

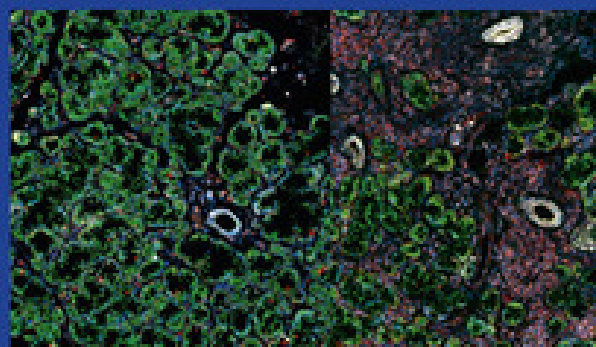
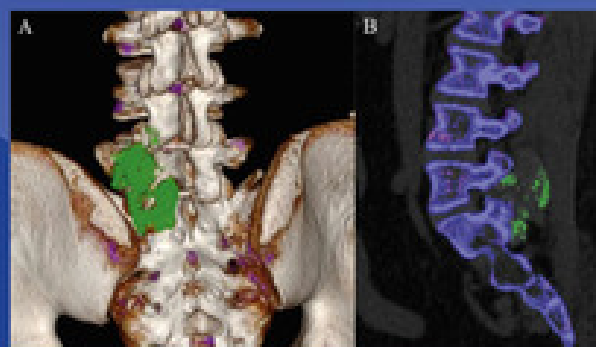
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Recommendations

Treating juvenile dermatomyositis to target: Paediatric Rheumatology European Society/Childhood Arthritis and Rheumatology Research Alliance-endorsed recommendations from an international task force

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ABSTRACT

Objectives: Despite the recent prognostic improvement, a sizeable proportion of patients with juvenile dermatomyositis (JDM) respond suboptimally to contemporary therapies. This study aimed to develop recommendations for treating JDM to target.

Methods: A Steering Committee formulated a set of provisional recommendations based on evidence derived from a systematic literature review and a retrospective chart review of patients. These were discussed, amended, and voted on by an international Task Force, including 28 paediatric rheumatologists, 2 specialists in neuromuscular diseases, 1 dermatologist, 1 physical therapist, 1 research nurse, 2 patients with JDM, and 1 parent of a patient with JDM. Items that achieved at least an 80% majority vote were accepted as final recommendations.

Results: Although the literature review did not reveal trials that compared a treat-to-target strategy with a nonsteered approach, it provided indirect evidence about specific end points that could serve as targets that facilitated development of recommendations. The group reached consensus on 7 overarching principles and 12 recommendations. It was agreed that both patients/parents and treaters should share decisions in setting treatment targets and therapeutic strategies, with inactive disease as the preferred target and minimal disease activity an alternative

one. Inactive disease is targeted to be achieved within 12 months after treatment start. Interim targets include minimal and moderate clinical improvement within 6 weeks and 3 months, respectively, and normalisation of muscle strength within 6 months. High-dose glucocorticoids remain fundamental in the initial management, but progressive tapering and discontinuation within 12 months through optimisation of concomitant immunomodulatory therapy was advised. A research agenda was formulated.

Conclusions: The Task Force developed recommendations for treating JDM to target, being aware that the evidence is not strong and needs to be expanded by future research. Implementation of the recommendations in clinical practice will help to reach optimal outcomes for JDM.

INTRODUCTION

Juvenile dermatomyositis (JDM) is the most common idiopathic inflammatory myopathy in childhood. It is a multisystem vasculopathic inflammatory disease of presumed autoimmune aetiology that involves primarily the skin and the skeletal muscles [1,2]. Despite the markedly reduced mortality rates and improvement of functional outcomes for JDM over the last 30 years, there are still many patients who respond suboptimally to available therapies and experience chronic disease activity. These patients are at risk of developing cumulative disease-related or treatment-related damage and physical disability, which may have a profound impact on their quality of life [3–7].

In recent years, 2 randomised controlled trials of traditional and novel medications have been conducted in JDM [8,9]. In addition, there have been several consensus initiatives aimed to provide expert advice on the optimal management of JDM and to harmonise the therapeutic approaches internationally [10–16]. This progress has been paralleled by the development and validation of novel standardised assessment tools (Table 1) [17–21]. However, the treatment remains largely empiric and widely variable across paediatric rheumatology centres. In addition, the optimal therapeutic goals and the ideal timing of their achievement are not well established.

Studies in adults with rheumatoid arthritis have shown that patient outcomes are improved if low levels of disease activity are targeted by frequent adjustments of therapy according to quantitative indices, regardless of the therapeutic agent chosen (so-called treat-to-target [T2T] strategy) [22–25]. In recent years, the T2T approach has been introduced in the management of juvenile idiopathic arthritis and childhood-onset systemic lupus erythematosus [26–30]. Since a similar effort has not yet been made for JDM, an international Task Force (TF) was convened to reach a consensus on a set of recommendations aimed at defining a T2T strategy for JDM.

METHODS

The project was initiated by authors AR and BMF, who invited paediatric rheumatologists from Europe and North America (CH, LMC, AMR, and LGR), selected on the basis of publication records, expertise in treating JDM and previous participation in similar activities, to form a Steering Committee (SC). The first online meeting of the SC (May 11, 2023) was aimed to discuss the unmet needs in the treatment of JDM and the potential for adopting a T2T strategy. It was unanimously agreed that defining therapeutic goals and an appropriate targeted treatment approach would be beneficial, but there was concern that underlying evidence could be lacking. It was, therefore, decided that – in accordance with the European Alliance of Associations for Rheumatology (EULAR) standardised operating procedures for recommendation efforts [31,32] – a

comprehensive systematic literature review (SLR) to address the current evidence in JDM was a necessary initial step of the process. After the formulation of a series of search questions (Supplementary Table S1), the SLR was performed by authors SR, JMM, TB, and AIR-G, with the help of a research librarian.

The SLR results (which will be described in a separate manuscript) were presented to the SC at a subsequent online meeting (March 27, 2024). The literature search revealed that no randomised controlled trials that addressed a target-oriented, steered therapy in comparison with conventional management had been published in JDM. Indirect evidence on optimal therapeutic approaches was, however, available to inform the next stages of the process. On this basis, the SC formulated a provisional set of recommendations.

In the meantime, a TF including 22 additional paediatric rheumatologists practicing in various areas of the world (Africa, Asia, Australia, Europe, South America, and North America), 2 specialists in neuromuscular diseases, 1 dermatologist, 2 allied health professionals (1 physical therapist and 1 research nurse), 2 patients with JDM and 1 parent of a patient with JDM was gathered. These invitations were based on individuals' contributions to the field, experience with JDM clinical care and research, and deliberations among members of the SC.

The provisional overarching principles and recommendations were, then, shared with the TF members through an online consensus survey, which remained open from July 8 to August 28, 2024. Respondents were asked to provide their agreement or disagreement about each recommendation, as well as the reasons for disagreement and suggestions for changes. The survey was completed by 92.5% of TF members, and the percentage of agreement was >80% for 17 of the 19 proposed statements (Supplementary Tables S2 and S3). The results of the survey were examined by the SC at an online meeting (September 4, 2024). Based on the comments provided by the respondents, some recommendations were amended or reworded.

To provide a further background to the process from the real world of clinical practice, a retrospective chart review of patients with JDM followed up at 2 tertiary care paediatric rheumatology centres was performed by 4 authors (SR, JMM, TB, and AIR-G). The objective of this exercise (whose findings will be reported in a separate manuscript) was to gain insights into optimal targets [17–21,33,34] and timing of their achievement during the first 2 years after disease diagnosis. The key data obtained from the retrospective chart review are summarised in Supplementary Table S4.

The preliminary recommendations, as well as the results of SLR and retrospective chart review, were presented and discussed at a final consensus conference, which was convened in Genoa, Italy, on October 7–8, 2024 and was attended in person by all but 1 member of the TF. A modified nominal group technique [35] was used to ensure equal participation among TF members. Participants were randomly divided into 3 breakout

Table 1

Instruments and criteria used for assessment of disease activity and treatment response in JDM

	Items included														Ref
	PhGA	PaGA	CMAS	MMT8	hMC	Skin VAS	Skin DAS	Total DAS	EM VAS	CK	Muscle enzymes ^a	CHAQ	CHQ-PhS	Requirements for classification	
Criteria for clinically inactive disease															
PRINTO criteria	X		X	X						X				At least 3 of the following 4 criteria: CK ≤ 150 U/L, CMAS ≥ 48, MMT8 ≥ 78 and PhGA ≤ 0.2 cm of 10 cm	[19]
Modified PRINTO criteria	X		X	X						X				PhGA ≤ 0.2 cm of 10 cm and at least 2 of the following 3 criteria: CK ≤ 150 U/L, CMAS ≥ 48, MMT8 ≥ 78	[20]
JDMAI1 criteria	X	X			X	X								Total score ≤ 2.4	[18]
JDMAI2 criteria	X	X			X		X							Total score ≤ 5.2	[18]
Criteria for minimal (or low) disease activity															
JDMAI1 criteria	X	X			X	X								Total score ≤ 6.6	[18]
JDMAI2 criteria	X	X			X		X							Total score ≤ 8.5	[18]
2016 ACR/EULAR JDM response criteria															
Based on IMACS core set	X	X		X					X		X	X		Minimal ≥ 30	[21]
Based on PRINTO core set	X	X	X					X				X	X	Moderate ≥ 45 Major ≥ 70	

ACR, American College of Rheumatology; CHAQ, Childhood Health Assessment Questionnaire; CHQ-PhS, Physical Summary Score of the Child Health Questionnaire; CK, creatine kinase; CMAS, Childhood Myositis Assessment Scale; DAS, Disease Activity Score; EM, extramuscular; EULAR, European Alliance of Associations for Rheumatology; hMC, hybrid MMT8/CMAS; IMACS, International Myositis Assessment and Clinical Studies Group; JDM, juvenile dermatomyositis; JDMAI, Juvenile DermatoMyositis Activity Index; MMT8, Manual Muscle Testing 8; PaGA, Parent's global assessment of child's overall wellbeing; PhGA, Physician's global assessment of overall disease activity; PRINTO, Paediatric Rheumatology International Trials Organisation; VAS, visual analogue scale.

^a Muscle enzymes: CK, lactate dehydrogenase, aspartate aminotransferase, alanine aminotransferase, aldolase.

tables, moderated by 3 authors (AR, BMF, and AC), who did not vote. Extensive discussions took place during these sessions, and the suggested wording was reformulated as deemed appropriate, with majority votes where controversy emerged. The results obtained by the breakout groups were reported to the whole TF, which then discussed the proposals, amended them, and arrived at final wordings that were subjected to an electronic poll (Poll Everywhere) [36]. Items that achieved at least an 80% majority vote were accepted as final recommendations.

After the consensus meeting, all participants were asked to adjudicate via e-mail their level of agreement with each overarching principle and recommendation on a 0 to 10 scale (0 = no agreement at all; 10 = full agreement). The evaluation of the level of evidence (LoE) and strength of recommendation was based on the Oxford Evidence Based Medicine categorisation [37].

RESULTS

The individual statements that received a positive vote comprised 7 overarching principles and 12 recommendations. These items are shown in [Tables 2 and 3](#), respectively, together with the percentage of positive votes obtained at the consensus conference, LoE, strength of recommendation, and level of agreement, and are discussed in detail further.

The evidence base

The SLR led to retrieve 2714 studies from the scrutinised databases, with additional 47 abstracts identified through citation searching and 23 flagged by SC members. Of these, 300 underwent a full-text review process and 220 were included in the final review ([Supplementary Fig S1](#)). The SLR revealed that no randomised controlled trials had compared a targeted therapeutic approach with conventional therapy in JDM. There was, therefore, no direct evidence that a T2T strategy was superior to nontargeted management. However, indirect support regarding the time that should elapse before escalating treatment in a patient with persistently active disease was provided by 2 randomised clinical trials, noncontrolled therapeutic studies, observational investigations, and therapeutic recommendations formulated by expert panels [8–15,38–43]. Nevertheless, the LoE for the development of recommendations was anticipated to be low and mainly based on expert consensus.

Overarching principles

A. The treatment targets and the therapeutic strategy should be based on shared decisions between patients, caregivers and the multidisciplinary healthcare team.

It was acknowledged that involvement of the parents/caregivers and, where appropriate, the patient in therapeutic decision-making is crucial and may enhance adherence to treatment and potentially improve the outcome. Although it was highlighted that the management of patients with JDM should be ideally conducted by professionals with specific paediatric expertise, it was argued that not all children will have access to paediatric rheumatology care and that adolescents and young adults should also be considered. For these reasons, it was decided to remove the adjective paediatric, which was placed in the initial formulation before the multidisciplinary health care team. This item was endorsed unanimously.

B. Treatment approaches should be based on all of an individual patients' disease characteristics, taking into account all

available findings (including, but not limited to, laboratory findings, autoantibody profile, capillaroscopy findings, imaging, and histopathology features).

It is well established that considerable heterogeneity exists among patients with JDM in terms of clinical phenotype, disease severity, course, and outcomes. This variability implies that the therapeutic choices, optimal targets, and treatment strategy should be tailored to the prevalent forms of organ involvement and the overall level of disease activity. In recent years, it has been shown that this heterogeneity also involves the presence of a wide array of myositis-specific autoantibodies, which can be identified in ~60% of children with JDM and have been found to define homogeneous subgroups of patients with similar clinical features, response to therapy, and prognosis [44–48]. Several studies have demonstrated the diagnostic and prognostic value of nailfold capillaroscopy in JDM [49–57]. In recent years, magnetic resonance imaging (MRI) has played an important role, both in the diagnosis of JDM and in helping monitor myositis activity [58–60]. Muscle biopsy remains an important investigation in the diagnostic workup of a subset of patients with JDM, especially in the absence of skin rash or in an otherwise atypical presentation [61,62]. Histopathology findings may predict disease prognosis [63]. Because emerging evidence indicates that the abovementioned tools may influence the therapeutic approach, participants believed it important to include them all in the statement. However, concern was raised that some diagnostic methods may not be available in all centres, particularly in lower-resource countries. It was also argued that novel JDM biomarkers, such as galectin-9, CXCL10, and Siglec-1 expression on monocytes, may deserve consideration in the future [64,65]. For this reason, the sentence 'but not limited to' was incorporated. The term imaging was preferred over muscle MRI in the consideration of the growing adoption of muscle ultrasonography [66–68]. After merging these suggestions, this item achieved 97% of participants' votes.

C. The goals of treating patients with JDM are to control signs and symptoms, to prevent organ damage and disease complications, including calcinosis and lipodystrophy, and to prevent flares. These objectives should be coupled with avoidance of drug toxicity and treatment complications and with optimisation of physical function, mental wellbeing, growth and development, quality of life, education and social participation.

This item was unanimously endorsed by TF members, after several amendments. It was agreed to give priority to the control of inflammatory signs and symptoms, followed by prevention of organ damage and disease complications, especially calcinosis and lipodystrophy. All participants concurred that caution should apply particularly to systemic glucocorticoids, whose side effects may have a devastating impact in the paediatric age. Optimisation of mental wellbeing, as well as growth and development, was incorporated in the therapeutic goals to highlight the paediatric specificity of the recommendations.

D. The management of JDM requires an understanding of its diverse manifestations and should be tailored, using patient-centred, individualised treatment strategies and a multidisciplinary approach.

The original term targeted was replaced by tailored, which was deemed more appropriate to express the need to adapt the therapeutic approach to the particular disease phenotype. It was also emphasised that the management of patients with JDM requires the involvement of a multidisciplinary team of specialists. This item was unanimously endorsed.

Table 2
Overarching principles for T2T in JDM

Overarching principles	Positive votes at consensus conference (%)	Level of evidence	Strength of recommendation	Level of agreement (mean ± SD)
A. The treatment targets and the therapeutic strategy should be based on shared decisions between patients, caregivers and the multidisciplinary healthcare team.	100	5	D	10 ± 0.2
B. Treatment approaches should be based on all individual patients' disease characteristics, taking into account all available findings (including, but not limited to, laboratory findings, autoantibody profile, capillaroscopy findings, imaging, and histopathology features).	97	5	D	9.9 ± 0.5
C. The goals of treating patients with JDM are to control signs and symptoms, to prevent organ damage and disease complications, including calcinosis and lipodystrophy, and to prevent flares. These objectives should be coupled with avoidance of drug toxicity and treatment complications and optimisation of physical function, mental wellbeing, growth and development, quality of life, education, and social participation.	100	5	D	10 ± 0.2
D. The management of JDM requires an understanding of its diverse manifestations and should be tailored, using patient-centred, individualised treatment strategies and a multidisciplinary approach.	100	5	D	9.9 ± 0.4
E. Use of glucocorticoids remains key to achieve early disease control. The dosage should be progressively reduced to the lowest level needed to maintain disease control and, when possible, discontinued through the optimisation of concomitant immunomodulatory therapy.	94	2b	B	9.9 ± 0.3
F. Treat-to-target requires regular assessment of the patient and adjustment of therapy until the desired target is reached.	91	5	D	9.8 ± 0.4
G. Patient monitoring should include assessment of treatment acceptability, adherence, and adverse events.	88	5	D	9.7 ± 0.9

JDM, juvenile dermatomyositis; T2T, treat-to-target.
Level of evidence ranges from 1a (systematic review of randomised controlled trials) to 5 (expert opinion); strength of recommendation ranges from A (directly based on category I evidence) to D (directly based on category IV evidence or extrapolated recommendation from category II or III evidence); agreement: percentage of experts agreeing with the recommendation statement; level of agreement ranges from 0 (fully disagree) to 10 (fully agree).

E. Use of glucocorticoids remains key to achieve early disease control. The dosage should be progressively reduced to the lowest level needed to maintain disease control and, when possible, discontinued through the optimisation of concomitant immunomodulatory therapy.

High-dose glucocorticoids remain the mainstay of the initial management of JDM, especially in patients with acute or life-threatening manifestations. However, in consideration of the serious side effects related to the prolonged administration of glucocorticoids, the recommendation to decrease progressively their dosage to the minimum level that ensures maintenance of adequate disease control was widely supported. This goal may be facilitated by the concomitant administration of immunomodulatory drugs that have shown a glucocorticoid-sparing potential in JDM. This item obtained 94% of votes.

F. Treat-to-target requires regular assessment of the patient and adjustment of therapy until the desired target is reached.

Although the SLR had produced only indirect evidence for the utility of the T2T strategy in JDM, the large majority of TF members were in favour of including regular measurement of disease activity and adjustment of therapy with persistently active disease, in the overarching principles. Nonetheless, the value of broadening the concept of patient assessment to encompass, beside disease activity, the whole burden/impact of the disease on patient's daily life was underscored. This item achieved 91% of participants' votes.

G. Patient monitoring should include assessment of treatment acceptability, adherence, and adverse events.

During breakout sessions, a debate arose about whether this statement should be kept separated or merged with principle F. It was finally decided to leave it as a standalone principle. However, because treatment adherence can be affected by tolerability and side effects, the terms acceptability and adverse events were added to the original formulation. Furthermore, the previous word therapy was replaced with the broader term treatment to extend the advice to non-medication therapies, such as physical therapy. After these refinements, approval was granted by 88% of the participants.

Recommendations

1. The primary target for treatment of patients with JDM is the state of inactive disease, which means the absence of signs and symptoms of inflammatory disease activity in muscles and skin as well as in other organs/systems.

Thus far, no clinical trial in JDM has compared progression of cumulative organ damage or improvement in quality of life when inactive disease rather than another state is targeted. However, there is indirect evidence that disease-related damage or complications are more effectively prevented when inactive disease is achieved [3,4,55,56,69–71]. Considering that the recent therapeutic advances enable reaching clinical remission in the majority of patients with JDM [72,73], inactive disease was set as the primary therapeutic target. The definition of inactive disease was intended to be quite strict, that is, as complete absence of all signs and symptoms of inflammatory disease activity not only in the skin and muscles but also in other affected organs. Although 91% of voters agreed with this statement, concern was raised that setting the state of inactive disease as principal therapeutic target may imply that only physician-centred goals should be pursued. Many participants thought that restoring normal function may be equally

Table 3
Recommendations for T2T in JDM

Recommendations	Positive votes at consensus conference (%)	Level of evidence	Strength of recommendation	Level of agreement (mean $\pm$ SD)
1. The primary target for treatment of patients with JDM is the state of inactive disease, which means the absence of signs and symptoms of inflammatory disease activity in muscles and skin as well as in other organs/systems.	91	2b	C	9.7 $\pm$ 0.7
2. Minimal (or low) disease activity may be an acceptable primary target when inactive disease cannot be achieved, as in more refractory cases.	87	2c	D	9.6 $\pm$ 0.8
3. Setting the target, selecting the measurement tools and decisions about treatment should all be based on individual patient characteristics and agreed upon with the caregivers/patient.	94	5	D	9.8 $\pm$ 0.5
4. Disease activity must be assessed and documented regularly using standardised and validated assessment tools.	94	2c	C	9.9 $\pm$ 0.4
5. Initial target I. Within 6 wk after starting treatment, at least a minimal clinical improvement ^a should be achieved. In patients with life-threatening conditions, a significant clinical improvement should be targeted in a shorter time frame.	97	5	D	9.5 $\pm$ 0.7
6. Interim target II. Within 3 mo after starting treatment, at least a moderate clinical improvement ^b , as well as normalisation of muscle enzymes, should be achieved. Patients should be free of life-threatening conditions at this time.	88	5	D	9.2 $\pm$ 0.9
7. Interim target III. Normalisation of muscle strength should occur within 6 mo.	86	5	D	9.2 $\pm$ 0.9
8. The primary target, the state of inactive disease ^c , should be achieved within 12 mo.	100	5	D	9.6 $\pm$ 0.6
9. Glucocorticoids should be aggressively tapered as long as interim targets are reached; glucocorticoids should be discontinued, ideally, within 12 mo or sooner ^d .	100	2b	C	9.8 $\pm$ 0.5
10. Regular assessment is recommended to ensure that the patient is on the correct trajectory to reach the target. The frequency of assessments depends on the level of disease activity and severity and the presence of complications.	100	5	D	9.9 $\pm$ 0.3
11. Treatment should be adjusted until the desired target is achieved.	97	2b	C	9.9 $\pm$ 0.3
12. Once the primary target has been achieved, it should be sustained. Ongoing monitoring should occur to ensure maintenance of the target.	100	2b	C	10 $\pm$ 0.0

JDM, juvenile dermatomyositis; T2T, treat-to-target.

Level of evidence ranges from 1a (systematic review of randomised controlled trials) to 5 (expert opinion); strength of recommendation ranges from A (directly based on category I evidence) to D (directly based on category IV evidence or extrapolated recommendation from category II or III evidence); agreement: percentage of experts agreeing with the recommendation statement; level of agreement ranges from 0 (fully disagree) to 10 (fully agree).

^a As defined by a validated response criteria or 25% improvement in a composite disease activity measure or in the physician global assessment.

^b As defined by a validated response criteria or 50% improvement in a composite disease activity measure or in the physician global assessment.

^c As defined in recommendation 1.

^d If target is not achieved, favour additional/change of immunomodulatory treatment, over repeatedly increasing glucocorticoid dose.

important as reaching inactive disease in the management of patients with JDM. Furthermore, some experts would have preferred to name the primary target clinically inactive disease to highlight the potential role of biomarkers or imaging techniques in defining disease remission more reliably than clinical assessment. However, despite emerging evidence for biomarkers and MRI reflecting subclinical inflammation, at present, there is no established role for these methodologies in defining remission.

2. Minimal (or low) disease activity may be an acceptable primary target when inactive disease cannot be achieved, as in more refractory cases.

Although the TF did not intend to replace the target of inactive disease with that of low disease activity, it was recognised that stringent inactive disease, as defined in recommendation 1, may be difficult to achieve in some patients, especially those with long-standing or refractory disease. Low disease activity is differentiated from the state of inactive disease by the persistence of a few residual signs and symptoms. However, it is assumed that physical function and quality of life would not be substantially worse than in inactive disease and that progression of organ damage, while possibly not halted, would be minimal. This item achieved 87% of participants' votes.

3. Setting the target, selecting the measurement tools and decisions about treatment should all be based on individual patient characteristics and agreed upon with the caregivers/patient.

This recommendation emphasises the need to individualise the therapeutic target, the method used for its assessment, and the therapeutic decisions based on patients' characteristics, which include the severity and extent of muscle and skin disease, involvement of visceral organs, presence of disease-related complications, especially dystrophic calcinosis and lipodystrophy, and comorbidities (eg, osteoporosis, growth failure, hypertension, and infection). Therapeutic decision-making may be guided by published consensus-based recommendations and treatment plans for the management of JDM [10–15]. This item was approved by 94% of the participants.

4. Disease activity must be assessed and documented regularly using standardised and validated assessment tools.

There was unanimous consensus that the use of standardised and validated assessment tools is the best method to evaluate disease activity and response to therapy in JDM. During the consensus meeting, the relative measurement properties and suitability for the T2T strategy of the existing tools [17–21,74–76] were discussed. It was finally decided not to recommend the use of a specific instrument, but to leave the choice open for the clinician. Hence, the neutral term standardised and validated assessment tool was endorsed by 94% of voters.

5. Initial target I. Within 6 weeks after starting treatment, at least a minimal clinical improvement (as defined by a validated response criteria or 25% improvement in a composite disease activity measure or in the physician global assessment) should be achieved. In patients with life-threatening conditions, a significant clinical improvement should be targeted in a shorter time frame.

