LB	SII: 0/9	SAE: 0/1	RV: NA BCG: NA	Very limited evidence without increased rates for SII. Very limited evidence showing no adverse events to nonlive vaccines and reporting adequate serologic vaccination response.	4 2 poor
Sarilumab	1 CR [s182]	1 Preg (exposure NA) 1 LB	SII: NA	SAE: NA	RV: NA BCG: 0/1	Very limited evidence showing no adverse events to live vaccines including BCG (time of administration of BCG after the age of 6 mo).	4 1 poor

(continued on next page)

Table 4 (Continued)

Drug	No. of studies per study type [Ref] ^a	Total exposed pregnancies (n) (per time points); total live births (n) ^b	Serious infant infections with hospitalisation during first year of life (per total pregnancies)	Serious adverse events after nonlive vaccination within first year of life (per total infants vaccinated)	Serious adverse events after live vaccination within first year of life (per total infants vaccinated)	Comment ^c	Evidence: LoE ^d and study quality ^e
Tocilizumab	2 CR [s186,s187] 1 CSE [s190]	4 Preg (exposure NA) 4 LB	SII: 0/4	SAE: 0/4	RV: 0/1 BCG: 0/2	Very limited evidence without increased rates for SII. Very limited evidence without any adverse events to nonlive vaccines. One small case series without any adverse events to live vaccines (time of administration of BCG not documented).	4 3 poor
Ustekinumab	3 CS [s106,s195,s197] 2 CR [s141,s198]	50 Preg (PRE 1; T1 ≥46; T2 ≥46; T3≥40) 45 LB	SII: 3/45	SAE: 0/1	RV: NA BCG: 0/1	One cohort study with control group did not show an increased risk of SII. Very limited evidence without any adverse events to nonlive vaccines. Very limited evidence without any adverse events to BCG vaccination after the age of 6 mo.	2b 1 good 4 poor
Others	Anifrolumab, eculizumab, guselkumab, risankizumab, secukinumab, ixekizumab, and mepolizumab: No studies available					Due to the molecular structure analogous to most other biologics, a similar risk profile is expected.	5

BCG, Bacillus Calmette–Guérin; bDMARD, biologic disease-modifying antirheumatic drug; CCS, case-control study; C5i, complement protein C5 inhibitor; CR, case report; CS, cohort study; CSE, case series; CSS, cross-sectional study; IFNAR1, interferon α/β receptor subunit 1 inhibitor; ILi, interleukin inhibitor; LoE, level of evidence; NA, not available; PRE, exposure prepregnancy; RV, rotavirus; SAE, serious adverse event; SII, serious infant infection; SLR, systematic literature review; T1, exposure in first trimester; T2, exposure in second trimester; T3, exposure in third trimester; TNFi, tumour necrosis factor inhibitor.

^a The complete list of references is available in the Supplementary Material.

^b The number of pregnancies and live births may vary due to different data reporting in the different studies. The same applies to the number of reported outcomes, where the number of pregnancies or live births may differ compared with the reported denominator. The same applies to the number of exposed pregnancies during the various exposure periods, which may differ from the total number of reported pregnancies. As the exposure period was not recorded in all studies, the number of pregnancies that were exposed during at least this period is used.

^c Summary including evidence of the present SLR of all adverse infant outcomes. Where available, only high-quality studies were considered.

^d Level of evidence according to studies retrieved in the SLR of EULAR points to consider 2016 and the 2024 SLR Update as well as additional studies (including additional SLRs and MA).

^e Study quality rated as good, fair, and poor based on the Newcastle–Ottawa Scale; CR and CSE were rated as poor.

^f All studies with different TNFi and studies not differentiating between the different TNFi.

^g Studies not differentiating between the different TNFi (if different bDMARD including >80% TNFi).

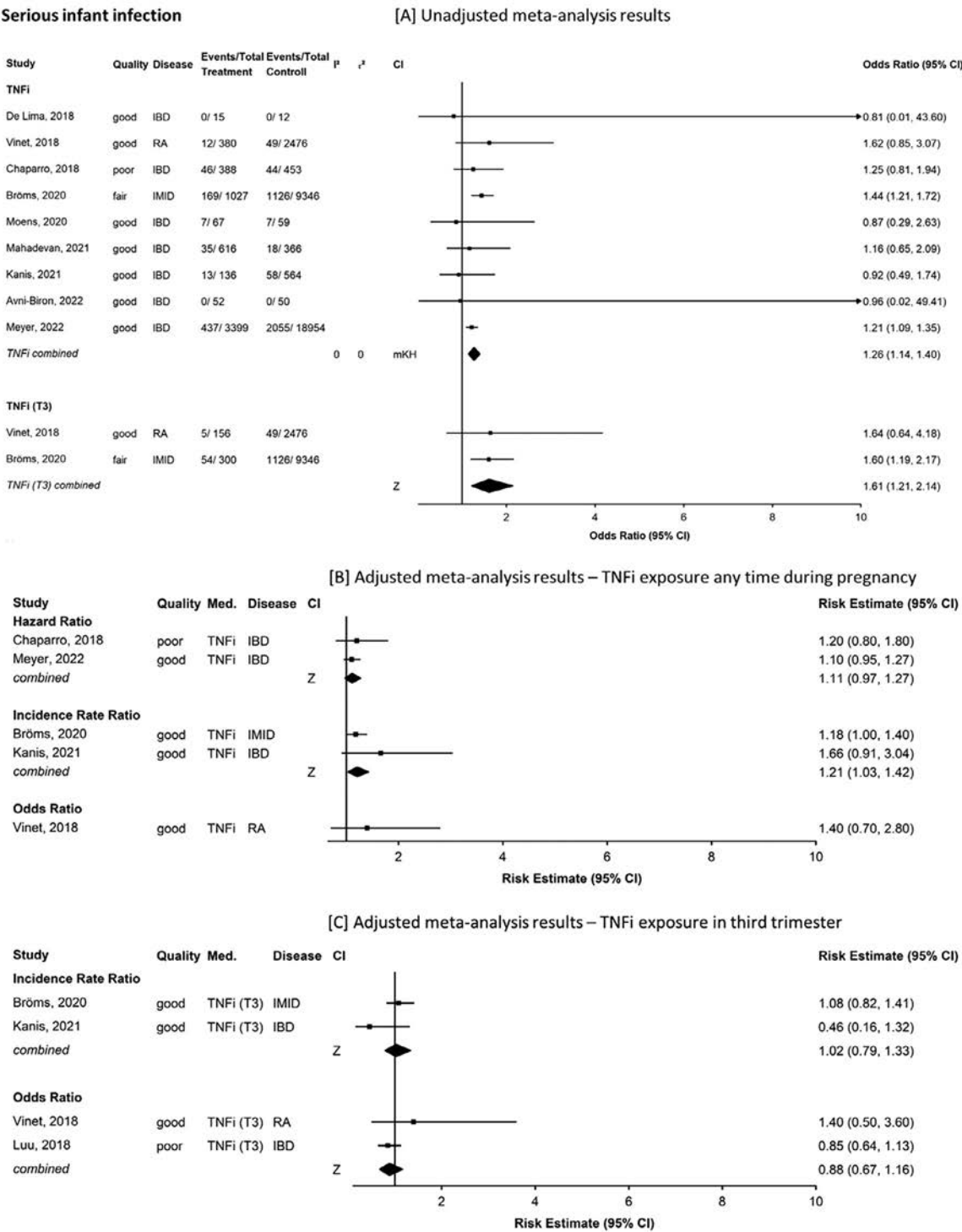


Figure. Serious infant infection risk associated with TNFi use during pregnancy investigated (A) unadjusted meta-analysis, (B) adjusted meta-analysis using TNFi exposure any time during pregnancy and (C) adjusted meta-analysis using TNFi exposure during third trimester. A fixed-effects normal-normal model with CIs based on normal approximation was used. Since no heterogeneity was estimated and the normal approximation was used, the results must be treated with caution. Combined effects of at least 3 studies: A random-effects normal-normal model, Paule Mandel estimates for τ , and Hartung–Knapp–Sidik–Jonkman (HKSJ) CIs were used. It was evaluated if the interval resulting from the HKSJ interval was shorter than an interval based on the DerSimonian–Laird method. If so, the variance correction method was used. If the resulting lower and/or upper bound from the CI of an estimate was smaller or larger, respectively, than the union of the combined study estimates, the resulting estimate was deemed uninformative and therefore removed. IBD, inflammatory bowel disease; IMiD, immune-mediated inflammatory disease; mKH, modified Knapp–Hartung; RA, rheumatoid arthritis; TNFi, tumour necrosis factor inhibitors; Z, CI based on fixed-effects normal-normal model.

inhibitors) but did not specify population or NSAID dosage (Table 5). One study reported comedication, noting corticosteroid use in 57.9% of cases [71]. Analysis of national health insurance data from South Korea revealed a 9% increased risk of oligohydramnios in women exposed to NSAIDs compared with unexposed women during the first 19 weeks of gestation (relative risk: 1.09; 95% CI: 1.01,

1.19) [71]. Further stratification into nonselective NSAIDs and selective COX-2 inhibitors showed aRRs of 1.08 (95% CI: 1.00, 1.18) and of 2.98 (95% CI: 1.70, 5.22), respectively. Despite a low overall rate, treatment duration had a significant impact, with evident risk after >10 days of exposure (aRR: 1.19; 95% CI: 1.00, 1.42). Data of the German teratology information service showed an increase in oligohydramnios risk with NSAID use in

Table 5
Effect of NSAID use on adverse fetal health outcomes: summary of results

Author, year [Ref] ^a	Study type	No. of pregnancies	NSAID type	Outcomes and results	Conclusions	Study quality ^b
Choi et al, 2023 [s25]	CS	n = 124,957	Both nonselective and COX-2 selective NSAIDs	Oligohydramnios 884/124,957 (0.7%), in comparison with unexposed women (aOR: 1.09; 95% CI: 1.01, 1.19)	Higher risk of oligohydramnios in women exposed to NSAIDs during pregnancy, especially with longer exposure times (>10 d). Increased risk with coxibs in comparison with nonselective NSAIDs.	Poor
Dathe et al, 2022 [s26]	CS	n = 1154 (T1 exposure) n = 1092 (T2/T3 exposure)	Both nonselective and COX-2 selective NSAIDs	Oligohydramnios T1: 29/1154 (2.5%) T2/T3: 41/1092 (3.8%) Limited to T2 exposure: 8/904 (0.9%) PDA T1: 10/1133 (0.9%) T2/T3: 15/1098 (1.4%) CDA T1: 0/1154 (0%) T2/T3: 5/1092 (0.5%) Risk estimates comparing T1 with T2/T3 exposure: no difference except for oligohydramnios in T2—RR: 5.1 (95% CI: 1.1, 24.0)	NSAIDs use in the second trimester limited to a few days is not associated with fetal health issues. Prolonged use in the advanced second trimester is associated with oligohydramnios.	Good

aOR, adjusted odds ratio; CDA, constriction of ductus arteriosus; CS, cohort study, COX, cyclooxygenase; NSAID, nonsteroidal anti-inflammatory drug; PDA, patent ductus arteriosus; RR, relative risk; T1, exposure in first trimester; T2, exposure in second trimester; T3, exposure in third trimester.

^a The complete list of references is available in the Supplementary Material.
^b Study quality rated as good, fair, and poor based on the Newcastle–Ottawa Scale.

the advanced second trimester (0.9% in exposed vs 0.2% in controls), but short-term use (few days) in the second trimester did not appear to increase the risk [72]. In this database, constriction of ductus arteriosus occurred in 0.5% of NSAIDs exposed women and patent ductus arteriosus in 1.4%. For the latter outcome, frequency was higher in PTB infants (6.8%).

Effect of NSAID use on maternal fertility

The PIO question regarding the impact of NSAIDs on fertility was added to this SLR. Five prospective cohort studies (3 in RMD, 2 in pain, and 1 in mixed RMD and pain; study quality 60% good, 40% poor) addressed a variety of outcomes including luteinised unruptured follicle syndrome, subfertility (absence of ongoing pregnancy or time-to-pregnancy >12 months), adjusted fecundability ratio, and ovarian reserve markers (antral follicle count, ovarian volume, and circulating hormone levels) (Table 6).

Luteinised unruptured follicle syndrome was more frequent in NSAID-exposed women. An independent subfertility risk with NSAID exposure was demonstrated for women with RA (adjusted hazard ratio for pregnancy: 0.66; 95% CI: 0.46, 0.94), after adjustment for disease activity and comedication [73]. One study showed a reduced adjusted fecundability ratio for naproxen with a dose-response effect [74]. No differences were revealed for ovarian reserve markers in juvenile idiopathic arthritis women versus controls [75]. In summary, the evidence indicated a negative impact of NSAIDs, especially naproxen, ibuprofen, and etoricoxib, on fertility, primarily affecting ovulation, not ovarian reserve.

ARDs in breastfeeding women

This SLR identified 42 articles, most of which addressed pharmacokinetic aspects with drug levels in breast milk in small

number of cases and follow-up of exposed infants. The cumulative evidence on ARDs in breastfeeding women summarises previous EULAR SLR data and new data, (Table 7) [4].

bDMARDs

Based upon the previous EULAR consensus, TNFi are considered compatible with breastfeeding, whereas non-TNFi bDMARDs with no data should be avoided if other therapy is available [4]. Most new data were available on bDMARDs, 245 cases with exposure to TNFi bDMARDs [4,57,63,76–82], and 69 cases with exposure to non-TNFi bDMARDs [59,83–98]. All reports showed that bDMARDs are undetectable or appear in minimal amounts (<0.5 µg/mL) in breast milk [76,78,81–83,86–89,91–98]. Limited data on bDMARDs (tocilizumab) in colostrum, the low volume of protein-rich milk produced during the first 5 days postpartum, revealed marginally higher levels compared with mature milk [92–94,99]. In infants of mothers starting treatment during lactation, bDMARDs (rituximab) were undetectable in the serum [87,89]. During follow-up of breastfed infants of mothers on various bDMARDs, no serious infections, serious adverse effects to vaccinations, or cytopenias were reported [57,59,63,76–80,83,85–91,93–98].

Traditional lactation-compatible conventional synthetic disease-modifying ARDs and anti-inflammatory drugs

The previously EULAR PtC lists lactation-compatible conventional synthetic disease-modifying ARDs (csDMARDs) [4], for which we found new data on hydroxychloroquine, tacrolimus, and mesalamine (active metabolite of sulfasalazine) revealing very low levels in human milk; 102 breastfed infants of mother on thiopurines showed no increased rate of infection in the first 12 months of life [76,100,101]. No new data have emerged on NSAIDs, IVIG, and colchicine [4].

Table 6
Effect of NSAID exposure on fertility: summary of results

Author, year [Ref] ^a	Study type	Population; No. of patients	NSAID type	Outcomes	Results	Conclusions	Study quality ^b
Tomioka et al, 2018 [s211]	CS	JIA n = 8 (n = 26 controls)	NA	LUF syndrome Antral follicle count Ovarian volume Hormones levels	LUF syndrome: 25% vs 0% in JIA and healthy controls Antral follicle count, ovarian volume, and hormones levels similar to controls	More LUF syndrome in patients with JIA on NSAIDs compared with controls (patients with JIA but without NSAIDs and HC), cofounded by disease activity. Ovarian reserve unaffected.	Good
Micu et al, 2011 [s212]	CS	RA and r-axSpA n = 14 (59 cycles) Low back pain n = 29 (29 cycles) (n = 446 controls)	Both nonselective NSAIDs and selective COX-2 inhibitors	LUF syndrome	LUF syndrome: 35.6% in patients with RMD (etoricoxib: 75% vs. diclofenac: 15%) 24.1% in patients with low back pain (etoricoxib: 56% vs. diclofenac: 14.3%) 3.4% in controls No LUF syndrome on ketoprofen, ibuprofen, celecoxib, and nimesulide	Higher frequency of LUF syndrome in patients on NSAIDs compared with unexposed women. LUF only occurred in cases with etoricoxib and diclofenac exposure.	Good
Brouwer et al, 2015 [s213]	CS	RA n = 60 (n = 185 unexposed)	Both nonselective NSAIDs and selective COX-2 inhibitors	Subfertility (no pregnancy or TTP >12 mo)	Subfertility: 58.3% and 37.2% in patients with RA on NSAIDs vs unexposed patients aHR for occurrence of pregnancy 0.66; 95% CI: 0.46, 0.94	Higher risk of subfertility in patients with RA on NSAIDs compared with unexposed patients	Good
McInerney et al, 2017 [s214]	CS	Pain n = 523 acetaminophen n = 884 ibuprofen n = 156 naproxen	Acetaminophen, aspirin, ibuprofen, and naproxen	aFR	Acetaminophen aFR: 1.04; 95% CI: 0.92, 1.18 Ibuprofen aFR: 1.00; 95% CI: 0.89, 1.11 Naproxen aFR: 0.78; 95% CI: 0.64, 0.97	Reduced fecundability with naproxen, with a dose-dependent effect	Poor
Jukic et al, 2020 [s215]	CS	Pain n = 150 acetaminophen n = 33 ibuprofen and naproxen	Acetaminophen, aspirin, ibuprofen, and naproxen	aFR	Acetaminophen aFR: 1.02; 95% CI: 0.73, 1.44 Ibuprofen and naproxen aFR: 1.00; 95% CI: 0.73, 1.46	No impact of acetaminophen, ibuprofen, and naproxen on fecundability.	Poor

aFR, adjusted fecundability ratio; aHR, adjusted hazard ratio; COX, cyclooxygenase; CS, cohort study; HC, healthy control; JIA, juvenile idiopathic arthritis; LUF syndrome, luteinised unruptured follicle syndrome; NA, not available; NSAID, nonsteroidal anti-inflammatory drugs; RA, rheumatoid arthritis; r-axSpA, radiographic axial spondyloarthritis; RMD, rheumatic and musculoskeletal disease; TTP, time-to-pregnancy.

^a The complete list of references is available in the Supplementary Material.
^b Study quality rated as good, fair, and poor based on the Newcastle–Ottawa Scale.

Glucocorticoids

In the previous EULAR consensus, GCs were considered compatible with breastfeeding [4]. We added data on both oral prednisone/prednisolone (4.0-15.0 mg/d) and intravenous methylprednisolone pulses (1000 mg) that appeared at very low concentrations in breast milk [102–106]. The maximum methylprednisolone levels occurred 1 to 2 hours after the application [103,104].

Drugs with limited data in lactation

We added new data on ARD in lactation. Case reports showed very low levels of sildenafil and bosentan in breast milk, and no harmful effects in breastfed infants [107,108]. The 2016 EULAR SLR and LactMed indicated that methotrexate is unlikely to be excreted into breast milk due to its lipid insoluble form at physiologic pH [4]. In 6 cases, methotrexate appeared at undetectable or very low levels in breast milk after both, high dose (given for malignancy) and low dose (≤25.0 mg weekly), and no evidence of harm during 9 months follow-up [109]. Very few cases have emerged on the use of the Janus kinase inhibitor (JAKi) tofacitinib in lactating women, showing low maximum drug concentration in breast milk samples (<0.1 µg/mL) and no severe adverse events in exposed infants [110–112]. Regarding

mycophenolate, data on breast milk were insufficient, and values of the estimated relative infant dose showed unacceptable variations [113,114]. As for cyclophosphamide, no new data have emerged [4,109].

Reproductive safety of ARDs in male patients

Two recent SLRs on this topic [115,116] were appraised, and an updated search from 2019 to 2023 identified 16 new articles (Table 8). Most of the new data came from 4 studies that evaluated the effect of methotrexate on male fertility. The study quality ranged from fair to good, with most new studies graded as good quality.

General findings

Treatment with various ARD, including azathioprine, mercaptopurine, colchicine, cyclosporine, hydroxychloroquine, chloroquine, filgotinib, IVIG, leflunomide, methotrexate ≤25.0 mg/wk, mycophenolate, NSAIDs, prednisone, prednisolone, sildenafil, sulfasalazine, tacrolimus, TNFi bDMARDs, and non-TNFi bDMARDs did not show a clinically relevant impact on fertility (semen parameters) or pregnancy outcomes.

Table 7

Lactation data on antirheumatic drugs: summary of results stratified by drug

Drug	No. of exposed cases	No. of studies per study type [Ref] ^a	Drug detected in breast milk	Dose	Drug detected in infant blood	Reported side effects in breastfed children	Evidence: LoE ^b and study quality ^c
Glucocorticoids							
Prednisone/prednisolone MP pulse	56 P 26 MP 30	SLR [s216] 3 CS [s217–s219] 2 CR [s220,s221]	Detected at low levels MP pulse: Cavg: 0.032–1.382 $\mu\text{g/mL}$ levels drop exponentially	Prednisone/prednisolone (4.0–15.0 mg) RID: 0.35–0.58/0.09–0.18 Milk:plasma ratio _{AUC 24h} : 0.5–0.6/0.02–0.03 MP pulse (1000 mg) RID 0.1–0.71 Milk:serum ratio _{AUC 24h} : 0.27	No data	No adverse events	2a 3 good 2 poor
NSAIDs							
Nonselective: e.g. ibuprofen Selective: e.g. celecoxib	53	SLR [s216]	Detected at very low levels, most reassuring data for short-half life agents, eg, ibuprofen	RID: <0.1–1.2	Not detected (celecoxib)	3–12 mo FU: no adverse events.	2a ibuprofen 4 celecoxib 5 etoricoxib poor
csDMARDs, immunosuppressive, and other drugs							
Antimalarials: HCQ and CQ	112 HCQ: 51 CQ: 61	SLR [s216] 1 CS [s222]	Detected at low levels Cavg: 0.4–1.4 $\mu\text{g/mL}$ (HCQ)	RID: 1.9–3.2 (HCQ) RID: 0.6–14 (CQ)	No data	No adverse events	2a HCQ 4 CQ poor 2a
Azathioprine and active metabolite (6-mercaptopurine)	241	SLR [s216] 1 CS [s223]	Not detected (n = 14) detected (n = 11)	RID: <1 Dose: <0.1% of paediatric transplant dose	Not detected (n = 16); detected at low level (n = 1)	No adverse events (n = 225), 12 mo FU: no increased infection rate with thiopurines + TNFi vs controls (n = 67); neutropenia (n = 1, possibly due to reduced TPMT activity).	good 2a
Bosentan	2	2 CR [s224]	Detected at very low concentrations Cavg 0.05 $\mu\text{g/mL}$	RID 0.24–0.33	No data	No adverse events (n = 2).	4 poor
Colchicine	154	SLR [s216]	Detected (n = 6)	RID <10	Not detected (n = 1)	No adverse events (n = 149).	2a poor
Cyclophosphamide	3	SLR [s216]	Detected (n = 1)	No data	No data	Neutropenia and bone marrow suppression (n = 2).	4 poor
Cyclosporine	76	SLR [s216]	Variable titres depending on fat content in sampled milk	RID: <2 Dose: <2% of paediatric transplant dose	Not detected (n = 12); detected (n = 2)	No adverse events	2a poor
IVIG	149	SLR [s216]	Expected to be poorly transferred into breast milk	No data	No data	No adverse events (n = 146) Transient rash (n = 1)	2a poor
Leflunomide	No studies available	SLR [s216]					5
Methotrexate	3	SLR [s216]	Very low transfer into breast milk due to lipid insoluble nature, MTX 25.0 mg/wk: below quantifiable limits	RID about 1	No data	9 mo FU: no adverse events [s216] (n = 1)	4 MTX ≤ 25 mg/wk poor
Mycophenolic acid products	10	SLR [s216] 1 CSE [s225] 1 CR [s226]	Detectable at variable levels: MPA (n = 2): undetectable to Cmax of 0.08 $\mu\text{g/mL}$ MMF (n = 1): Cmax of 0.13 $\mu\text{g/mL}$	Very heterogeneous data: RID: 0–83 (MPA), 0.02 (MMF)	No data	No adverse events (n = 7)	5 poor

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Table 7 (Continued)

Drug	No. of exposed cases	No. of studies per study type [Ref] ^a	Drug detected in breast milk	Dose	Drug detected in infant blood	Reported side effects in breastfed children	Evidence: LoE ^b and study quality ^c
Sildenafil	2	2 CR [s224,s227]	Detected at low concentrations (20.0 mg, 3×/d)—Cavg: 0.003 µg/mL; Cmax: 0.006 µg/mL)	RID 0.06-0.08	No data	No adverse events (n = 2)	4 poor
Sulfasalazine and active metabolite mesalamine	39	SLR [s216] 1 CS [s228]	Low levels (mesalamine) Cavg: 0.11 µg/mL Cmax: 1.7 µg/mL	RID: 0.003-0.09	Sulfasalazine not detected (n = 5), detected (n = 2), sulfapyridine ≤10% of maternal level (n = 6)	No adverse events in majority of exposed infants. Bloody diarrhoea (n = 1)	2a poor
Tacrolimus	167	SLR [s216] 1 CS [s229]	Variable titres depending on fat content in sampled milk, in n = 13 Cavg: 0.0004 µg/mL Cmax 0.0012 µg/mL	RID: 0.18 (0.12-0.35) Milk:plasma ratio: 0.12 (0.08-0.24) Dose <0.1% of paediatric transplant dose	Not detected (n = 28), detected level declining with time (n = 4)	No adverse events (n = 136)	2a poor
Avocapan, iloprost, and voclosporin: No studies available							5
tsDMARDs							
Tofacitinib	4	1 CSE [s230] 2 CR [s231,s232]	Detected in all samples Cavg: 0.01 µg/mL (5.0 mg twice daily), 0.01 µg/mL (10.0 mg twice daily) Cmax: 0.03 µg/mL (5.0 mg twice daily), 0.04 µg/mL (10.0 mg twice daily)	Maximal RID: 3.4 (based on peak milk levels, probably lower average) Milk:plasma ratio: 1.07 (0.47-1.68)	No data	No adverse events, eg, serious infections or SAE to mandatory nonlive vaccination and rotavirus live vaccination, at 3 mo FU: normal complete blood count with only mild thrombocytosis, normal immunoglobulin levels	4 poor
Apremilast, baricitinib, filgotinib, and upadacitinib: No studies available							5
TNFi bDMARDs							
adalimumab	245	SLR [s216]	Detected at minimal levels:	RID (CZP): 0.04-0.30	Not detected, serum	No adverse events, eg, similar risks of infection and rates of milestone achievements in exposed (TNFi or TNFi + immunomodulators) vs controls	2a
certolizumab	IFX: >71	6 CS [s106,s112,s114, s115,s223,s233]	IFX—Cmax: 0.15-0.74 µg/mL	Milk:plasma ratio: 0.001-0.05	concentration after <i>in utero</i> exposure		5 good
golimumab	ADA: >31		ADA—Cmax: 0.45-0.71 µg/mL		decreased despite drug exposure during breastfeeding		4 poor
infliximab	GOL: 1 CZP: >41	2 CR [s137,s138] 1 CSE [s140]	CZP—Cmax: 0.08-0.29 µg/mL				
Non-TNFi bDMARDs							
Abatacept	1	1 CR [s234]	Detected at minimal levels—Cmax: 0.256 µg/mL	RID: 1.30 Milk:serum ratio: 0.005	No data	12 mo FU: no adverse events, eg, serious infections or SAE to vaccines including rotavirus and BCG at 6 mo of age	4 poor
Anakinra	12	1 CS [s174] 1 CR [s177]	No data	No data	No data	9 mo FU: no adverse events, eg, serious infections or SAE to vaccines	2a poor
Belimumab	1	1 CR [s235]	Detected at minimal levels—Cmax: 0.17 µg/mL	RID: 3.67 Milk:serum ratio: 0.0041	No data	15 mo FU: no adverse events	4 poor
Canakinumab	5	1 CS [s174] 1 CR [s236]	Detected at minimal levels—Cmax: 0.023 µg/mL	RID: 0.08 Milk:serum ratio: 0.015	Not detected (n = 1)	2 y FU: no adverse events	2a poor
Rituximab and ocrelizumab	23 RTX: 21 OCR: 2	3 CS [s237–s239] 2 CR [s165,s236]	Detected at minimal levels—Cmax: 0.004-0.6 µg/mL	RID: 0.01-0.10 Milk:serum ratio: 0.0001-0.005	Not detected (n = 7)	Normal B cell count and immunoglobulin levels (n = 6), no adverse events (n = 3)	2a 1 fair 4 poor

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Table 7 (Continued)

Drug	No. of exposed cases	No. of studies per study type [Ref] ^a	Drug detected in breast milk	Dose	Drug detected in infant blood	Reported side effects in breastfed children	Evidence: LoE ^b and study quality ^c
Tocilizumab and sarilumab	6	4 CR [s182,s186,s187,s240] 1 CSE [s190]	Detected at minimal levels— Cmax: 0.22–0.39 µg/mL (colostrum), 0.148 µg/mL (mature milk)	RID: 1.30 Milk:serum ratio: 0.01–0.11 (colostrum), 0.001–0.01 (mature milk)	Not detected (n = 1)	6–9 mo FU: no adverse events, eg, serious infections or SAE to vaccines; nonlive vaccines (n = 4) and live vaccine BCG (n = 1)	4 poor
	21	3 CS [s106,s195,s223] 2 CR [s198,s201]	Detected at minimal levels— Cmax: 0.014–3.2 µg/mL	Milk:serum ratio: 0.001–0.005	Infant serum concentration after <i>in utero</i> exposure decreased despite drug exposure during breastfeeding	12 mo FU: no adverse events	2a 4 good 1 poor
Anifrolumab, eculizumab, guselkumab, mepolizumab, secukinumab, and ixekizumab: No studies available							

ADA, adalimumab; BCG, Bacillus Calmette–Guérin; bDMARD, biologic disease-modifying antirheumatic drug; Cavg, average concentration; C5i, complement protein C5 inhibitor; Cmax, maximum concentration; CQ, chloroquine; CR, case report; CS, cohort study; csDMARD, conventional synthetic disease-modifying antirheumatic drug; CSE, case series; CZP, certolizumab; FU, follow-up time; GOL, golimumab; HCQ, hydroxychloroquine; IFNAR1, interferon α/β receptor subunit 1 inhibitor; IFX, infliximab; ILi, interleukin inhibitor; IVIG, intravenous immunoglobulin; LoE, level of evidence; MMF, mycophenolate mofetil; MP, methylprednisolone pulse; MPA, mycophenolic acid; MTX, methotrexate; NSAID, nonsteroidal anti-inflammatory drugs; OCR, ocrelizumab; P, prednisone/prednisolone; RID, relative infant dose (given as %); RTX, rituximab; SAE, serious adverse event; SAR, sarilumab; SLR, systematic literature review; SSZ, sulfasalazine; TCZ, tocilizumab; TNFi, tumour necrosis factor inhibitor; TPMT, thiopurine methyltransferase.

^a The complete list of references is available in the Supplementary Material.
^b Level of evidence based on EULAR SLR 2016 [4], 2024 SLR Update and LactMed.
^c Study quality rated as good, fair, and poor based on the Newcastle–Ottawa Scale; CR and CSE were categorised as poor.

Methotrexate

New evidence supports the safety of weekly methotrexate doses (≤ 25.0 mg/wk) on semen parameters, sperm DNA fragmentation index, and reproductive hormones [117,118]. A large retrospective cohort study involving over 171 paternal methotrexate-exposed pregnancies found no increased risk of major congenital malformations, LBW, or PTB (relative risk: 0.67; 95% CI: 0.21, 1.55) [117].

TNFi bDMARDs

Evidence from studies involving more than 1500 cases indicates that TNFi do not impair semen parameters or lead to adverse birth outcomes. Notably, some studies reported an improvement in semen parameters following TNFi exposure [115,116]. The risk for adverse birth outcomes with TNFi was not elevated (relative risk: 1.14; 95% CI: 0.81, 1.57) [117].

Non-TNFi bDMARDs

Based on very limited data, no negative effects on fertility and pregnancy outcomes were reported for non-TNFi bDMARDs such as abatacept, canakinumab, ixekizumab, ustekinumab, tocilizumab, and anakinra [116,118].

Targeted synthetic disease-modifying ARDs

A recent randomised controlled trial involving 249 patients demonstrated no measurable impact on semen parameters or sex hormones after at least 13 weeks of filgotinib (200.0 mg/d) [119]. Data on pregnancy and offspring outcomes was reported on only 3 pregnancies resulting in 3 healthy offsprings. Limited to no information is available for the other JAKi.

Other medications

Thiopurines (azathioprine and 6-mercaptopurine) and calcineurin inhibitors (cyclosporine and tacrolimus) showed no adverse effects on semen parameters or APO [115]. Limited evidence suggests that antimalarials (hydroxychloroquine and chloroquine) and mycophenolate have no negative effect on sperm quality [115,116]. Studies on teriflunomide (active metabolite of leflunomide), including 63 cases, reported no APO.

Colchicine does not negatively impact semen parameters at therapeutic doses and was not associated with an increased risk of spontaneous abortions [115]. Sildenafil improves sperm motility and morphology and is safe for use in men planning to conceive [120].

Sulfasalazine has been associated with reversible asthenozoospermia and decreased sperm counts [115,116]. Regarding pregnancy outcomes, data on sulfasalazine are reassuring [116].

Exposure to cyclophosphamide has been linked to reduced sperm counts and a dose-related risk for irreversible infertility, particularly at doses ≥ 4000.0 mg/m² [115].

DISCUSSION

This SLR provided crucial insights to the EULAR TF for the 2024 update of recommendations on ARDs in reproduction, pregnancy, and lactation. The SLR included data on the safety of ARDs concerning miscarriage, malformation, and breastfeeding from 2015 onwards, and it additionally covered additional pregnancy outcomes, newly approved drugs, other RMD drugs, and the impact of ARDs on male fertility and offspring outcomes. Among the 255 included studies, 86% focused on maternal exposure to drugs during pregnancy. The broad search strategy aimed to capture as much information as possible, including

Table 8

Male exposure to antirheumatic drugs and reproductive safety: summary of results stratified by drug

Study, year [Ref] ^a	Type of study; No. total exposed cases and controls	Drug	Impact on fertility: sperm quality (sperm concentration, sperm motility, and sperm DNA fragmentation index), fecundability, and reproductive hormones	Impact on adverse pregnancy outcomes: MC, elective termination of pregnancy, SB, PTB, SGA, LBW, BDs	Evidence: LoE ^b and study quality ^c
Prednisone, prednisolone	4165				2b
Perez-Garcia et al, 2020 [s241]	SLR (7 studies) exp: 4165 ctr: 1,011,691	Prednisone and prednisolone	One study showed no adverse effect on human testis (after 75.0 mg cortisone, n = 4). One study reported slightly elevated FSH and LH (n = 36).	No adverse drug-related effects on pregnancy outcomes. No increased rates of MC, PTB, SGA, and BD.	Fair
NSAIDs	861				2b
Wesseling et al, 2020 [s242]	CS exp: 812 ctr: 944 (HC)	Ibuprofen (200.0 mg) and naproxen (220.0 mg)	Ibuprofen and naproxen used at low dose do not impair fecundability.	NA	Fair
Perez-Garcia et al, 2020 [s241]	SLR (5 studies) exp: 49 ctr: 29	Acetylsalicylic acid, ibuprofen, indomethacin, and naproxen	Limited evidence showed no adverse effect on fertility.	NA	Fair
csDMARDs, immunosuppressive, and other drugs					
Antimalarials	50				2c
Perez-Garcia et al, 2020 [s241]	SLR (4 studies) exp: 37	Chloroquine	No adverse effect on sperm quality. No adverse effect on reproductive hormones.	NA	Poor
Mouyis et al, 2019 [s243]	SLR (2 studies on pregnancy outcome) exp: 13 1034	Hydroxychloroquine	NA	No adverse drug-related effects on pregnancy outcomes.	Poor
Calcineurin inhibitors	1034				2b
Perez-Garcia et al, 2020 [s241]	SLR (23 studies) exp: 851 ctr: 417,793	Cyclosporine, sirolimus, and tacrolimus	Majority of data did not report relevant sperm abnormalities. No negative effect on reproductive hormones.	No adverse drug-related effects on pregnancy outcomes.	Fair
Mouyis et al, 2019 [s243]	SLR (3 studies) exp: 183 339	Cyclosporine	Limited evidence showed no adverse effect on fertility.	No adverse drug-related effects on pregnancy outcomes.	Fair
Colchicine	339				2c
Perez-Garcia et al, 2020 [s241]	SLR (11 studies) exp: 158	Colchicine	Inconsistent reports with confounding by disease activity and severity. Therapeutic doses of colchicine are not likely to affect sperm motility and production.	No increased risk of spontaneous abortions.	Fair
Cyclophosphamide	315				2b
Tiseo et al, 2019 [s244]	CS exp: 12	Cyclophosphamide IV (median cumulative dose: 10.5 g)	Patients with nonazoospermic SLE had increased sperm DFI without evident gonadal dysfunction, an abnormality confounded by indication.	NA	Fair
Perez-Garcia et al, 2020 [s241]	SLR (21 studies) exp: 303 ctr: 88	Cyclophosphamide	Dose-dependent negative effect on sperm quality (azoospermia and teratospermia). Negative effect on reproductive hormones.	Data confounded by treatment indication and use of comedications, especially for use in malignancies.	Fair
Leflunomide and metabolite	85				2c
Andersen et al, 2022 [s78]	CSS exp: 63 ctr: 364 (HC)	Teriflunomide	NA	No adverse drug-related effects on pregnancy outcomes.	Fair