This statement was subject to intense debate. Some attendees argued that the application of validated response criteria in clinical practice is time-consuming and proposed the alternative use of the simpler physician global assessment. There was much interest for composite disease activity measures, such as the Juvenile DermatoMyositis Activity Index (JDMAI) [17], which were thought to be feasible for regular assessments in routine clinics. A 6-week time frame was considered too long for the

most severe patients (eg, those with intestinal vasculitis or rapidly progressive interstitial lung disease). Conversely, other participants objected that the proposed magnitude and time of improvement could be too ambitious for some patients. In the end, it was widely agreed that although setting a therapeutic goal that fit all patients is unrealistic; it is, nevertheless, fundamental to prompt an early therapeutic change if a satisfactory improvement is not observed within 6 weeks after treatment start. This item was approved by 97% of the participants.

6. Interim target II. Within 3 months after starting treatment, at least a moderate clinical improvement (as defined by a validated response criteria or 50% improvement in a composite disease activity measure or in the physician global assessment), as well as normalisation of muscle enzymes should be achieved. Patients should be free of life-threatening conditions at this time.

As for interim target I, there was strong discussion about this proposal for interim target II. Some TF members wanted to eliminate the requirement of muscle enzymes normalisation within 3 months as it may be difficult to attain and does not apply to amyopathic disease. Some participants were in favour of keeping patients with more aggressive disease separated from those with mild-to-moderate involvement. Others argued that setting diverse targets for different degrees of disease severity could make the recommendations too complex and cumbersome. To reconcile these disparities, a sentence was added to underscore the need to ensure that life-threatening disease manifestations should no longer be present at 3 months after treatment start. After this change, the item was endorsed by 88% of the participants.

7. Interim target III. Normalisation of muscle strength should occur within 6 months.

Some participants contended that a 6-month time frame is excessively brief for patients who present with extreme muscle weakness, for whom a 9-month interval could be more acceptable. Consensus was finally reached to maintain the proposed timeline for all disease phenotypes. It was, however, advised that in case complete recovery of muscle strength is not obtained by the suggested time point, the treating physician should evaluate whether not only the patient needs an intensification of drug regimen but also a focused physiotherapy intervention could help to improve energy and stamina or whether persistent muscle weakness is due to glucocorticoid-induced myopathy. In patients with long-standing disease, the possible contribution of muscle damage or atrophy should be accounted for. Approval of this item was provided by 86% of the participants, with contrary votes being mostly explained by the disagreement about the 6-month time frame.

8. The primary target, the state of inactive disease (as defined in recommendation 1), should be achieved within 12 months.

This statement was endorsed unanimously. Indeed, all participants agreed that the target of inactive disease should be set at 12 months. It was emphasised that normalisation of muscle strength is not necessarily equivalent to complete abrogation of muscle inflammation.

9. Glucocorticoids should be aggressively tapered as long as interim targets are reached; glucocorticoids should be discontinued, ideally, within 12 months or sooner (if target is not achieved, favour additional/change of immunomodulatory treatment, over repeatedly increasing glucocorticoid dose).

Although all participants agreed on the merit of addressing glucocorticoid tapering and to consider it as a treatment target,

this statement was discussed in depth and modified several times, due to divergence about schemes of glucocorticoid tapering. Bearing in mind that the T2T strategy is not intended to provide indications for use of specific treatments, it was finally agreed to make a general statement that underlined the key importance of aggressive tapering and discontinuation of glucocorticoids. As a cautionary note, the need for verifying attainment of interim targets before decreasing glucocorticoid dosage was included. However, the concept that aggressive glucocorticoid tapering should be pursued was strengthened by indicating that the ideal timing for glucocorticoid discontinuation is 12 months after their start. The final version of the statement was endorsed unanimously.

10. Regular assessment is recommended to ensure that the patient is on the correct trajectory to reach the target. The frequency of assessments depends on the level of disease activity and severity and the presence of complications.

Owing to the clinical heterogeneity of JDM, the intervals between clinical evaluations vary in relation to the disease phenotype, level of disease activity, and presence of complications or comorbidities. In patients with profound muscle weakness and extensive skin rash or life-threatening disease manifestations, such as interstitial lung disease or intestinal vasculitis, frequent evaluations (even daily) of the disease status and course are required to ensure the necessary therapeutic adjustments in a timely manner. Patients with less severe disease require less frequent evaluations, which may occur weekly to every 3 months, depending on the degree of disease activity. Patients in sustained remission can be assessed at looser intervals, approximately every 4 to 6 months, to ensure maintenance of the target reached and, in those who are still receiving therapy, to watch the occurrence of adverse events and avoid overtreatment. This recommendation was approved by 100% of the participants.

11. Treatment should be adjusted until the desired target is achieved.

Indirect evidence indicates that early clinical improvement or the achievement of inactive disease is associated with better long-term outcome [3,4]. It is, thus, likely that pursuing the best possible target through treatment adjustments improves prognosis. Several TF members emphasised the value of non-pharmacologic interventions, particularly physiotherapy and rehabilitation, occupational therapy, optimisation of bone health and muscle fitness, management of fatigue, and psychological support. This recommendation was supported by 97% of the participants.

12. Once the primary target has been achieved, it should be sustained. Ongoing monitoring should occur to ensure maintenance of the target.

There are rationale and evidence, in JDM, that sustained/persistent remission leads to an optimal quality of life, enhances physical function, and stops progression of organ damage [3,4,55,69–71,77,78]. Conversely, an increase in disease activity during follow-up may lead to reduced quality of life and progression of cumulative damage [79,80]. Maintenance of the treatment target does not necessarily imply maintenance of treatment. Unfortunately, there is currently a lack of evidence-based data or guidelines to aid in the withdrawal of medications after disease remission in JDM. Furthermore, no reliable clinical, biomarker, or imaging indicators are currently available to identify patients at higher risk of experiencing disease flare after treatment discontinuation. This item was unanimously approved.

Adjudication of the level of agreement after the consensus meeting

The level of agreement on overarching principles and recommendations adjudicated by the TF members after the Genoa consensus meeting was very high, as all items achieved an average score greater than 9, and only 2 items had an average score lower than 9.5 (Tables 2 and 3).

DISCUSSION

We present in this study the overarching principles and specific recommendations for the T2T strategy in JDM, which were developed by a TF including a broad range of international experts and were endorsed by the Paediatric Rheumatology European Society (PReS) and the Childhood Arthritis and Rheumatology Research Alliance (CARRA). These were based on published evidence available to date, as well as on a retrospective review of 2 patient series, and were derived from an online consensus survey and extensive discussions conducted using a modified nominal group technique approach. Considering the wide international representation within the TF and the excellent level of agreement reached for all statements, the result of the effort will likely gain widespread acceptance.

The recommendations are primarily intended to provide guidance on general treatment approaches, with the aim of allowing for an optimal outcome of JDM in the individual patient. A remarkable difference with the previous treatment recommendations or treatment plans is the absence of indications or advice regarding specific agents or classes of medications – apart from the suggestion about tapering and discontinuation of glucocorticoid therapy and avoidance of long-term glucocorticoid administration. Hence, these recommendations should be applicable in all regions and countries, irrespective of medication availability. Although the recommendations are primarily aimed at paediatric rheumatology practitioners and other health professionals involved in the care of patients with JDM, they may also be of interest to official bodies, such as health authorities or payers, who may wish to explore clinical practice in their environment, as well as to clinical trialists and regulatory agencies, owing to the growing interest of pharmaceutical industries in trials evaluating a targeted therapeutic approach in comparison with conventional therapy in JDM.

The process was initiated by a SC, which followed the EULAR standardised operating procedures for the development of recommendations [31,32]. A large TF of 36 additional members was convened, which comprised 28 paediatric rheumatologists, 2 specialists in neuromuscular diseases, 1 dermatologist, 1 physical therapist, 1 research nurse, 2 patients with JDM, and 1 parent of a JDM patient. Ensuring input from a multidisciplinary group of paediatric specialists and allied health professionals was an important strength of the project. Participation of patients and parents was also critical to incorporate the view of these stakeholders and ensure that their needs were addressed, particularly when considering T2T potential side effects and quality of life as well as prevention of damage and disability.

Ideally, treatment recommendations should be based on available evidence. However, clinical trials focused on the comparison between continued treatment modification to reach a prespecified treatment target and a nontargeted approach do not exist for JDM. Although the SLR and the retrospective chart review provided indirect evidence about specific end points that could serve as targets, the proposed recommendations can only

be regarded as consensus-based expert opinion and, therefore, require additional research efforts.

Several TF members argued that the recommendations did not account for potential financial constraints or access to costly therapies. However, studies in adult patients with rheumatoid arthritis have shown that a good outcome can be obtained in a large proportion of patients with easily accessible and affordable therapies, as long as a strategic treatment approach is implemented [22,23,81]. A similar issue was raised regarding the availability of certain investigations, such as MRI, myositis-specific autoantibodies, or capillaroscopy, which may be valuable to define the best therapeutic approach. This problem was accounted for by recommending consideration for their results, but only when obtainable.

The TF agreed unanimously that a pivotal aspect in the care of JDM is the shared decision-making with the patient and the caregivers. There was also full consensus that control of sign and symptoms is the main objective in the treatment of JDM. However, other key goals should be pursued concomitantly, including prevention of organ damage, disease flares, and complications. A further item specific to paediatric patients is the optimisation of linear growth and pubertal development. The TF emphasised that care of adolescent patients be tailored in accordance with the broader process of transition from paediatric to adult rheumatology care. Parent/patient representatives put a particular emphasis on restoring physical and emotional functioning and wellbeing as well on avoidance of medication side effects.

The TF set the state of inactive disease as the primary target for treatment, with minimal (or low) disease activity as alternative goal. Although some experts highlighted the potential superiority of more stringent targets, such as remission by biomarkers or muscle MRI over clinical assessment, the majority considered that at present remission should be defined on clinical grounds through the use of one of the existing criteria, as the evidence for other methods is still limited.

Beside the overall therapeutic goals, interim targets as well as the timing for their achievement after treatment start were set. These included minimal and moderate clinical improvement within 6 weeks and 3 months, respectively, normalisation of muscle strength within 6 months, and inactive disease within 12 months. Although some consensus conference participants argued that the time frame of initial targets was too long for the most severe patients, whereas others warned that reaching a normal muscle strength within 6 months could prove difficult in patients with profound muscle impairment, all proposals were approved, with the commitment of verifying the feasibility of the targets by future investigations in clinical settings. In setting the timeline for treatment adjustments, the variability of time of onset of effect of some medications used to treat JDM should be taken into account.

It was recognised that the initial management of JDM relies on high-dose glucocorticoids, especially in patients with the most severe presentations. However, in consideration of the devastating side effects related to the prolonged administration of glucocorticoids in children, the indication for their gradual tapering till reaching the minimum dosage required to maintain the disease control and, when possible, discontinuation through the optimisation of concomitant immunomodulatory therapy was incorporated. The ideal time for their discontinuation was set at 12 months. The established treatment targets and the optimal time of their achievement are outlined in [Supplementary Figure S2](#).

In conclusion, the recommendations to treat JDM to target have been developed by an international TF. Their implementation in clinical practice may improve patient care and outcomes

Box Research agenda

- > Implementation of randomised trials aimed to show the superiority of a targeted treatment approach based on treat-to-target over a nontargeted approach
- > Acceptance and applicability of treat-to-target strategies in standard clinical practice
- > Acceptance and feasibility of treat-to-target strategies in low-resource countries
- > Evaluation of the impact of treat-to-target in terms of cost, safety, and treatment burden compared with usual care
- > Impact of parent/patient evaluation, particularly in the presence of marked fatigue, in the assessment of targets
- > Impact of diagnostic delay in the achievement of targets
- > Clarification of the role of myositis-specific autoantibodies in designing treatment strategies and defining targets
- > Clarification of the role of capillaroscopy findings in designing treatment strategies and defining targets
- > Role of emerging biomarkers (eg, interferon-related biomarkers) in the assessment of targets
- > Role of imaging methods in definition and assessment of targets, especially remission of muscle disease
- > Role of muscle histopathology in designing treatment strategies and defining targets
- > Analysis of the optimal modalities of tapering and/or withdrawing treatments in patients reaching inactive disease
- > Development and validation of widely accepted outcome measures for skin disease
- > Evaluation of additive targets meaningful to patients and parents, such as restoring normal physical and emotional function and wellbeing

for patients with JDM. The research agenda should prioritise testing of the recommendations in randomised controlled trials that compare a targeted therapeutic approach with conventional treatment and other objectives listed in the [Box](#).

Competing interests

All authors declare they have no competing interests related to the present work.

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Contributors

AC, AR, AMR, BMF, CHH, FB, LGR, LJM, and SR developed the online consensus surveys, organised and facilitated the consensus meeting, and wrote the first version of the manuscript. AIR-G, JMM, SR, and TB performed the systematic literature review and the retrospective chart review. AMH, ANM, BB-M, CB, CF, CM, CPa, CPI, ES, GT, HS, LC, LRW, MA, MJ, MMK, NAH, NT, OK, PL, RC-M, RM, RN, SC, SET, SK, SM, SS, and TM participated as voting members of the consensus meetings, contributing to decisions regarding items to be included in the overarching principles and recommendations. All authors reviewed and approved the final draft of the manuscript.

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Patient and public involvement statement

Patients and parents from Europe and the USA actively participated in the design and conduct of this research. The Task Force included 1 parents and 2 young adults with juvenile dermatomyositis, now older than 18 years, providing patient-centred perspectives. They participated in the online consensus survey and attended the in-person consensus meetings with full, equal voting rights. They were involved in all discussions that led to the final formulations of overarching principles and recommendations. Patients and parents reviewed and approved the manuscript and are appropriately included as named authors.

Patient consent for publication

Not applicable.

Ethics approval

The study protocol was approved by the Ethics Committee of Regione Liguria on 18/09/2023, number 209/2023.

Provenance and peer review

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Supplementary materials

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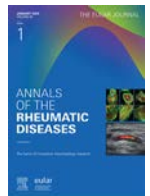
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Review

Primary Sjögren's Disease: a review of unmet need, outcome measures, therapeutic advances and health economic impacts. Lessons from the NEw Clinical Endpoints in primary Sjögren's Syndrome: an Interventional Trial based on stratifying patients (NECESSITY) Innovative Health Initiative (IHI)

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ABSTRACT

Primary Sjögren's disease (pSjD) is an autoimmune rheumatic disease involving exocrine glands and associated with high symptom burden (dryness, fatigue, pain), systemic features and salivary gland dysfunction. B-cell hyperactivity is common, with an increased risk of mucosa-associated lymphoid tissue lymphoma. This review describes the unmet need, scientific validity of outcome measures, optimisation of clinical trial design, therapeutic advances and how clinical improvement relates to health-related quality of life, additional quality-adjusted life years and economic benefit in pSjD. It derives from the EU-funded Necessity IHI Academic-Industry collaborative Consortium project while also drawing on work by the European Alliance of Associations for Rheumatology Sjögren's task force and others. The NECESSITY Consortium, formed within the framework of the Innovative Health Initiative (IHI), comprises 20 academic partners, 1 patient group partner and 4 industry partners (NECESSITY; <https://necessity-h2020.eu>). Patient leaders have been closely involved, with expert advice obtained from the European Medicines Agency and the United States Food and Drug Administration during the development phase of a new outcome measure, the Sjögren's Tool for Assessing Response composite response criteria. This tool is now undergoing validation through the NECESSITY IHI clinical trial and industry-sponsored trials.

WHAT IS ALREADY KNOWN ON THIS TOPIC

- Several phase 2 trials have met their primary endpoint with regards to improvement in systemic disease activity measured by the ESSDAI or ClinESSDAI.

WHAT THIS STUDY ADDS

- Significant progress has been made in the design and implementation of clinical trials of novel and effective therapies to address the substantial unmet needs in pSjD.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- Some of these trials have also demonstrated improvement in patient symptoms, but further work is required to optimise inclusion criteria, trial design and outcome tool usage including

patient reported outcome measures and composite endpoints and demonstrating clinical and economic benefit to persuade payers and regulators to approve novel therapies.

- Patient engagement and partnership has been a key element of both academic and commercial studies in pSjD and was an important aspect of the NECESSITY IHI project.

INTRODUCTION

Primary Sjögren's disease (pSjD) is a systemic autoimmune disease characterised by inflammation of exocrine secretory glands [1] with a prevalence estimated at 38.95 cases/100,000 [2] and incidence at 6.92 new cases/100,000 person-years. The female to male incidence ratio is 9.29:1 with a peak diagnosis incidence at 56 to 60 years old [3,4].

Over 90% of patients have dry eye/dry mouth symptoms and 70% to 80% have chronic fatigue/joint/limb pain [5–8]. An estimated 20% to 40% of patients have extraglandular manifestations (such as inflammatory arthritis, lymphadenopathy and constitutional features, cutaneous involvement, interstitial lung disease, neurological/muscle and haematological involvement, etc), and 5% to 10% develop lymphoma, typically salivary gland (SG) mucosa-associated lymphoid tissue (MALT) B-cell lymphoma [9,10].

Hypergammaglobulinemia with polyclonal B-cell activation is frequent. Antinuclear antibodies (ANAs) are detected in 60% to 80% of patients and can predate disease onset by decades [11]. Anti-Ro (SSA) (majority anti-Ro60 + /Ro52 + [12]) and anti-La (SSB) antibodies occur in one-third of patients and anti-Ro without anti-La in another third. Rheumatoid factor (RF) is also frequently elevated. Autoantibodies and hypergammaglobulinemia are associated with systemic complications and increased lymphoma risk [13–15].

Patients with uncomplicated glandular-predominant pSjD have a slowly progressive disease course without a clearcut increase in mortality, in contrast to patients with systemic disease/lymphoma [16–19]. Nevertheless, glandular disease alone can be associated with significant morbidity [20].

This review explores the elements required for clinical trials in pSjD and the unmet needs and challenges in delivering novel therapies for pSjD to market.

PATHOGENESIS

pSjD is characterised by activation of the innate immune system, with type I interferon (IFN) production in response to an unknown trigger, involvement of pattern recognition receptor and Toll-like receptor pathways [21], and focal periductal immune cell infiltration into exocrine glands [22–28]. Inflammatory mediators, such as B-cell activating factor (BAFF) and tumour necrosis factor (TNF) released by sources including dendritic cells and macrophages, contribute to B-cell activation, hypergammaglobulinemia and autoantibody formation. Approximately 25% of patients develop tertiary lymphoid structures (TLS) (also referred to as ‘ectopic germinal centre like structures’) in the SGs, with microenvironmental stromal and epithelial cells playing a critical role in their formation [29–32].

Chemokine (C-X-C motif) ligand-13 (CXCL-13), mainly expressed by stromal cells and follicular helper T (Tfh) cells, has an essential role in B-cell migration into lymphoid aggregates and TLS in inflamed SGs [33–35]. Increased levels of proinflammatory cytokines (IFN- γ , interleukin [IL]-21) secreted by T cells are associated with severity of disease [36]. IFN- γ , produced by natural killer cells and T helper (Th)1 cells, stimulates production of BAFF by antigen-presenting cells, in turn activating B cells, promoting the permanence of autoreactive B cells and promoting lymphoma development [25]. The cytokine IL-21, produced by Tfh cells, promotes B-cell differentiation into plasma cells and production of autoantibodies. Levels of IL-21 correlate with the degree of SG lymphocytic infiltration, as well as autoantibody production and B-cell hyperactivity [37,38]. Another cytokine, IL-22, also has a key role in TLS organisation [39,40].

Genome-wide association studies [41] have identified loci associated with pSjD. Specifically, the human leukocyte antigen region, but also loci containing IFN regulatory factor 5 (IRF5), signal transducer and activator of transcription 4 (STAT4) and IL-12A linked to IFN activation, B-lymphoid kinase and chemokine (C-X-C motif) receptor 5 (CXCR5) (receptor for CXCL-13

and marker of Tfh cells) linked to B-cell activation and TNFAIP3 interaction protein 1 (TNIP1). Khatri et al. [42] identified other associations including the tyrosine kinase 2 (TYK2) loci. TYK2 mediates signalling of key cytokines such as type 1 IFNs, IL-23, and IL-12. A TYK2 inhibitor, deucravacitinib, is currently being evaluated in systemic lupus erythematosus (SLE) [43,44] and pSjD (<https://clinicaltrials.gov/study/NCT05946941>).

CURRENT STANDARD OF CARE

National/International management guidelines have been developed [45–48]. Patient engagement and education is critical. Dry eyes are managed with preservative-free artificial tears, lubricating ointments, topical nonsteroidal anti-inflammatory drugs (NSAIDs) or corticosteroid/cyclosporin/tacrolimus eye drops and/or punctal silicone plugs. Similarly, for dry mouth, saliva substitutes (moisturising sprays/gels, saliva stimulating tablets and alcohol-free rinses) are used. Pilocarpine (or cevimeline) stimulates tear and saliva production.

Hydroxychloroquine is used to treat fatigue/arthritis and inhibits B-cell hyperactivity, although evidence of clinical efficacy is limited [49,50]. Low dose glucocorticoids are typically recommended empirically for systemic complications.

Conventional disease-modifying antirheumatic drugs (DMARDs) are used for systemic features, although without definitive evidence from randomised controlled trials. Access to biologic DMARDs depends on local rules. Lymphoma is treated conventionally with chemotherapy-rituximab combinations.

CLINICAL TRIAL DESIGN

Without an effective therapy most trials will be double-blind randomized superiority studies against a placebo, or standard of care, with predefined improvement criteria. Extension or cross-over studies, used predominantly to assess longer term safety, can be incorporated.

Platform trials may be considered both for early-phase (phase 2, exploratory) or later phase (phase 3, confirmatory). In such randomised multistage adaptive trials, multiple treatments can be assessed against a common control. Key features include the options of adding new investigational arms during the trial, terminating an investigational arm at an interim analysis based on predefined criteria for lack of activity, or, incorporating an investigational treatment that has met its primary endpoint into a new standard of care arm, which would become the new reference arm.

In terms of duration, a minimum of 12 weeks, depending on the study medication, but generally, 24 weeks or longer is preferred for biologic trials in which beneficial effects may take some time to manifest (unless predictive biomarkers are developed in the future) and for placebo responses to level out.

Inclusion and exclusion criteria

The ACR – European League Against Rheumatism (ACR-EULAR) Classification Criteria [51] and the Revised American-European Consensus Group (AECG) Criteria [52] have >90% concordance, and both have been used in clinical trials [53]. Key elements of inclusion and exclusion criteria for a number of pSjD clinical trials are summarised in Table 1 [54–77].