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Table 8 (Continued)

Study, year [Ref] ^a	Type of study; No. total exposed cases and controls	Drug	Impact on fertility: sperm quality (sperm concentration, sperm motility, and sperm DNA fragmentation index), fecundability, and reproductive hormones	Impact on adverse pregnancy outcomes: MC, elective termination of pregnancy, SB, PTB, SGA, LBW, BDs	Evidence: LoE ^b and study quality ^c
Kieseier and Benamor, 2014 [s82]	CS exp: 19	Teriflunomide	NA	No adverse drug-related effects on pregnancy outcomes.	Poor
Perez-Garcia et al, 2020 [s241]	SLR (1 study) exp: 1	Leflunomide	NA	No adverse drug-related effects on pregnancy outcomes.	Poor
Mouyis et al, 2019 [s243]	SLR (2 studies) exp: 2 1143	Leflunomide	NA	No adverse drug-related effects on pregnancy outcomes.	Poor
Methotrexate					2b
Perez-Garcia et al, 2023 [s245]	CS exp: 25 ctr: 25 (HC)	Methotrexate (16.0-18.0 mg/wk)	No adverse effect on sperm quality (SC, SM, DFI). Very low concentrations of MTX-polyglutamates in spermatozoa.	NA	Good
Grosen et al, 2022 [s246]	CSS exp: 14 ctr: 40 (HC)	Methotrexate (12.5 mg/wk)	No negative effect on reproductive hormones. No adverse effect on sperm quality (SC, SM, DFI). No negative effect on reproductive hormones.	NA	Good
Meserve et al, 2021 [s247]	CS exp: 171 ctr: 5607 (DC)	Methotrexate	NA	No adverse drug-related effects on pregnancy outcomes, adjusted risk estimates showed no increased risk of PTB, LBW, and BD.	Good
Salama et al, 2019 [s248]	CR exp: 1	Methotrexate (10.0 mg/wk)	Normal sperm quality (SC, SM, and DFI). No negative effect on reproductive hormones.	NA	Poor
Perez-Garcia et al, 2020 [s241]	SLR (14 studies) exp: 932 ctr: 2,379,668	Methotrexate	No adverse effect on sperm quality in most exposed patients. No negative effect on reproductive hormones.	No adverse drug-related effects on pregnancy outcomes; adjusted risk estimates showed no increased risk of MC, PTB, SGA, and BD.	Fair
Mycophenolate					2b
Perez-Garcia et al, 2020 [s241]	299 SLR (4 studies) exp: 295 ctr: >1308	Mycophenolate acid products: mycophenolate mofetil and mycophenolate sodium	NA	No adverse drug-related effects on pregnancy outcomes.	Fair
Mouyis et al, 2019 [s243]	SLR (1 study on fertility) exp: 4 >2782	Mycophenolate mofetil	Very limited evidence did not suggest an adverse effect on fertility.	NA	Poor
Sulfasalazine					2b
Norgard et al, 2022 [s88]	CS exp: 2168 ctr: 7732 (DC)	5-ASA drugs: mesalazine (94%), sulfasalazine (58%), olsalazine (1%-2%), and basalazide (0.3%-1%)	NA	No adverse drug-related effects on pregnancy outcomes, adjusted risk estimates showed no increased rates of PTB, SGA, and BD.	Good
Chatzimeletiou et al, 2021 [s249]	CR exp: 1	Sulfasalazine	SM, DFI normal; abnormal sperm form in 93% (1 patient, analysis of cryoconserved sperm sample)	No BD.	Poor
Perez-Garcia et al, 2020 [s241]	SLR (22 studies on fertility) exp: 329	Aminosalicylic acid and similar agents	Abnormal sperm quality reported in 40%-100% (decreased SC and SM). Sperm quality usually resolved after discontinuation of drug.	NA	Fair

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Table 8 (Continued)

Study, year [Ref] ^a	Type of study; No. total exposed cases and controls	Drug	Impact on fertility: sperm quality (sperm concentration, sperm motility, and sperm DNA fragmentation index), fecundability, and reproductive hormones	Impact on adverse pregnancy outcomes: MC, elective termination of pregnancy, SB, PTB, SGA, LBW, BDs	Evidence: LoE ^b and study quality ^c
Mouyis et al, 2019 [s243]	SLR (12 studies on pregnancy outcome) exp: >284 1647	Sulfasalazine	NA	No adverse drug-related effects on pregnancy outcomes.	Fair
Thiopurines Meserve et al, 2021 [s247]	CS exp: 461 ctr: 5607 (DC)	Azathioprine and 6-mercaptopurine	NA	No adverse drug-related effects on pregnancy outcomes, adjusted risk estimates showed no increased risk of PTB, LBW, and BD.	2b Good
Perez-Garcia et al, 2020 [s241]	SLR (13 studies) exp: 1186 ctr: 1,013,251	Azathioprine and 6-mercaptopurine	No adverse effect on sperm quality (SC, SM, and DFI) in majority of exposed patients, including off vs on cases. No negative effect on reproductive hormones.	No adverse drug-related effects on pregnancy outcomes.	Fair
tsDMARDs Reinisch et al, 2023 [s250]	204 RCT exp: 120 ctr: 120 (DC)	Filgotinib (200.0 mg/d, 13 wk)	No adverse effect on sperm quality (SC, SM). No negative effect on reproductive hormones.	3 healthy live born infants without BD.	1b-4 Good
Perez-Garcia et al, 2020 [s241]	SLR (1 study) exp: 63 1610	Tofacitinib	NA	No adverse drug-related effects on pregnancy outcomes.	Poor
TNFi bDMARDs Meserve et al, 2021 [s247]	CS exp: 1082 ctr: 5607 (DC)	Adalimumab, certolizumab, golimumab, etanercept, and infliximab	NA	No adverse drug-related effects on pregnancy outcomes; adjusted risk estimates showed no increased risk of PTB, LBW, and BD.	1b Good
Perez-Garcia et al, 2020 [s241]	SLR (23 studies) exp: 528 ctr: 412,365	Adalimumab, certolizumab, golimumab, etanercept, and infliximab	No adverse effect on sperm quality (SC, SM, and DFI). Improved sperm quality after initiation of TNFi therapy. No negative effect on reproductive hormones.	No adverse drug-related effects on pregnancy outcomes.	Fair
Non-TNFi bDMARDs Abatacept Perez-Garcia et al, 2020 [s241]	10 SLR (1 study) exp: 10	Abatacept	NA	No adverse drug-related effects on pregnancy outcomes.	4 Poor
Anakinra Perez-Garcia et al, 2020 [s241]	6 SLR (1 study) exp: 6	Anakinra	NA	No adverse drug-related effects on pregnancy outcomes.	4 Poor
Canakinumab Perez-Garcia et al, 2020 [s241]	5 SLR (1 study) exp: 5	Canakinumab	NA	No adverse drug-related effects on pregnancy outcomes.	4 Poor
Ixekizumab Egeberg et al, 2022 [s191]	75 CSE, pharmacovigilance exp: 75	Ixekizumab	NA	No adverse drug-related effects on pregnancy outcomes.	4 Poor
Rituximab Mouyis et al, 2019 [s243]	11 SLR (1 study) exp: 11	Rituximab	NA	No adverse drug-related effects on pregnancy outcomes.	4 Poor

(continued on next page)

Table 8 (Continued)

Study, year [Ref] ^a	Type of study; No. total exposed cases and controls	Drug	Impact on fertility: sperm quality (sperm concentration, sperm motility, and sperm DNA fragmentation index), fecundability, and reproductive hormones	Impact on adverse pregnancy outcomes: MC, elective termination of pregnancy, SB, PTB, SGA, LBW, BDs	Evidence: LoE ^b and study quality ^c
Secukinumab Perez-Garcia et al, 2020 [s241]	54 SLR (1 study) exp: 54	Secukinumab	NA	No adverse drug-related effects on pregnancy outcomes.	4 Poor
Tocilizumab Hoeltzenbein et al, 2016 [s183]	15 CSE, pharmacovigilance exp: 13	Tocilizumab	NA	No adverse drug-related effects on pregnancy outcomes.	4 Poor
Perez-Garcia et al, 2020 [s241]	SLR exp: 2	Tocilizumab	NA	No adverse drug-related effects on pregnancy outcomes.	Poor
Ustekinumab Meserve et al, 2021 [s247]	219 CS exp: 132 ctr 5607 (DC)	Ustekinumab	NA	No adverse drug-related effects on pregnancy outcomes; adjusted risk estimates showed no increased risk of PTB, LBW, and BD.	2b Good
Mahadevan et al, 2021 [s194]	CSE, pharmacovigilance exp: 87	Ustekinumab	NA	No adverse drug-related effects on pregnancy outcomes.	Poor

5-ASA, 5-aminosalicylic acid; FSH, follicle-stimulating hormone; BD, birth defect; bDMARD, biologic disease-modifying antirheumatic drug; CM, congenital malformation; CR, case report; CS, cohort study; csDMARD, conventional synthetic disease-modifying antirheumatic drug; CSE, case series; CSS, cross-sectional study; ctr, control; DC, diseased control; DFI, sperm DNA fragmentation index; exp, exposed cases; HC, healthy control; LBW, low birth weight; LH, luteinising hormone; LoE, level of evidence; MC, miscarriage; NA, not available; NSAID, nonsteroidal anti-inflammatory drug; PTB, preterm birth; RCT, randomised controlled trial; SC, sperm concentration; SGA, small-for-gestation age; SLE, systemic lupus erythematosus; SLR, systematic literature review; SM, sperm motility; TNFi, tumour necrosis factor inhibitor; tsDMARD, targeted synthetic disease-modifying antirheumatic drug.

^a The complete list of references is available in the Supplementary Material.

^b Level of evidence according to studies retrieved in included SLR and the 2024 SLR Update.

^c Study quality rated as good, fair, and poor based on the Newcastle–Ottawa Scale; CR and CSE were categorised as poor. Quality of studies of the SLR rated as low, fair, or good were converted into poor, fair, and good. RoB2 showing low risk of bias was converted into good.

diverse patient groups apart from RMDs, such as those with IBD or multiple sclerosis, in whom ARDs or its active metabolites (eg, teriflunomide and 6-mercaptopurine) or bDMARDs throughout pregnancy were used.

Teratogenic drugs comprise methotrexate, cyclophosphamide, and mycophenolate that should be discontinued before conception, although cyclophosphamide and mycophenolate may be used in the second or third trimester for organ-threatening or life-threatening disease, as most children exposed during these trimesters were born healthy [4,121–124]. Regarding leflunomide or teriflunomide, data suggest no major teratogenic effects, even with unknown or low washout rates [31,36,38,125], with a prospective pharmacovigilance database suggesting discontinuation 3.5 months before conception [125]. Other drugs that should be stopped before conception include those with insufficient data in pregnancy, for example, apremilast, avacopan, bosentan, all JAKi (baricitinib, tofacitinib, upadacitinib, and filgotinib), mepacrine, and voclosporine.

Current evidence remains reassuring for csDMARDs already considered compatible with pregnancy in the previous EULAR consensus [4]. These drugs include antimalarials, azathioprine, colchicine, cyclosporine, sulfasalazine, and tacrolimus, all showing favourable safety profiles without increasing the risk for APOs.

Further, bDMARDs, particularly TNFi, have a largely safe profile during pregnancy. Our meta-analyses with adjusted risk estimates did not show significant increases in malformations, PTB, SGA, LBW, or SII in pregnancies exposed to TNFi, consistent with previous meta-analyses [126]. Crude risk estimates did show an increased risk for these outcomes, highlighting the importance of considering disease activity and severity [55,127–129]. In addition, TNFi use was not associated with miscarriage and stillbirth. Data on non-TNFi biologics in pregnancy are limited and often lack comparison with unexposed diseased controls, but existing evidence does not indicate significant concerns about adverse pregnancy or infant outcomes [54,57,61,62,70,84]. Transient B cell depletion or cytopenias may occur in neonates exposed *in utero* to rituximab or belimumab, with recovery within 6 months [54,62,130,131]. Concerns about immunologic changes, including adverse vaccination effects in infants exposed *in utero* to bDMARDs, exist due to transplacental passage. However, no serious adverse events were reported with nonlive vaccines, and immunologic responses were similar to unexposed infants [54]. In addition, no serious adverse events were linked to live rotavirus vaccines, consistent with a recent larger cohort study [67]. BCG vaccination safety in bDMARDs-exposed infants is a concern in the first 6 months but is considered safe thereafter [68,70].

Regarding oral GC in pregnancy, this SLR confirmed no increased rate of major birth defects [4,13,14,62,132], but there are other relevant dose-related risks for the mother and the newborn [133–136]. A large retrospective study showed an increased risk of serious maternal infections at daily doses of 10.0 mg [133]. Our meta-analysis, including studies adjusted for disease severity, revealed an association of prednisone with PTB [109,134,135,137,138]. A cumulative dose above 300.0 mg prednisone within the first 20 weeks of gestation or an average daily dose above 5.0 to 10.0 mg was associated with increased PTB risk [134–136,139]. GC cross the placental barrier, yet the placental enzyme 11 β -hydroxysteroid dehydrogenase type 2 significantly limits fetal exposure to active prednisolone [102]. Two studies indicated normal developmental milestones, but conflicting infection rates in infants exposed to maternal GC [13,14].

Data on NSAIDs showed that a continuous periovulatory use can reduce fecundability [73,74]. Data on first trimester NSAID use, including prospective studies, did not show increased risks of major birth defects or miscarriage [4,15,16,18,140]. Most reassuring data were available for ibuprofen, whereas data on COX-2 inhibitors were limited [15,16,18,19,140]. Short-term (7–10 days) NSAID use in the second trimester did not reveal substantial fetal risks [71,72,141].

Among the vasodilatory drugs used in RMDs, data in pregnancy are available for sildenafil. Sildenafil is not teratogenic and was investigated in clinical trials for various pregnancy-related conditions like fetal growth restriction, pre-eclampsia, and preterm labour [43–45]. Limited data on the use of sildenafil in maternal pulmonary hypertension showed no maternal or fetal drug-related adverse effects [142]. However, for the treatment of fetal growth restriction, sildenafil may increase the risk of persistent pulmonary hypertension [43].

Evaluations of drug safety during breastfeeding are based on pharmacokinetic studies and clinical follow-ups. Despite limitations in study size, most studies showed very low drug levels in breast milk and no reported harm to infants. The relative infant dose (RID) is used to assess risk, with a cutoff of 10% indicating breastfeeding compatibility [143]. In fact, none of the drugs analysed showed an average RID above 10%. Most studies addressed bDMARDs in breastfeeding, all showing no to minimal transfer into breast milk and no adverse events in infants [57,59,63,76–79,83–86,88–90,92–95,97,98]. Drugs with limited data, such as methotrexate, sildenafil, and bosentan, also displayed low levels in breast milk without evidence of infant harm [107–109]. Given the overall benefits of breastfeeding, these findings support a risk-benefit assessment in breastfeeding patients, especially when alternative treatments are lacking [144].

Data on the impact of ARDs on male fertility are limited and often of poor quality, with disease activity itself known to negatively affect sperm quality [145–147]. Recent studies on methotrexate and filgotinib set new standards for research in this area [119,148]. Compared with filgotinib, however, data on methotrexate are more comprehensive, both in terms of sperm parameters and birth outcomes. Sulfasalazine may temporarily impair fertility without affecting offspring outcomes. It is advisable to switch to alternative medications to prevent disease flares, which could further impact fertility. Cyclophosphamide is the only ARD known to cause irreversible infertility at high doses, necessitating fertility preservation [149]. The most reassuring evidence for male fertility and birth outcomes was available for TNFi bDMARDs [115].

There are significant limitations of this SLR that need to be mentioned, such as the heterogeneity and varying quality among the included studies, for example, small sample size, insufficient data on drug exposure time during pregnancy, or unknown outcomes. However, classical scores of study quality rather focus on efficacy of a drug than on safety [4]. In addition, most studies were lacking adequate control groups, particularly unexposed populations with the same disease, making it difficult to distinguish the effects of medication from the underlying disease. This issue is crucial, as patients with RMDs have a higher risk of APOs, and treatment may mitigate disease activity, affecting these outcomes [1–3]. Therefore, well-designed prospective studies are needed, comparing drug exposure in diseased populations with unexposed controls while adjusting for confounders like disease severity, comorbidities, maternal age, and comedication. In addition, further studies on ARDs during breastfeeding and in male patients planning a family are required.

In conclusion, the updated SLR provides valuable insights about the relative safety of ARDs in reproduction, pregnancy, and lactation and supports the update of the EULAR recommendations.

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Contributors

FF, AF, YM, APS, LR, and SH developed the search strategies. FF, APS, LR, SH, IC, and LFP-G extracted the data for the SLR. MK performed the meta-analyses; FF, YM, APS, LR, SH, and LFP-G wrote the first version of the manuscript. All authors revised the manuscript critically for important intellectual content and gave final approval of the version to be published.

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All data relevant to the study are included in the article or uploaded as online supplemental information.

Patient and public involvement

Patient and/ or public were not involved in the design, conduct, reporting or disseminating of the study.

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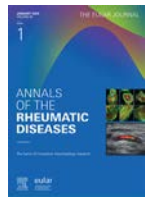
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Original Research

Satisfaction with telemedicine versus in-person visits in rheumatology: a noninferiority randomised controlled trial

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ARTICLE INFO

ABSTRACT

Objectives: We tested whether a rheumatology telemedicine visit was noninferior to an in-person visit for patient satisfaction and care effectiveness, including for subgroups of age, sex, race and ethnicity, income, and employment status.