A requirement for anti-Ro antibodies provides diagnostic homogeneity and is a marker for B-cell activation and type I IFN signature. Other trials may use classification criteria alone, allow enrolment of 10% to 20% of patients without anti-Ro

Table 1
Eligibility criteria and general trial design in pSjD

Publication	Therapy	Entry criteria	Trial design	Primary outcome	Number of patients
Gottenberg et al 2014 [50]	HCQ	AECG +	HCQ 400 mg once daily or PBO	≥30% improvement in 2/3 ESSPRI domains at 24 wk	120 (HCQ = 56, PBO = 64)
Devauchelle-Pensec et al 2014 [54]	RTX (anti-CD20)	AECG +, ≥50 mm/100 mm VAS on ≥2 of: global, pain, fatigue, dryness, recent onset ≤10 years, anti-Ro + or other, or ≥1 systemic feature	RTX 1 g IV + MPRED 100 mg IV at wk 0 and 2 or PBO + MPRED 100 mg IV	≥30-mm improvement in ≥2 of 4 VAS at 24 wk	120 (RTX = 63, PBO = 57)
Bowman et al 2017 [55]	RTX	AECG +, anti-Ro +, fatigue, and dryness both ≥5/10 on NRS, USF >0	RTX 1 g IV at 0, 2, 24, and 26 wk or PBO. All participants received IV MPRED and oral prednisolone	≥30% reduction in either fatigue or dryness VAS at 48 wk	133 (RTX = 67, PBO = 66)
Fisher et al 2018 [56]					
Pontarini et al 2024 [57]					
St Clair et al 2018 [58]	Baminercept	AECG +, SSF ≥0.1 plus 1 of: fatigue, pain, specified systemic feature	Baminercept 100 mg S/C weekly or PBO	Change in SSF at 24 wk	52 (2:1) (baminercept = 33, PBO = 19)
Dörner et al 2019 [59]	Ianalumab (anti-BAFF + B-cell lysis)	AECG +, anti-Ro + or ANA + and RF +, ESSDAI ≥6	Ianalumab 3 mg/kg or 10 mg/kg IV or PBO	Change in ESSDAI at 12 wk	27 (3 mg/kg = 6, 10 mg/kg = 12, PBO = 9)
Felten et al 2021 [60]	Tocilizumab (anti-IL6R mAb)	AECG +, Ro +, ESSDAI ≥5	Tocilizumab 8 mg/kg IV or PBO monthly × 6	ESSDAI improvement of ≥3 points at 24 wk AND no worsening	110 (tocilizumab = 55, PBO = 55)
Juarez et al 2021 [61]	Seletalisib (oral PI3Kδ inhibitor)	AECG +, ESSDAI ≥5, anti-Ro + OR anti-La +, USF >0	Seletalisib 45 mg/d or PBO	Change in ESSDAI at 12 wk	27 (seletalisib = 13, PBO = 14)
van Nimwegen et al 2020 [62]	Abatacept (costimulation blocker CD80/CD86/CD80/CD28)	AECG +, ESSDAI ≥5, duration ≤7 y, SG biopsy +	Abatacept 125 mg S/C weekly or PBO	Between-group change in ESSDAI at 24 wk	80 (abatacept = 40, PBO = 40)
Baer et al 2021 [63]	Abatacept	ACR/EULAR +, ESSDAI ≥5, anti-Ro +	Abatacept 125 mg S/C weekly or PBO	Change in ESSDAI at 169 d	187 (abatacept = 92, PBO = 95)
Fisher et al 2020 [64]	Iscalimab (anti-CD40)	AECG +, anti-Ro +, or ANA + and RF +, ESSDAI ≥6, SSF >0	Cohort 1: iscalimab 3 mg/kg S/C × 4 or PBO Cohort 2: iscalimab 10 mg/kg IV × 4 or PBO	Safety and change in ESSDAI at 12 wk	Cohort 1: 12 (iscalimab = 8, PBO = 4) Cohort 2: 32 (iscalimab = 21, PBO = 11)
van der Heijden et al 2020 [65]	HCQ + LEF	AECG +, ESSDAI ≥5, SG biopsy +	LEF 20 mg once daily + HCQ 400 mg once daily (200 mg once daily if low weight) or PBO + PBO	Change in baseline adjusted ESSDAI at week 24	29 (LEF + HCQ = 21, PBO + PBO = 8)
Hamkour et al 2023 [66]					
Posada et al 2021 [67]	RSLV-132 (RNase Fc fusion protein)	AECG +, anti-Ro52/60 +, IFN signature +	RSLV-132 10 mg/kg IV weekly then alternate weeks for 12 wk, or PBO	IFN gene expression (clinical: change in ESSDAI and ESSPRI) at day 99	28 (RSLV-132 = 20, PBO = 8)
Bowman et al 2022 [68]	Ianalumab	AECG +, anti-Ro +, SSF >0.1, ESSDAI ≥6, ESSPRI ≥5	Ianalumab 5 mg or 50 mg or 300 mg S/C or PBO MPRED 250 mg IV before 1 st injection	Change in ESSDAI at 24 wk; ESSDAI dose response	190 (1:1:1:1) (300 mg = 47, 50 mg = 47, 5 mg = 47, PBO = 49)
Mariette et al 2022 [69]	Belimumab + RTX	AECG +, ESSDAI ≥5, oral dryness NRS ≥5, USF >0 or SSF >0.05	Belimumab 200 mg S/C or PBO to wk 24 or 51 AND RTX 1000 mg IV at wk 8 and 10 or PBO, then 16 wk follow-up	Safety to wk 68	86 (2:2:2:1) (belimumab S/C = 24; RTX IV = 25; sequential belimumab + RTX = 24, PBO = 13)
Price et al 2022 [70]	Filgotinib, lanraplenib, tirabrutinib (JAK-1, SYK and BTK inhibitors, respectively)	AECG +, pSjD or associated-SjD, ESSDAI ≥5, anti-Ro + or anti-La +	Oral filgotinib 200 mg, or lanraplenib 30 mg, or tirabrutinib 40 mg or PBO daily	Composite endpoint of CRP and VAS at wk 12	150 (1:1:1:1) (filgotinib = 38, lanraplenib = 37, tirabrutinib = 39, PBO = 36)
He et al 2022 [71]	Low dose IL-2	AECG +, ESSDAI ≥5, ≥50 mm/100 mm on ≥2 of 3 VAS of dryness, fatigue, pain, raised IgG, or parotid enlargement or systemic involvement	Recombinant human IL-2 1 mIU S/S or PBO alternate days for 2 wks then a 2-wk break, repeated × 3, then 12 wk follow-up	≥3 point reduction in ESSDAI at 24 wk	60 (IL-2 = 30, PBO = 30)

(continued on next page)

Table 1 (Continued)

Publication	Therapy	Entry criteria	Trial design	Primary outcome	Number of patients
Bentley et al 2023 [72]	RO5459072 (petesicatib) (oral cathepsin-S inhibitor)	AECG +, anti-Ro + and/or anti-La +, ESSDAI ≥ 5 AND ESSPRI ≥ 5	Petesicatib 200 mg/d or PBO	ESSDAI improvement of ≥ 3 points at wk 12	75 (petesicatib = 38, PBO = 37)
St Clair et al 2024 [73]	Dazodalibep (anti-CD40L)	ACR-EULAR +, SjD (21.6% with RA, 10.1% with SLE) Population 1: ESSDAI ≥ 5 ; Population 2: ESSPRI ≥ 5 AND SSF ≥ 0.01 AND ESSDAI < 5	Dazodalibep 1.5 g IV or PBO. Crossover at 24 wks	Population 1: Change in ESSDAI at wk 24 Population 2: Change in ESSPRI at wk 24	Population 1: 74 (dazodalibep = 36, PBO = 38) Population 2: 99 (dazodalibep = 54, PBO = 55)
Fisher et al 2024 [74]	Iscalimab	ACR/EULAR +, anti-Ro +, SSF ≥ 0.1 mL/min Cohort 1: ESSDAI ≥ 5 AND ESSPRI ≥ 5 Cohort 2: ESSDAI < 5 AND ESSPRI ≥ 5 AND IDEEL ≥ 30	Iscalimab 150 mg or 300 mg or 600 mg S/C or PBO	Dose response change in ESSDAI at wk 24 in cohort 1, change in ESSPRI at wk 24 in cohort 2	cohort 1 = 173 (150 mg = 44, 300 mg = 43, 600 mg = 43, PBO = 43) cohort 2 = 100 (600 mg = 50, PBO = 50)
Dörner et al 2024 [75]	Remibrutinib (oral BTK inhibitor)	ACR-EULAR +, anti-Ro + ESSDAI ≥ 5 AND ESSPRI ≥ 5 AND USF > 0	Remibrutinib 100 mg once daily or 100 mg twice a day or PBO	Change in ESSDAI at wk 24	73 (1:1:1) (100 mg twice a day = 24, 100 mg once daily = 25, PBO = 24)
Xu et al 2024 [76]	Telitacicept (inhibits BAFF/BLyS and APRIL)	ACR/EULAR +, anti-Ro +, ESSDAI ≥ 5	Telitacicept 160 mg S/C or 240 mg weekly or PBO for 24 wk	Change in ESSDAI at wk 24	42 (1:1:1) (160 mg = 14, 240 mg = 14, PBO = 2)
Gottenberg et al 2024 [77]	Nipocalimab (anti-neonatal FcR mAb)	ACR-EULAR +, ClinESSDAI ≥ 6 , anti-Ro +, ≥ 1 abnormal laboratory marker	Nipocalimab 5 mg/kg or 15 mg/kg IV or PBO	Change in ClinESSDAI at wk 24	163 (1:1:1) (15 mg/kg = 54, 5 mg/kg = 53, PBO = 56)

ACR, American College of Rheumatology; AECG, American-European Consensus Group; ANA, antinuclear antibody; BAFF/BLyS, B-cell activating factor/B lymphocyte stimulator; BTK, Bruton's tyrosine kinase; CRP, C-reactive protein; ESSDAI, EULAR Sjögren's Syndrome Disease Activity Index; ESSPRI, EULAR Sjögren's Syndrome Patient Reported Index; EULAR, European League Against Rheumatism; FcR, Fc receptor; HCQ, hydroxychloroquine; IDEEL, Impact of Dry Eye on Everyday Life Scale; IL, interleukin; IV, intravenous; JAK, Janus kinase; LEF, leflunomide; mAb, monoclonal antibody; MPRED, methylprednisolone; NRS, numeric rating scale; PBO, placebo; PI3K δ , phosphoinositide 3-kinase delta; pSjD, Sjögren's disease; R, receptor; RA, rheumatoid arthritis; RF, rheumatoid factor; RTX, rituximab; S/C, subcutaneous; SG, salivary gland; SLE, systemic lupus erythematosus; SSF, stimulated salivary flow rate; SYK, spleen associated tyrosine kinase; USF, unstimulated salivary flow rate.

antibodies or allow other antibody combinations, eg, positive RF and ANA.

There is some evidence that patients with a shorter disease duration, eg, ≤ 7 years since diagnosis, may be more likely to respond to biologic therapy [62,68,78], but this has not been consistently reproduced [54] and there is no expert consensus. Age limits, eg, ≥ 18 and ≤ 75 to 80 years, are set for regulatory (lower limit) or pragmatic reasons (upper limit). Unstimulated/ stimulated whole salivary flow rate >0 , or stimulated ≥ 0.05 mL/min, or ≥ 0.1 mL/min excludes individuals with no residual salivary flow (who may not show improvement if this is due to glandular damage). Trials have differed in whether unstimulated or stimulated salivary flow, or both, improve [55,65,74]. Concomitant medications typically allow stable daily doses of prednisolone ≤ 10 mg, hydroxychloroquine and variably other DMARDs, typically for at least 4 weeks prior to baseline. Washout periods for biologic therapies are typically based on pharmacokinetic/pharmacodynamic data (eg, 5 half-lives).

Inclusion/exclusion criteria also need to match outcomes. Restrictive inclusion criteria are not always better at demonstrating treatment efficacy and may limit recruitment [79,80] and future labelling.

Patient stratification

Ioannidis et al. [81] proposed a ‘type I’ pSjD (~20% of their patients) with low complement C4 levels and palpable purpura who had a higher risk of lymphoma, while patients without these features were classified as low risk (type II Sjögren’s syndrome) (~80% of their patients).

Tarn et al. [82] used cluster analysis of symptom questionnaire scores to identify 4 patient subgroups: low symptom burden, high symptom burden (HSB), dryness dominant with fatigue (DDF), and pain dominant with fatigue. Differences in lymphocyte counts, autoantibody positivity, serum IgG, κ -free light chain, $\beta 2$ -microglobulin, and CXCL-13 concentrations were observed between these subgroups, along with differentially expressed peripheral blood transcriptomic modules. Reanalysis of the JOQUER (hydroxychloroquine vs placebo), TRACTISS (rituximab vs placebo) and tocilizumab versus placebo clinical trials suggested a treatment effect with hydroxychloroquine and tocilizumab in the HSB subgroup and with rituximab in the DDF subgroup compared with placebo [83,84] consistent with their biological profiles (high[er] IL-6 activity in HSB and high B-cell activities in DDF). McCoy et al. [85] and Lee et al. [86] identified similar subgroups in other international cohorts.

Soret et al. [87] used a combination of clinical and molecular signatures in the PRECISEADS European pSjD cohort to identify 4 clinical-molecular clusters with differences in IFN signature, B-cell activity and clinical features. Further studies [88] indicate that the transcriptomically ‘healthy-like’ cluster are less likely to respond to biologic therapies and perhaps should be excluded from these clinical trials.

Nguyen et al. [89] performed hierarchical cluster analysis with 26 variables including demographics, systemic disease, symptom scores, and biological parameters to stratify eligible patients from 2 large combined French cohorts into 3 distinct subgroups of patients: B-cell active disease and low symptom burden (BALS); high systemic disease activity (HSA); and low systemic disease activity with high symptom burden. The 3 clusters displayed differential expression of IFN signatures (higher in BALS and associated with a greater risk of new

immunosuppressant treatment), CXCL-13 levels and markers of T- and B-cell activation, confirming distinct pathophysiological mechanisms. Almost all patients with lymphoma were in the BALS and HSA clusters. Although the BALS cluster had low symptom burden, this progressed over time.

Although several groups have identified differences in disease features between biologic females and males in SjD and the potential influence of sex hormones on the response to therapy [90], the predominance of females has limited this as a useful stratifying biomarker in SjD clinical trials.

Primary and other clinical/imaging efficacy endpoints

pSjD is a heterogenous disease with both measurable systemic and subjective patients reported outcomes. Therefore, the EULAR Sjögren’s Syndrome Disease Activity Index (ESSDAI) and EULAR Sjögren’s Syndrome Patient Reported Index (ESSPRI) were developed to assess both elements in a complementary manner (see below).

Table 2 provides a list of double-blind randomised controlled trials (RCTs) published as full articles in peer-reviewed journals since 2014, when ESSDAI and ESSPRI started to be widely used, along with 1 recent trial of nipocalimab presented in detail at ACR Convergence 2024. It does not include 2 negative trials of anti-TNF therapy [92,93], 1 negative trial of anakinra [94] and 2 pilot studies of rituximab [95,96] published prior to this date. It excludes 2 trials published as abstracts only [97,98] and 1 single-blind study of iguratimod [99] (see also [70,100]).

Systemic disease: ESSDAI

The ESSDAI measures systemic activity in pSjD across 12 domains [101]. An ESSDAI score of 1 to 4 equates to mild, 5 to 13 moderate, and ≥ 14 high systemic disease activity. It does not assess glandular function or subjective patient symptoms. The ESSDAI has been used as a primary endpoint in multiple clinical trials [59–65,68,71–76,102]. ESSDAI has some limitations in that improvement does not automatically translate to symptom improvement, as there is some lack of specificity, with a high placebo response, and most ESSDAI domains will be negative in any given individual.

Generally, a minimum inclusion ESSDAI score of ≥ 5 at baseline is also necessary to demonstrate a treatment effect of at least 3 points (‘ESSDAI responder’). This is also relevant for composite measures that include the Clinical EULAR Sjögren’s Syndrome Disease Activity Index (ClinESSDAI) are being used (see Sjögren’s Tool for Assessing Response [STAR] and Composite of Relevant Endpoints for Sjögren’s Syndrome [CRESS] below). In a standard of care cohort, ESSDAI scores varied over time, with a longer period in ESSDAI low disease activity (ESSDAI <5) associated with improved SG outcomes [103].

A glossary is available [104], and a pragmatic approach is taken to items such as computed tomography (CT) scans, neurophysiology, lung function etc—if there is no clinical indication to perform these, they are scored as negative.

The ClinESSDAI [105] was developed to evaluate disease activity independent of B-cell biomarkers to avoid data collinearity and to detect change independently of the biological effect of the drug. It has been used as a primary endpoint [77] and as part of composite measures (see below). Some domains of the ClinESSDAI may be less likely to respond to therapy, but this does not affect its utility [106].

Table 2
Improvement in primary outcome measures

Publication	Therapy	Primary outcome met	ESSDAI	ESSPRI	HRQoL	Salivary flow	Other selected outcomes
Gottenberg et al 2014 [50]	Hydroxychloroquine	No	No	No	SF-36 = No	USF = No	PROFAD-SSI = No, HADS = No
Devauchelle-Pensec et al 2014 [54]	Rituximab	No	No	No (inferred)	SF-36 = No	USF = No	VAS = NS; fatigue improved at wk 6 ($P < .001$) and wk 16 ($P = .012$) but not at wk 24 (primary endpoint). Improvement in ≥ 2 of 4 VAS at wk 6 only ($P = .036$), PGA = No, PhGA = No
Bowman et al 2017 [55]	Rituximab	No	No	No	SF-36 = No, EQ-5D = No	USF = P $P = .0015$ at wk 48, SSF = No	VAS = No, PROFAD-SSI = No, SGUS = improvement, SG B-cell infiltration = stabilised. CRESS = P ($P = .026$)
Fisher et al 2018 [56]							
Arends et al 2021 [137]							
Pontarini et al 2024 [57]							
St Clair et al 2018 [58]	Baminercept	No	No	No (inferred)	SF-36 = No	USF = No, SSF = No	Schirmer I: right eye = P ($P = .036$), left eye = No ($P = .662$), OSS = No, PGA = No, PhGA = No
Dörner et al 2019 [59]	Ianalumab	No	No/NS	No/NS	SF-36 = No	USF = NS, SSF = NS	Variable/nonsignificant trends to improvement for most outcomes: MFI = NS, PGA = NS, PhGA = NS, Fatigue VAS = NS, OSS = NS
Felten et al 2021 [60]	Tocilizumab	No	No	No	SF-36 = No	USF = No	FACIT-F = No, HADS = No
Juarez et al 2021 [61]	Seletalisib	No	No/NS	No/NS		USF = No, SSF = No	PhGA = No, PROFAD-SSI = NS. Study was terminated early due to low recruitment so trends in improvement were not statistically significant
van Nimwegen et al 2020 [62]	Abatacept	No	No/NS ($P = .039$ only at wk 12)	No/NS (only for MCII, $P = .031$ at wk 12, $P = .001$ at wk 24)	SF-36 = No, EQ-5D = No	USF = No, SSF = No	MFI = No, PGA = No, PhGA = NS ($P = .022$ only at wk 12), WPAI = No, FSFI = P ($P = .042$)
Baer et al 2021 [63]	Abatacept	No	No	No		USF = No, SSF = No	PhGA = No, PGA = No
Fisher et al 2020 [64]	Iscalimab	Yes	Yes (Cohort 2: $P = .009$)	No/NS	SF-36 = NS	USF = NS, SSF = NS	MFI = NS. Some trends to improvement but a small study (cohort 2: $n = 32$)
van der Heijden et al 2020 [65]	Hydroxychloroquine + Leflunomide	Yes	Yes ($P = .0078$)	No/NS ($P = .079$)	SF-36 = NS (social function and mental health improved)	USF = P ($P = .014$), SSF = NS ($P = .098$)	ESSPRI Fatigue = P ($P = .044$), MFI = N, PGA = NS ($P = .059$), PhGA = P ($P = .036$)
Posada et al 2021 [67]	RSLV-132	No	No	No		USF = No, SSF = No	FACIT-F = NS ($P = .05$), ≥ 6 point increase in FACIT-F = P ($P = .002$), PROF mental component = P ($P = .04$), DSST = P ($P = .02$)
Bowman et al 2022 [68]	Ianalumab	Yes	Yes ($P < .025$ in 4 of 5 models)	No	SF-36 = No	USF = No, SSF = P ($P = .037$)	FACIT-F = No, PGA = No, PhGA = P ($P = .022$)
Mariette et al 2022 [69]	Belimumab + rituximab	No	No/NS	No		USF = P (P not stated) SSF = NS	ClinESSDAI = NS
Price et al 2022 [70]	Filgotinib, lanraplenib, tirabrutinib	No	No	No		USF = No, SSF = No	

(continued on next page)

Table 2 (Continued)

Publication	Therapy	Primary outcome met	ESSDAI	ESSPRI	HRQoL	Salivary flow	Other selected outcomes
He et al 2022 [71]	Low dose IL-2	Yes	Yes ($P = .004$)	No/NS	SF-36 = NS: mental component score = P ($P = .01$)	SGUS = No	MFI = No, SGUS = No, STAR = P ($P = .001$), dryness VAS = P ($P = .006$, fatigue VAS = P ($P = .005$), pain VAS ($P = .38$), DLCO = P (sub-group $n = 12$ with ILD, $P = .003$)
Bentley et al 2023 [72]	RO5459072 (petesica-tib)	No	No	No	SF-36 = No	USF = No, SSF = No	
St Clair et al 2024 [73]	Dazodalibep	Yes	Yes (Population 1: $P = .0167$)	Population 2: $P = .0002$		SSF = N/NS (pop1 $P = .133$, pop2 $P = .217$)	Population 2: Dryness VAS = P ($P = .0066$, Fatigue VAS = P ($P = .0022$) Pain VAS = P ($P = .0010$), FACIT-F = P ($P = .0095$), PGA = NS ($P = .1781$), OSDI = NS ($P = .1936$)
Fisher et al 2024 [74]	Iscalimab	Yes	Yes (Cohort 1 $P = .0041$)	NS (Cohort 2 $P = .12$)		USF = P /NS (Cohort 1 150 mg $P = .012$; Cohort 2 600 mg $P = .033$) SSF = P /NS (Cohort 2 600 mg $P = .005$)	Dryness VAS = P /NS (Cohort 2 600 mg $P = 0.016$), FACIT-F = NS, PGA = NS, PhGA = NS, IDEEL = NS, PROFAD-SSI = NS, SSSD = P /NS ($p = 0.0029$ for the 600mg dose in cohort 1)
Dörner et al 2024 [75]	Remibrutinib	Yes	Yes ($p = 0.003$)	No	EQ-5D = No	USF = NS ($P = .121$)	FACIT-F = No, PGA = No, PhGA = No, STAR = P /NS (post hoc analysis: 24/49 responders at wk 24 on remibrutinib compared to 4/24 for PBO)
Xu et al 2024 [76]	Telitacicept	Yes (160 mg dose)	Yes/NS (160 mg, $P < .05$, 240 mg $P = .056$)	No	SF-36 = No	USF = No	ClinESSDAI = NS (160 mg $P = .004$; 240 mg $P = .139$), MFI = P ($P < .05$), PGA = No, PhGA = No
Gottenberg et al 2024 [77]	Nipocalimab	Yes	Yes (15 mg/kg ClinESSDAI $P = .002$)	No	N/A		ESSDAI = P , PhGA = P , STAR = P , CRESS = P , DAL = P ($P < .05$)

ClinESSDAI, Clinical EULAR Sjögren's Syndrome Disease Activity Index; CRESS, Composite of Relevant Endpoints for Sjögren's Syndrome; DAL, Disease Activity Level, DLCO, Diffusing Capacity for Carbon Monoxide; DSST, Digit Symbol Substitution Test; EQ-5D, EuroQoL-5 Dimension; ESSDAI, EULAR Sjögren's Syndrome Disease Activity Index; ESSPRI, EULAR Sjögren's Syndrome Patient Reported Index; FACIT-F, Functional Assessment of Chronic Illness Therapy-Fatigue; FSFI, Female Sexual Function Index; HADS, Hospital Anxiety and Depression Scale; HRQoL, health-related quality of life; IDEEL, Impact of Dry Eye on Everyday Life Questionnaire; IL, interleukin; ILD, interstitial lung disease; MCII, minimal clinically important improvement; MFI, Multidimensional Fatigue Inventory; N/A, not applicable; No, negative; NS, nonsignificant change/significant at $P < .05$ at some timepoints only/borderline/debatable; OSDI, Ocular Surface Disease Index; OSS, Ocular Staining Score; P, positive at primary endpoint visit; PGA, patient global assessment; PhGA, physician global assessment; PROF, Patient-Reported Outcome Fatigue; PROFAD-SSI, Profile of Fatigue and Discomfort – Sicca Symptoms Inventory; SF-36, Short Form-36; STAR, Sjögren's Tool for Assessing Response; SG, salivary gland; SGUS, salivary gland ultrasound; SSF, stimulated salivary flow; USF, unstimulated salivary flow; VAS, visual analogue scale; WPAI, Work Productivity and Activity Impairment Questionnaire.

Patient reported outcome measures: ESSPRI/alternatives

The Profile of Fatigue and Discomfort – Sicca Symptoms Inventory (PROFAD-SSI) captures the cardinal symptoms of pSjD (dryness, fatigue and pain) [7,107]. This subsequently led to the development and validation of the ESSPRI [8], which gives equal weighting to these 3 domains, averaging them to produce an overall score. Scoring is based on a 0 to 10 numeric rating scale and records symptom severity over the previous 2 weeks, accounting for day-to-day variability of symptoms. A score of ≥ 5 is generally chosen as an eligibility criterion when improvement in this measure is being evaluated (≥ 1 point or 15% reduction compared with baseline). The overall dryness score gives the same result as the mean of separate oral and ocular dryness scores [108].

The ESSPRI has been used in clinical trials with variable results, with one challenge being the high placebo response. One option to address this is through more stringent response criteria [109]. A contemporaneous symptom diary may be an alternative [110]. A patient diary app is also being evaluated in the NECESSITY IHI study [111]. Dryness-specific questionnaires are also available [112,113].

Many other questionnaires have been used to measure fatigue [114]. The Patient Reported Outcome Measurement Information System (PROMIS) questionnaire includes a fatigue domain and generates comparable results in pSjD patients to the fatigue domain of ESSPRI [115] and potentially to Functional Assessment of Chronic Illness Therapy-Fatigue (FACIT-F) [116] but does not include a dryness domain. A study of fatigue in rheumatoid arthritis suggests that a single fatigue visual analogue scale performs as well as longer questionnaires [117]. Although a few clinical trials have assessed fatigue as a primary outcome, it has generally been a secondary outcome or included in composite tools [118].

In attempting to assess improvement in both ESSDAI and ESSPRI, some trials have included only participants with both an ESSDAI ≥ 5 and an ESSPRI ≥ 5 , whereas others have recruited separate cohorts, eg, ‘cohort 1’: ESSDAI ≥ 5 and ‘cohort 2’: ESSPRI ≥ 5 and ESSDAI < 5 (Table 1). Whether the ESSPRI responds to biologic therapies in the same way in both cohorts is under investigation. Pain and fatigue can also be due to concomitant fibromyalgia, and this may not respond to biologic therapies.

Glandular function

Unstimulated and/or stimulated salivary flow rates are typically used as objective measures for SG function. The methodology was developed by Navazesh and Kumar in 2008 [119] and, with training, can be performed without specialist expertise. Stimulated salivary flow can be performed using different techniques including diluted citric acid or chewing a standardised formulation of paraffin gum (the latter method is generally preferred for safety and reliability reasons).

Schirmer I (lachrymal function/tear flow) test uses a standardised blotting paper strip placed in the temporal corner of the lower lid margin for 5 minutes. It does not require a slit-lamp and so can be performed without optometry/ophthalmology input. Whitcher et al. [120] describe the detailed methodology of the Ocular Staining Score, which uses lissamine green to stain areas affected by ocular dryness. They also reference the van Bijsterveld and Oxford grading scales that use the more irritant rose bengal dye and other techniques such as the Tear Film Breakup Time.

Tests should be performed in a quiet, moderate environment, ideally at the same time of day at each visit. Eye drops/drinking

are avoided for at least 1 to 2 hours beforehand. Stimulatory medications such as pilocarpine are avoided for 12 hours before each assessment.

SG ultrasound scanning and other imaging modalities

Major SG ultrasonography (SGUS) in pSjD can demonstrate inhomogeneity of the gland and associated fibrous aspects with good reliability and validity [121–124]. There is a moderate association between SGUS and clinical activity (ESSDAI), histological findings, biological tests, glandular function, and patient reported outcome measures (PROMs) [125,126], with the strongest correlation with salivary flow. New probes and assessment of minor salivary/lachrymal glands may also be helpful [127,128].

The 2 most widely used scoring systems are the Hocevar [129] and OMERACT scores [130], also associated with disease duration in pSjD [131]. The sensitivity to change in SGUS has been evaluated in 3 large RCTs: TEARS [132], ASAP [133], and TRACTISS [56] and in a smaller study of iganalimumab [134], with variable improvement in the intervention group. SGUS improvement required the smallest estimated sample size compared with other potential primary outcome measures [79].

SGUS may be a useful prognostic tool for lymphoma development in pSjD [135,136] and can guide core needle biopsy in lymphoma diagnosis [137]. Deep learning and artificial intelligence may lead to improved methods of reading the grey scale or Doppler images and increase reliability [138–140].

Fluorodeoxyglucose-positron emission tomography/CT and MRI scanning are established techniques to diagnose lymphoma in pSjD [141–143].

Composite measures: STAR and CRESS

Given the limitations of individual measures such as ESSDAI or ESSPRI, as described above, the NECESSITY IHI has developed the STAR, a composite responder index for use in clinical trials as a comprehensive efficacy endpoint [144] (Table 3). The development of STAR was data- and expert-driven and involved patients throughout. Data were analysed from 9 clinical trials testing drugs with diverse mechanisms of action. Subgroup identification by Virtual Twins identified responder subsets, and an effect size analysis was performed to determine measurements that changed with treatment. Seventy-six experts and 20 patients from Europe, North America and Asia participated in a Delphi process to construct the STAR endpoint and determine its clinical relevance and meaningfulness to both patients and clinicians. Of the STAR options, 227 were evaluated for sensitivity and specificity to change. Several rounds of voting by experts and patients led to the selection of a candidate STAR.

The candidate STAR includes 5 domains (systemic activity, symptoms, lachrymal gland function, SG function, biologic parameters) measuring the key facets (core set) of pSjD. For validation, a meta-analysis was performed after separating positive and negative trials. The candidate STAR showed good sensitivity and specificity to change and a greater response rate in the treatment arm in positive trials than in negative trials. Two versions, one with an improvement of ClinESSDAI of 3 points (STAR3) and another with an improvement of ClinESSDAI of 5 points (STAR5) (ie, comparable to ACR20 and ACR50 for rheumatoid arthritis or SRI4 and SRI6 for lupus) are currently being validated in a post hoc analysis of recent interventional trials as well as being the primary endpoint in the NECESSITY clinical trial evaluating the effect of hydroxychloroquine and mycophenolate and hydroxychloroquine and leflunomide against placebo.

Table 3
Candidate STAR (cSTAR) and CRESS

Domain	Definition of response	cSTAR (points)	CRESS (points)
Systemic activity (cSTAR)	Decrease of ClinESSDAI ≥ 3	3	
Systemic activity (CRESS)	ClinESSDAI score < 5		1
Patient-reported outcome	Decrease of ESSPRI ≥ 1 point or $\geq 15\%$	3	1
Lachrymal gland function (mean of both eyes)	Schirmer I: If abnormal score at baseline: increase ≥ 5 mm from baseline or Ocular Staining Score: If abnormal score at baseline: decrease ≥ 2 points from baseline or If both normal score at baseline: no change to abnormal	1	1
Salivary gland function	Unstimulated Whole Salivary Flow (UWSF): If score > 0 at baseline: increase $\geq 25\%$ from baseline If score is 0 at baseline: any increase in UWSF from baseline or Ultrasound: Decrease $\geq 25\%$ in total Hoyer score from baseline	1	1
Biological	IgG: decrease $\geq 10\%$ or Rheumatoid factor (RF): decrease $\geq 25\%$ (Total RF or RF-IgM (IU/mL))	1	1
Responder definition (cSTAR)		≥ 5 points	
Responder definition (CRESS)			≥ 3 points

ClinESSDAI, Clinical EULAR Sjögren's Syndrome Disease Activity Index; CRESS, Composite of Relevant Endpoints for Sjögren's Syndrome; ESSPRI, EULAR Sjögren's Syndrome Patient Reported Index; STAR Sjögren's Tool for Assessing Response.

A similar measure (CRESS), with the same domains and components (although ClinESSDAI is scored differently and domain weightings are different), was developed and validated independently in the Netherlands [91] and is also being evaluated in the NECESSITY clinical trial (Table 3). In CRESS, unlike STAR, all domains are scored equally (1 point per item), and the domain measuring systemic disease activity is considered to be achieved by a state of low disease activity (ClinESSDAI < 5) rather than an improvement of ClinESSDAI. Patients are classified as CRESS responders when they reach ≥ 3 of 5 points.

CRESS was validated using data from 3 independent RCTs. In 2 of these, post hoc analysis using CRESS resulted in higher response rates in patients treated with abatacept and rituximab, respectively, than in those given placebo. CRESS also resulted in decreased placebo response rates compared with the ESSDAI minimal clinically important improvement. CRESS is the primary outcome measure in the ANISE-II trial of anifrolumab [145] and in a study of efgartigimod in pSjD [146]. Furthermore, CRESS is included as a secondary endpoint or in post hoc analysis of several other trials [146,147].

EUROPEAN MEDICINES AGENCY (EMA) AND FOOD AND DRUG ADMINISTRATION (FDA) ADVICE ON (CANDIDATE) STAR

The NECESSITY IHI consortium met with the EMA Innovative Task Force on July 27, 2020 through the Qualification of Novel Methodologies for Drug Development process. The EMA recommendations were to clarify the statistical methodology for STAR development, provide a detailed description of the endpoint components, include data demonstrating that the endpoint is more responsive and more meaningful than using current measures (eg, ESSDAI or ESSPRI), clarify the objectives of the clinical trial to ensure appropriate design of the study, and initiate scientific advice consultation with the EMA before finalising the protocol of the clinical trial. A follow-up meeting with the EMA Committee for Medicinal Products for Human Use took place on October 25, 2021. Following this, the EMA published a Letter of Support on March 15, 2022 'to encourage the further

development and validation of the STAR. A qualification opinion will be considered when the results of the final validation step will be available'.

A Critical Path Innovation Meeting was held with the United States FDA on February 23, 2023 (<https://www.fda.gov/drugs/novel-drug-approvals-fda/critical-path-innovation-meeting-cpim-topics-held-date>). The FDA comments mainly reflected their existing general guidance on PROMs [148]. Specifically, their preference is for an immediate (eg, 24 hour or 'today') recall period (as in a patient daily diary format) as opposed to the 2-week recall period of the ESSPRI. A preference was also expressed for multifaceted measures of patient symptoms rather than a single item rating scale. The FDA recommends the use of continuous rather than dichotomous outcomes. It was observed that, in the broader pSjD patient population, a change in biological test parameters may not correlate with clinical outcomes.

SECONDARY AND EXPLORATORY BIOMARKER/HISTOLOGICAL ENDPOINTS

Measurement of serum immunoglobulin levels, RF, anti-Ro, and anti-La have been indicators of levels of B-cell reactivity in clinical trials (Table 4). Elevated IgG and lower complement levels also contribute to the ESSDAI score. Many, if not most, studies report changes in some or many of these biomarkers.

CXCL-13 is an important chemokine to attract T and B cells into lymphoid follicles and autoimmune tissues. It has been the most widely utilised exploratory serum biomarker in pSjD clinical trials (Table 4 and [58,69]). Whether CXCL-13 reduction links to clinical improvement will likely depend on the mechanism of drug action.

Higher baseline BAFF levels were found in nonresponders to rituximab [149], and serum BAFF measurements may be of broader utility, for example, in relation to pharmacokinetic analysis [69] (see also Table 4).

Tfh and peripheral Th cells are key mediators in autoreactive B-cell differentiation through inducible T-cell co-stimulator (ICOS)–ICOS ligand interaction and IL-21 production. Verstappen et al [150] demonstrated in a study of abatacept that Tfh cell numbers were reduced along with a decrease of ICOS

Table 4

Biomarkers with potential for response to therapy

Publication	Therapy	Immunoglobulins	RF	ESR	Complement C3 and C4	Other conventional blood biomarkers	CXCL-13	Soluble BAFF/BLyS	Other exploratory biomarkers
Gottenberg et al 2014 [50]	HCQ	No change		Reduced					
Devauchelle-Pensec et al 2014 [54]	Rituximab	All reduced				B2M reduced		NS	
Bowman et al 2017 [55]	Rituximab	All reduced	Reduced		Increased				<i>CXCL13, IL21, IL21R</i> (reduced RNA expression); cTfh cells (reduction inferred from RNA expression)
Pontarini et al 2024 [57]									circulating B and T-cell counts (variable); BAFF, LIGHT, IP-10
St Clair et al 2018 [58]	Baminercept	IgG no change	No change		C3 no change; C4 increased	Anti-Ro, anti-La no change	Reduced		no change
Dörner et al 2019 [59]	Ianalumab					FLC $\pm$ reduction		Variable	CD19+ reduced
Felten et al 2021 [60]	Tocilizumab	IgG, IgA, IgM all NS		NS	NS/variable				
Juarez et al 2021 [61]	Seletalisib	IgG and IgM reduced; IgA no change							Cell counts and SG histology (reduced infiltrates)
van Nimwegen et al 2020 [62]	Abatacept	IgG reduced; IgA and IgM no change	Reduced	No change	C3 and C4 no change				
Baer et al 2021 [63]	Abatacept	IgG and IgA reduced	Reduced		C4 reduced	κ -FLC reduced	Reduced		
Fisher et al 2020 [64]	Iscalimab		NS		C3 and C4 no change	Cohort 2: anti-Ro60, anti-Ro52, anti-La, ANA all NS; FLC no change	Cohort 2: Reduced		
van der Heijden et al 2020 [65]	HCQ + Leflunomide	IgG reduced	Reduced		C3 and C4 no change				IFN signature, CXCL10, galectin-9 (reduced)
Hamkour et al 2023 [66]									
Posada et al 2021 [67]	RSLV-132	No change		No change	C3 and C4 no change	Anti-Ro no change			IFN-inducible gene expression (increased)
Bowman et al 2022 [68]	Ianalumab	IgG reduced	Reduced					Increased	CD19+ B -cell counts (reduced)
Mariette et al 2022 [69]	Belimumab + rituximab	IgG, IgA and IgM all NS (trend to reduction)	NS (trend to reduction)		C3 and C4 no change	FLC trend to reduction; anti-Ro, anti-La and B2M (no change)	Reduced	Increased	B-cell depletion (SG and periphery)
Price et al 2022 [70]	Filgotinib, lanraplenib, tirabrutinib	Reduced (filgotinib predominant)	Reduced (filgotinib predominant)			CRP reduced (filgotinib predominant)			Filgotinib predominant changes in B-cell subtypes and IFN signature
He et al 2022 [71]	Low dose IL-2	IgG and IgA reduced	No change	No change		Anti-Ro and anti-La no change		NS	Tregs, B-cell subtypes, cytokines (variable changes)

(continued on next page)

Table 4 (Continued)

Publication	Therapy	Immunoglobulins	RF	ESR	Complement C3 and C4	Other conventional blood biomarkers	CXCL-13	Soluble BAFF/BlyS	Other exploratory biomarkers
Bentley et al 2023 [72]	RO5459072 (pete-sicatib)	IgG and IgM reduced						NS	cathepsin-S mass (increased); related molecules and immune cell numbers (NS)
St Clair et al 2024 [73]	Dazodalibep		Reduced				Reduced		
Fisher et al 2024 [74]	Iscaimab	IgG and IgM reduced	Reduced			FLC reduced	Reduced		
Dörner et al 2024 [75]	Remibrutinib	IgG and IgM reduced; IgA no change				Anti-Ro60, anti-Ro52, and anti-La reduced	Reduced		Transcriptomic changes
Xu et al 2024 [76]	Telitacicept	IgG, IgA and IgM reduced			C4 reduced; C3 NS				CD19 +, T-cell subsets (NS)
Gottenberg et al EULAR 2024 [77]	Nipocalimab	IgG reduced				Anti-Ro60, anti-Ro52 and anti-La reduced			

ANA, antinuclear antibody; B2M, β 2-microglobulin; BAFF, B-cell activating factor; BlyS, B lymphocyte stimulator; CRP, C-reactive protein; cTfh, circulating T follicular helper; CXCL-13, chemokine (C-X-C motif) ligand-13; ESR, erythrocyte sedimentation rate; FLC, free light chain; HCO, hydroxychloroquine; IL, interleukin; IFN, interferon; IP-10, INF- γ inducible protein-10; NS, nonsignificant change/significant at $P < .05$ at some timepoints only/borderline/debatable; RF, rheumatoid factor; SG, salivary gland; Treg, regulatory T-cell.

expression in parotid gland tissue and circulating Tfh cells [150]. This was associated with a reduction in ESSDAI score and in serum levels of IL-21, CXCL-13 and anti-Ro and anti-La antibody levels. Similar findings were seen in patients who had received rituximab [151]. Pontarini et al. [152] demonstrated enrichment of Tfh and Th cells in SGs and peripheral blood in pSjD and particularly in TLS.

Patients with pSjD have increased serum and SG IFN levels/ expression. Type I and type II IFN signatures classify pSjD patients particularly in relation to MALT lymphoma risk (see above). Measuring IFN signature and related protein expression (including MxA, CXCL-10 and Galectin-9) may, therefore, be a potential biomarker in pSjD clinical studies [66,153]. Conversely, in a study of RSLV-132, an RNase Fc fusion protein, improvement in fatigue was significantly correlated with increased expression of selected IFN-inducible genes [67].

Salivary gland histological change can also be an important biomarker and indicator of pharmacological mechanism/drug effect [69]. Pontarini et al. [57] analysed longitudinal SG biopsies collected as part of the TRACTISS study of rituximab versus placebo in pSjD, and they also reference previous studies by other groups. Differences in CD20/aggregate area fractions, CD20 and B/T-cell density were more sensitive than the focus score in discriminating between the rituximab and placebo arms at 48 weeks. Significant improvement was seen in the prevalence of TLS in the rituximab arm after 48 weeks, but not at 16 weeks, suggesting that repeated cycles of B-cell depletion are required to prevent TLS formation. Downregulation in SG T-cell and complement activation and restoration of glandular epithelium metabolic function were associated with CRESS/STAR improvement and improvement in unstimulated salivary flow rates.

DISEASE-RELATED DAMAGE

Two tools are available to assess disease-related damage: the Sjögren’s Syndrome Disease Damage Index (SSDDI) [154] and the Sjögren’s Syndrome Damage Index [155]. Generally, damage accrual in pSjD appears limited and develops slowly [156], thereby limiting its use as an outcome measure in clinical trials. Although predictive factors/biomarkers for lymphoma development exist or have been proposed [157], they are not necessarily specific or sensitive to change over the relatively short duration of a clinical trial. This highlights the need for a specific score able to measure lymphoma risk. This project (SCOLYSS) is part of the NECESSITY consortium activities.

HEALTH-RELATED QUALITY OF LIFE & HEALTH ECONOMIC CONSIDERATIONS

PSjD patients have significantly reduced health-related quality of life (HRQoL) with debilitating symptoms of dryness, fatigue and pain leading to depression, anxiety and loss of work productivity [158]. Patients struggle with social isolation, financial difficulties and poor nutrition [159]. Systemic disease activity, however, correlates poorly with reduced HRQoL [160], with the main predictors being pain/fatigue and anxiety/depression [161–163]. These studies have mainly used the generic Short Form-36 (SF-36) and EuroQoL-5 Dimension (EQ-5D) questionnaires as there are currently no FDA-endorsed pSjD-specific HRQoL measures. pSjD patients have higher health-related direct and indirect costs, greater reduction of work productivity

and unpaid homecare/caring for dependents, and a greater likelihood of early retirement [164–169].

Health economic studies are also critical in informing the cost-effectiveness of therapies and health policy. One widely used approach to assessing the health economic impacts and cost-effectiveness of therapy is cost-utility analysis. To measure the ‘effectiveness’ element, the National Institute for Health and Care Excellence (NICE) in the United Kingdom recommends the generic 3-level EQ-5D (EQ-5D-3L) questionnaire [170] to calculate quality-adjusted life years (QALYs). This has also been accepted by health technology assessment agencies of other countries [171,172]. The SF-36 questionnaire can be used to calculate QALYs by converting the SF-36 scores to the preference-based SF-6D equivalent [173].

Each response by an individual to the EQ-5D questionnaire (or SF-6D) generates a ‘health state’, and this can be converted into QALYs for the individual and study population using large published ‘value sets’ [174]. The incremental cost-effectiveness ratio can then be estimated by comparing the cost of the novel therapy minus the cost of existing therapy against the effectiveness of the novel therapy minus the effectiveness of existing therapy [$\text{cost}(\text{exp}) - \text{cost}(\text{control}) / \text{QALY}(\text{exp}) - \text{QALY}(\text{control})$]. The health system may then take a view on what threshold ‘cost per QALY’ is worthwhile to fund. For example, NICE quotes a value of £20,000 to £30,000 per annum cost per QALY as the threshold for cost-effectiveness, although this may not be the only consideration for drug approval. The decision on what costs to include in the analysis (direct healthcare costs, indirect costs, work disability, societal costs, etc) will have a major impact on these calculations. NICE, for example, generally only includes drug and NHS costs in their analyses.

Disease-specific quality of life measures can be used in some circumstances if there is justification that they are more relevant than generic measures and if data exists to ‘map’ the results onto the generic questionnaire values. Although regulators prefer generic measures, they may not capture important disease-specific domains. Consequently, disease-specific tools such as the Primary Sjögren’s Syndrome Quality of Life Questionnaire and the Sjögren’s Related Quality of Life Instrument have been developed with the aim of better characterising the substantial disease-specific impact of the condition on patients and family members [175,176].

In terms of health resource utilisation (‘direct healthcare costs’), few healthcare systems collect this data automatically. It can be collected through a patient recall questionnaire [177] such as the Client Service Receipt Inventory [178], the annotated cost questionnaire [179] and the economic component of the Stanford Health Questionnaire [180]. Costs can be allocated using local or national tariffs and adjusted when costs vary between countries.

Of the 9 studies analysed as part of the STAR development in NECESSITY, 5 collected SF-36 data and 1 also collected EQ-5D-5L data and the Work Productivity and Activity Impairment questionnaire (Table 2). Although 3 of these studies demonstrated a statistically significant improvement in the ESSDAI score, improvement in the ESSPRI, EQ-5D and SF-36 were at best modest. If there is no improvement in patient symptoms or in HRQoL, then it will be challenging to demonstrate cost-effectiveness using conventional approaches.

In many healthcare systems in which patients are not reimbursed for specific aspects of medical or dental care or for over the counter or other medical items, the direct healthcare costs are likely to be underestimated. The same is true for indirect

costs such as loss of work activity, etc, that are often not reported/recorded as a matter of routine.

STATISTICAL ANALYSIS

Key statistical considerations for clinical trials in pSjD include the determination of the response rate in the control group and the targeted treatment difference (effect size). The response rate in the control group may be expected to be similar whether the control group includes a placebo or consists of standard of care. When developing the STAR composite measure, analyses of prior trials showed differential response rates of approximately 25% [181].

The target effect size is also a crucial element in planning a new trial. When investigating the treatment effect with STAR in the development study, following a Delphi approach by 78 experts and 20 patients, 3 trials were considered positive, 3 ‘positive/in between’ and 3 negative. For the 3 positive trials, the pooled odds ratio was 5.59 (95% CI, 1.90–16.5) and for the 6 positive or ‘positive/in between’ was 3.29 (95% CI, 2.07–5.22). The absolute difference of proportion was 30.3% (95% CI, 19.6–41.1%) overall for the 6 positive plus ‘positive/in between’ studies when combined and 46.2% (95% CI, 27.8–64.5%) for the 3 positive studies only. The NECESSITY trial was powered for an absolute difference of 30%, corresponding to an odds ratio of 3.5, given the anticipated control response rate of 30%, and therefore close to the pooled odds ratio observed overall for the 6 positive and ‘positive/in between’ trials.

Other statistical issues are not specific to pSjD trials and include the handling of missing outcome data, intention to treat versus per protocol analysis, and the consideration of intercurrent events by defining relevant estimands as outlined in the ICH-E9(R1) addendum [182].

Safety issues are broadly generic (eg, infection risk). The ethics of performing repeated minor labial SG biopsies for research purposes has also been considered [183].

PATIENT PERSPECTIVES AND ENGAGEMENT

In a survey of 3000 members of the United States Sjögren Foundation by McCoy et al. [161], 74% of patients reported a social burden from living with pSjD. Fatigue, dryness and pain were top priorities for treatment along with reduction in lymphoma risk. Sex life, social activities and work were the main areas reported to be important for quality of life. Healthcare costs, particularly dental care costs, were a financial concern.

Vieira et al. [184], on behalf of Sjögren Europe, emphasised that fatigue and cognitive dysfunction cause the greatest patient reported disability and remain a major unmet need. Perella et al. [185] used social media posts to assess the impact of Sjögren’s disease on peoples’ lives.

During the course of 3 workshops held by the NECESSITY IHI consortium, the key patient issues highlighted for clinical trials were inclusion of an interim analysis so that patients can leave the study early if it is futile; lip biopsies, as an invasive procedure, should be optional and may limit recruitment; participant information sheets should be clear about the potential of receiving a placebo, should signpost psychological support where possible, emphasise the importance of contraceptive advice at all stages, and keep patients informed before, during and after the study with clear and well-written information including a summary of the study outcome suitable for a lay reader.

Patients are keen to play an active part in clinical trial design [186]. Guidelines from the EMA, FDA, World Health

Organization, and others are available on how to fully engage patients in clinical trial design through to regulatory approval (Appendix 2).

REGULATOR AND PAYER PERSPECTIVES

During the concluding (third) NECESSITY IHI workshop on June 12, 2024, a session on payer and regulator priorities was held with Professor Loic Guillevin, Professor Emeritus of the Paris Descartes Faculty and Member of the National Academy of Medicine and Dr Tove Ragna Reksten, Novartis Pharmaceuticals, formerly of the Norwegian Medical Products Agency. The discussion highlighted the importance of cost-effectiveness, clear efficacy, well-defined comparator populations, and robust patient-reported outcomes to demonstrate benefit for patients. Early engagement with Health Technology Assessment (HTA) bodies and the use of validated tools and modelling to demonstrate long-term value and efficacy across all relevant patient subpopulations was recommended.

The usefulness of a combined outcome measure like STAR, the PICO (Patient, Intervention, Comparison, Outcome) model and the importance and challenges of following European Union HTA regulations were also highlighted. pSjD is a chronic condition rather than an acute life-threatening disease and might score lower than the latter in terms of national priorities. New therapies would need to demonstrate clear efficacy against unmet needs in pSjD, with evidence of improvement in patient wellbeing/quality of life using validated scoring systems.

QALYs and/or survival benefits are seen as vital to payers, and market access will involve negotiations between pharmaceutical companies and relevant authorities who hold health budgets. ESSPRI is seen as too subjective, and more robust tools may be required. Any biomarkers that correlate with ESSPRI scores showing clinical effectiveness would be welcome. Combining ESSPRI and ESSDAI scores can capture group differences, hence the interest in measures such as STAR. Modelling clinical benefit over time to demonstrate long-term value would be advisable, with robust studies to support modelling assumptions. Generic tools that enable comparison across diseases are preferred by payers. Considerable effort is required to capture quality of life and assign utility values. Understanding efficacy data and response differences among subpopulations is also important as is the inclusion of patient voices to understand disease symptoms and impact.

PAEDIATRIC POPULATIONS

pSjD is rare in children and has a different clinical presentation than adult-onset pSjD, with less dryness and more frequent systemic features including fever, lymphadenopathy, recurrent parotid gland swelling, and more severe histological changes in the salivary glands [187–189]. As a consequence of its rarity, no paediatric double-blind RCTs have been undertaken.

FURTHER WORK AND UNMET NEED

Arends et al. [190,191] recommended the use of composite measures and additional PROMs, how restricting eligibility impacts recruitment, the potential for patient stratification and the consequences of placebo and nocebo responses and how to mitigate these.

Vitali et al. [192] proposed longer trial duration, better phenotypic characterisation of patient subgroups, subgroup-specific

therapeutic approaches, enrolling patients at an earlier stage and the need for better validated biomarkers.

Further understanding of pathogenesis and the reasons for poor correlations between symptoms, extraglandular features and glandular function would be helpful.

Composite measures such as STAR and CRESS, even though they include a PROM (ESSPRI), do not require symptomatic improvement to define a responder, ie, there is not always improvement from the patient's perspective and this needs to be considered when interpreting trial results.

The FDA and EMA have highlighted the importance of demonstrating an improvement in patient wellbeing, and payers also want to see studies demonstrating cost-effectiveness. This is likely to be a challenge, given the lack of existing therapy other than low-cost over the counter symptomatic remedies.

CONCLUSIONS

There is a clear unmet need for better therapy in pSjD given the reduced HRQoL, high symptom burden and systemic features in many patients. There has been significant progress, with studies demonstrating improvement in systemic disease activity and, in some cases, symptomatic improvement. Therapies must make people feel better to be licensed, and demonstration of cost-effectiveness will enable funding approval. These elements are under active consideration and provide optimism that we may have effective immune-based therapies within the next decade. The NECESSITY IHI consortium is a successful example of a collaborative project involving a broad group of stakeholders to achieve significant progress towards treating pSjD patients.