Methods: This multicenter noninferiority trial randomised patients to 1 in-person or telemedicine visit. The primary outcome was the proportion of high visit satisfaction (9 or 10 on a 0–10 scale). The primary analysis tested whether patient satisfaction with telemedicine was noninferior to in-person care (10% noninferiority margin). Secondary and exploratory outcomes included preference for next visit type, satisfaction in subgroups, and immunosuppressant toxicity monitoring. We performed modified intent-to-treat (mITT) and per-protocol analyses.

Results: Among 651 randomised patients (in-person, n = 323; telemedicine n = 328), 500 (76.8%) had a visit defining the mITT population. Satisfaction with telemedicine was not noninferior to in-person; 77.0% telemedicine, 90.1% in-person, difference 13.1% (90% CI, 7.7%–18.5%). Per-protocol analysis results were consistent. More participants in the in-person group compared with those in telemedicine preferred the same visit type for their next visit vs a different visit type/no preference (55.6% in-person, 19.1% telemedicine, $P < .0001$, mITT analysis). Men were equally satisfied with both visit types (90.0%), while women were more likely to be satisfied with in-person visits (90.2% vs 74.7%). Toxicity monitoring rates were higher in in-person vs telemedicine (eg, hepatic function: 92.1% vs 66.3%, $P = .0001$, per-protocol analysis).

Conclusions: Among a large group of diverse patients, satisfaction with telemedicine was not noninferior to in-person rheumatology visits. More participants preferred in-person visits in the mITT and per-protocol analyses. Appropriate toxicity monitoring was lower in telemedicine vs in-person groups.

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WHAT IS ALREADY KNOWN ON THIS TOPIC

- Telemedicine has played an important role in delivering rheumatology care during the COVID-19 pandemic. Previous observational studies and small randomised clinical trials reported no significant differences in patient satisfaction between usual care and telemedicine services in rheumatology.

WHAT THIS STUDY ADDS

- This study rigorously assesses patients' acceptance of and satisfaction with telemedicine and its effects on healthcare processes.
- In this large, multisite randomised trial, we demonstrated that patient satisfaction with telemedicine is not noninferior to in-person visits for outpatient rheumatology care. Overall, satisfaction with telemedicine was high at 77%, but only about 1 in 5 people preferred that their next visit be via telemedicine.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- Telemedicine may not be universally suitable for all people with rheumatic diseases, such as for individuals who take high-risk medications that require frequent monitoring. Future research should focus on elucidating patient subgroups that may prefer or benefit most from telemedicine.
- These findings will inform whether a redesign of telemedicine practice is necessary to ensure care delivery is consonant with the needs of diverse individuals living with rheumatic and musculoskeletal diseases.

INTRODUCTION

The COVID-19 pandemic led to a dramatic uptake in telemedicine, including in rheumatology [1–3]. Data are mixed about whether outpatient rheumatology care delivered via synchronous remotely delivered telemedicine visits by phone or video occurring in the patient's own home is comparable to in-person care for important outcomes such as patient satisfaction [1,4–6], preference for type of visit [2,7,8], and monitoring for disease activity and medication side effects [1,9,10]. The standard-of-care for many rheumatic diseases is to use immunosuppressive drugs [11,12] that require close monitoring for side effects, which may be challenging via telemedicine [13,14]. There is a knowledge gap in the comparative effectiveness of telemedicine vs in-person visits to deliver appropriate care, especially for patients on immunosuppressive drugs [3,15,16].

Telemedicine has been advocated as a means of providing healthcare for medically vulnerable groups [17–19], including those with comorbidities such as rheumatic conditions. While telemedicine may have an important role in delivering rheumatology care, certain groups experience healthcare disparities [20]. Age, sex, race, ethnicity, and other socioeconomic factors shape telemedicine access [21,22]. However, little is known about whether vulnerable populations with rheumatic diseases are receiving adequate medical care as telemedicine has expanded. There is a need to rigorously assess patients' acceptance of and satisfaction with telemedicine and its effects on health outcomes.

We aimed to determine if one key aspect of quality of care [23,24], patient satisfaction with a telemedicine visit [4–7,25], was noninferior to in-person visits in rheumatology. We secondarily evaluated satisfaction with care among subgroups characterised by age, sex, race, ethnicity, and social determinants of health, including income and employment status, and determined the effect of telemedicine vs in-person care on preference

for the next visit type. The receipt of appropriate laboratory monitoring for high-risk medications was an exploratory outcome. These findings will inform whether a telemedicine redesign is necessary to ensure care delivery is consonant with the needs of diverse individuals living with rheumatic and musculoskeletal diseases.

METHODS

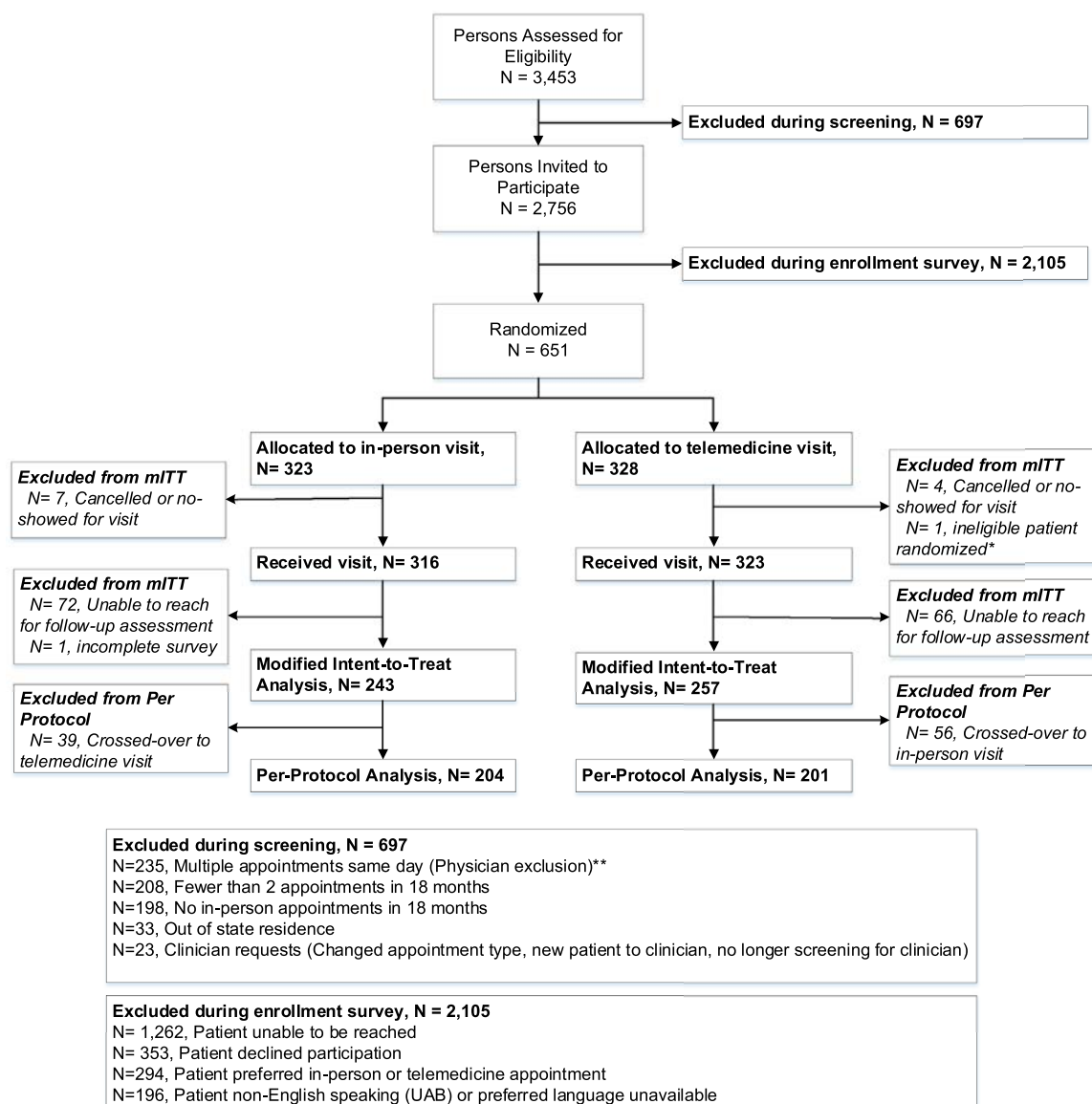
Trial design and oversight

We conducted a parallel-group, randomised, single-blind, noninferiority trial in rheumatology clinics at the University of Alabama at Birmingham (UAB) and the University of California San Francisco (UCSF) comparing patient satisfaction with in-person vs synchronous remote home-based telemedicine visits via phone-only or video conferencing only for outpatient rheumatologic care. Outcomes were collected via phone survey and electronic health record (EHR) review after the visit. We tested whether satisfaction with telemedicine was noninferior to in-person care using a prespecified *a priori* noninferiority margin of a 10% difference in the proportion of high satisfaction with the visit. Investigators selected this noninferiority margin based on the prespecified expectation that a 10% difference (ie, a 1-point difference on a 0 to 10 Consumer Assessment of Healthcare Providers and Systems [CAHPS] [23] survey satisfaction scale) between the 2 groups would represent a clinically important difference. We chose the 10% noninferiority margin *a priori* based on the literature, as scores of 9 or 10 in the CAHPS are considered standard top-box scores for satisfaction [25,26]. This minimally important difference in patient satisfaction corresponds to the smallest change in satisfaction scores that patients perceive as meaningful. Namely, the detection of differences of 10 percentage points in top-box scores from CAHPS survey data is clinically meaningful in assessing care quality [27,28]. Thus, we hypothesised that satisfaction with in-person rheumatology visits would be 10% higher (noninferiority margin) or more than telemedicine, with the alternative hypothesis that telemedicine is noninferior to in-person visits given a margin of 10%.

Institutional Review Boards at the participating institutions approved the protocol (Supplementary Appendix S1); the trial was registered on ClinicalTrials.gov (NCT04704544), and results were reported in accordance with the Consolidated Standards of Reporting Trials (CONSORT) 2010 statement guidelines [29]. We obtained a waiver of informed consent predicated on the study's minimal risk. At the end of the study, participants were debriefed about their study participation. While patients were not involved in the design or conduct of this research, this project was conducted in response to the call for more research on telemedicine use in the practice of rheumatology by the American College of Rheumatology in 2020 [30]. Funders of this study were not involved in the study design, data collection, analysis, data interpretation, or writing of the manuscript.

Participants

Participants were recruited between August 2021 and November 2022. Eligible participants were (1) adults aged 18 years and older with a rheumatic disease and (2) had 2 or more rheumatology clinic visits in the last 18 months, including at least 1 in-person visit. Potentially eligible patients were identified through an EHR search for rheumatologic conditions (see Supplementary Table S1). Patients were excluded based on EHR



*1 individual was randomized in error and subsequently excluded as they were followed by a clinician that did not conduct telemedicine visits.

**Individuals were excluded if they had multiple appointments with different clinicians or for other studies on the same day

Figure 1. Consolidated Standards of Reporting Trials diagram showing patient enrollment, randomisation, and follow-up. mITT, modified intent-to-treat; UAB, University of Alabama at Birmingham.

review if they had conditions that might indicate more severe rheumatic disease diagnosed in the past 6 months (eg, active vasculitis or ongoing treatment for lupus nephritis, neuropsychiatric lupus, myositis, or interstitial lung disease based on a review of the most recent rheumatology visit note), a rheumatic disease requiring recent hospitalisation since the most recent rheumatology visit, or were not appropriate for participation due to the need for a specific visit type (eg, patient requiring an in-person visit for a procedure or imaging study based on the most recent rheumatology visit note or appointment list, respectively).

Potentially eligible participants were contacted by phone 2 to 3 weeks prior to their upcoming previously scheduled outpatient rheumatology appointment and invited to complete an eligibility/enrollment survey. Patients were excluded during this phone call if they (1) were unable to be reached; (2) reported new or worsening symptoms since a previous rheumatology clinic visit

for which the patient expressed a desire for an in-person visit, or there was an expected in-office procedure (eg, joint injection); (3) expressed a specific preference to be seen by telemedicine or in-person, or they lacked access to a telephone; or (4) preferred a spoken language that was unavailable (ie, non-English speakers at UAB) (Fig 1). The baseline survey also ensured that participants resided in Alabama for UAB participants and California for UCSF participants to ensure compliance with state laws governing the reimbursement of telemedicine.

In a postvisit phone survey, we collected self-report data on covariates, including education level, health literacy [31,32], employment status, and income. We reviewed EHR records to capture demographic information, including sex (as reported in the EHR), age, address, current use of immunosuppressive/immunomodulatory therapies (see [Supplementary Table S2](#)), and the type of the most recent rheumatology visit, either telemedicine or in-person. Race and ethnicity were based on self-

reports when available from the survey and otherwise derived from the EHR. Race categories included Native American, American Indian, or Alaska Native; Asian; Black; White; and race unknown. Ethnicity was reported as Hispanic, not Hispanic, or unknown. Neighbourhood-level vulnerability and socioeconomic status were assessed with 2020 Social Vulnerability Index (SVI) [33] scores (0–1 national percentile scale, with higher values indicating higher vulnerability) and 2020 Area Deprivation Index (ADI) [34] scores (1–100 on a national scale, with higher values indicating higher disadvantage) based on patients' home addresses, respectively [35]. We ascertained the presence of autoimmune disease by EHR review conducted by 2 independent reviewers with adjudication by a third reviewer if needed. We used a hierarchical approach to recategorise people with multiple rheumatic conditions, adapted from previous studies [36]. For instance, medical records review evidence of diagnoses of Sjögren's syndrome, and rheumatoid arthritis was categorised as having rheumatoid arthritis since this was presumed to be rheumatoid arthritis with secondary Sjögren's syndrome.

Randomisation and masking

Participants were randomly assigned 1:1 stratified by site within the practices of rheumatologists to receive either a telemedicine or an in-person visit for their next rheumatology visit using a computer-generated list of random numbers implemented in REDCap [37] of variable block length. The allocation sequence was concealed until participants were enrolled and assigned to the interventions. Participants were blinded to the allocation process and were not aware that they were enrolled in a study or that their next rheumatology visit type would be randomly assigned. In addition, all participants were then assigned to the opposite type of visit for their second subsequent rheumatology visit. Since we received a waiver of informed consent, participants, clinicians, and investigators were not aware of which patients were included in the study and thus were blinded to the randomisation assignment. We were unable to blind study coordinators who conducted the outcomes assessments.

Interventions

After randomisation, participants were scheduled to have a home-based telemedicine (ie, synchronous remotely delivered visit in their own homes, either by phone or video) [38] or an in-person visit based on their assigned group for their next scheduled rheumatology visit. On the day of their appointment, for those assigned to telemedicine, clinicians contacted patients by either phone or video call at the discretion of the clinician. However, because clinicians were blinded to patients' study participation, a patient assigned to either group may have had their visit type modified at the discretion of the clinician or patient after randomisation. The study was designed as a clinical trial in a pragmatic fashion with implementation as part of routine rheumatology care.

Outcomes

The primary outcome was visit satisfaction, assessed via a phone survey conducted approximately 36 to 72 hours postvisit using the validated 11-point visit satisfaction scale from the CAHPS [23] survey. The primary outcome of patient satisfaction was chosen because it is patient-centric and has been shown to be associated with a range of patient-oriented clinical outcomes,

such as quality of care, treatment adherence, and a positive relationship with medical providers [39–42]. High postvisit satisfaction was defined as a score of 9 or 10 on a 0 to 10 satisfaction scale, with higher scores associated with higher satisfaction [25,26]. Patient satisfaction was calculated as the percentage of highly satisfied participants in each group. For this primary analysis, we compared patient satisfaction as a categorical variable rather than a continuous measure because a continuous measure of satisfaction typically has a ceiling effect [43], and high satisfaction scores defined as a categorical variable are frequently used when assessing satisfaction [5,25,43]. We performed modified intent-to-treat (mITT) and per-protocol analyses to account for those that crossed over.

Preference for the next visit type [25] was a secondary outcome categorised as the same as the group allocation, different than the group allocation, or no preference. We assessed the effectiveness of care by evaluating the rate of appropriate laboratory monitoring according to recommended guidelines for certain medications [13,44,45] (see [Supplementary Table S2](#)).

Sample size

For the primary outcome of satisfaction, we used a 1-sided score test (Farrington and Manning [46]). A sample size of 198 survey completers per group with a 1-sided alpha of .05 would achieve 80% power to detect a difference of 0% between 2 proportions when the noninferiority margin is 10%, where under the null hypothesis, the high satisfaction (proportion of participants rating the visit as 9 or 10 on a satisfaction scale of 0–10) is 70% in the telemedicine group and 80% in the in-person group. We expected a 20% to 25% dropout rate based on our previous study [25], and we inflated the sample size to allow for subgroup analysis.

Statistical analysis

We summarised variables using mean and SD or median and IQR. Two-sample *t*-tests and chi-square tests (Fisher's exact test, if appropriate) were used to examine differences between variables. Because the noninferiority margin was specified on a difference scale, we estimated treatment comparisons for the primary outcome as risk differences using generalised linear models [47,48]. In noninferiority design studies, intervention nonadherence can bias assessments away from the null hypothesis by making the groups more similar than in reality. Thus, we analysed the outcomes of individuals randomised using both a mITT (as randomised with completed visits and for whom primary outcome was available) and per-protocol (receiving the assigned visit type) analyses [49]. We explored the heterogeneity of treatment effects using prespecified subgroups of age, sex, race/ethnicity, income, and employment status. We conducted several sensitivity analyses. First, we compared the levels of satisfaction between the video-only and phone-only groups of those who received a telemedicine visit in the per-protocol sample since the type of telemedicine visit received was contingent upon receiving a telemedicine visit. Second, we evaluated the primary outcome of satisfaction by site, as regional tendencies and practices might influence satisfaction. Regarding the outcome of high satisfaction, our *a priori* outcome of high satisfaction included scores of 9 or 10 as top-box values [26], but we conducted a sensitivity analysis that considered scores of 10 as high satisfaction and all other scores as unsatisfied [26]. Lastly, we also considered patient satisfaction as a continuous variable rather than a categorical variable. All analyses were performed

using SAS 9.4 (SAS Institute) or R 4.3.2 (R Foundation for Statistical Computing).

RESULTS

Demographics and clinical characteristics

Participants were screened for eligibility between August 2021 and November 2022, and all visits for the assessment of the primary endpoint occurred between August 2021 and January 2023, after the COVID-19 lockdowns had ceased. Study enrollment was stopped when the prespecified number of participants who fully completed the primary outcome was reached ($N = 500$). Figure 1 displays the CONSORT diagram, including reasons for exclusion [29]. Of the 3453 potentially eligible patients screened, 651 individuals were randomised, including 598 (91.9%) at UAB and 53 (8.1%) at UCSF, and 639 (98.2%) attended a visit. Participants were excluded during the prerandomisation screening for having multiple appointments on the same day ($n = 235$), having fewer than 2 visits in the last 18 months ($n = 208$) or no in-person visit in the same timeframe ($n = 198$), residing out of state ($n = 33$), or due to clinician requests ($n = 23$). One individual was excluded after randomisation for later being found to be ineligible for the study. Of the 639 participants (98%), among those randomised to receive a visit, we were unable to reach or complete the follow-up phone survey in 139 (21.8%) individuals. We contacted all participants who attended their visit and reached 501 individuals (78.4%), of whom 500 completed the survey and were analysed for the primary outcome in the mITT analysis. The 500 survey completers comprised 243 randomised to the in-person group and 257 randomised to the telemedicine group.

Randomised participants who completed the surveys included 420 (84.0%) women and 179 (35.8%) Black individuals, with a mean age of 55.3 (SD, 13.7) years (Table 1). A majority of participants in both groups had completed some college or less (58.2%) and reported adequate health literacy [31,32] (84.8%), while participants in the telemedicine group were slightly more likely to report being employed (42.8% vs 33.7%, $P = .04$). The mean national ADI and SVI scores were 58.6 (SD, 29.6) and 0.5 (SD, 0.3), respectively, suggesting that most individuals resided in areas associated with moderate socioeconomic disadvantage and social vulnerability.

Primary outcome: satisfaction with visit type

Patient satisfaction was high in both groups (90.1% for the in-person group and 77.0% for the telemedicine group) (Table 2). The unadjusted risk difference in visit satisfaction for telemedicine vs in-person visits was 13.1% (90% CI, 7.7%–18.5%), which contained the 10% noninferiority margin. Thus, rheumatology telemedicine visits were not noninferior to in-person visits for patient satisfaction. In addition, although we defined patient satisfaction as a categorical variable for our primary analysis, in a sensitivity analysis, we considered patient satisfaction as a continuous variable. Consistent with the primary analysis findings, we confirmed a statistically significant difference between groups, with a mean (SD) satisfaction score of 9.6 (0.9) vs 9.2 (1.5) for in-person vs telemedicine visit type, respectively ($P = .004$; Table 2). In the prespecified subgroups of age, sex, race, income, and employment status, we found only significant heterogeneity in treatment effects by sex; men were equally satisfied with both modes of delivery (90% for both in-person and telemedicine), while women were more likely to be

Table 1

Demographic characteristics of participants who completed phone surveys and were included in the modified intent-to-treat analysis

Characteristics	In-person ($n = 243$), n (%)	Telemedicine ($n = 257$), n (%)
Age (y), mean (SD)	56.3 (13.7)	54.4 (13.7)
Age (y) group		
<45	56 (23.1)	69 (26.9)
≥45 to <65	111 (45.7)	126 (49.0)
≥65	76 (31.3)	62 (24.1)
Sex, female	203 (83.5)	217 (84.4)
Race and ethnicity		
White	134 (55.1)	130 (50.6)
Black	86 (35.4)	93 (36.2)
Native American, Asian, Hispanic	23 (9.5)	33 (12.8)
Education, some college or more		
Less than high school graduate	10 (4.1)	20 (7.8)
High school graduate or GED	48 (19.8)	56 (21.8)
Some college	79 (32.5)	78 (30.4)
4-y degree	58 (23.9)	48 (18.7)
More than 4-y college degree	33 (13.6)	41 (16.0)
Health literacy, inadequate ^a	27 (11.1)	33 (12.8)
Employment status, employed ^b	82 (33.7)	110 (42.8)
Annual income		
Low, <\$29,999	34 (14.0)	48 (18.7)
Medium, \$30,000–79,999	47 (19.3)	41 (16.0)
High, ≥\$80,000	53 (21.8)	51 (19.8)
SVI, mean (SD)	0.5 (0.27)	0.5 (0.28)
ADI ranking, state decile, mean (SD) ^c	4.4 (3.0)	4.6 (2.9)
ADI ranking, national percentile, mean (SD) ^d	58.6 (29.6)	61.0 (29.5)
Rheumatic conditions		
Autoimmune inflammatory joint diseases ^e	116 (47.7)	113 (44.0)
CTD/vasculitis ^f	96 (39.5)	104 (40.5)
Crystal arthritis ^g	15 (6.2)	11 (4.3)
Other ^h	10 (4.1)	12 (4.7)
Medication use ⁱ		
Conventional synthetic DMARDs ^j	167 (68.7)	187 (72.8)
Biologic DMARDs ^k	80 (32.9)	77 (30.0)
Targeted synthetic DMARDs ^l	10 (4.1)	8 (3.1)
Urate-lowering therapy ^m	18 (7.4)	18 (7.0)
Osteoporosis therapy ⁿ	15 (6.2)	8 (3.1)
Other medications (colchicine, IVIG)	20 (8.2)	17 (6.6)

ADI, Area Deprivation Index; CTD, connective tissue diseases; DMARD, disease-modifying antirheumatic drug; GED, general educational development; IVIG, intravenous immunoglobulin; SVI, Social Vulnerability Index.

Race was missing for 1 participant; education was missing for 29 individuals; health literacy was missing for 16 individuals; employment status was missing for 25 individuals; income was missing for 226 individuals; SVI was missing for 18 individuals; and ADI national and state was missing for 21 individuals.

^a Health literacy was assessed with the question “How confident are you filling out medical forms by yourself?” and “inadequate” was defined by grouping the following answers: “Somewhat,” “A little bit,” and “Not at all.”

^b Employed is full-time, part-time, or temporary work.

^c State decile from 1 (least disadvantaged) to 10 (most disadvantaged).

^d National percentile from 1 (least disadvantaged) to 100 (most disadvantaged).

^e Autoimmune inflammatory joint diseases include rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, reactive arthritis, and undifferentiated spondyloarthropathy.

^f CTD/vasculitis includes systemic lupus erythematosus, scleroderma, vasculitis, myositis, Sjögren’s syndrome, systemic connective tissue disease (unspecified), and Raynaud’s.

^g Crystal arthritis includes gout and pseudogout.

^h Other includes osteoporosis or osteopenia and osteoarthritis.

ⁱ Categories are not mutually exclusive.

^j Conventional synthetic DMARDs include azathioprine, chloroquine, cyclophosphamide, cyclosporine, hydroxychloroquine, leflunomide, methotrexate, mycophenolate/mycophenolic acid, sulfasalazine, and tacrolimus.

^k Biologic DMARDs include abatacept, adalimumab, anakinra, belimumab, canakinumab, certolizumab, etanercept, golimumab, guselkumab, infliximab, ixekizumab, ofatumumab, rilonacept, risankizumab, rituximab, sarilumab, secukinumab, tildrakizumab, tocilizumab, and ustekinumab.

^l Targeted synthetic DMARDs include apremilast, baricitinib, tofacitinib, and upadacitinib.

^m Urate-lowering therapy includes allopurinol and febuxostat.

ⁿ Osteoporosis therapy includes abaloparatide, alendronate, denosumab, ibandronate, teriparatide, and zoledronic acid.

Table 2

Patient experience with in-person and telemedicine visits, modified intent-to-treat, and per-protocol analyses

	Modified intent-to-treat			Per-protocol		
Outcomes	In-person (n = 243)	Telemedicine (n = 257)	P	In-person (n = 204)	Telemedicine (n = 201)	P
Primary outcome						
Satisfaction rate, score ≥ 9	219 (90.1)	198 (77.0)	.0001	185 (90.7)	151 (75.1)	<.0001
Satisfaction, mean (SD)	9.6 (0.9)	9.2 (1.5)	.004	9.6 (0.9)	9.1 (1.5)	.001
Secondary outcomes						
Preference for next visit			<.0001			<.0001
Same type	135 (55.6)	49 (19.1)		116 (56.9)	45 (22.4)	
Different type	40 (16.5)	123 (47.9)		33 (16.2)	86 (42.8)	
No preference	68 (28.0)	85 (33.1)		55 (27.0)	70 (34.8)	
Exploratory outcomes						
Appropriate toxicity monitoring						
Conventional synthetic DMARDs ^a	n = 107	n = 102		n = 89	n = 80	
<i>Hepatic function panel</i>	93 (86.9)	73 (71.6)	.006	82 (92.1)	53 (66.3)	.0001
<i>CBC</i>	94 (87.9)	80 (78.4)	.09	84 (94.4)	60 (75.0)	.0005
<i>Creatinine</i>	96 (89.7)	81 (79.4)	.05	85 (95.5)	61 (76.3)	.0004
IL-6 receptor inhibitors ^b	n = 2	n = 3		n = 2	n = 2	
<i>Hepatic function panel</i>	2 (100.0)	2 (66.7)	1.0	2 (100.0)	1 (50.0)	1.0
<i>CBC</i>	2 (100.0)	2 (66.7)	1.0	2 (100.0)	1 (50.0)	1.0
<i>Creatinine</i>	2 (100.0)	2 (66.7)	1.0	2 (100.0)	1 (50.0)	1.0
JAK inhibitors ^c	n = 9	n = 8		n = 9	n = 8	
<i>Hepatic function panel</i>	7 (77.8)	5 (62.5)	.6	7 (77.8)	5 (62.5)	.6
<i>CBC</i>	7 (77.8)	6 (75.0)	1.0	7 (77.8)	6 (75.0)	1.0
<i>Creatinine</i>	7 (77.8)	6 (75.0)	1.0	7 (77.8)	6 (75.0)	1.0
CD20 inhibitors ^d	n = 10	n = 10		n = 9	n = 8	
<i>CBC</i>	9 (90.0)	9 (90.0)	1.0	8 (88.9)	7 (87.5)	1.0

CBC, complete blood count; DMARD, disease-modifying antirheumatic drug; IL-6, interleukin-6; JAK, Janus tyrosine kinase.

Data are represented as n (%) unless otherwise stated. P values are for a null hypothesis of no difference between groups. Medications included in this table were limited to those that require frequent laboratory toxicity monitoring (eg, hydroxychloroquine and chloroquine excluded from DMARDs). P < .05 in bold font.

^a Conventional synthetic DMARDs include azathioprine, leflunomide, methotrexate, mycophenolate/mycophenolic acid, and sulfasalazine.^b IL-6 receptor inhibitors include tocilizumab and sarilumab.^c JAK inhibitors include upadacitinib, tofacitinib, and baricitinib.^d CD20 inhibitors include rituximab and ofatumumab. Medication use and laboratory monitoring were assessed by electronic health record review; the presence of appropriate monitoring was defined according to published guidelines. For instance, if laboratory monitoring is recommended approximately every 3 months, we assessed for the presence/absence of laboratory testing in the time period starting 30 days prior to the visit and 90 days after the visit.

highly satisfied with in-person visits (90.2% vs 74.7%) (Fig 2). Due to the homogeneity in ethnicity, this subgroup was ultimately not included in the subgroup analysis.

Of the 500 individuals who completed the surveys, 405 (81.0%) received the visit type to which they were randomised (204 in the in-person group [84.0%] and 201 in the telemedicine group [78.2%]; Fig 1). This per-protocol population did not have any significant differences in sociodemographic characteristics. By arm, 39 participants crossed over to telemedicine in the in-person group, and 56 in the telemedicine group crossed over to in-person ($P = .095$). Based on the proportion of high satisfaction in the per-protocol population (90.7% in the in-person group and 75.1% in the telemedicine group), the unadjusted difference in satisfaction between the in-person and telemedicine groups was 15.6% (90% CI, 9.6%–21.7%). Because the CI included the noninferiority margin, the per-protocol analysis confirmed that rheumatology telemedicine visits were not noninferior to in-person visits for patient satisfaction.

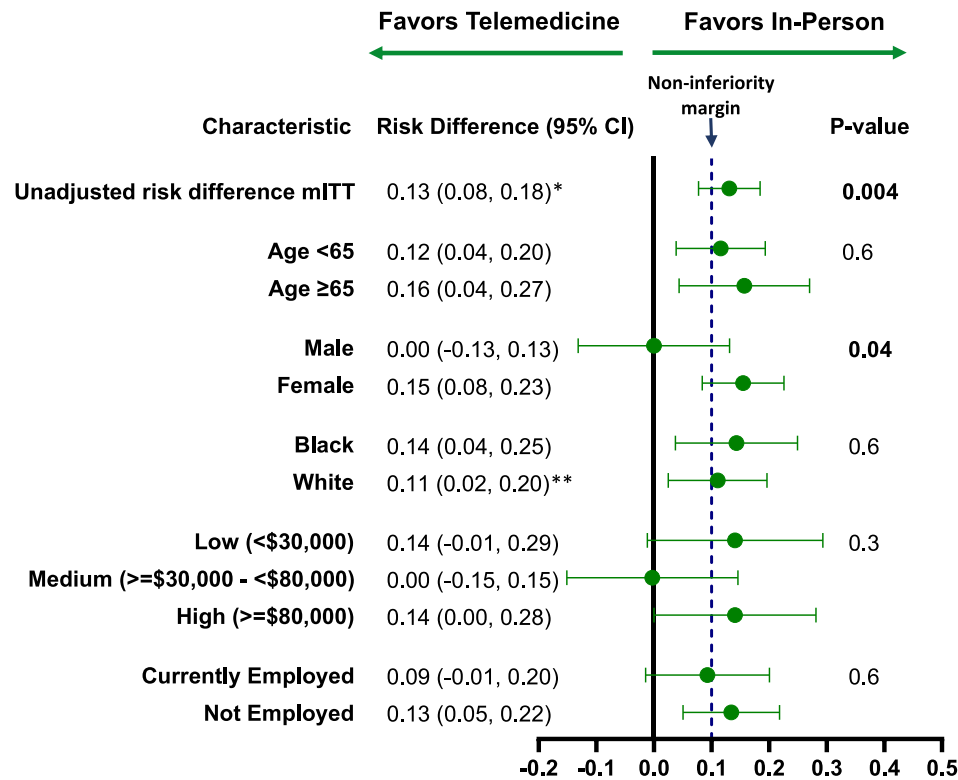
In the sensitivity analysis evaluating differences in satisfaction among those who received only a telemedicine visit, 111 (55.2%) individuals had only phone, 87 (43.3%) had some video, and 3 (1.5%) were missing the type of telemedicine visit. The satisfaction rate was similar in the video (74.7%) and phone (75.7%) groups (unadjusted risk difference 0.96% [90% CI, –9.1% to 11.26%]). In another sensitivity analysis, when we evaluated the primary outcome of satisfaction by site, the results were similar in magnitude and in the same direction. For

example, the unadjusted risk difference for UAB was 12.8% (90% CI, 7.2%–18.4%) and for UCSF, 14.9% (90% CI, –4.3% to 33.2%). When we considered only scores of 10 as high satisfaction, the results were consistent with our primary finding that telemedicine was not noninferior to in-person visits based on a noninferiority margin of 10% (65.0% satisfaction for telemedicine, 74.5% for in-person; 90% CI, 2.8%–16.2%, mITT analysis).

Preference for visit type and laboratory monitoring for high-risk medications

In the mITT population, the proportion of participants who indicated they preferred the same type of visit for their next visit was significantly greater in the in-person group compared with the telemedicine group (55.6% vs 19.1%, $P < .0001$) (Table 2). Only 17.8% of all participants preferred that their next visit be telemedicine.

Appropriate medication laboratory monitoring for conventional synthetic disease-modifying antirheumatic drugs (DMARDs) use (azathioprine, leflunomide, methotrexate, mycophenolate/mycophenolic acid, and sulfasalazine) according to current guidelines [13,44] was significantly different between groups. The in-person group had a higher likelihood of appropriate laboratory monitoring than the telemedicine group for the monitoring of hepatic function (per-protocol: 92.1% vs 66.3%, $P = .0001$), complete blood count (per-protocol: 94.4% vs



*Unadjusted risk difference for the primary outcome was conducted using a 90% confidence interval.
**Non-Hispanic White.
Race missing for 1 individual; income missing for 226 individuals; employment status missing for 25 individuals.

Figure 2. Risk differences and 95% CIs for high satisfaction (scores of 9 or 10) rate in the telemedicine group vs in-person group for modified intent-to-treat (mITT) analysis. *P* values for subgroup analyses tested whether the difference in proportions of high satisfaction between treatment groups is the same within each subgroup.

75.0%, *P* = .0005), and creatinine (per-protocol: 95.5% vs 76.3%, *P* = .0004) (Table 2).

DISCUSSION

In this large, multisite, randomised trial, we found that satisfaction was high in both in-person and telemedicine visits for people with established rheumatic diseases. While approximately 90% of in-person group participants were satisfied with their visit, a similarly high proportion (77%) of patients were also satisfied with their telemedicine-delivered care. However, using a noninferiority margin of 10%, we did not find noninferiority of telemedicine relative to in-person care with respect to high patient satisfaction. In addition, we observed higher satisfaction with telemedicine vs in-person in men compared with women. Among participants who received a telemedicine visit, satisfaction was similar in the video and phone groups. To our knowledge, this is the largest randomised trial to evaluate whether telemedicine visits are noninferior to in-person visits for important outcomes, including high satisfaction with the visit, visit type preference, and appropriate medication toxicity monitoring in rheumatology.

Several prior randomised studies have investigated the effectiveness of telemedicine in rheumatology care [3] in terms of disease activity [4,6,50], patient satisfaction [4–6,25], and acceptance of telemedicine [25,51], among others [3,52]. Most of these studies tested novel telemedicine methods as a supplement to usual care rather than evaluating the effectiveness of standard-of-care delivered either by telemedicine or in-person

visits. Findings from recent randomised clinical studies assessing telemedicine for rheumatology care vary, with 2 studies reporting no significant differences in patient satisfaction between usual care and telemedicine services [4,6] and 2 others reporting higher satisfaction with telemedicine compared with standard in-person care. However, the latter studies were limited by small sample sizes (*n* = 122 and *n* = 55, respectively) [1,5]. Many prior randomised studies of telemedicine-delivered care were limited by the high risk of bias, largely due to the inability to blind study participants or clinicians [3] and the small sample size [1,5]. Our study builds on these challenges by enrolling a large number of participants and blinding both participants and clinicians to visit allocation.