Competing interests

SJB reports consultancy for Abbvie, Aera, Amgen, Argenx, Aurinia, Bain, BMS, EcoR1, Galapagos, Iqvia, J&J, Kiniksa, Novartis, Scitaris. RS reports consulting fees/honoraria from GSK, Bristol Myer Squib, Boehringer, Amgen, Novartis, Janssen Pfizer and Roche; support for meeting attendance from Amgen and GSK. SA is consultant for Argenx, BMS, Galapagos and Novartis. GV is consultant for Argenx, Aurinia. VD-P reports a role in advisory boards for Abbvie, Chugai, Novartis and financial support from Abbvie, Lilly, Novartis. Chiara Baldini reports consulting fees/honoraria from Amgen, GSK, Johnson & Johnson, Sanofi, Novartis, Aurinia. MB reports unrestricted grants from Janssen Pharmaceuticals, GSK and BMS and is an advisory board member and/or has received speaker fees from Novartis, Amgen, Ono Pharmaceutical, GSK, UCB, sGTx. EP is in consultancy for GSK. WH is an employee and shareholder of Novartis Pharma AG. JM is an employee of Novartis Services Inc and stockholder of Novartis Corporation. PG is an employee and stockholder of Novartis Pharma AG. DC reports research grants from Novartis, GSK, Servier, BMS, Roche-Chugai, CSL Behring, Astra-Zeneca. BAF reports consultancy: Novartis, BMS, Servier, Galapagos, Roche, UCB, Sanofi, Janssen, AZ, Otsuka, Amgen, Kiniksa. BAF reports funding from Janssen, Servier, Galapagos, Celgene. FB is an employee and stockholder of Candel Therapeutics. W-FN reports consultancy for Amgen, Bristol Myers Squibb, Novartis, Johnsons & Johnsons Innovations, Sanofi, Argenx, IQVIA, Quotients, Resolve Therapeutics, EQT & Veloxis. J-E G is involved with Abbvie, BMS, Chugai, Galapagos, Gilead, Lilly, Janssen, Novartis, Pfizer, Roche, Sanofi. HB reports grants from BMS, AstraZeneca, Novartis. HB reports consultancy in BMS, AstraZeneca, Novartis, Galapagos, Argenx, UCB. TD is involved

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Appendix 2: Relevant Guidelines

EMA guidelines on good clinical practice taking patient perspectives into greater consideration (<https://www.ema.europa.eu/en/events/ich-e6r3-good-clinical-practice-workshop-patients-and-consumers-pcwp-and-healthcare-professionals-hcpwp-working-parties>).

EMA guidelines on patient involvement in regulatory processes (https://www.ema.europa.eu/en/documents/other/laborary-patient-journey-centrally-authorized-medicine_en.pdf)

EMA patient engagement (<https://www.ema.europa.eu/en/partners-networks/patients-consumers>). The European Patients' Academy on Therapeutic Innovation [EUPATI] toolbox (<https://toolbox.eupati.eu/guidance/>)

FDA recommendations on patient engagement in clinical trial design from the earliest stage of development (<https://www.fda.gov/about-fda/oncology-center-excellence/patient-focused-drug-development>)

The International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) (<https://www.ich.org/>)

The Patient Engagement Management Suite (pemsuite.org) / Patient Focused Medicines Development (<https://patientfocusedmedicine.org/>)

World Health Organization guidelines on best practice for clinical trials (<https://www.who.int/publications/i/item/9789240097711>).

FDA and EMA guidance on clinical trials in Systemic Lupus Erythematosus (exemplar): https://www.fda.gov/media/71150/downloadhttps://www.ema.europa.eu/en/documents/scientific-guideline/draft-guideline-clinical-investigation-medical-products-treatment-systemic-lupus-erythematosus-cutaneous-lupus-and-lupus-nephritis_en.pdf

FDA guidance on patient reported outcome measures in clinical trials:

<https://www.fda.gov/media/77832/download>

FDA guidance on clinical trials of chronic fatigue syndrome and their relevance to clinical trials in Sjögren's Disease:

<https://www.fda.gov/files/drugs/published/Chronic-Fatigue-Syndrome-Myalgic-Encephalomyelitis—Developing-Drug-Products-for-Treatment.pdf>

EUnetHTA – Endpoints used for relative effectiveness assessment of pharmaceuticals: Clinical Endpoints – 2015 https://www.eunetha.eu/wp-content/uploads/2018/03/composite_endpoints.pdf

EMA/750178/2017: Essential considerations for successful qualification of novel methodologies https://www.ema.europa.eu/en/documents/other/essential-considerations-successfulqualification-novel-methodologies_en.pdf

EMA/CHMP/SAWP/72894/2008: Qualification of novel methodologies for drug development: guidance to applicants

EUnetHTA guideline on Endpoints used in REA: Clinical endpoints. https://www.eunetha.eu/wp-content/uploads/2018/02/WP7-SG3-GL-clin_endpoints_amend2015.pdf

EUnetHTA guideline on Endpoints used in REA: HRQoL. <https://www.eunetha.eu/wp-content/uploads/2013/01/Health-related-quality-of-life.pdfEUnetHTA guideline on Endpoints used in>

WHO Guidance for best practices for clinical trials. 9th September 2024. <https://www.who.int/publications/i/item/9789240097711>

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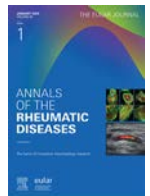
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Rheumatoid arthritis

Epitopes targeted by autoantibodies in presymptomatic individuals predict early rheumatoid arthritis

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ABSTRACT

Objectives: To determine anticitrullinated protein antibody (ACPA) responses to novel peptides predicting the clinical outcomes of treatment-naïve early rheumatoid arthritis (RA) in the pre-symptomatic stage.

Methods: We analysed monoclonal ACPAs derived from RA patients, including a characterised protective ACPA (clone E4), along with plasma samples collected from 520 presymptomatic individuals, of whom 244 were also sampled at diagnosis of RA, and 530 population controls in Sweden. The validation cohort (The Nordic Rheumatic Diseases Strategy Trials and Registries, NORD-STAR) consisted of 690 treatment-naïve early RA patients. Responses to citrullinated or native alpha-enolase (ENO1) or peptidylarginine deiminase 4 (PAD4) peptides were analysed by bead-based multiplex flow immunoassay. Clinical outcomes included C-reactive protein (CRP) and the 28-joint disease activity score (DAS28) with its components: tender joint count (TJC), swollen joint count (SJC), and erythrocyte sedimentation rate (ESR).

Results: Monoclonal ACPAs displayed distinct binding patterns to ENO1 and PAD4 peptides. A time-dependent increase of ACPA response to citrullinated peptides was observed in the pre-symptomatic stage towards onset. In the presymptomatic (0.2–5 years before onset) and early RA stage, ACPA responses to several ENO1 and PAD4 peptides were associated with less severe RA, assessed as lower levels of CRP and DAS28 and its components. In early RA, the association was more pronounced in rheumatoid factor (RF)-negative patients based on lower SJC. In pre-symptomatic individuals, ACPA responses widely predicted lower disease activity in early RA and were more pronounced in 5 selected peptides.

Conclusions: Antibody responses to certain citrullinated epitopes are associated with lower disease activity in treatment-naïve early RA and appear years before symptom onset of RA.

WHAT IS ALREADY KNOWN ON THIS TOPIC

- Promiscuous anticitrullinated protein antibodies (ACPAs) are common in rheumatoid arthritis (RA) and are specific for the citrulline side chain.
- A subset of ACPAs is protective in RA.
- ACPAs are associated with a lower number of swollen joints in early RA.

WHAT THIS STUDY ADDS

- ACPA responses to certain citrullinated epitopes of alpha-enolase (ENO1) and peptidylarginine deiminase 4 (PAD4) are associated with lower disease activity in early RA.
- In presymptomatic individuals, ACPA responses resembling the fine specificity of protective ACPAs predict disease activity at the early RA stage.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- A group of ACPA responses associated with a protective phenotype from the presymptomatic stage to early RA calls for an update on the clinical interpretation of the serological markers.
- Determination of ACPA fine specificities could supplement the clinical anti-second-generation cyclic citrullinated peptide (anti-CCP2) or anti-rheumatoid factor (RF) test to increase the precision of RA diagnosis and the accuracy of prediction of disease activity.

INTRODUCTION

Certain autoantibodies, such as anticitrullinated protein antibodies (ACPAs) and rheumatoid factors (RFs), are present in the circulation in individuals years before the onset of rheumatoid arthritis (RA) [1]. Their repertoire and concentration increase as the disease onset approaches [2]. The typical ACPA, denoted promiscuous ACPA, is highly specific for citrulline side chains

exposed on a protein surface but has limitations in binding due to negative interactions of surrounding side chains within the epitopes [3,4]. This leads to a highly restricted detection of citrullinated epitopes *in vivo* but with a larger binding spectrum *in vitro*, especially for the cyclic peptide structure with more accessible citrulline in the centre for antibody binding, as in the anti-second-generation cyclic citrullinated peptide (anti-CCP2) assay [5,6].

The anti-CCP2 assay has shown that promiscuous ACPAs have high specificity (>98%) and sensitivity (>70%) for RA [7]. For a long time, it has, therefore, been generally assumed that these antibodies are pathogenic, and pathogenicity has been observed when tested using monoclonal or polyclonal ACPAs *in vitro* [8–11]. Lately, however, several monoclonal promiscuous ACPAs have been shown to protect against experimental arthritis [5,12,13]. In our recent study, an ACPA clone, denoted E4, was able to protect against arthritis by forming immune complexes with alpha-enolase (ENO1) and interacting with the downstream inhibitory receptor FCGR2B on macrophages, inducing a regulatory phenotype [5]. Shortly afterwards, it was shown that the presence of ACPAs, together with the absence of RF in early RA, was associated with fewer swollen joints and lower systemic inflammation [14]. Therefore, it is important to be able to utilise certain epitopes to detect a set of ACPA responses in connection with the protective function and to investigate any predictive value for clinical outcomes in the early or even presymptomatic stage of RA [15].

ENO1 is a glycolytic enzyme present in a large number of cell types and tissues. Its function depends on localisation; for example, it can serve as a tumour suppressor when present in the nucleus or a plasminogen receptor when located on the cell surface [16]. In addition, it has been shown that ENO1 protein is highly expressed in monocytes/macrophages and excessively present in RA synovial fluid [17]. Although the exact function of citrullinated ENO1 in RA is still unclear, its diagnostic and predictive potential in RA remains a topic of interest [18], especially with new evidence showing the formation of the immune

complex of protective ACPAs and ENO1 [5]. In RA, the epitope “peptide 1” of citrullinated ENO1 (CEP1) can be recognised by 37% to 63% of RA patients, making it one of the most detected epitopes [18]. However, the amino acid sequence of human ENO1 consists of 17 arginines, whereas CEP1 only encompasses the first 2 arginines from the N-terminus, replaced by citrulline.

Another key autoantigen in RA is peptidylarginine deiminase 4 (PAD4) [19], a crucial enzyme that citrullinates arginine side chains and plays an important role in many key physiological processes, including the formation of neutrophil extracellular traps (NETs) [20]. PAD4 can undergo autocitrullination, which may not necessarily modify its enzymatic function or activity [21,22] but may convert itself into a target of ACPAs. However, the extent and importance of this phenomenon in RA are not well understood.

The function of ACPAs depends on their specificity *in vivo*. Promiscuous ACPAs from the blood of RA patients have been shown to recognise a wide spectrum of citrullinated peptides *in vitro*, which may partly reflect their specificity *in vivo*. Since ACPAs can be detected in blood long before the clinical onset of RA, this raises the question of whether their peptide specificities are associated with clinical diagnosis and prediction in combination with the disease outcomes of RA. We have investigated not only the binding of various monoclonal ACPAs to a panel of citrullinated type II collagen (COL2) peptides but also other joint-related peptides in early RA cohorts using an established bead-based multiplex immunoassay [23,24]. Here, we developed a new panel of peptides covering different ENO1 and PAD4 epitopes and analysed the immunoglobulin (Ig) G responses to the panel in a Swedish cohort consisting of early RA patients.

METHODS

Subjects

A case-control study was performed within the Medical Biobank of northern Sweden and used as the discovery cohort in this study. The cohorts within the Medical Biobank are population-based, and all adult individuals residing in the county of Västerbotten are continuously invited to participate. Recruitment, blood sampling, and storage conditions (at –80°C) have been described previously [1]. The register of patients attending the Department of Rheumatology, Umeå, and fulfilling the 1987

American College of Rheumatology classification of RA [25] and with a known date for the onset of symptoms of joint disease was coanalysed with those of the Medical Biobank. Consequently, 520 individuals (148 men and 372 women, designated as “presymptomatic individuals”) were identified as having donated blood samples before the onset of any symptoms of joint disease. Among the individuals identified as presymptomatic, 244 (75 males and 169 females) had also provided blood samples when attending the clinic at the time of being diagnosed with RA, thus creating paired samples. The median time (Q1-Q3) between the onset of symptoms and a diagnosis of RA was 0.6 (0.3-1.0) years. All cases were classified as either “nonsmokers” or “ever-smokers.” A total of 530 control subjects (175 men and 355 women) were randomly selected from the same Biobank cohorts as the presymptomatic individuals and matched for sex, age, and area at the time of blood sampling (‘sex’ refers to biological classification of participants as male or female, as recorded in medical records). Baseline data and serum samples from patients with treatment-naïve early RA (N = 690) according to European Alliance of Associations for Rheumatology (EULAR)/American College of Rheumatology (ACR) classification criteria included in the randomised phase IV Nordic Rheumatic Diseases Strategy Trials and Registries (NORD-STAR) trial were analysed as a validation cohort at diagnosis before treatment initiation [26–29]. Briefly, NORD-STAR inclusion criteria were age 18 years and older with treatment-naïve RA, symptom duration less than 24 months, moderate to severe disease activity, and RF or ACPA positivity or increased C-reactive protein (CRP). The subjects are described in Table 1.

The Regional Ethics Committee at the University Hospital, Umeå, Sweden, approved this study (2013-347-31), and all participants gave their written informed consent when donating samples to the Medical Biobank and at the early arthritis clinic. The Swedish Ethical Review Authority approved the current NORD-STAR study (Dnr 2022-02326-02), with the inclusion of samples obtained from Sweden, Denmark, Norway, and the Netherlands for the current study. All participants gave their written informed consent before entering the study.

Monoclonal antibodies derived from RA patients

The acquisition and generation of all monoclonal antibodies in the present study (E4, “L” series, and 3F3WT) have been previously described [5,6]. Briefly, single B-cell clones based on

Table 1
Demographic data for study subjects

Characteristics of subjects	Discovery cohort			Validation cohort
	Presymptomatic individuals (N = 520) n/N (%)	Early RA patients (n = 244) n/N (%)	Population controls (N = 530) n/N (%)	Early RA patients (N = 690) n/N (%)
Females, (%)	71.5	69.3	67	69
Age (y), median (Q1-Q3)	55 (50-60)	61 (53-67)	51 (50-60)	56 (46-66)
Smoking ever	337/520 (65)	166/243 (68)	222/522 (42)	418/688 (61)
HLA-DR SE	390/511 (76)	190/240 (79)	165/299 (55)	NA
RF	189/513 (37)	188/244 (77)	33/251 ^a (13)	512/687 (75)
Anti-CCP2	213/519 (41)	187/241 (78)	16/530 (3)	563/689 (82)
Anti-CCP2 + RF	146/513 (29)	168/244 (69)	2/251 (1)	466/685 (68)
Predating time (y), median (Q1-Q3)	4.5 (2.1-7.7)			

NA, not available; Q, quartile; RA, rheumatoid arthritis; RF, rheumatoid factor; CCP2, second-generation cyclic citrullinated peptide; HLA-DR SE, human leukocyte antigen-DR shared epitope.
^a RF was analysed in 251 of the population controls.

CCP2 reactivity were isolated from RA patients in the Rheumatology Department of Leiden University Medical Center; the B cell receptors (BCRs) were sequenced and constructed with identical human IgG1 or mouse IgG2b constant domains. The E4NG is a variant of the E4 wild-type (E4WT) with the Fab glycosylation sites mutated, but the citrulline specificity is maintained. The E4m antibody is a mutant of the E4 antibody (W48M and S51A in paratope), lacking the citrulline specificity [5], and therefore, it is used as a negative control for E4. All antibodies were produced in Expi293F cells, purified by affinity chromatography, dialysed, and stored in phosphate buffered saline (PBS) at -20°C .

Multiplex bead-based flow immunoassay

The multiplex bead-based flow immunoassay was performed as described previously [15,23]. Cyclic citrullinated or native peptides derived from ENO1 and PAD4 (Vacara AB), as well as control peptides such as CCP4 and CEP1 (Vacara AB) (Supplementary Table S1), were coupled to magnetic beads. Samples (plasma or serum) were diluted 1:100 by Dulbecco's phosphate buffered saline (DPBS), randomised on assay plates in singlicates, preblocked, and incubated with peptide-coupled beads. Bound IgGs were detected with phycoerythrin-conjugated anti-human IgG Fc γ (Jackson ImmunoResearch Laboratories Inc) by measuring the median fluorescence intensity (MFI) using the Bioplex-200 instrument (Bio-Rad). Monoclonal antibodies diluted in different concentrations were assayed similarly, and in case they had the mouse IgG2b constant domain, phycoerythrin-conjugated anti-mouse IgG Fc γ (Jackson ImmunoResearch Laboratories Inc) was used for detection. Data integrity and/or result comparability between the plates was ensured by assay calibrators and control samples. In the validation cohort, 18 of the 708 assayed samples were excluded due to technical reasons, resulting in 690 samples entering data analyses.

Assays for anti-CCP2 antibodies and RF

In the discovery cohort, the detection of anti-CCP2 antibodies and RF in plasma were performed using enzyme-linked immunoassays according to the manufacturer's instructions (Euro-Diagnostica AB) as previously described [1]. The cutoff for anti-CCP2 positivity was set at 25 arbitrary units (AU)/mL and at 20 AU/mL for RF. Anti-CCP2 was considered low when >25 and <75 AU/mL and high when ≥ 75 AU/mL. In the validation cohort, anti-CCP2 and RF were analysed according to laboratory standards at each contributing hospital. Patients with ≥ 20 International Units (IU)/mL anti-CCP antibodies or RF in serum were considered anti-CCP2 or RF-positive, respectively.

Statistics

In the discovery cohort, we measured diagnostic accuracy using sensitivity and specificity with corresponding CIs to quantify uncertainty. Cutoffs for specifying antibody positivity/negativity were determined by maximising the Youden index for RA cases vs controls with respect to antibody concentration under the additional condition that specificity must be at least 0.95. For each RA manifestation, we compared antibody-positive/negative groups with respect to manifestation score as follows. First, we performed an overall sign test, where each antibody contributed a "+" if the mean antibody level was higher in the cases than in controls and a "-" if otherwise. This was followed up using the Mann–Whitney U-test for each individual

antibody. For investigating trends between groups with an increasing number of positive antibodies, the Jonkheere-Terpstra trend test was used. When comparing proportions, we used large-sample tests based on a normal approximation unless otherwise stated. For the RA cases, sensitivity and specificity were compared between pairs of tests following a 2-step procedure outlined in Roldán-Nofuentes [30], where one first tests the joint equality of sensitivity and specificity. Should the joint test yield a significant result, sensitivity and specificity should be separately compared post hoc between tests. We used all available cases; however, a power calculation where we assume an exposure (antibody positivity) rate of 5% in controls and 15% in cases, assuming a .05 level of significance and 95% power, yields a sample size of 231 cases and controls, respectively. Thus, even the early RA patient sample comes with sufficient power.

For the validation cohort, we used the continuous values (MFI) for the antibodies and implemented the method described in Dinse et al [31] to accommodate our analysis values below the limit of detection (LoD). In short, in order to apply an existing statistical methodology that correctly incorporates censored values into statistical analyses (Cox regression), we had to transform the data from left-censored (ie, values below the LoD) to right-censored. The Cox regression is able to deal very well with right-censored data and produces unbiased estimates of the association between risk factors and outcome as a hazard ratio. For this, we first applied a scale reversal by calculating for each antibody individually the difference between an arbitrary constant value, M , that was chosen to exceed all antibody values (in this case, $M = 13,000$) and the respective antibody (ie, M -Antibody values). All original left-censored antibody values $\leq$ LoD were thus transformed into M -Antibody values and treated as right-censored when they exceeded M -LoD. Further, the transformed (M -Antibody) values were treated in the Cox model as the modelled outcome and the indicators of disease activity (tender joint count [TJC] 28, swollen joint count [SJC] 28 categories, etc) as the risk factor of interest, "implying a role reversal when the analyte is a putative cause of a health outcome," as in the study by Dinse et al [31]. Further, the association estimated from the Cox model can be interpreted as the odds ratio (OR), and ORs > 1 are indicative of a worse outcome (in our case, indicative of pathogenicity), and ORs < 1 are indicative of a better health outcome (in our case, indicative of protection). The effect on the 28-joint disease activity score (DAS28), erythrocyte sedimentation rate (ESR), and CRP was interpreted to be slightly/moderately/strongly indicative of protection/pathogenicity based on the OR and the p value. For SJC28 and TJC28, the patients were categorised into 3 groups ($C1 = 0-3$; $C2 = 4-10$; $C3 \geq 10$ affected joints), and the effect was interpreted to be slightly/moderately/strongly indicative of protection/pathogenicity based on the number of significant differences when these groups were compared.

RESULTS

Reactivity of ACPAs to ENO1 and PAD4 epitopes

We analysed the reactivity of a series of earlier defined monoclonal antibodies derived from RA patients (E4, "L" clones, and 3F3WT) with cyclic citrullinated or unmodified peptides derived from ENO1 and PAD4, along with often used peptides, such as CCP4 and CEP1, as controls (Supplementary Table S1). The results from multiplex bead-based immunoassay show that in contrast to most of the used antibody clones that recognised

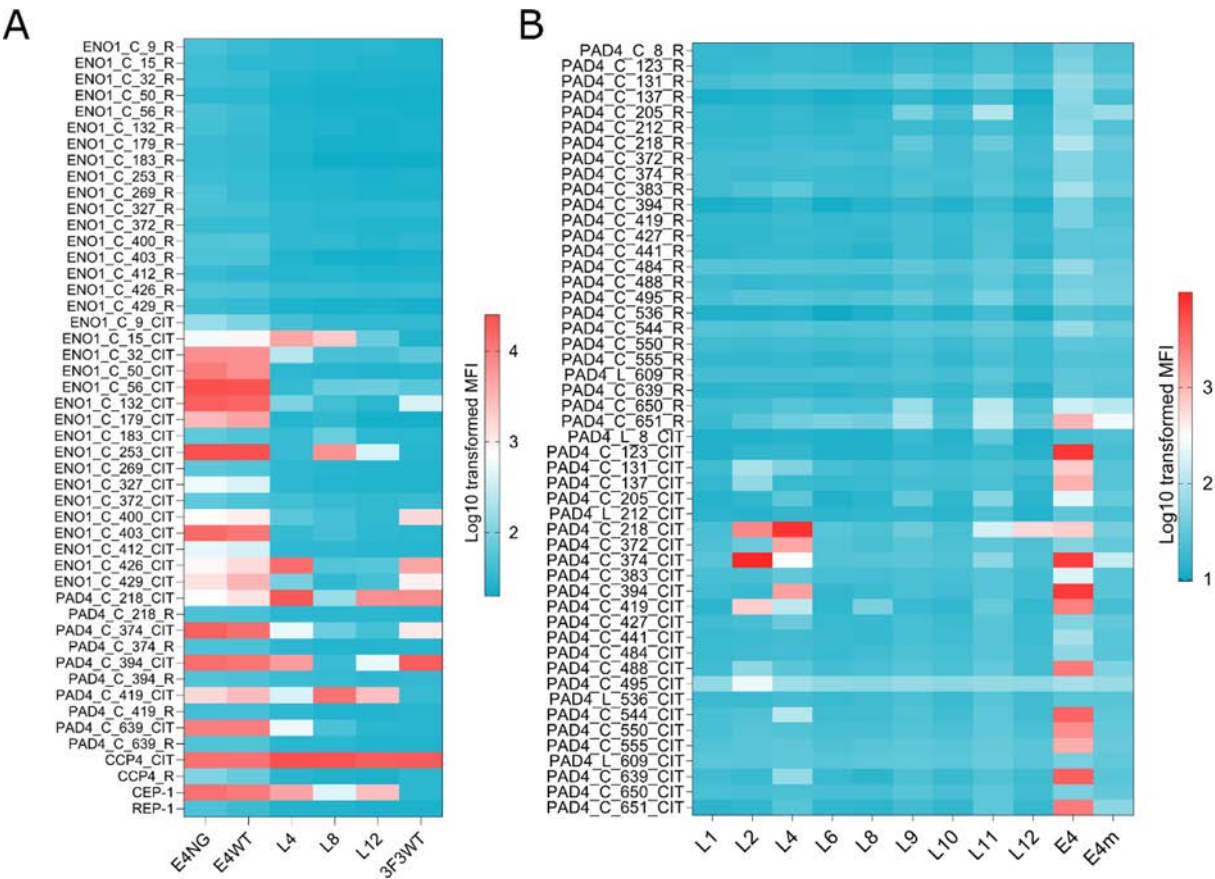


Figure 1. A, and B, Reactivity of monoclonal antibodies to citrullinated cyclic peptides derived from human alpha-enolase (ENO1) and peptidylarginine deiminase 4 (PAD4). The heatmaps represent the log10-transformed median fluorescence intensity (MFI) results at an antibody concentration of 1.0 µg/mL. Antibodies are denoted on the x-axis; antibodies used are (A) human immunoglobulin (Ig) G1 and (B) mouse IgG2b. E4, “L” series, and 3F3WT are monoclonal anticitrullinated protein antibodies derived from rheumatoid arthritis patients. Peptides are denoted on the y-axis. C, cyclic peptide; L, linear peptide; R, unmodified peptide with arginine; CIT, citrullinated peptide; E4m, mutated E4 lacking citrulline specificity; E4NG, non-Fab glycosylated E4; E4WT, wild-type E4 with Fab glycosylation; CCP4, fourth-generation cyclic citrullinated peptide; CEP1, citrullinated alpha-enolase peptide 1; REP1, alpha-enolase peptide 1.

only a very limited number of citrullinated epitopes on both ENO1 and PAD4, E4 bound to many citrullinated epitopes, indicating that E4-like protective ACPAs could have a distinct pattern for epitope recognition compared with other “nonprotective” ACPAs (eg, L2) (Fig 1).

Time-dependent increase of plasma IgG responses to citrullinated epitopes on ENO1 and PAD4 in presymptomatic individuals towards RA onset

The discovery cohort of presymptomatic individuals and early RA patients (patient characteristics are in Table 1) was analysed for plasma IgG responses with the 56-plex. Overall, the antibody responses to all citrullinated peptides were elevated in a time-dependent manner (Fig 2A,B, and Supplementary Tables S2 and S3). Upon the RA onset, the most frequent IgG response in this panel was against the CCP4 peptide, with a 75.4% sensitivity and 95.3% specificity. Among the ENO1 peptides, the most frequent one was ENO1_C_426_CIT (71.3% sensitivity and 94.5% specificity), followed by ENO1_C_9_CIT (68.3% sensitivity and 94.9% specificity) and ENO1_C_132_CIT (65.2% sensitivity and 94.7% specificity). For the selected PAD4 peptides (Fig 2A), the most frequent one was PAD4_C_394_CIT (72.5% sensitivity and 94.7% specificity), followed by PAD4_C_218_CIT (71.7% sensitivity and 94.7% specificity). In contrast, the responses to arginine peptides were at a minimal level, rarely above those in

the control population, which is expected as background cross-reactivity.

When analysing the relationship between antibody responses, we found that most antibodies were positively correlated with each other both at the presymptomatic stage and in patients, whereas the correlations were clearly stronger in the presymptomatic samples (Fig 2C).

ACPA fine specificities are associated with lower SJC in early RF-negative RA

The association between ACPA responses to the peptides (positive or negative) and clinical outcomes in early RA (<1 year after onset, n = 244) was analysed based on different disease activity variables, including DAS28 and its components, such as the TJC, SJC, and ESR, as well as CRP (Fig 3). Without stratification for RF positivity (Fig 4A and Supplementary Table S4), several ACPA responses to the peptides, such as ENO1_C_9_CIT, ENO1_C_327_CIT, ENO1_C_426_CIT, and CCP4, were found to associate with a lower SJC or TJC, whereas another peptide, ENO1_C_269_CIT, was associated with a higher ESR. Notably, RF was not associated with any of these disease activity variables, and CCP2 positivity was associated only with a lower SJC in early RA. There were no differences between groups having low or high anti-CCP2 levels for any of the outcomes (Supplementary Table S4). By

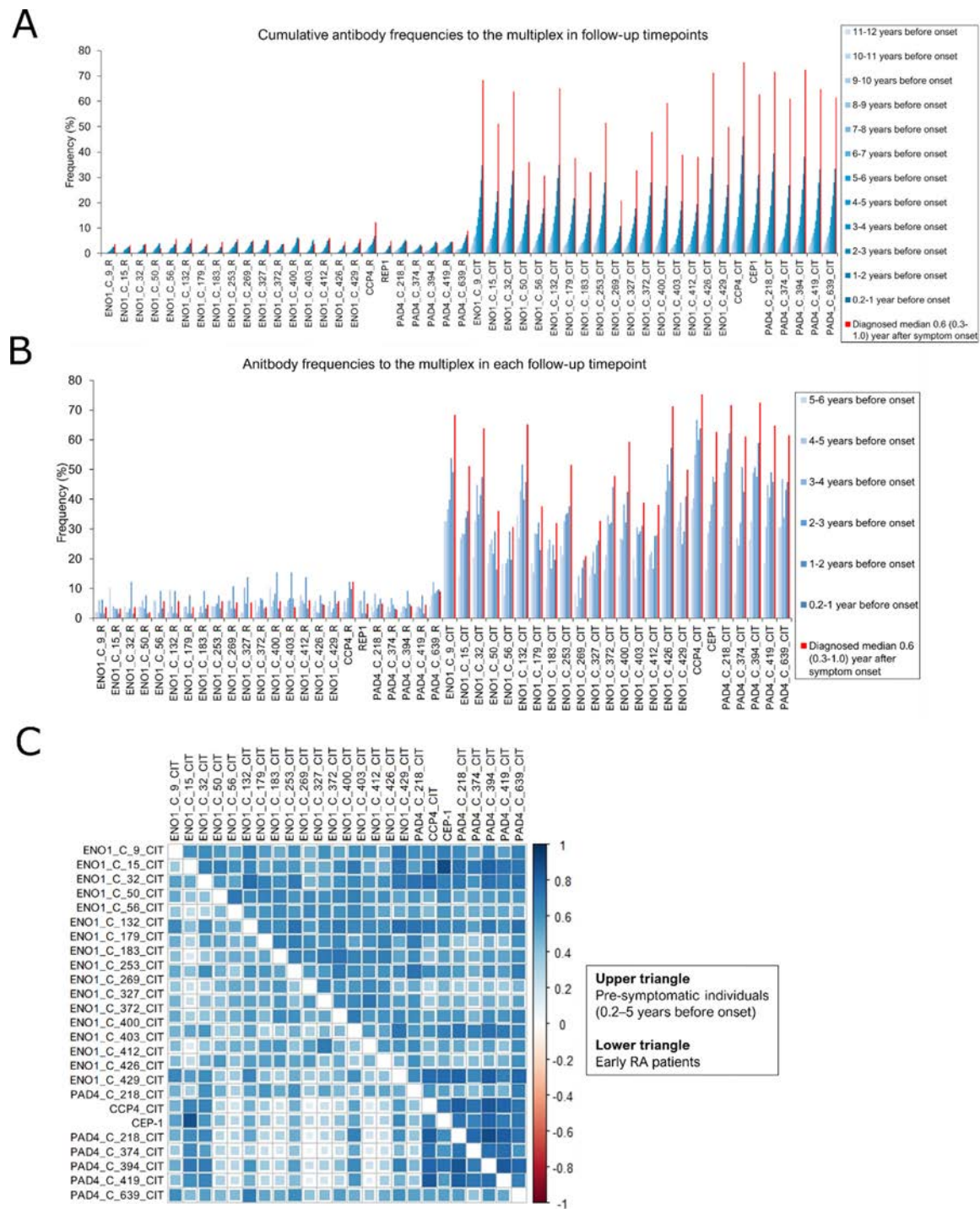


Figure 2. Immunoglobulin (Ig) G responses to citrullinated (CIT) or native/arginine (R) epitopes of alpha-enolase (ENO1) and peptidylarginine deiminase 4 (PAD4) in presymptomatic individuals and early rheumatoid arthritis (RA) patients in the discovery cohort. A, Cumulative frequency of antibody responses to ENO1 and PAD4 epitopes in plasma samples (N = 520) from the presymptomatic individuals during the predating time (from 12 to less than 0.2 years before onset). The frequency of patients with early RA (n = 244; diagnosed median [Q1-Q3] years after symptom onset, 0.6 [0.3-1.0]) stratified for time intervals is shown as a comparison. B, Individual frequency of antibody responses to ENO1 and PAD4 epitopes in plasma samples from the presymptomatic individuals during the predating time (from 6 to less than 0.2 years before symptom onset) (n = 335). The frequency of patients with early RA (n = 244; diagnosed median [Q1-Q3] years after symptom onset, 0.6 [0.3-1.0]) is shown as a comparison. The median fluorescence intensity (MFI) cutoff for positivity was determined as 95% specificity in population controls. The y-axis is the frequency of positivity (%). Response to CCP4, citrullinated alpha-enolase peptide 1 (CEP1), and their respective noncitrulline control peptides (CCP4_R and REP1) are shown for comparison. C, Correlation between different IgG responses to CIT epitopes based on MFI values in presymptomatic individuals (upper triangle, 0.2-5 years before onset) and early RA patients at disease onset (lower triangle). C, cyclic peptide; L, linear peptide; R, unmodified peptide with arginine; CIT, citrullinated peptide; CCP4, fourth-generation cyclic citrullinated peptide; CEP1, citrullinated alpha-enolase peptide 1.

stratifying these early RA patients with RF status (Fig 4A and Supplementary Table S5), we could observe an association with a lower SJC in RF-negative patients having ACPA fine specificities of 8 citrullinated peptides, aside from CCP2, compared with those not having those antibodies. The largest

differences were found in PAD4_C_218_CIT, ENO1_C_13_CIT, ENO1_C_426_CIT, and PAD4_C_394_CIT (Fig 4A and Supplementary Table S5), and 3 of these peptides (3/8) were also found to have an association with a lower DAS28. In addition, the TJC, ESR, or CRP was not significantly different in the

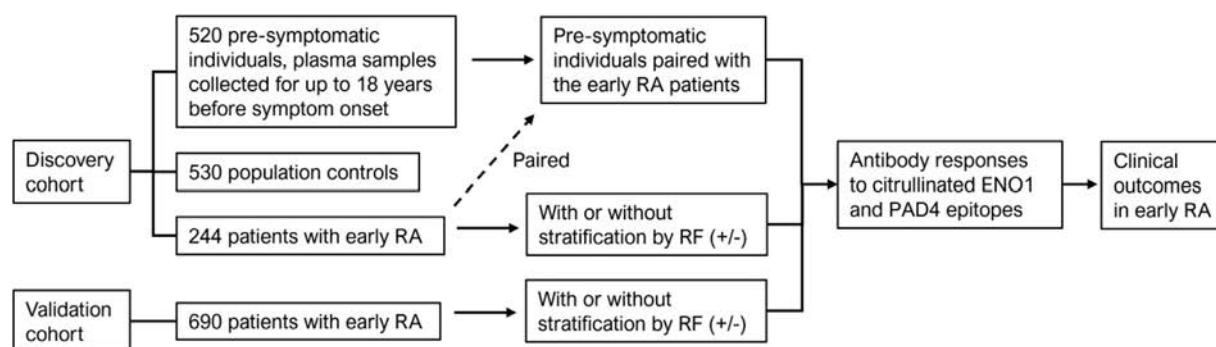


Figure 3. Flowchart of the analysis strategy for relationships (1) between antibody responses in presymptomatic individuals and their clinical outcomes after RA onset and (2) between antibody responses in early rheumatoid arthritis (RA) patients and their clinical outcomes in the discovery and validation cohorts. ENO1, alpha-enolase; PAD4, peptidylarginine deiminase 4; RF, rheumatoid factor.

RF-negative group with or without the measured ACPA fine specificities, although the tendency for a “protective” association was commonly observed (Fig 4A and Supplementary Table S5). In the RF-positive group, ACPA response to ENO1_C_179_CIT and ENO1_C_269_CIT was found to have an association with a higher DAS28 and ESR, respectively (Fig 4A and Supplementary Table S5). The results were confirmed in the treatment-naïve early RA validation cohort (Fig 4B and Supplementary Tables S6–S9). In short, the ACPAs in RF-negative RA patients were indicative of protection.

ACPA fine specificities in presymptomatic stage predict future onset outcomes

To investigate whether ACPAs in presymptomatic individuals could predict future RA severity, we looked at the discovery cohort’s RA cases ($n = 119$) who had donated samples during the period of 0.2 to 5 years before disease onset. The paired samples from the presymptomatic phase were directly stratified by ACPA responses to the citrullinated peptides (positive or negative), and their association with the clinical outcomes in early RA were analysed (Fig 5 and Supplementary Table S10). Surprisingly, no ACPA responses could be found to show a “pathogenic” association with early RA clinical outcomes. In contrast, many were associated with a lower SJC, TJC, DAS28, ESR, or CRP at RA diagnosis. In fact, patients positive for responses to most citrullinated peptides within the panel in their presymptomatic stage showed overall tendencies of having lower disease severity at disease onset. Among these “protective” associations, several could be highlighted in more than 1 clinical variable, such as ENO1_C_15_CIT, ENO1_C_132_CIT, ENO1_C_426_CIT, CCP4, PAD4_C_419_CIT, and PAD4_C_639_CIT. Notably, ACPA responses to ENO1_C_426_CIT, PAD4_C_218_CIT, and PAD4_C_419_CIT in RF-negative early RA were also associated with a lower SJC as described above (Fig 4). Additionally, no associations between clinical manifestations and the anti-CCP2 response or RF status were found, neither between groups with high nor low anti-CCP2 responses (Fig 5 and Supplementary Table S10). Stratification of RF positivity was not achievable for presymptomatic individuals due to the small sample size at different time points and even lower RF positivity among the presymptomatic samples.

New biomarkers predict disease activity at RA onset

To evaluate whether our biomarkers could aid the clinical anti-CCP2 test in the prospect of providing a diagnostic/

predictive value, we selected 5 candidate peptides with ACPA responses that were associated with less severe RA outcomes in the presymptomatic stage or early RA, including ENO1_C_132_CIT, ENO1_C_426_CIT, PAD4_C_218_CIT, PAD4_C_419_CIT, and PAD4_C_639_CIT, named “Citrullinated Peptides for RA Prediction and Diagnosis 5-set (CitPreDi5).”

We first compared the frequencies of positive ACPA responses with these peptides in early RA patients, presymptomatic individuals, and population controls (Fig 6A and Table 2). In comparison with the anti-CCP2 test as a reference (77.6% sensitivity in early RA, 41% sensitivity in presymptomatic individuals, at 98% specificity), CitPreDi5 provided comparable sensitivities (77.0% in early RA and 42.9% in presymptomatic individuals) with a 94.7% specificity when 2 to 5 (≥ 2) ACPA responses to CitPreDi5 were detected (Table 2), extended by a frequency of 88.3% in RF-positive early RA patients, 39.3% in RF-negative early RA patients, and 53.7% in presymptomatic individuals with 0.2 to 5 years before onset (Fig 6A).

In anti-CCP2-negative cases, we could identify 31.5% of early RA patients and 29.1% (95% CI, 24%–34%) of presymptomatic individuals positive for at least 1 of the 5 ACPA responses (Table 3). Although the specificity was lower (87.0%), the CitPreDi5 test captured both early RA patients and presymptomatic individuals more often than the controls, as demonstrated by the nonoverlapping CIs (Table 3). In the case when samples were positive for 2 to 5 (≥ 2) ACPA responses to CitPreDi5, we could still detect 11.1% of early RA patients and 10.1% of presymptomatic individuals with a specificity of 96% (Table 3). There were significantly more presymptomatic individuals than controls who identified as testing positive with the CitPreDi5 test. Despite similar proportions (10%–11%) of test-positives in the early RA and presymptomatic groups, there was no statistical significance between early RA and controls due to the low patient number ($n = 54$) (Table 3).

Next, we investigated whether the CitPreDi5 test could demonstrate predictive value in presymptomatic individuals of their disease activity at diagnosis. The clinical outcomes of presymptomatic individuals (0.2–5 years before diagnosis) with ≥ 1 , ≥ 2 , ≥ 3 , ≥ 4 , or 5 positive ACPA responses to CitPreDi5 were compared with those of individuals with no response to CitPreDi5 (0). Those who were positive for ACPA responses to certain numbers of CitPreDi5 peptides showed significantly lower disease activity with a clear trend depending on the number of ACPA responses (Fig 6B). Analysis of the trend of having a positive antibody response for CitPreDi5 showed a significant decrease in DAS28, TSJ, SJC, ESR, and CRP ($p < .002$ –.030, Jonckheere–Terpstra test). Notably, the individuals positive for

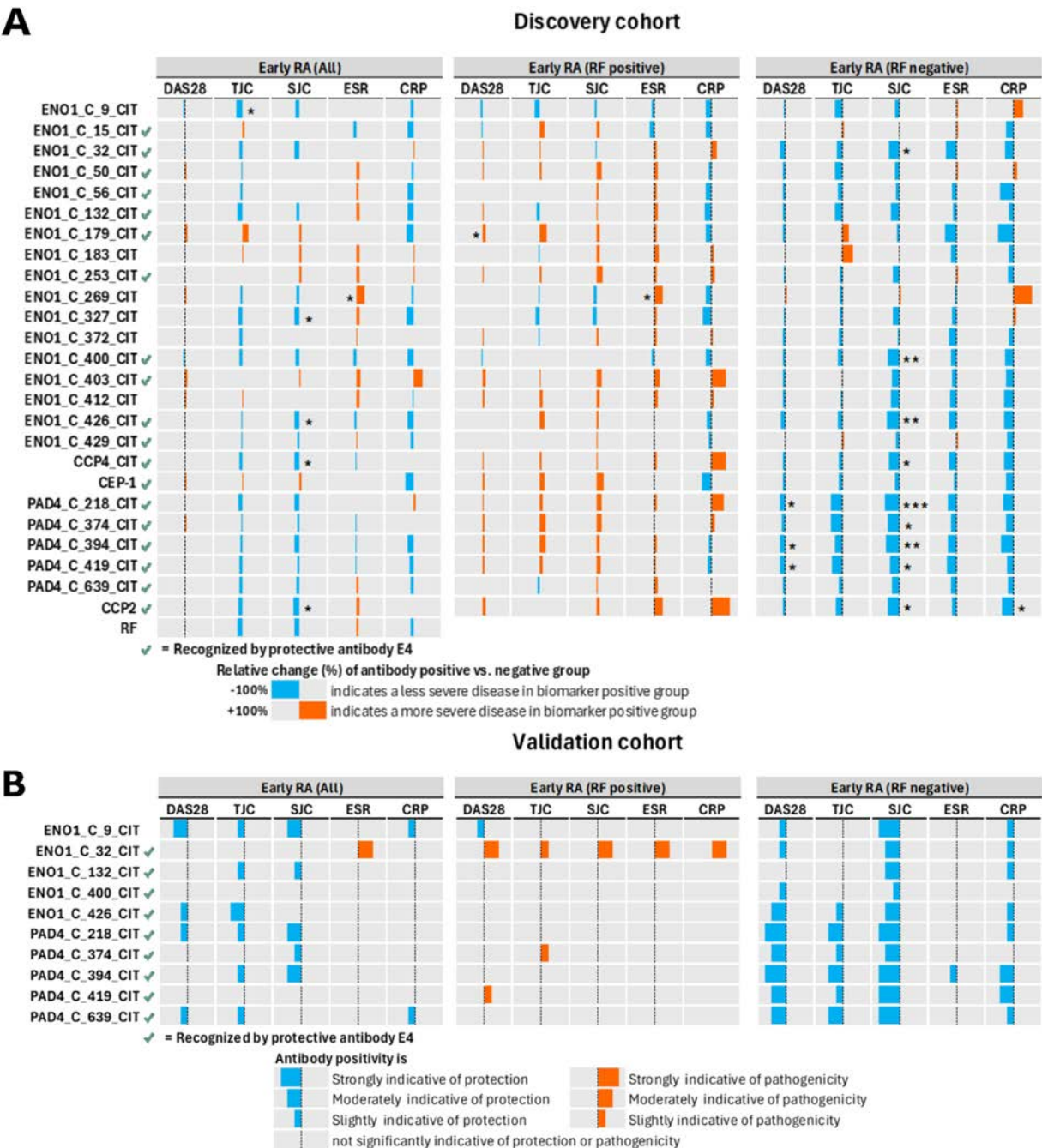


Figure 4. Associations of the serological biomarkers with clinical presentation (28-joint disease activity score [DAS28], tender joint count [TJC], swollen joint count [SJC], erythrocyte sedimentation rate [ESR], and C-reactive protein [CRP]) in early rheumatoid arthritis (RA) patients in the discovery (A) and validation (B) cohorts. A, Changes in clinical presentation in early RA patients are positive for the serological biomarkers compared with those who are negative. Biomarkers, including immunoglobulin Gs against the alpha-enolase (ENO1), peptidylarginine deiminase 4 (PAD4), and control peptides, as well as anti-second-generation cyclic citrullinated peptide (anti-CCP2) antibodies and rheumatoid factor (RF) in early RA patients (diagnosed median [Q1-Q3] years after symptom onset, 0.6 [0.3-1.0]), were determined, and each patient categorised as being positive or negative for each of the biomarkers. Results are shown as relative change (%), and the Mann-Whitney U-test (the overall differences were first validated by a sign test [results not shown]) was used when the means of the DAS28, TJC, SJC, ESR, or CRP data were compared between biomarker-positive and -negative groups. * $p < .05$, ** $p < .01$, *** $p < .001$. Early RA (All) $n = 244$; early RA (RF-positive) $n = 188$; early RA (RF-negative) $n = 56$, of which 22 were anti-CCP2 positive. B, The effect of selected antibodies in the early RA validation cohort. Early RA (All) $N = 690$; early RA (RF-positive) $n = 512$; early RA (RF-negative) $n = 175$; RF missing in $n = 3$. A decrease indicates a less severe disease in the biomarker-positive group. In addition, peptide epitopes recognised by the protective antibody E4 are indicated next to the peptide names. C, cyclic peptide; CIT, citrullinated peptide; R, unmodified peptide with arginine; CCP4, fourth-generation cyclic citrullinated peptide; CEP1, citrullinated alpha-enolase peptide 1.

≥ 4 or 5 ACPA responses in the CitPreDi5 test were found to have the lowest disease activity at diagnosis. In addition, despite the tendency, the anti-CCP2 test in the presymptomatic individuals did not show a similar association with any of the analysed clinical outcomes at RA onset (Figs 5 and 6B).

DISCUSSION

We have taken advantage of the epitope specificities of human monoclonal ACPAs with known protective and nonprotective functions to analyse plasma samples from presymptomatic

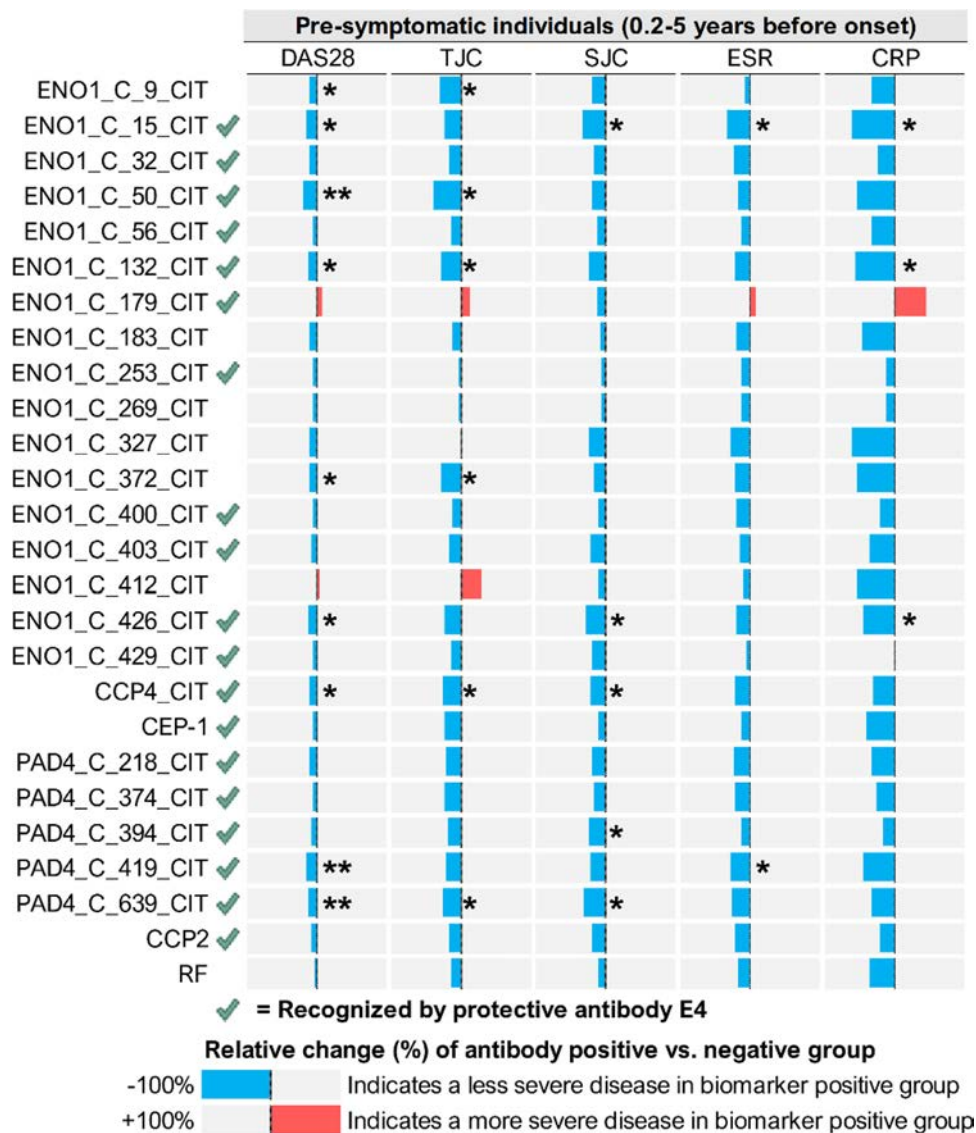


Figure 5. Change in the mean clinical presentation (28-joint disease activity score [DAS28], erythrocyte sedimentation rate [ESR], and C-reactive protein [CRP]) at rheumatoid arthritis diagnosis of presymptomatic individuals positive for the serological biomarkers compared with those who were negative in the discovery cohort. Biomarkers, including immunoglobulin Gs against the alpha-enolase (ENO1), peptidylarginine deiminase 4 (PAD4), or control peptides, as well as anti-CCP2 antibodies and rheumatoid factor (RF) in presymptomatic individuals (0.2-5 years before the onset, n = 117 for DAS28 and ESR; n = 116 for SJC and TJC; and n = 82 for CRP), were determined, and each individual was categorised as being positive or negative for each of the biomarkers. Results are shown as relative change (%), and the Mann–Whitney U-test (the overall differences were first validated by a sign test [results are not shown]) was used when the means of the DAS28, TJC, SJC, ESR, or CRP data were compared between biomarker-positive and -negative groups. A decrease indicates a less severe disease in the biomarker-positive group, and an increase indicates a more severe disease in the biomarker-positive group. In addition, peptide epitopes recognised by the protective antibody E4 are indicated next to the peptide names. **p* < .05, ***p* < .01. C, cyclic peptide; CEP-1, citrullinated ENO1; CIT, citrullinated peptide.

individuals and early RA patients. In line with a recent study [14], we confirmed that the association of ACPAs with a disease-protective response, such as a lower SJC, was the strongest in RF-negative early RA. Strikingly, without the need to stratify RF, we found that presymptomatic ACPA responses (0.2-5 years before the RA diagnosis) to a set of peptides, some of which are exclusively recognised by protective ACPAs (eg, clone E4), could predict a lower disease activity for the forthcoming RA onset. From these “protection-associated” peptides, we developed a new ACPA test encompassing 5 peptides (ENO1_C_132_CIT, ENO1_C_426_CIT, PAD4_C_218_CIT, PAD4_C_419_CIT, and PAD4_C_639_CIT), named “CitPreDi5,” which could predict the expected severity of disease.