Greater satisfaction with medical care is associated with better outcomes and greater treatment adherence [53–55]. Patient demographics, including sex, are known to influence satisfaction with telemedicine [56]. In addition, women, compared with men, historically have a greater utilisation of telemedicine services when adjusted for total healthcare services [57]. Our findings indicated the presence of an interaction effect of treatment group by sex. However, contrary to previous observational studies in which women expressed greater satisfaction with telemedicine, including clinical explanations about their condition [56,58], we found that men were equally satisfied with both modes of delivery, but women were more likely to be highly satisfied with in-person visits.

Clear guidelines exist on the need for laboratory monitoring in individuals taking certain high-risk medications such as DMARDs [13,44]. However, data on whether the receipt of

appropriate monitoring is affected by the delivery of care via telemedicine are lacking. Our findings that telemedicine, compared with in-person care, is associated with lower rates of appropriate laboratory monitoring in the setting of high-risk DMARD use are notable, given that a majority of people with rheumatic diseases are treated with such therapies [13,45,59]. This suggests that extra vigilance is required to ensure that individuals participating in telemedicine visits receive appropriate laboratory monitoring for DMARDs.

We chose a noninferiority design based on the assumption that satisfaction with telemedicine would be slightly less but not unacceptably lower than traditional in-person visits [60]. A study of individuals followed in pulmonary, endocrine, and rheumatology clinics at a Veterans Administration hospital evaluated the effectiveness of telemedicine on satisfaction with visit convenience and physician's patient-centred communication [54]. This study confirmed noninferiority but used a larger noninferiority margin of 15% [54]. Using a more generous noninferiority margin of 15% in our study did not change our study conclusion that high satisfaction with telemedicine was not noninferior to in-person care. All of the upper bounds in both mITT and per-protocol analyses exceeded 15%. A sensitivity analysis using a satisfaction score of only 10 to define high satisfaction also did not lead to the noninferiority of telemedicine compared with in-person care. A previous randomised noninferiority study by members of our team also demonstrated noninferiority in satisfaction with video-based vs telephone-based telemedicine for participants followed in multidisciplinary clinics (eg, cardiology, family medicine, and rheumatology) [25]. To our knowledge, only one other randomised noninferiority study has been conducted in people with rheumatic conditions, which investigated the effectiveness of telemedicine follow-up conducted by a nurse or a rheumatologist to assess disease activity in people with rheumatoid arthritis, with a noninferiority margin of 0.6 change in the Disease Activity Score in 28 joints [4]. The authors demonstrated noninferiority for disease activity but did not report a difference in satisfaction between groups [4].

Our study significantly adds to the body of literature by comparing the effect of telemedicine with in-person care on important patient-reported and objective outcomes, including appropriate laboratory monitoring for medication-related toxicity. Another strength is its pragmatic design, which involves intervention implementation as part of routine rheumatology care with existing staff and workflows. Our study is generalisable to people with diverse types of rheumatic disease who may be considering periodic telemedicine visits for routine follow-up. Among individuals receiving high-risk medications such as DMARDs, extra attention is required if they engage in telemedicine to ensure they receive appropriate laboratory monitoring [13].

Despite its novel design and methodologic strengths, we recognise some limitations. Similar to previous studies [61], we did not restrict the population to patients who might be considered optimal candidates for either telemedicine or in-person care in an effort to maximise generalisability and since clear indications for one type of visit over another do not yet exist. In addition, we do not have clinical information regarding disease activity and disease duration among those randomised. Although patients were excluded if they reported new or worsening symptoms, this exclusion could potentially introduce a residual confounder since we cannot determine whether patients were evenly distributed between groups based on disease activity or duration. Moreover, since we report data related to a single visit, we cannot comment on potential changes in satisfaction with

telemedicine among individual patients over the course of their chronic conditions. Since the trial was designed pragmatically in an effort to understand the real-world performance of different visit types, clinicians were not aware of patient participation in the study, and they were not asked to record data on any potential technical difficulties that might occur with telemedicine visits. Following the study's conclusion, reimbursement for phone-only visits has changed, which may affect the generalisability of our findings among those with phone-only visits. The study was conducted at 2 sites, with 1 site contributing over 90% of participants. Differences in site enrollment were associated with variable startup times and recruitment rates. We observed an approximately 22% loss of follow-up among those who were randomised and received a visit, and because our primary and secondary outcomes were assessed using surveys, we were not able to collect outcomes from those who did not respond to surveys. Among those in whom we assessed outcomes, recall bias may have affected the results, though this is likely minimal since most surveys were conducted within a week postvisit. Notably, when we considered patient satisfaction as a continuous variable, we confirmed a statistically significant difference between groups ($P = .004$). Our study was single-blinded; although both patients and their clinicians were blinded to the study visit allocation, we were unable to blind study coordinators who conducted the outcomes assessments to group allocation. The COVID-19 pandemic-related challenges resulted in fluctuations in the conduct and public perception of telemedicine and may have affected satisfaction with the type of visit. In addition, the findings of optimal medication toxicity monitoring should be interpreted with caution, as this finding is limited by small sample sizes for most other medications except for DMARDs.

In this large, multisite randomised trial, patient satisfaction with telemedicine assessed soon after the visit was not shown to be noninferior to in-person visits for outpatient rheumatology care. Overall, satisfaction with telemedicine was high at 77%, but only about 1 in 5 people preferred that their next visit be telemedicine. Satisfaction with telemedicine differed between men and women. Telemedicine should be considered among patients who would benefit most from it. Telemedicine may not be universally unsuitable for all people with rheumatic diseases, and extra care may be indicated for individuals participating in telemedicine visits who take high-risk medications that require frequent monitoring. Future studies should focus on elucidating patient subgroups that may prefer or benefit most from telemedicine.

Competing interests

LEJ reports financial support was provided by Rheumatology Research Foundation. JY reports financial support was provided by National Institute of Arthritis and Musculoskeletal and Skin Diseases. JML and JRC reports financial support was provided by National Institute of Arthritis and Musculoskeletal and Skin Diseases. MID reports financial support was provided by Rheumatology Research Foundation. The other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Contributors

LEJ collected data, cleaned and analysed the data, and drafted the paper. JY designed the study, designed data collection tools, monitored data collection for the whole trial, interpreted the data, and revised the paper. JML cleaned and analysed the data, interpreted the data, and revised the paper. KGS designed the study, interpreted the data, and revised the paper. KA collected data and revised the paper. JRC designed the study, interpreted the data, and revised the paper. SG designed the study, interpreted the data, and revised the paper. MM designed the study, interpreted the data, and revised the paper. DHC designed the study, interpreted the data, and revised the paper. DP collected data and revised the paper. GC designed the study, interpreted the data, and revised the paper. MID designed the study, initiated the collaborative project, designed data collection tools, monitored data collection for the whole trial, wrote the statistical analysis plan, interpreted the data, and revised the paper. MID is the guarantor. All authors approved the final version to be published.

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Patient consent for publication

Not applicable.

Ethics approval

Institutional Review Boards at the participating institutions approved the study (IRB-300006553).

Provenance and peer review

Not applicable.

Data availability statement

Data are available upon reasonable request.

Patient and public involvement

While patients were not involved in the design or conduct of this research, this project was conducted in response to the call for more research in telemedicine use in the practice of rheumatology by the American College of Rheumatology in 2020.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.ard.2025.03.003](https://doi.org/10.1016/j.ard.2025.03.003).

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Images in rheumatology

The game-changer: ultrasound examination of distal interphalangeal joint swelling and nail psoriasis

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A 38-year-old man with psoriasis of the nails and the genital folds since he was 33 years, receiving topical therapy and methotrexate 12.5 mg/wk, was referred to our rheumatology unit for suspected psoriatic arthritis (PsA). The patient reported a 2-year history of pain in the first interphalangeal (IP) and the second distal interphalangeal (DIP) joints of both the hands. Physical examination revealed swelling and tenderness in the first IP and second DIP joints associated with psoriatic nail dystrophy. Blood tests showed a slight increase of inflammatory markers with negative rheumatoid factor and anticitrullinated peptide antibodies. Ultrasound examination revealed hypoechogenicity and increased vascularisation of the extensor tendon (ET) insertion on the nail matrix of the first and second fingers bilaterally, with inflammatory involvement of the overlying subcutaneous tissue, no signs of synovitis or other enthesitis were found in hands, knees, ankles, or heels. PsA was confirmed and treatment with guselkumab was introduced.

After 2 months, the ultrasound follow-up demonstrated a marked reduction in inflammation at the nail matrix and enthesitis of the ET of the second finger bilaterally, with near-complete resolution of inflammation in the first fingers.

Psoriatic nail disease is a common feature of PsA, and its association with DIP joints arthritis has been extensively reported [1,2]. However, DIP swelling represents a diagnostic challenge due to the ET complex anatomy at the level of distal phalanx, which accounts for several structures: the DIP joint, the nail matrix, and the ET fibres insertions [3] (Fig). Therefore, as illustrated in this case, a comprehensive approach integrating high-frequency ultrasound with clinical examination is crucial for accurately identifying the affected structures and excluding other conditions, such as isolated soft tissue inflammation or bone disease. Furthermore, in case of isolated ET complex involvement, an integrated clinical and ultrasonographic evaluation is a key strategy for diagnosing PsA and promptly initiating a treatment.

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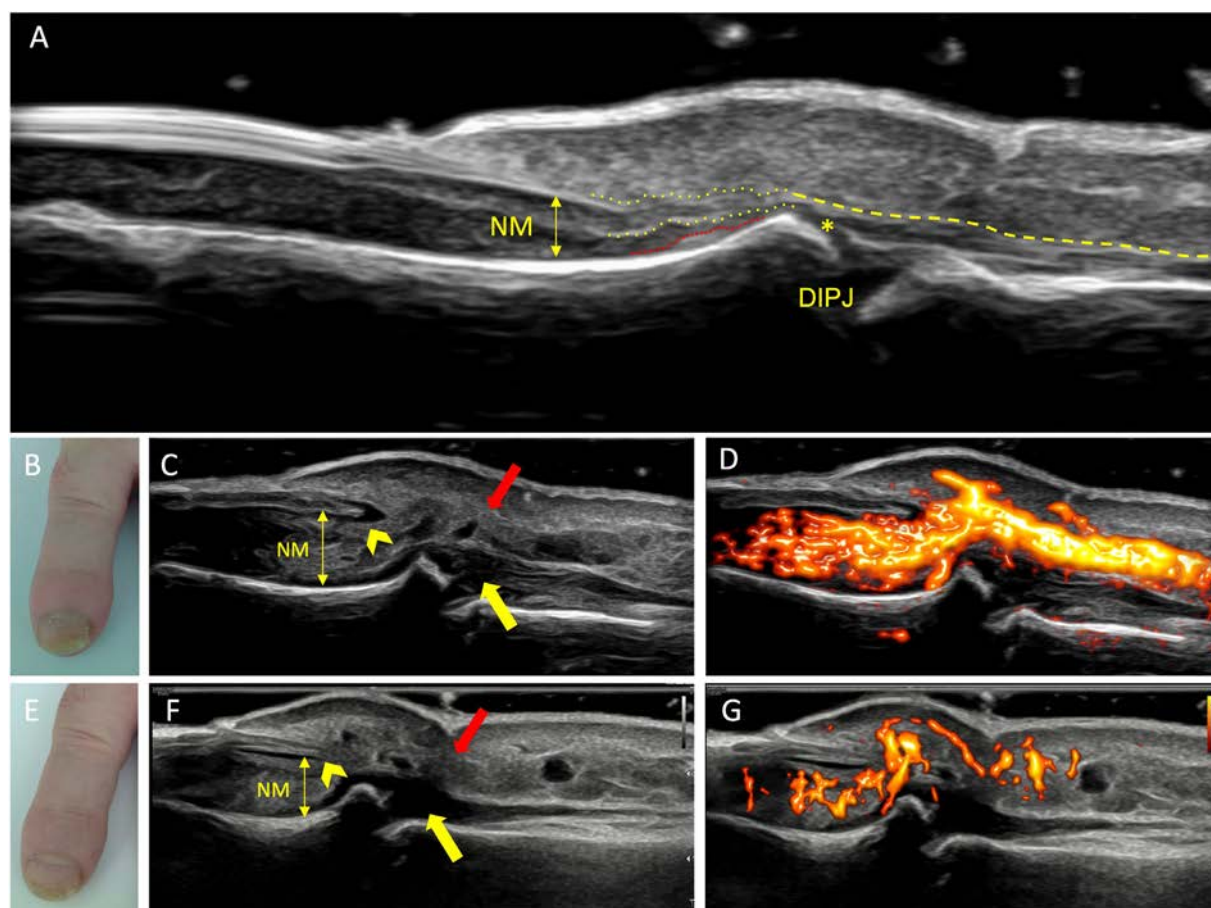


Figure. (A) Longitudinal US scan of the dorsal aspect of finger ET complex and DIPJ of a healthy subject. The dashed line follows the superficial margin of the ET, which splits into fibres inserting on the proximal side of the DP (asterisk), fibres (yellow dotted lines) extended to the NM and fibres inserting into the DP beyond the DIPJ (red dotted line). (B) Picture of the second finger at baseline. (C,D) Longitudinal US scan of the dorsal side of the ET complex at baseline. The insertion of the ET into the DP (yellow arrow) and the nail (arrowhead) appears thickened and hypoechoic, as well as the NM, with evidence of SCTE (red arrow). PD signal is significantly increased. (E) Picture of the same finger after 3 months of treatment with GUS. (F,G) Longitudinal US scan of the dorsal side of the same ET complex after 3 months of treatment. The insertion of the ET (yellow arrow) and the NM show reduced thickening compared with baseline, with improvement in SCTE. PD signal is notably decreased compared with baseline. DIPJ, distal interphalangeal joint; DP, distal phalanx; ET, extensor tendon; GUS, guselkumab; NM, nail matrix; PD, power-Doppler; SCTE, subcutaneous tissue oedema; US, ultrasound.

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

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Letter

Prognosis of older patients with multimorbidity who do not meet the definition of difficult-to-treat rheumatoid arthritis but have uncontrolled disease activity

Difficult-to-treat (D2T) rheumatoid arthritis (RA) remains an unmet need despite significant advances in the management of RA. We previously analysed 1709 patients with RA in 2018 in our institution and revealed that 10.1% were classified as D2T RA based on the EULAR definition [1]. Recently, we investigated the 5-year outcomes of these patients and reported that 45.0% of them escaped from D2T RA [2]. In this process, we also identified 77 patients (5%) in 2018 who were in moderate or high disease activity but were not classified as D2T RA, because they had not used 2 or more biological/targeted synthetic disease-modifying antirheumatic drugs with different mechanisms of action [3], and their characteristics were elderly (mean age, 77.5 years) and had several comorbidities. In this letter, we report the 5-year prognosis of the patients with moderate or high disease activity without fulfilment of D2T RA definition.

Of the 77 patients in 2018, 17 were excluded mainly due to insufficient information. Figure A shows the 5-year outcomes of the remaining 60 patients. During the 5 years, 25 (42%) patients were no longer able to visit our hospital by public transportation due to decline in activities of daily living (dropout), and 7 (15%) patients died, resulting in only 28 patients (47%) who continued to visit our hospital. Among them, 16 (27%) achieved remission or low disease activity by both disease activity score for 28 joints-erythrocyte sedimentation rate (ESR) and clinical disease activity index in 2023, whereas 12 (20%) remained in the uncontrolled disease activity group. Nine of the 12 patients still did not meet the D2T RA criteria in 2023. When we performed the same analysis by using only clinical disease activity index, because ESR tends to be high in the aged population, the total number of patients with non-D2T RA with moderate or high disease activity was 16. We could follow up 11 patients with enough information until 2023: 3 (27%) achieved remission or low disease activity, 2 (18%) remained moderate or high disease activity, 3 (27%) were dropout, and 3 (27%) were dead, suggesting the results showed similar poor outcomes.

Disease activity score for 28 joints–ESR and health assessment questionnaire-disability index of the 28 patients

significantly improved from 2018 to 2023 (Fig. B). When we compared the clinical characteristics of patients by outcomes in 2023, patients who achieved remission or low disease activity had a higher proportion of treatment changes other than glucocorticoids (remission/low, moderate/high, dropout, vs dead: 63%, 50%, 12%, vs 29%, respectively; $P = .004$), but there were no significant differences in age, sex, seropositivity of rheumatoid factor and anticyclic citrullinated peptide antibody, rheumatic disease comorbidity index, and disease activity score in 2018 (Table). On the contrary, patients who achieved remission or low disease activity were less likely to experience admission due to infection during the 5 years (13%, 50%, 40%, vs 86%, respectively; $P = .008$). Logistic regression analysis revealed that treatment change and an infection history were predictors with opposite effects for achieving remission or low disease activity (odds ratio [OR], 5.08; 95% CI, 1.24–20.7; $P = .02$; OR, 0.10; 95% CI, 0.02–0.61; $P = .01$) and for poor prognosis including dropout and death (OR, 0.16; 95% CI, 0.04–0.59; $P = .006$; OR, 3.10; 95% CI, 0.88–10.9; $P = .08$) (Fig. C).

Our study revealed that more than 50% of patients who did not meet the D2T RA definition but had uncontrolled disease activity have poor prognosis during the subsequent 5 years, especially if they have not changed their treatment. Their prognosis appears to be worse than that of patients with D2T RA, as we recently reported [2], highlighting the difficulties in managing aged patients with multimorbidities. The types of comorbidities are not specific to Japan, a superaged society, but are common worldwide, such as chronic kidney disease (45%), pulmonary disease (45%), hypertension (37%), and fractures (25%). Multiple morbidities are associated with serious infections [4] and high mortality in RA [5], suggesting that management of comorbidities is crucial. On the contrary, treatment modification, especially initiation of biological disease-modifying antirheumatic drugs, was an independent predictor for achieving treatment target, emphasising that a treat-to-target strategy should be carefully considered in such aged patients with comorbidities based on the individual risk–benefit balance. Indeed, stringent disease control may prevent the development of comorbidities [6]. Thus, our data provide the important insights to discuss the appropriate strategy in older patients in such ageing societies. The accumulation of data from all over the world is necessary to develop optimal management strategies.