According to current classification criteria, patients with RA can be divided into several subgroups depending on the presence of anti-CCP antibodies and/or RF. RF positivity has been

shown to be associated with more destructive RA [32], whereas a recent study suggested that CCP2 positivity was associated with a low SJC and TJC in the absence of RF [14]. Initial studies on diagnostic peptides for ACPA detection focused on the citrullinated profilaggrin peptide (CCP1), whose epitope is not tissue-specific and widely present under physiological conditions [33], and later on synthetic CCP2, which is still the gold standard in the clinical diagnosis of RA. Diagnosis and initiation of appropriate treatment can be delayed in approximately 30% of patients who are negative for both RF and anti-CCP2, and nearly 20% of these patients still possess ACPA responses to a variety of citrullinated peptides [33]. Therefore, it is likely that more patient subgroups can be identified by deciphering the subsets of ACPAs and combining these findings with RF status [15], which may provide better implications for the timing and accuracy of diagnosis and treatment recommendations.

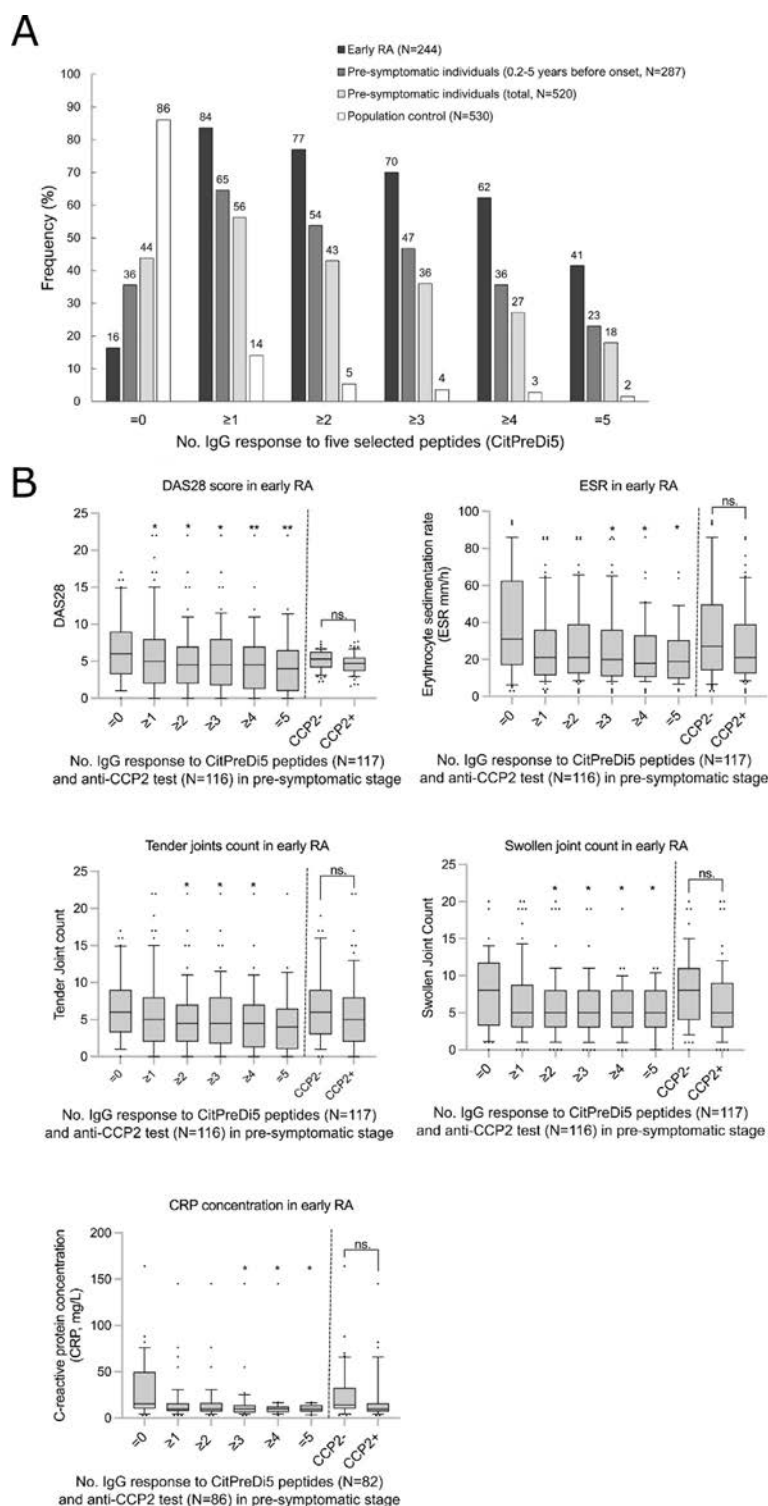


Figure 6. Diagnostic and predictive value of new biomarkers for anticitrullinated protein antibody (ACPA) responses. **A**, Frequencies of ACPA immunoglobulin (Ig) G responses to 5 peptides (Citrullinated Peptides for RA Prediction and Diagnosis 5-set [CitPreDi5]) in early rheumatoid arthritis (RA) patients, pre-symptomatic individuals, and population controls in the discovery cohort. **B**, Early RA clinical outcomes were associated with the number of ACPA responses from presymptomatic individuals (0.2-5 years before onset, $n = 117$ for the 28-joint disease activity score [DAS28] and its components: the tender joint count [TJC], swollen joint count [SJC], and erythrocyte sedimentation rate [ESR]; and $n = 82$ for C-reactive protein [CRP]), and similar data were included for the anti-second-generation cyclic citrullinated peptide (anti-CCP2) test ($n = 116$ for DAS28 and its components: the TJC, SJC, and ESR; and $n = 86$ for CRP) as a reference. For each clinical measure, the comparisons were separately made between the individuals with ≥ 1 , ≥ 2 , ≥ 3 , ≥ 4 , or 5 positive ACPA responses to the CitPreDi5 peptides vs the individuals with no response to the peptides (0); data were analysed with the Mann–Whitney U-test (2-tailed) and presented as median with 10th to 90th percentiles; outliers are shown as dots in groups. $*p < .05$, $**p < .01$, ns, not significant.

The CitPreDi5 peptides from our panel showed comparable specificity (95%) and sensitivity (77%) to the clinical anti-CCP2 test when the samples were positive for at least 2 of the CitPreDi5 peptides. In our analysis of presymptomatic individuals, the anti-CCP2 test was incapable of predicting future clinical outcomes, whereas the CitPreDi5 captured a subset of patients with lower disease activity at diagnosis. The functional role of ACPAs in RA is still an important unanswered question. Although the specificity of the citrulline side chain of ACPAs is highly conserved between mice and humans, the context is also important. Antibodies may play different roles if operating together; thus, complexes between promiscuous ACPAs and RF might add additional effects, and it is also possible that more

specific ACPAs that cross-react with cartilaginous joints could have a pathogenic role. We hypothesise that our results reflect a subset of protective ACPAs that have a similar epitope fingerprint to the E4 antibody [5]. In fact, most human-derived ACPA clones, tested in different laboratories, have been found to exert certain levels of arthritis protection in animal models [5,12,13]. Here, ACPA responses to an epitope fingerprint associated with protection were present at least 10 years before RA diagnosis. Although their concentration could remain low in the presymptomatic stage, these antibodies correlated more strongly with each other than in early RA (Fig 2C), indicating that a wider B-cell repertoire that produces epitope-specific antibodies (eg, antibodies with more specific binding to cartilage), potentially

Table 2
Comparison of the anti-CCP2 test and the new ACPA test, where positivity for at least 2 of the CitPreDi5 peptides was considered a positive test result. Sensitivity and specificity are shown with 95% CIs

Discovery cohort	Early RA (n = 244)		Presymptomatic (N = 520)		Controls (N = 530)	
	Test positive n/N (%; 95% CI)	Test negative n/N (%; 95% CI)	Test positive n/N (%; 95% CI)	Test negative n/N (%; 95% CI)	Test positive n/N (%; 95% CI)	Test negative n/N (%; 95% CI)
RF	188/244 (77; 72, 82)	56/244 (23)	189/513 (37; 33, 41)	324/513 (63)	33/251 (13)	218/251 (87; 83, 91)
CCP2	189/244 (77; 72, 82)	55/244 (23)	213/519 (41; 37, 45)	306/519 (59)	16/530 (3)	514/530 (97; 96, 98)
RF+ and/or CCP2+	210/244 (86; 82, 90)	34/244 (14; 10, 18)	256/519 (49; 45, 54)	263/519 (51; 46, 55)	47/525 (9; 7, 11)	478/525 (91; 89, 93)
≥2 Ab+ ^b	188/244 (77; 72, 82)	56/244 (23)	223/520 (43; 39, 47)	297/520 (57)	28/530 (5)	502/530 (95; 93, 97)
≥3 Ab+ ^c	171/244 (70; 64, 76)	73/244 (30)	187/520 (36; 32, 40)	333/520 (64)	19/530 (4)	511/530 (96; 95, 98)

ACPA, anticitrullinated protein antibody; CitPreDi5, Citrullinated Peptides for RA Prediction and Diagnosis 5-set; RA, rheumatoid arthritis; RF, rheumatoid factor; CCP2, second-generation cyclic peptide; Ab, antibody.

^a Ab+, positive ACPA response to CitPreDi5 peptides (ENO1_C_132_CIT, ENO1_C_426_CIT, PAD4_C_218_CIT, PAD4_C_419_CIT, and PAD4_C_639_CIT)
^b The overall test p values are $p = .086$ (vs CCP2, no post hoc); $p = .015$ (vs RF+, post hoc: sensitivity $p = .88$, specificity $p = .0072$); $p = 1.7 \times 10^{-6}$ (vs RF+ and/or CCP2+, post hoc: sensitivity $p = .000038$, specificity $p = .017$). Should a Bonferroni correction be applied to the 6 overall tests, the overall result for ≥ 2 Ab+ vs RF+ would be considered nonsignificant.
^c The overall test p values are $p = .0019$ (vs CCP2, post hoc: sensitivity $p = .0013$, specificity $p = .66$); $p = .000062$ (vs RF+, post hoc: sensitivity $p = .034$, specificity $p = .00047$); $p = 2.3 \times 10^{-13}$ (vs RF+ and/or CCP2+, post hoc: sensitivity $p = 6.8 \times 10^{-9}$, specificity $p = .00010$).

pathogenic, arises shortly before onset, outweighing the protective antibodies and leading to clinical RA. This is consistent with the concept of epitope spreading before disease onset, where antibody reactivity broadens with enhanced specificities over time, and such dynamic change may explain why presymptomatic individuals exhibit more intercorrelated antibody responses, while early RA reflects a more individualised immune profile. It is intriguing to think that during the decades of the presymptomatic stage, the low level of protective ACPAs could act as a sentinel to eliminate abnormal citrullination but may not be potent enough to combat the dramatic disease development around the onset when hypercitrullination takes place and epitope-specific pathogenic antibodies become predominant [34]. Nevertheless, we were able to confirm that a subset of presymptomatic ACPAs is associated with less severe RA at diagnosis based on CRP, DAS28, and its components, the SJC, TJC, and ESR, which may have implications for future disease management.

When examining the fine specificities of a series of patient-derived monoclonal ACPAs on ENO1 and PAD4 epitopes, a distinct pattern exhibited by the protective ACPA E4 showed that E4 itself was sufficient to interact with most of the citrullinated epitopes on ENO1 and PAD4, covering those that were associated with less severe RA, whereas the others, such as the “nonprotective” ACPAs (ie, clone L2) [5], had more restricted access to most of the ENO1 or PAD4 epitopes. However, this difference in the binding pattern was reversed on citrullinated COL2 in our recent study, in which ACPAs, such as L2, could access more epitopes, whereas E4 became more selective [5]. By comparing the sequences of peptides, it is clear that the Cit-Gly

motif within the epitope plays a critical role in such a pattern [23]. In our previous study [3], the used citrullinated COL2 peptides and CEP1 contained the Cit-Gly motif in their epitopes, and in crystallography, we observed additional polar contacts, ie, the hydrogen bonding between the Cit-Gly glycine and the carboxamide group, but the impact of such interaction on the E4 Fab-peptide complex is unknown. In this study, it is clear that E4 could strongly interact with many PAD4 or ENO1 epitopes that do not possess the Cit-Gly motif, unlike the L2 or L4 antibody. With this observation, we deemed that Cit-Gly is not required or has only a minimal impact on E4 for epitope binding. Nonetheless, we could conclude from the sequences of all tested peptides that a common subtype of promiscuous ACPAs (eg, clone L2 or L4) heavily relies on the Cit-Gly motif for interaction, whereas E4 does not require a glycine neighbouring citrulline for interaction, and such a difference in binding preference may have an impact on the function of the antibodies.

Seronegative RA patients experience a longer time from clinically documented joint swelling to clinical diagnosis of RA compared with seropositive patients [35]. For seronegative patients, the current practice of diagnosing RA relies on the other parameters in the classification criteria [25,26] and physicians’ experience, which raises, eg, the SJC and TJC required to achieve diagnosis. Thus, seronegative patients may present more severe manifestations than the seropositive ones at early RA on a group level. This may influence the interpretation of our results when comparing our biomarkers’ predictive/diagnostic value between seropositive and seronegative RA patients. The results of the RF-negative group are less affected by that. We envision that CitPreDi5 could improve the RA diagnosis/prediction of

Table 3
Performance of the anti-CitPreDi5 test in anti-CCP2-negative individuals. Positivity for at least 1 or 2 (≥ 1 or ≥ 2) of the 5 peptides was considered positive. Sensitivity and specificity are shown with 95% CIs

Discovery cohort	Early RA (n = 54)		Presymptomatic (n = 306)		Controls (n = 514)	
	Test positive n/N (%; 95% CI)	Test negative n/N (%; 95% CI)	Test positive n/N (%; 95% CI)	Test negative n/N (%; 95% CI)	Test positive n/N (%; 95% CI)	Test negative n/N (%; 95% CI)
RF	20/54 (37; 24, 50)	34/54 (63; 50, 76)	43/303 (14; 10, 18)	260/303 (86; 82, 90)	31/243 (13; 9, 17)	212/243 (87; 83, 91)
≥1 Ab+	17/54 (31; 19, 44) ^a	37/54 (69)	89/306 (29; 24, 34) ^a	217/306 (71)	67/514 (13)	447/514 (87; 84, 89)
≥2 Ab+	6/54 (11; 3, 19)	48/54 (89)	31/306 (10; 7, 14) ^a	275/306 (90)	21/514 (4)	493/514 (96; 94, 98)

Ab+, positive anticitrullinated protein antibody response to CitPreDi5 peptides (ENO1_C_132_CIT, ENO1_C_426_CIT, PAD4_C_218_CIT, PAD4_C_419_CIT, and PAD4_C_639_CIT).

CitPreDi5, Citrullinated Peptides for RA Prediction and Diagnosis 5-set; RA, rheumatoid arthritis; RF, rheumatoid factor.

^a The proportion positive is significantly different compared with the population control.

presymptomatic but at-risk individuals (eg, family history, predisposition of genetic and environmental risk factors, elevated inflammatory markers, or RF presence etc), as upon the appearance of symptoms, having ACPAs in circulation associated with a protective phenotype indicates a less severe early RA. This stratification provides an opportunity for rheumatologists and individuals to intervene more timely and efficiently through better monitoring, disease management, lifestyle adjustment, or even early treatment.

A strength of the study is the unique collection of samples from presymptomatic individuals. The lack of access to similar independent material is a limitation of the study, while the use of an independent early RA cohort for validation confirmed the results and provided evidence to establish generalisability. We also acknowledge that multiple test corrections may affect the significance of some biomarkers with borderline *p* values. However, the overall trend remains clear and evident, and several biomarkers continue to show significant associations.

To conclude, in early RA patients and presymptomatic individuals who subsequently developed RA, we identified ACPA responses to certain epitopes derived from ENO1 and PAD4 that were associated with lower disease activity, expanding the notion that the majority of ACPA responses could be protective in RA, especially in the presymptomatic stage. To our knowledge, this is the first study showing the relationship between epitope-specific protective ACPA responses from either the presymptomatic or early stage of RA and clinical manifestations in early RA. The CitPreDi5 test could also contribute to the development of a more precise assay supplementing the anti-CCP2 test for RA diagnosis/prediction.

Competing interests

RH is a founder, YH and OS are employees, and EL is part of the steering group of Vacara AB. Vacara provided reagents for the multiplex flow immunoassay used in the study and has contracted with Karolinska Institutet and Sahlgrenska University Hospital for a collaboration project. Vacara has an approved US patent on protective antibodies to citrullinated proteins and has submitted a patent application for the invention of this study. DN has received consulting fees from BMS, Lilly, MSD, Novartis, Pfizer, and UCB. TU has received honoraria from Pfizer, Galapagos, and UCB. SR-D has a royalty agreement with Vacara. RH has received consulting fees from Lipum AB. For the remaining authors (LJ, MLA, LC, AL, MHL, BG, KH-P, MN, KL, JL, AR, GG, MØ, and IG), no competing interests were declared.

Ethics approval

For the discovery cohort, the Regional Ethics Committee at the University Hospital, Umeå, Sweden approved this study (2013-347-31), and all participants gave their written informed consent when donating samples to the Medical Biobank and at the early arthritis clinic. For the validation cohort, the Swedish Ethical Review Authority approved the current NORD-STAR study (Dnr 2022-02326-02), with the inclusion of samples obtained from Sweden, Denmark, Norway and The Netherlands for the current study. All participants gave their written informed consent before entering the study.

Provenance and peer review

Not commissioned, externally peer-reviewed.

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Contributors

YH: Conceptualisation, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Validation, Visualisation, Writing – original draft; OS: Conceptualisation, Data curation, Funding acquisition, Methodology, Validation, Visualisation, Writing – original draft; LJ: Formal analysis, Visualisation, Writing – review & editing; MLA: Data curation, Formal analysis, Writing – review & editing; LC: Investigation, Methodology, Writing – review & editing; AL: Formal analysis, Writing – review & editing; EL: Methodology, Writing – review & editing; TU: Resources; MLH: Resources; BG: Resources; KH-P: Resources; MN: Resources; KL: Resources; JL: Resources; DN: Resources; AR: Resources; GG: Resources; MØ: Resources; IG: Funding acquisition, Writing – original draft; SR-D: Data curation, Formal analysis, Funding acquisition, Resources, Supervision, Writing – original draft; RH: Conceptualisation, Funding acquisition, Investigation, Resources, Supervision, Writing – original draft.

TU, MLH, BG, KH-P, MN, KL, JL, DN, AR, GG, and MØ (contribution in the original NORD-STAR study): Conceptualisation, Funding acquisition, Investigation, and Writing. All authors revised the work critically for important intellectual content, provided final approval of the version to be published, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

YH and OS contributed equally to this paper. RH is responsible for the overall content as guarantor.

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Patient consent for publication

All participants gave their written informed consent.

Patient and public involvement

Besides providing samples, patients or the public were not involved in this study.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ard.2025.04.013.

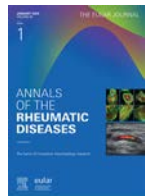
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Rheumatoid arthritis

Spatiotemporal distributions and regional disparities of rheumatoid arthritis in 953 global to local locations, 1980-2040, with deep learning-empowered forecasts and evaluation of interventional policies' benefits

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ABSTRACT

Objectives: To investigate global to local socioeconomic-driven distributions and inequalities in burdens of rheumatoid arthritis (RA) and to forecast long-term burdens.

Methods: We analysed the prevalence, incidence, mortality, disability-adjusted life years (DALYs), years of life lost (YLLs), and years lived with disability (YLDs) of RA across 953 locations worldwide, as well as their inequalities and ideal frontiers. A deep-learning pipeline was developed to forecast long-term burdens with scenario simulations.

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Results: In 2021, RA affected 17.9 million people globally, with a 13.2% increase in incidence rate from 1990–2021, trending younger and broader. The age-standardised death rate fell 32.7% from 1980 to 2021, but global DALYs nearly doubled from 1990 to 2021. In 2021, among 652 subnational regions, West Berkshire in the UK had the highest age-standardised incidence rate (35.1; 95% uncertainty interval [UI]: 30.8–39.8). Zacatecas in Mexico had the highest age-standardised DALY rate (112.6; 95% UI: 87.2–142.7). Regions with a high sociodemographic index (SDI) bore the heaviest burden, with regional inequalities aggravating from 1990 to 2021. Over 90% of areas lagged in RA frontiers of multiple indicators. Japan uniquely showed declining trends (1990–2021), exemplified by Tokyo's age-standardised DALY rate dropping by 22.4% since 1990, unlike that in other high SDI regions. Implementing smoking control policies is forecasted to reduce RA-related deaths by 16.8% and DALYs by 20.6% among male patients in high-smoking regions like China.

Conclusions: Demographic changes and uneven health infrastructure exacerbated RA burdens and disparities worldwide, with high SDI areas hardest hit while low SDI regions saw increases. Trend analysis empowered targeted policies such as localised smoking control to address these inequities.

WHAT IS ALREADY KNOWN ON THIS TOPIC

- RA disproportionately impacts women, aging populations, and high-income regions, with established links to smoking, aging, and comorbidities. Existing studies primarily focus on national trends, using traditional models like ARIMA, which struggle with nonlinear dynamics and subnational disparities.

WHAT THIS STUDY ADDS

- This study integrates the largest spatiotemporal RA dataset (953 global to subnational locations, 1980–2021) and introduces a deep learning framework (iTransformer) to forecast burdens until 2040. It uncovers subnational inequalities, quantifies intervention impacts and highlights paradoxical patterns like Japan's declining DALYs despite high SDI.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- The findings advocate for localized policies and resource allocation, particularly in low-middle SDI regions. The novel forecasting model advances methodologies for complex disease burden analysis, while Japan's success underscores the value of early diagnosis and biologic therapies, informing global clinical and public health strategies.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disorder primarily affecting joints, causing inflammation, pain, and joint damage [1]. Unlike osteoarthritis, RA involves systemic inflammation and is linked to comorbidities such as cardiovascular disease and interstitial lung disease, complicating management and increasing mortality risk [2,3]. Treatment focuses on disease-modifying antirheumatic drugs, including conventional, biologic, and targeted synthetic options [4]. Long-term drug use can cause side effects such as liver [5] and kidney [6] damage, adding to the RA disease burden. RA remains a leading cause of disability worldwide, with prevalence estimates ranging from 0.2% to 1% across populations [1]. Existing epidemiological studies have documented the disproportionate impact of RA on women [7], ageing populations [8], and high-income regions [9], yet critical gaps persist in understanding its spatiotemporal evolution, subnational disparities, and long-term trajectory under demographic and policy shifts.

Previous research has predominantly focused on national-level trends, overlooking granular subnational variations that drive inequities in healthcare access and outcomes. Moreover, conventional forecasting models, such as autoregressive integrated moving average (ARIMA) [10], often fail to capture complex nonlinear relationships and dynamic interactions between socioeconomic, demographic, and epidemiological factors. While the association of RA with smoking [11,12] and ageing [13] is well-established, few studies have quantified the potential benefits of targeted interventions such as tobacco control at regional levels or explored why economically similar regions exhibit divergent disease burdens.