CONTRIBUTORS

ST, TT, and YK designed the study. ST was involved in data collection and wrote the first draft of the manuscript. All authors edited the manuscript and approved the final version.

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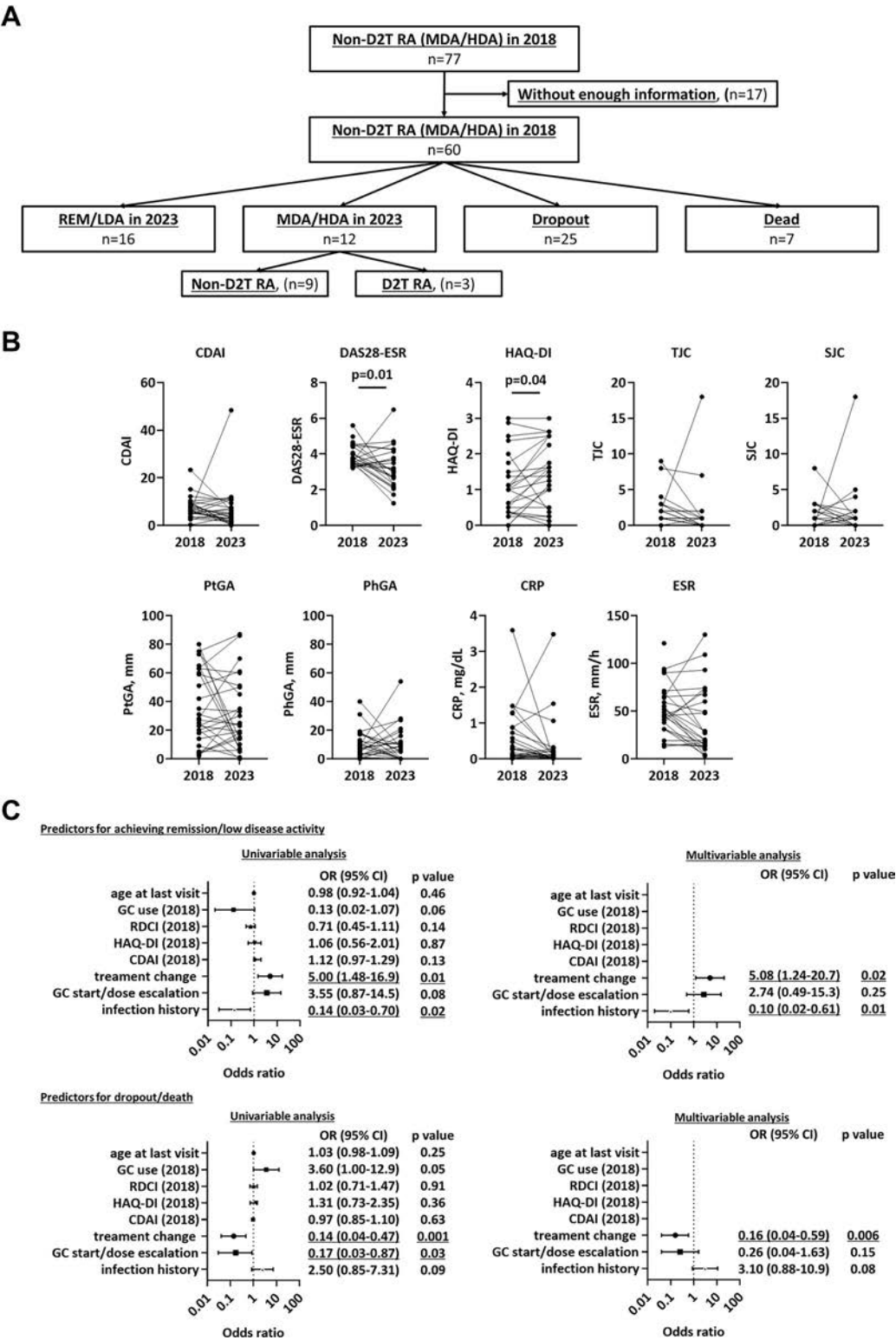


Figure. Prognosis of non-D2T RA with uncontrolled disease activity. (A) Five-year outcome of non-D2T RA with uncontrolled disease activity. (B) Changes in disease activity from 2018 to 2023. Wilcoxon signed-rank sum test. ESR and DAS28-ESR, n = 26. (C) Logistic regression analysis for the predictors of remission or low disease activity and dropout or death. CDAI, clinical disease activity index; CRP, C-reactive protein; DAS28, disease activity score for 28 joints; D2T, difficult-to-treat; ESR, erythrocyte sedimentation rate; GC, glucocorticoid; HAQ-DI, health assessment questionnaire-disability index; HDA, high disease activity; LDA, low disease activity; MDA, moderate disease activity; PhGA, physician global assessment; PtGA, patient global assessment; RA, rheumatoid arthritis; RDCI, rheumatic disease comorbidity index; REM, remission; SJC, swollen joint count; TJC, tender joint count.

Table
Clinical characteristics patients of non-D2T RA with MDA/HDA

	All (N = 60)	REM/LDA (n = 16)	MDA/HDA (n = 12)	Dropout (n = 25)	Dead (n = 7)	<i>P</i> ^a	<i>P</i> ^b	<i>P</i> ^c
Age at RA diagnosis (y)	63.8 (55.0-72.8)	63.8 (52.1-65.6)	61.9 (54.6-61.9)	67.6 (56.3-74.1)	56.2 (55.0-64.8)	.45	.59	.80
Age at last visit (y)	82.6 (77.8-88.1)	82.6 (73.5-88.1)	82.2 (74.6-83.8)	83.3 (79.3-88.1)	80.0 (74.3-88.2)	.85	.78	.70
Female	54 (90.0)	16 (100)	11 (91.7)	22 (88.0)	5 (71.4)	.16	.26	.43
RF positive	52 (86.7)	13 (81.3)	11 (91.7)	23 (92.0)	5 (71.4)	.36	.78	.61
Anti-CCP positive	46/59 (78.0)	10/16 (62.5)	10/12 (83.3)	22/25 (88.0)	4/6 (66.7)	.21	.28	.40
RDCI (2018)	2.0 (1.0-3.0)	1.5 (0.8-3.0)	3.0 (2.0-3.0)	2.0 (1.0-3.0)	3.0 (2.0-3.0)	.26	.21	.10
Hypertension	22 (36.7)	5 (31.3)	4 (33.3)	9 (36.0)	4 (57.1)	.73	.82	1.00
Ischaemic heart disease	4 (6.7)	0 (0)	0 (0)	2 (8.0)	2 (28.6)	.08	.30	—
Stroke	6 (10.0)	0 (0)	1 (8.3)	4 (16.0)	1 (14.3)	.36	.26	.43
Diabetes mellitus	8 (13.3)	1 (6.3)	2 (16.7)	3 (12.0)	2 (28.6)	.51	.68	.56
Liver disease	4 (6.7)	1 (6.3)	0 (0)	1 (4.0)	2 (28.6)	.14	.81	1.00
Pulmonary disease	27 (45.0)	7 (43.8)	9 (75.0)	9 (36.0)	2 (28.6)	.13	.12	.14
Depression	1 (1.7)	1 (6.3)	0 (0)	0 (0)	0 (0)	.58	.47	1.00
Fracture	15 (25.0)	3 (18.8)	3 (25.0)	8 (32.0)	1 (14.3)	.76	.92	1.00
Gastrointestinal ulcer	6 (10.0)	1 (6.3)	1 (8.3)	3 (12.0)	1 (14.3)	.93	.86	1.00
CKD (2018)	27 (45.0)	4 (25.0)	6 (50.0)	15 (60.0)	2 (28.6)	.13	.17	.24
Admission history due to infection until 2023	24 (40.0)	2 (12.5)	6 (50.0)	10 (40.0)	6 (85.7)	.008 ^d	.03 ^e	.04
Treatment change 2018-2023 (other than GC)	21 (35.0)	10 (62.5)	6 (50.0)	3 (12.0)	2 (28.6)	.004 ^f	.002 ^e	.70
bDMARD initiation	7 (11.7)	6 (37.5)	1 (8.3)	0 (0)	0 (0)	.002 ^f	<.001 ^e	.18
bDMARD switching	6 (10.0)	2 (12.5)	3 (25.0)	1 (4.0)	0 (0)	.2	.05	.62
JAKi initiation	2 (3.3)	0 (0)	2 (16.7)	0 (0)	0 (0)	.05	.04	.18
JAKi switching	1 (1.7)	0 (0)	1 (8.3)	0 (0)	0 (0)	.32	.20	.43
bDMARD/JAKi dose escalation	5 (8.3)	2 (12.5)	2 (16.7)	0 (0)	1 (14.3)	.11	.20	1.00
MTX dose escalation	2 (3.3)	1 (6.3)	1 (8.3)	0 (0)	0 (0)	.44	.21	1.00
csDMARD addition	8 (13.3)	4 (25.0)	1 (8.3)	2 (8.0)	1 (14.3)	.41	.27	.36
GC dose escalation	10 (16.7)	5 (31.3)	3 (25.0)	1 (4.0)	1 (14.3)	.07	.07	1.00
Treatment attenuation	14 (23.3)	3 (18.8)	3 (25.0)	6 (24.0)	2 (28.3)	.97	.92	1.00
Treatment (2018)								
TNFi	7 (11.7)	3 (18.8)	1 (8.3)	3 (12.0)	0 (0)	.72	.57	.61
IL-6i	2 (3.3)	0 (0)	2 (16.7)	0 (0)	0 (0)	.05	.04	.18
CTLA-4Ig	13 (21.7)	2 (12.5)	4 (33.3)	6 (24.0)	1 (14.3)	.63	.46	.35
JAKi	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	—	—	—
csDMARD	36 (60.0)	11 (68.8)	4 (33.3)	15 (60.0)	6 (85.7)	.13	.13	.13
MTX use	26 (43.3)	7 (43.8)	5 (41.7)	13 (52.0)	1 (14.3)	.40	1.00	1.00
PSL use	16 (26.7)	1 (6.3)	3 (25.0)	9 (36.0)	3 (42.9)	.11	.06	.29
Treatment (2023, n = 28)								
TNFi	3 (10.7)	3 (18.8)	0 (0)	—	—	.24	.24	.24
IL-6i	5 (17.9)	4 (25.0)	1 (8.3)	—	—	.36	.36	.36
CTLA-4Ig	6 (21.4)	2 (12.5)	4 (33.3)	—	—	.35	.35	.35
JAKi	2 (7.1)	0 (0)	2 (16.7)	—	—	.18	.18	.18
csDMARD	12 (42.9)	7 (43.8)	5 (41.7)	—	—	1.00	1.00	1.00
MTX use	11 (39.3)	6 (37.5)	5 (41.7)	—	—	1.00	1.00	1.00
PSL use	5 (17.9)	2 (12.5)	3 (25.0)	—	—	.62	.62	.62
Disease activity (2018)								
DAS28-ESR	3.7 (3.5-4.1)	3.7 (3.6-4.0)	3.6 (3.4-3.6)	3.8 (3.5-4.0)	3.8 (3.5-4.1)	.91	.78	.48
CDAI	6.4 (4.8-8.7)	7.7 (5.8-9.5)	5.5 (4.4-7.1)	5.4 (4.7-7.9)	8.2 (5.0-12.5)	.26	.26	.16
HAQ-DI	1.0 (0.38-1.8)	1.0 (0.3-1.6)	0.6 (0.3-1.1)	1.0 (0.4-1.9)	0.9 (0.6-2.1)	.58	.44	.50
Disease activity (2023), n = 28								
DAS28-ESR ^g	3.0 (2.4-3.7)	2.7 (2.1-2.9)	4.1 (3.7-4.4)	—	—	—	—	<.001
CDAI	4.6 (2.2-8.0)	3.1 (1.4-4.5)	9.0 (5.3-11.5)	—	—	—	—	.001
HAQ-DI	1.2 (0.3-1.9)	1.1 (0.3-1.7)	1.6 (0.4-2.3)	—	—	—	—	.66

Data are shown by n (%) or median (interquartile range). Kruskal–Wallis analysis with Bonferroni post hoc correction or Mann–Whitney U analysis.

bDMARD, biological disease-modifying antirheumatic drug; CCP, cyclic citrullinated peptide; CDAI, clinical disease activity index; CKD, chronic kidney disease; CRP, C-reactive protein; csDMARD, conventional synthetic disease-modifying antirheumatic drug; DAS28, disease activity score for 28 joints; D2T, difficult-to-treat; ESR, erythrocyte sedimentation rate; GC, glucocorticoid; HAQ-DI, health assessment questionnaire-disability index; HDA, high disease activity; JAKi, Janus kinase inhibitor; LDA, low disease activity; MDA, moderate disease activity; MTX, methotrexate; RA, rheumatoid arthritis; RDCI, rheumatic disease comorbidity index; REM, remission; RF, rheumatoid factor.

^a Comparison between 4 groups (REM/LDA vs MDA/HDA vs dropout vs dead).

^b Comparison between 3 groups (REM/LDA vs MDA/HDA vs dropout/dead).

^c comparison between 2 groups (REM/LDA vs MDA/HDA).

^d REM/LDA vs dead.

^e REM/LDA vs dropout/dead (*P* < .05) by Bonferroni post hoc correction).

^f REM/LDA vs dropout.

^g n = 26.

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Competing interests

ST has received speaking fees from AbbVie, Asahi-Kasei, Astellas, Ayumi, Bristol-Myers Squibb, Eisai, Eli Lilly, Janssen, Mitsubishi-Tanabe, Pfizer, Taisho, and UCB. TT has received consulting fees from AbbVie, Eli Lilly Japan, Gilead Sciences, Mitsubishi Tanabe Pharma, and Taisho Pharmaceutical Holding and speaking fees from AbbVie, Astellas Pharma, AstraZeneca, Chugai Pharmaceutical, Daiichi Sankyo, Eisai, Eli Lilly Japan, Gilead Sciences, Janssen Pharmaceutical, Mitsubishi Tanabe Pharma, and Pfizer Japan. YK has received research grants from AbbVie, Eisai, Sanofi, Chugai, Mitsubishi-Tanabe, and Taisho; has received scholarship grant from Asahi-Kasei, Eisai, Boehringer Ingelheim, and Taisho; and has received speaking fees from AbbVie, Asahi-Kasei, Astellas, Ayumi Pharmaceutical Corporation, Boehringer Ingelheim, Bristol-Myers Squibb, Chugai, Eisai, Eli Lilly, Glaxo Smith Kline, Novartis, Pfizer, Janssen, UCB, and Gilead Sciences.

Patient consent for publication

Written informed consent was waived in accordance with Japanese regulations.

Ethics approval

This study was approved by the ethics committee of the Keio University School of Medicine (approval No. 20130506). All the investigations were conducted in accordance with the principles of the Declaration of Helsinki.

Provenance and peer review

Peer reviewed.

Data availability statement

The data that underlying this article cannot be shared publicly due to the privacy of individuals who participated in the study. The data will be shared if a reasonable request is made to the corresponding author.

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Letter

Anifrolumab in childhood-onset systemic lupus erythematosus: a promising option after belimumab failure

Dear Editor,

In recent years, the development of novel therapeutics for systemic lupus erythematosus (SLE) has grown due to significant advances in research. Biological therapies such as belimumab and anifrolumab have gained prominence and are recommended by international guidelines for managing moderate to severe SLE, particularly in patients unresponsive to conventional therapies and in severe cutaneous manifestations [1]. In childhood-onset SLE (cSLE), treatment recommendations rely on expert consensus and available evidence [2] while also considering off-label therapies proven effective in adults. Although belimumab is approved for children, the latest paediatric guidelines do not yet include anifrolumab despite promising results in adults.

This manuscript presents the case of an adolescent treated with anifrolumab after the failure of belimumab, with continued favourable progression.

A 13-year-old Caucasian female weighing 45 kilograms with no significant medical history presented in January 2024 with butterfly-shaped erythema, asthenia, and anorexia. Physical examination revealed scalp desquamation, chilblain lupus lesions on the feet, and millimetric haemorrhagic macules on the right fifth finger pulp.

She was initially assessed at another centre, where cSLE was diagnosed based on blood tests, including leukopenia (2600/ μ L), lymphocyte count at the lower limit of normal (1400/ μ L), hypocomplementemia (C3: 65 mg/dL, C4: 4 mg/dL), positive antinuclear antibodies (ANA) (1:640) and positive double-stranded DNA antibodies (anti-dsDNA), and a skin biopsy confirming cutaneous lupus with vacuolar interface dermatitis, lymphocytic infiltrate, and mucin deposition. Treatment was started with oral glucocorticoids (GC) at 30 mg/d and strict photoprotection. Due to a failure to taper GC, hydroxychloroquine (HCQ) at 200 mg/d was added, along with topical GC and tacrolimus 0.1%.

She presented to our centre a few weeks later for follow-up, where a comprehensive workup revealed leukopenia (leucocytes: 3890/ μ L), hypocomplementaemia (C3: 77 mg/dL, C4: 4 mg/dL), elevated liver enzymes (glutamate-pyruvate transaminase (GPT): 80 U/L), an elevated erythrocyte sedimentation rate (ESR) (46 mm/h), ANA positivity (1:320), and positive anti-

dsDNA antibodies (>600 IU/mL, determined by indirect immunofluorescence using *Crithidia luciliae*). Possible mild renal involvement was considered (protein-to-creatinine ratio: 215 mg/g, normal renal function, no haematuria or hypertension); therefore, a renal biopsy was not indicated. This decision was made after consulting with the nephrology department and coming to a consensus with the parents. Other assessments, including ophthalmological examination, capillaroscopy, pulmonary function tests, and cardiological evaluation, showed no abnormalities. Given these findings, together with the systemic symptoms and inadequate GC response, the decision was made to initiate subcutaneous belimumab at 200 mg/wk.

Over the following 2 months, attempts to reduce the GC dose failed due to poor cutaneous progression, requiring an increase in HCQ to 400 mg/d, which is in line with the European Alliance of Associations for Rheumatology (EULAR) recommendations, allowing higher doses in moderate to severe disease [1]. Six months after disease onset, due to steroid dependency, lack of cutaneous response to belimumab, and persistent inflammatory activity, belimumab was replaced with intravenous anifrolumab (300 mg/mo), maintaining all other treatments unchanged. This decision, supported by the Treatment of Uncontrolled Lupus via the Interferon Pathway-2 (TULIP-2) trial [3], followed the adult dosing regimen, considering the patient's symptoms and weight. This off-label use was formally approved by written parental consent. Marked cutaneous improvement was observed after the first dose, with ongoing clinical and laboratory improvements, including normalisation of ESR and transaminase levels, as well as a reduction in anti-dsDNA antibody levels and partial recovery of complement (C3, C4) levels. These changes enabled complete GC withdrawal after 4 anifrolumab administrations and full remission of cutaneous symptoms. No adverse effects related to anifrolumab were observed (Fig).

Current evidence suggests that overproduction of type I interferon is associated with the pathogenesis of SLE, leading to immune dysregulation and tissue damage [4]. Anifrolumab, a humanised immunoglobulin G1 kappa monoclonal antibody targeting the Interferon Alpha and Beta Receptor Subunit 1 (IFNAR1), has demonstrated efficacy across multiple trials; however, information regarding its use in cSLE is limited [5]. Notably, the BLOSSOM study is currently underway to evaluate anifrolumab's safety and efficacy in cSLE patients [6].

In our case, biological therapies were introduced early due to insufficient response to conventional treatments, particularly for severe cutaneous manifestations.

In our case, biological therapies were introduced early due to persistent cutaneous disease activity and steroid dependency. Following EULAR 2023 recommendations, belimumab was chosen for its steroid-sparing effect and efficacy in mucocutaneous

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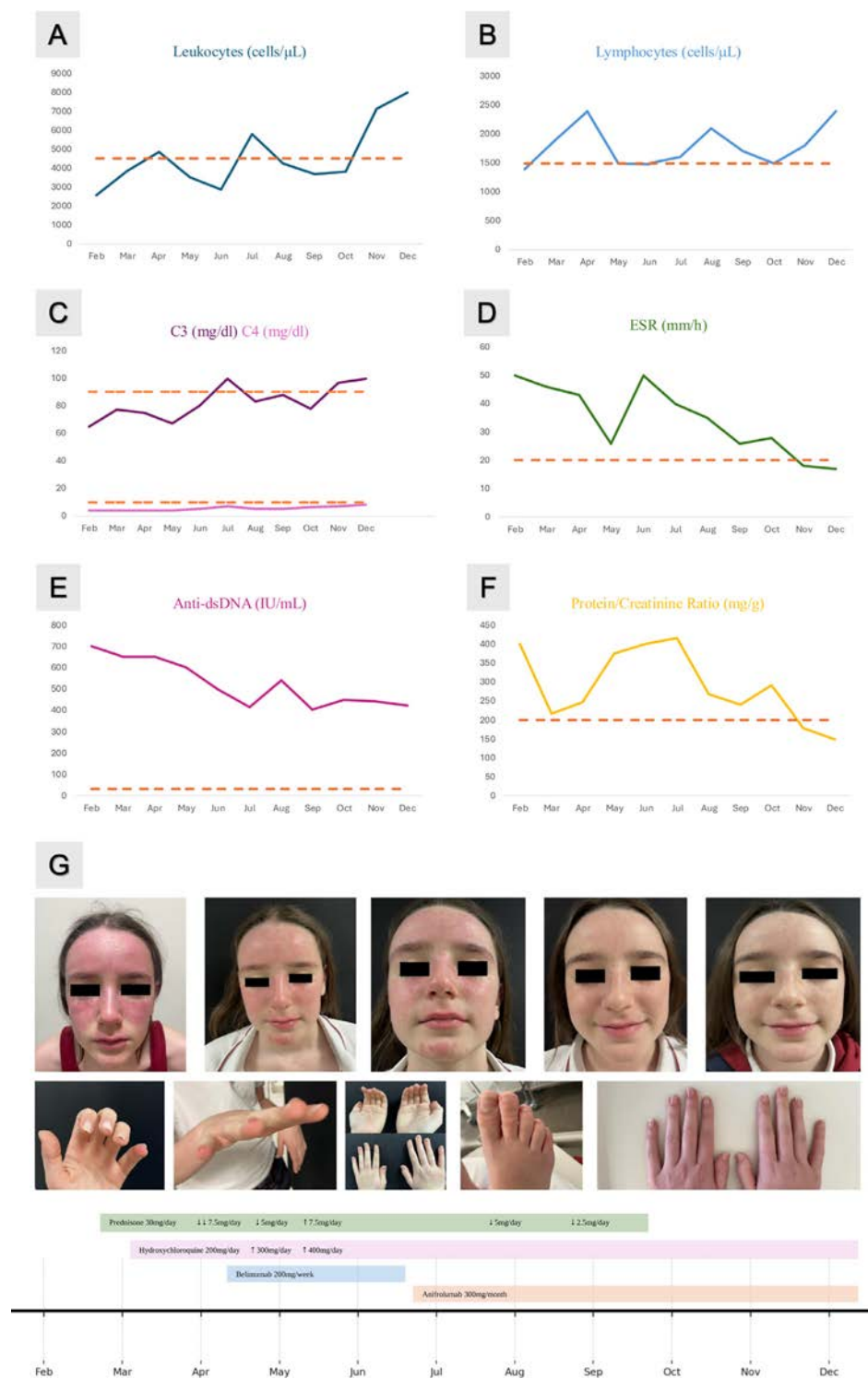


Figure. The patient’s clinical and analytical progression throughout the follow-up. Leukocyte (A), lymphocyte (B), erythrocyte sedimentation rate (ESR) (D), and renal function (F) levels have normalised, with marked improvement evident since the introduction of anifrolumab at the end of June. While complement (C) and anti-dsDNA (E) antibody levels remained positive, they significantly improved. Clinically, the patient has shown progressive improvement in cutaneous manifestations (G) over time, ultimately achieving complete remission. The timeline highlights the evolution of these manifestations in relation to the different treatments administered. dsDNA: double-stranded DNA.

lupus, particularly in cases refractory to HCQ and GC. Unlike previous guidelines, EULAR 2023 does not require the failure of conventional immunosuppressants before initiating a biologic, acknowledging that belimumab and anifrolumab are equally valid options for treatment escalation. Moreover, as disease activity remained high and GC tapering was unsuccessful, anifrolumab was introduced without waiting for the usual

evaluation period, following EULAR’s guidance to adapt therapy in refractory SLE cases [1].

Anifrolumab proved effective after belimumab failure, achieving complete cutaneous remission and normalisation of ESR, leucocyte counts, and transaminase levels. Renal involvement showed a slight improvement in proteinuria while remaining overall stable. Although anti-dsDNA antibodies remained

positive and complement levels decreased, their improvement suggests stabilisation with continued treatment.

As of today, the patient remains under treatment with anifrolumab and HCQ, leading a normal life free from limitations or the need for GC, with high satisfaction reported by both the patient and her parents.

Although further studies are needed to evaluate its efficacy, safety, and optimal dosing in the paediatric population, anifrolumab may prove to be a promising option for cSLE.

Competing interests

EC-A reports a relationship with GlaxoSmithKline that includes funding grants. The other authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Contributors

DAB, EBM, and EC-A were responsible for the study conception and design, as well as the acquisition, analysis, and interpretation of the data for the work. EBM and EC-A were involved in data collection. DAB prepared the original draft of the manuscript, and all authors (DAB, EBM, and EC-A) were involved in writing, reviewing, and editing. EBM and EC-A provided supervision throughout the project. DAB handled project administration. DAB is the guarantor of the content of the manuscript. All authors have read and agreed to the submitted version of the manuscript.

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Patient consent for publication

Written informed consent for publication was obtained from both the patient and her parents, including consent for the use of clinical photographs.

Ethics approval

Ethical approval was not required for this study as it involved a retrospective review of clinical data and no direct interventions with the patient.

Provenance and peer review

Not commissioned; externally peer reviewed.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the author, Delia Argüelles Balas, used ChatGPT in order to assist with the translation and improve the style and fluency of the manuscript. After using this tool, the author reviewed and edited the content as needed and takes full responsibility for the content of the published article.

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Correspondence

Correspondence to 'Evaluating the limitations of random forest and SHAP in predicting treatment responses in systemic lupus erythematosus'

Bachali et al [1] explored the responsiveness of systemic lupus erythematosus (SLE) patients to iberdomide based on molecular endotypes. Their study employed a random forest (RF) model to predict treatment responses to the highest dose of iberdomide (0.45 mg once daily), utilising Shapley Additive Explanations (SHAP) analysis to identify significant factors influencing these predictions.

This paper raises significant concerns about the application of RF models in conjunction with SHAP, emphasising the model-specific nature of these methodologies, which can lead to erroneous conclusions. It is essential for Bachali et al [1] to understand the fundamental theoretical principles of machine learning. While supervised machine learning models such as RF require labelled training data (known as 'ground truth' or 'gold standard' values) to evaluate prediction accuracy of patient response to iberdomide, the feature importances derived from these models lack such reference standards for validation. Ground truth refers to information that is regarded as real or true, typically obtained through direct measurement, such as clinically validated SLE disease activity scores or molecular markers of treatment response. For example, in this study, the actual observed clinical responses of SLE patients to iberdomide treatment would constitute the ground truth data for evaluating the model's predictive performance.

This distinction is crucial; high prediction accuracy might indicate strong model performance but does not necessarily validate the reliability of the feature importances. Thus, a model can produce accurate predictions while still misrepresenting the significance of individual features.

The absence of universally accepted ground truth values means that different models utilise varied methodologies for calculating feature importances, which can introduce significant biases in the results. Numerous studies—over 100 peer-reviewed articles [2–6]—have documented that the feature importances derived from models are often severely biased, which can lead to erroneous conclusions about the data's underlying relationships, as detailed in the supplementary document accompanying this study.

Furthermore, the use of the function `'explain=SHAP(model)'` implies that SHAP does not merely calculate feature importance but may also inherit—and potentially exacerbate—any biases present in the original model. This can result in an amplified distortion of feature importance, misleading researchers who rely on these measures for interpreting their results [7–9].

In light of these concerns, this paper advocates for the adoption of nonlinear and nonparametric robust statistical methods such as Spearman's correlation, Kendall's tau, Goodman-Kruskal gamma, Somers' D, and Hoeffding's D, each accompanied by *P* values to assess statistical significance. These methods offer a more reliable means of evaluating relationships between variables because they do not rely on the same assumptions as parametric methods and are less sensitive to outliers and skewed data distributions. Employing these robust statistical techniques could lead to more accurate interpretations of feature importance and treatment response, ultimately strengthening the conclusions drawn from studies of SLE patients and their responsiveness to iberdomide.

Our analytical framework deliberately employs multiple complementary approaches to enhance the validity and clinical utility of our findings. Rather than relying solely on correlation coefficients, we implement a multifaceted strategy that strengthens the robustness of our conclusions.

This comprehensive approach combines standardised correlation analyses with unstandardised effect measures, providing multiple lines of evidence that reinforce each other. For analysing SLE patient responses to iberdomide, we triangulate evidence using: (1) standardised correlation coefficients to assess relationship strengths, (2) unstandardised absolute differences in disease activity scores for direct clinical interpretation, (3) changes in molecular marker levels that maintain their biological meaning, and (4) cross-validation techniques to ensure reproducibility of findings.

By integrating these diverse analytical approaches, we not only enhance the validity of our findings but also provide clinically meaningful measures that directly translate to patient care. This methodological triangulation helps mitigate the limitations of any single analytical approach while offering complementary perspectives on the relationships between molecular endotypes and treatment response. Such a multifaceted strategy aligns with best practices in clinical research by providing both statistical rigour and practical clinical utility.

Competing interests

The author has no conflict of interest.

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According to ScholarGPS, Yoshiyasu Takefuji holds notable global rankings in several fields. He ranks 54th out of 395,884 scholars in neural networks (AI), 23rd out of 47,799 in parallel computing, and 14th out of 7,222 in parallel algorithms. Furthermore, he ranks highest in AI tools and human-induced error analysis, underscoring his significant contributions to these domains.

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Response

Response to: Correspondence on ‘Evaluating the limitations of random forest and SHAP in predicting treatment responses in systemic lupus erythematosus’

We thank Dr Takefuji for his interest in our manuscript ‘Responsiveness of systemic lupus erythematosus subjects to iberdomide based on molecular endotypes’ [1] and would like to address the concerns he raised. His major concern relates to our application of a random forest (RF) machine learning classifier in conjunction with Shapley Additive Explanations (SHAP) feature importance analysis ‘...to predict treatment responses to the highest dose of iberdomide...’ and ‘to identify significant factors influencing these predictions’. Although it is important to assess the appropriateness and utility of all bioinformatic tools and the accuracy of their predictions, in this circumstance, it appears that Dr Takefuji may not have fully comprehended the purpose of these analyses in the context of our manuscript.

The overall goal of this work was to identify subsets of lupus patients based on baseline gene expression profiles and determine whether these profiles showed patterns of molecular change over time with iberdomide treatment and how these changes were reflected in clinical response. Initially, we identified patient subsets at baseline using k-means clustering of gene module enrichment carried out using gene set variation analysis (GSVA). To determine the optimal number of clusters for k-means, we employed both the silhouette and elbow methods, which guided the selection of the most appropriate number of patient subsets. Two subsets (C and E) defined by baseline gene expression profiles showed clinical responsiveness defined by the Systemic Lupus Erythematosus (SLE) Responder Index 4 (SRI-4) metric to iberdomide [2]. The baseline GSVA scores of each gene module in each subset were then compared to molecular profiles after various periods of iberdomide treatment by Hedge’s *g* effect size; in addition, changes in gene module expression over time were visualised using violin plots with pairwise t-test comparisons to provide an intuitive and statistical representation of distributional shifts. Using this approach, we were able to define changes in gene module expression in the clinical responders in patients in subset E and to a lesser extent in those in subset C. An RF model was then applied to validate

these findings. Contrary to Dr Takefuji’s claim, the classes used in this supervised analysis represented valid ‘ground truths’ derived from a controlled clinical trial—specifically, gene expression profiles measured at baseline and week 24 in patients who were already known to be clinical responders based on the SRI-4 metric. These labels were directly tied to real clinical measurements and outcomes, consistent with the definition of ‘ground truth’. Importantly, the RF model was constructed independently for each patient subset (E and C) using only the gene expression data from clinical responders within each subset to compare molecular profiles at baseline versus week 24. The objective was not to *predict* future treatment responses but to *confirm* and further characterise the molecular changes over time in patients who had already exhibited a clinical response to iberdomide. Feature importance analysis, including SHAP values, was employed to identify the gene modules most associated with these longitudinal changes, thereby complementing the earlier findings based on GSVA analyses and Hedge’s *g* effect size. Thus, while supervised models typically rely on labelled data to predict unknown outcomes, in this context, the RF model was used to validate molecular trajectories within clinically defined responders, supporting the reproducibility and biological relevance of the observed gene module dynamics.

In summary, the RF model was employed to confirm the results obtained with other analytic tools. As opposed to the claim of Dr Takefuji, the classes of this supervised analysis were ‘ground truths’, namely gene expression at baseline and week 24 separately in clinical responders and nonresponders. SHAP analysis was carried out to identify the gene module features that contributed most to classification, using this model as confirmation of the previous analysis of longitudinal gene expression changes by Hedge’s *g* effect size. Thus, Dr Takefuji’s claim that the RF model was used to predict treatment responses is incorrect, but rather this model was used to confirm changes in molecular features important in classification of patients known to be clinical responders or nonresponders.

Dr Takefuji asserts that our application of RF with SHAP feature importance analysis was flawed as it lacked ‘ground truth’ values, stating that ‘ground truth refers to information that is regarded as real or true, typically obtained through direct measurement...’ This concern is not appropriate as the ‘ground truth’ values used for RF classification were the RNA-seq gene expression profiles of each patient at baseline and at 24 weeks. Furthermore, the analysis was targeted as the patients were first selected based on clinical response or nonresponse, such that the response status was known

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beforehand and, thus, could also be considered as ‘ground truth’ information.

Additional concerns were raised in relation to feature importance calculation using SHAP as Dr Takefuji states, ‘...different models utilise varied methodologies for calculating feature importances, which can introduce significant biases in the results’. Furthermore, he cites an article asserting that SHAP is not appropriate for explainable artificial intelligence because ‘...the goal of SHAP is to measure the relative importance of features and not so much to compute the exact value of feature importance’ [3]. We fully acknowledge the relative and model-dependent nature of SHAP values; however, it is precisely this model-specific attribution that makes SHAP a powerful and widely accepted tool for interpretability in complex machine learning models. In our study, SHAP was not used to claim exact causal influence of each gene module or to assert an absolute biological effect size. Rather, it was employed as an *orthogonal validation tool*—an additional lens to examine the features (gene modules) that contributed most to the classification of known clinical responders’ molecular profiles over time. These SHAP-derived feature importance scores were not interpreted in isolation but in the context of our prior effect size-based analysis using Hedge’s *g*, as well as GSVA trends over time. Moreover, our SHAP analysis was applied within well-controlled experimental settings: a supervised classification problem in which the model was trained on gene expression data from clinical responders at baseline and week 24 within each patient subset (E and C). We acknowledge the relative nature of calculating SHAP feature importance values, which is carried out in the context of a specific machine learning model. However, SHAP is generally considered to be the most effective means of calculating feature importance in machine learning models [4–6]. Moreover, we did not intend the feature importance calculations to represent the exact contribution of each gene module at baseline to the molecular profile at week 24 of treatment. Rather, in this instance, SHAP served as an orthogonal approach to identify the gene modules (features) among those tested that contributed most to the molecular profiles of responders over 24 weeks of treatment.

At the end of his correspondence, Dr Takefuji proposes that his own analytical framework, which ‘...deliberately employs multiple complementary approaches to enhance the validity and clinical utility of our findings’ would be a better strategy to employ. In response, we would note that we did use multiple, complementary approaches to increase the body of evidence for our characterisation of gene expression-based patient subsets that are most likely to respond to iberdomide therapy. These included comparison of clinical features and SRI-4 response levels across subsets as well as correlation of clinical features with disease severity, direct comparison of molecular features (gene modules) across patient subsets, longitudinal analysis to calculate the effect size of the change of each gene module in each subset after treatment and finally, RF and SHAP analysis to confirm the relationship of changes in gene expression to clinical response over time. In conclusion, while we realise that there are a multitude of approaches that could have been utilised in this work, we believe that our orthogonal analytical strategies were employed appropriately and with sound reasoning behind them and, thus, that the results are valid and should be

considered when evaluating the effectiveness of iberdomide for the treatment of lupus patients.

Competing interests

All authors declare they have no competing interests.

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