This study addressed these gaps by integrating the largest spatiotemporal dataset on RA to date, spanning 953 global to local locations from 1980 to 2021, with a novel deep learning framework to forecast burdens until 2040. We employed an enhanced Inverted Transformer (iTransformer) model, uniquely tailored for multivariate time-series forecasting, to overcome the limitations of traditional linear methods and enable scenario simulations for policy evaluation. By analysing socioeconomic inequalities, frontiers, and decomposition of epidemiological factors, we reveal how demographic ageing, population growth, and uneven healthcare infrastructure exacerbate RA burdens differently across regions. Furthermore, we dissect paradoxical patterns, such as Japan's success in reducing disability-adjusted life years (DALYs) despite a high sociodemographic index (SDI), and quantify the potential impact of smoking control policies in high-risk areas such as China.

METHODS

Data sources and study design

The Global Burden of Disease (GBD) 2021 report analysed health losses from 371 diseases and injuries across 204 countries and territories, with a focus on RA across 20 age groups and 953 subnational locations. Data on incidence, prevalence, mortality, DALYs, years of life lost (YLLs), and years lived with disability (YLDs) were extracted from The Institute for Health Metrics and Evaluation Global Health Data Exchange, which was expressed as numbers, percentages, and rates per 100,000 population, stratified by age, sex, and location. The GBD 2021 study incorporated 56,604 data sources, including vital registration, verbal autopsy, surveys, censuses, surveillance systems, and cancer

registries, among others [14], which enabled the calculation of cause-specific fatal metrics (death and YLLs) across locations, sexes, and age groups from 1980 to 2021. In addition, 75,459 data sources including scientific literature, household surveys, epidemiological surveillance, illness registries, and clinical informatics, were extracted to establish nonfatal metrics (incidence, prevalence, DALYs, and YLDs) between 1990 and 2021. To address incomplete data and regional unreliability (notably in underresourced/conflict zones), we applied spatiotemporal smoothing methods like Gaussian process regression (GPR) and Bayesian hierarchical modelling (BHM) to leverage data from adjacent regions or periods, generating continuous estimates despite gaps. Mixed-effects models addressed biases from incomplete reporting, recall errors, and coding inconsistencies. Data completeness was rigorously evaluated using methods such as death distribution models, followed by bias correction techniques to address underenumeration. The accuracy of cause-of-death information was critically evaluated by comparing death certificate quality and verbal autopsy data, enabling systematic adjustments to account for misclassification. Overall, our integrated Bayesian hierarchical approach synthesised these multiple data streams, ensuring internally consistent and reliable estimates that can inform global burden assessments and guide health policy decisions despite inherent challenges in data quality and availability. This study follows Guidelines for Accurate and Transparent Health Estimates Reporting (GATHER) guidelines [15], with indicator definitions, bias correction methods, and calculation methods (including socioeconomic inequalities, frontiers, and decomposition) listed in Appendix 1 and supplementary materials shown in Appendix 2. Additional data details can also be found at <https://www.healthdata.org/gbd/methods-appendices-2021>. In the results, rates are presented per 100,000 individuals, with uncertainty intervals (UIs) derived from 1000 posterior distribution draws. Analyses used R (v4.2.0) and Python (v3.9.19). All data were anonymised and aggregated, eliminating the need for ethics approval or consent. Patient and public did not participate in the writing of the article.

Attentive deep learning-based forecasting and scenario simulations summary

We tailored forecasts for all-cause mortality, DALYs, YLDs, and YLLs using modified iTransformer [16], a state-of-the-art deep learning algorithm designed for multivariate time series. From 1990 to 2021, the model inputs included incidence rates for GBD level 3 causes, geographical SDI (gross domestic

product [GDP] was used in China), and population data. The step-by-step forecasting and scenario simulation settings, formula, and protocols are detailed in Appendix 1. We divided inputted data in half as training and test sets based on locations, age, and sex (Appendix 1). In the training set, these inputs were standardised to ensure a uniform influence on the model’s output. The model was well-configured for optimisation. Modelling and optimisation were independently conducted on the training set with 5-fold validation (10% of the training set was withheld as a validation set in each fold), while the test set was isolated for rigorous performance testing with multiple metrics. These metrics included mean absolute percentage error (MAPE) and mean squared percentage error (MSPE). To ensure an optimal forecast horizon, the iTransformer used an historical 13-year period (1990-2002) to fit and forecast the next 19 years (2003-2021), allowing us to extend accurate forecasts to 2040. Subsequently, long-term forecasting under the current trend as a reference scenario was conducted 5 times to measure uncertainty, which is also known as sensitivity analysis.

Unlike traditional models like ARIMA (which rely on linearity and stationarity assumptions), the iTransformer leverages its unique architecture, including a self-attention mechanism, to dynamically prioritise time-series data points based on their predictive relevance. This capability allows it to capture complex nonlinear relationships and long-range dependencies that are often missed by models assuming observation independence. In addition, the iTransformer processes entire data sequences simultaneously, rather than incrementally as ARIMA does, which enables it to model future volatility more effectively by recognising underlying patterns such as trend shifts, seasonal fluctuations, or sudden shocks. Its ability to learn collectively from all data points means that the model can integrate and adjust to changes in the dynamic trends of disease burden, ensuring more adaptive and accurate forecasts. Furthermore, the iTransformer architecture is highly scalable and efficient, supporting the integration of high-dimensional inputs, such as multiple risk factors, without the need for extensive manual tuning. This end-to-end learning approach not only streamlines the forecasting process but also mitigates issues related to data noise and missing values, which are common challenges in real-world datasets. In summary, by overcoming the limitations inherent in traditional time-series models such as ARIMA [10,17,18], which relies on linearity and stationarity, the iTransformer offers a robust alternative that is better suited for capturing the complex, evolving nature of RA disease burden.

Table 1
Global numbers and rates of rheumatoid arthritis incidence in different age groups

Age	Incidence number 1990	Incidence number 2019	Incidence number 2021	Incidence number 1990-2019	Incidence number 1990-2021	Incidence number 2019-2021
<20	41,652 (27,793-58,947)	56,734 (38,335-79,929)	57,851 (39,170-81,750)	36.2% (37.9-35.6)	38.9% (36-43)	2% (0.7-3.2)
20-54	280,445 (230,796-335,293)	500,658 (412,839-595,981)	508,186 (417,808-605,687)	78.5% (78.9-77.7)	81.2% (74.4-89)	1.5% (0.8-2.2)
55+	166,172 (130,096-207,067)	406,214 (325,296-497,393)	434,283 (347,463-531,756)	144.5% (150-140.2)	161.3% (151.7-170.8)	6.9% (6-7.9)
All ages	488,269 (435,015-545,895)	963,606 (868,989-1,073,306)	1,000,319 (902,687-1,114,213)	97.4% (99.8-96.6)	104.9% (98.2-111.4)	3.8% (3.2-4.3)
Age	Incidence rate 1990	Incidence rate 2019	Incidence rate 2021	Incidence rate 1990-2019	Incidence rate 1990-2021	Incidence rate 2019-2021
<20	1.8 (1.2-2.6)	2.2 (1.5-3.1)	2.2 (1.5-3.1)	17.5% (19-17)	19% (16.5-22.5)	1.3% (0-2.5)
20-54	11.7 (9.6-13.9)	13.5 (11.1-16)	13.5 (11.1-16.1)	15.3% (15.5-14.8)	15.5% (11.2-20.5)	0.2% (-0.5-0.9)
55+	24.7 (19.4-30.8)	28.9 (23.1-35.4)	29.2 (23.4-35.8)	16.7% (19.4-14.7)	18.1% (13.7-22.4)	1.2% (0.3-2.1)
All ages	9.2 (8.2-10.2)	12.4 (11.2-13.9)	12.7 (11.4-14.1)	35.9% (37.6-35.4)	38.5% (34-42.9)	1.9% (1.3-2.4)
Age-standardised	10.4 (9.3-11.6)	11.8 (10.6-13.1)	11.8 (10.6-13.1)	12.7% (13.9-12.1)	13.2% (11.8-14.7)	0.4% (0-0.8)

Changes over time (1990-2019, 1990-2021, and 2019-2021) shown as percentage (95% uncertainty interval).

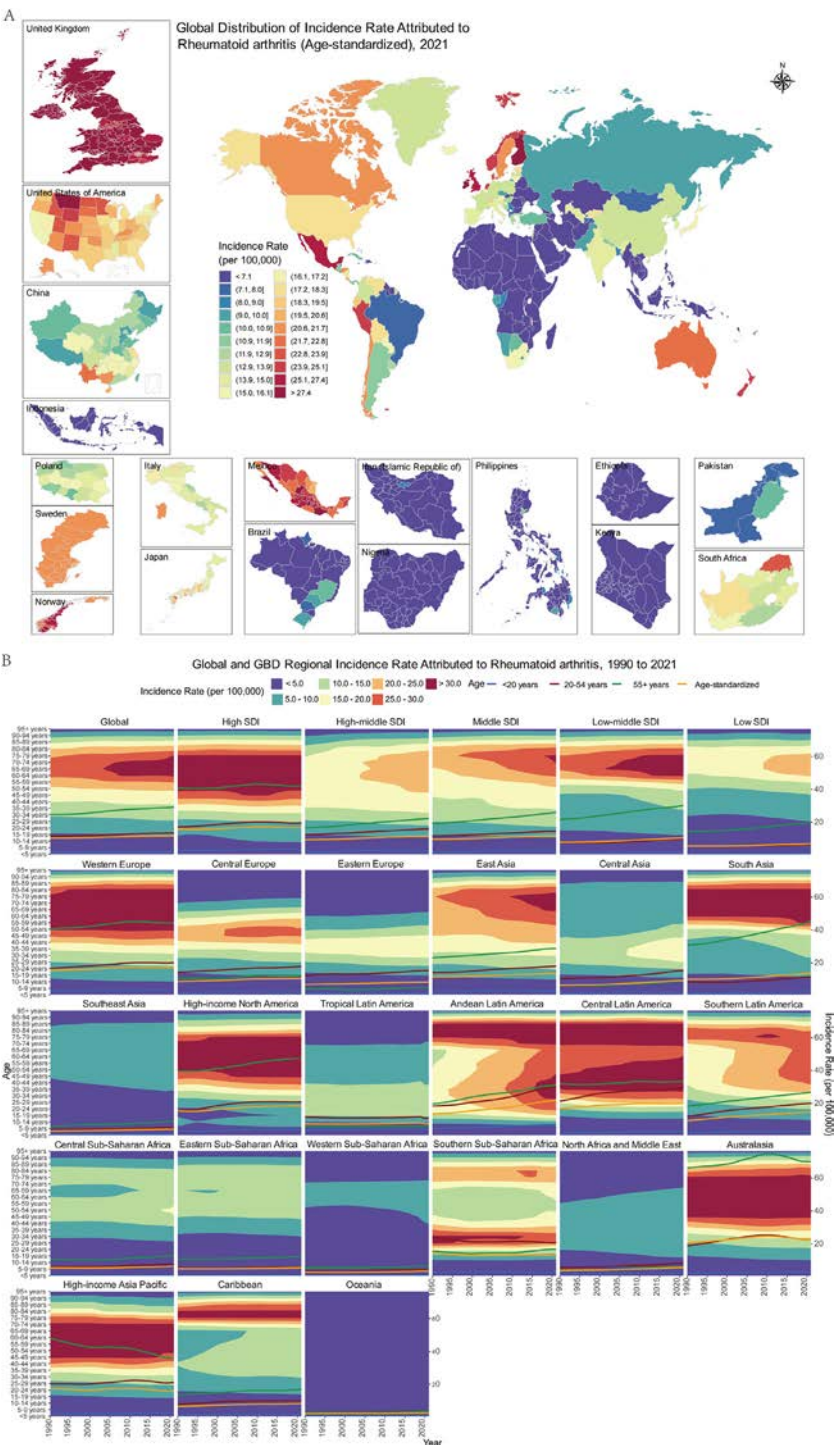


Figure 1. (A) Geographic distribution of age-standardised incidence rates in 2021. (B) The age-specific temporal patterns of age-standardised incidence rate in global and regional RA, ALL, 1990-2021. GBD, Global Burden of Disease; RA, rheumatoid arthritis; SDI, socio-demographic index.

RESULTS

Global, regional, and subnational trends of incidence and prevalence in 953 locations

The age-standardised incidence rate in 2021 was 11.8 (95% UI: 10.6-13.1), marking a 13.2% (95% UI: 11.8-14.7) increase from 1990 to 2021 (Table 1). Higher incidence rates were seen in Western Europe and North America and lower rates in Africa (Fig 1A). Among 204 countries and territories, Ireland had the highest age-standardised incidence rate at 35.1 (95% UI: 31.8-38.8) in 2021 with an increase of 22.5% (95% UI: 15.2-29.9%) compared with 1990. Oman displayed the fastest growth in age-standardised incidence rate in the past 31 years, with an

increase of 78.6% (95% UI: 68.2-89.9%). Among 652 local administrative units, West Berkshire in the UK displayed the highest age-standardised incidence rate at 35.1 (95% UI: 30.8-39.8) in 2021, with the female age-standardised incidence rate reaching 49.7 (95% UI: 44.9-55.2). In 2021, there were 17,924,667 (95% UI: 15,973,178-20,303,303) prevalent cases of RA globally. The age-standardised prevalence rate increased by 14.4% (13.0-15.8%) from 1990 to 2021 (Appendix 2).

RA incidence was highest among those over 55 globally. However, after 2015, the age of onset of RA showed a trend towards younger age groups, with an increasing proportion of patients aged 20 to 54, especially in high SDI areas (Fig 1B). Regional variations existed, such as a bimodal pattern in Latin America (Appendix 2). In Latin America, the onset age of RA

Table 2
Global numbers and rates of RA DALYs in different age groups

Age	DALY number 1990	DALY number 2019	DALY number 2021	DALY number 1990-2019	DALY number 1990-2021	DALY number 2019-2021
<20	41,937 (29,682-58,089)	42,897 (27,097-63,316)	43,219 (26,942-65,407)	2.3% (−8.7 to 9)	3.1% (−11.7 to 13.6)	0.8% (−2.3 to 3.3)
20-54	634,013 (443,026-889,144)	1,144,563 (780,503-1,648,397)	1,157,872 (785,078-1,661,873)	80.5% (76.2-85.4)	82.6% (74.1-89.3)	1.2% (−0.3 to 2.6)
55 +	869,749 (712,817-1,064,419)	1,779,368 (1,418,619-2,232,238)	1,874,211 (1,474,492-2,343,724)	104.6% (99-109.7)	115.5% (103.7-124.2)	5.3% (2.9-7.8)
All ages	1,545,699 (1,201,479-1,977,786)	2,966,828 (2,235,445-3,856,285)	3,075,303 (2,310,381-3,974,046)	91.9% (86.1-95)	99% (89.5-106.1)	3.7% (1.8-5.5)
Age	DALY rate 1990	DALY rate 2019	DALY rate 2021	DALY rate 1990-2019	DALY rate 1990-2021	DALY rate 2019-2021
<20	1.9 (1.3-2.6)	1.6 (1-2.4)	1.6 (1-2.5)	−11.7% (−21.2 to −6)	−11.7% (−24.3 to −2.6)	0.1% (−2.9 to 2.6)
20-54	26.4 (18.4-37)	30.8 (21-44.3)	30.7 (20.8-44.1)	16.6% (13.8-19.7)	16.5% (11-20.7)	−0.1% (−1.6 to 1.3)
55 +	129.5 (106.2-158.5)	126.6 (100.9-158.8)	126.1 (99.2-157.7)	−2.3% (−5 to 0.1)	−2.6% (−8 to 1.3)	−0.3% (−2.6 to 2)
All ages	29 (22.5-37.1)	38.3 (28.9-49.8)	39 (29.3-50.4)	32.2% (28.1-34.3)	34.5% (28.1-39.3)	1.7% (−0.1 to 3.5)
Age-standardised	36.4 (28.7-46)	36.1 (27.2-46.8)	35.9 (27-46.5)	−1% (−5.3 to 1.8)	−1.4% (−6.6 to 2.4)	−0.4% (−2.2 to 1.3)

Changes over time (1990-2019, 1990-2021, and 2019-2021) shown as percentage (95% uncertainty interval). DALY, disability-adjusted life year; RA, rheumatoid arthritis.

presents a bimodal pattern, with ages above 70 and between 30 and 60 years old (Fig 1B, Appendix 2). For example, in most sub-regions of Mexico, especially Chiapas, Hidalgo, and Mexico City, the age distribution of female RA patients showed a bimodal distribution (Appendix 2).

Global, regional, and subnational trends of mortality and disabilities in 953 locations

In 2021, RA caused 37,330 deaths globally (95% UI: 31,060-43,136) (Table 2). The age-standardised death rate in 2021 decreased by 32.7% (95% UI: 44.9 to 17.8%) from 1980. DALYs for RA nearly doubled over the past 31 years. The 55+ age group had the highest DALY rate, over 4 times that of other groups. Nationally in 2021, Mexico recorded the highest age-standardised DALY rate at 87.4 (95% UI: 68.5-110.5) (Fig. 2A). Among 652 local administrative units, Zacatecas in Mexico had the highest age-standardised DALY rate at 112.6 (95% UI: 87.2-142.7), marking a 6.5% increase (95% UI: −3.1 to 17.1%) from 1990 (Fig. 2B, Appendix 2). Compared with 1990, the global age-standardised YLL rate dropped by 31.7% (95% UI: 38.2 to 25.3%) in 2021, while the global age-standardised YLDs increased by 14.1% (95% UI: 12.4-15.7%) in 2021. Mexico and Honduras had higher YLLs, while Ireland and Peru reported increased YLDs (Appendix 2).

Disparities and SDI-linked inequalities in RA burden

Over the past 31 years, most regions experienced an upwards trend in RA incidence (Fig 3A; Appendix 2). COVID-19 impacted RA burdens, with Central Latin America showing an unusual decline in 2020 compared to 2019 (Appendix 2). RA incidence in women remained higher across most age groups (Fig 3B; Appendix 2). However, in 2021, males aged >75 years in regions such as East Asia, Central Asia, South Asia, and Sub-Saharan Africa had higher incidence rates (Fig 3B; Appendix 2) compared with 2019. DALY rates for RA fluctuated globally (Fig 3C; Appendix 2), with females generally bearing a higher burden. Exceptions included young and elderly males in South-east Asia (Fig 3D; Appendix 2).

Globally, significant absolute and relative SDI-related inequalities were observed, with a disproportionately higher burden

shouldered by countries with high and high-middle SDI (Fig 3E, F) [19]. The slope index of inequality (SII) related to the global incidence rate in 2021 increased by 60.95% compared with the global SII in 1990. The SII of DALY rates increased by 62.55% from 1990 to 2021. In 2021, high SDI regions showed a sharp SII of 12.87 (95% UI: 2.96-22.77) in incidence rate, with Finland, Ireland, and New Zealand as the 3 most unequal countries. The DALY rate SII in high SDI regions was 22.60 (95% UI: −5.26 to 50.46) (Fig 3E, F). Differing from the absolute inequalities, relative SDI-related inequality results indicated that high-middle SDI regions have higher regional inequality (Appendix 2).

Frontier analysis of RA also reveals a positive relationship between the SDI and disease burden (Fig 3G, H; Appendix 2). As SDI increased, the effective difference (EF) and variance also increased, which indicated the burden of RA disease was seriously neglected before. Once SDI exceeded 0.5, the frontier trend of RA changes significantly. From 1990 to 2021, most countries and regions moved farther from the ideal disease burden line for both incidence and DALY rates. In 2021, only 1 country reached the frontier level for incidence and 19 countries did so for DALY rate (Fig 3G, H). Ireland, Finland, and the UK had higher EFs for incidence rate (range: 25-35, Figure 3G), while Mexico, Honduras, and Ireland had higher EFs in DALYs (range: 75-85, Fig 3H).

Countries with high SDI: a comparison between Japan and the UK

Of the 18 countries with subregional data, the UK had the highest incidence in 2021. West Berkshire in the UK was the most severe of 652 local administrative units (Appendix 2). Furthermore, the UK had the third highest incidence of EF in frontiers analysis in 2021 (Fig 3). Although RA burden was more severe in high SDI countries, we found that even in the same SDI region, cross-country inequalities existed, based on the inequality analysis (Fig 3). For example, Japan and the UK are both island nations with constitutional monarchies, renowned for blending rich traditions with modern innovation, and they both face shared challenges such as ageing populations and balancing historical heritage with contemporary progress. However, Japan and the UK exhibited contrasting patterns in RA disease burden, making them an ideal comparative pair (Fig 4). Incidence rates

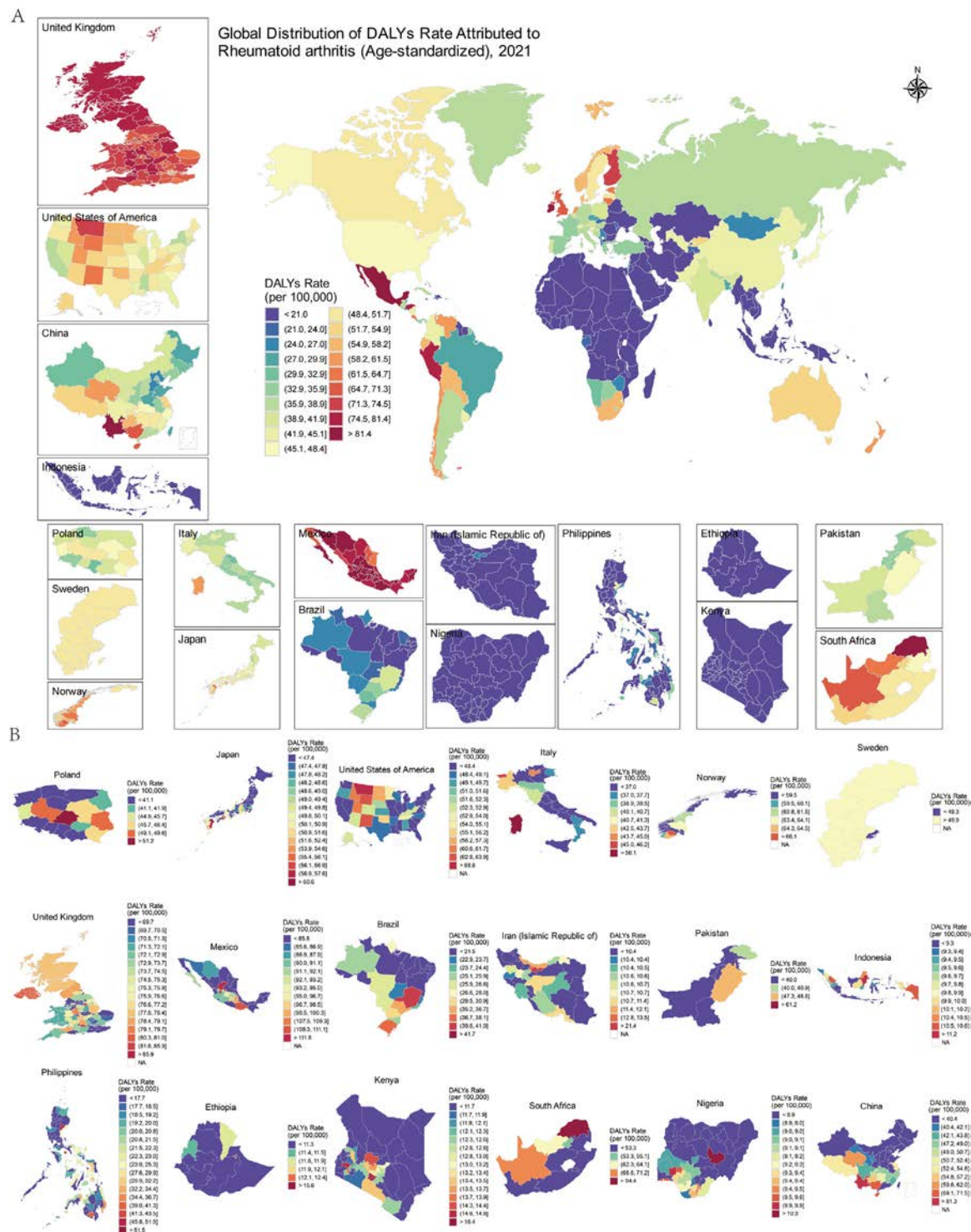


Figure 2. Geographic distribution of age-standardised DALY rates in 2021 at national (A) and subnational levels (B). DALY, disability-adjusted life year.

in the UK increased in most subregions since 2016 (Fig 4C, D), and DALY rates were higher in 2021 than in 1990 (Fig 4H). Of 153 UK subregions, 151 experienced increased EF, with West Berkshire maintaining relatively high DALYs over 31 years (Fig 4G). The UK area, particularly northern counties, showed a high disease burden. Middlesbrough had a high age-standardised DALY rate of 89.4 (95% UI: 67.8-116.7). Women in Middlesbrough had an age-standardised DALYs rate of 122.1 (95% UI: 91.9-159.8). It is worth noting that the DALY rates in all subnational regions of the UK were higher than the world average in 2021. The age-standardised DALY rate of Kensington and

Chelsea was 52.8 (95% UI: 37.5-73.6) in 2021, which was the lowest level within the UK (Appendix 2).

In Japan, age-standardised incidence rates decreased in most subregions from 2010 to 2019 (Fig 4A, B), with 7 subregions reaching or surpassing the frontier in 2021 (Fig 4A). The age-standardised incidence rate in Japan was 25.1 (95% UI: 21.6-29.0) in 2021. Postpandemic, >90% of countries saw increased RA burden, although Japan's DALY rates declined consistently (Fig 3; Appendix 2). The age-standardised DALY rate of Tokyo was 42.3 (95% UI: 30.5-56.3) in 2021, which dropped 22.4% (95% UI: 30.3 to 15.0%) from

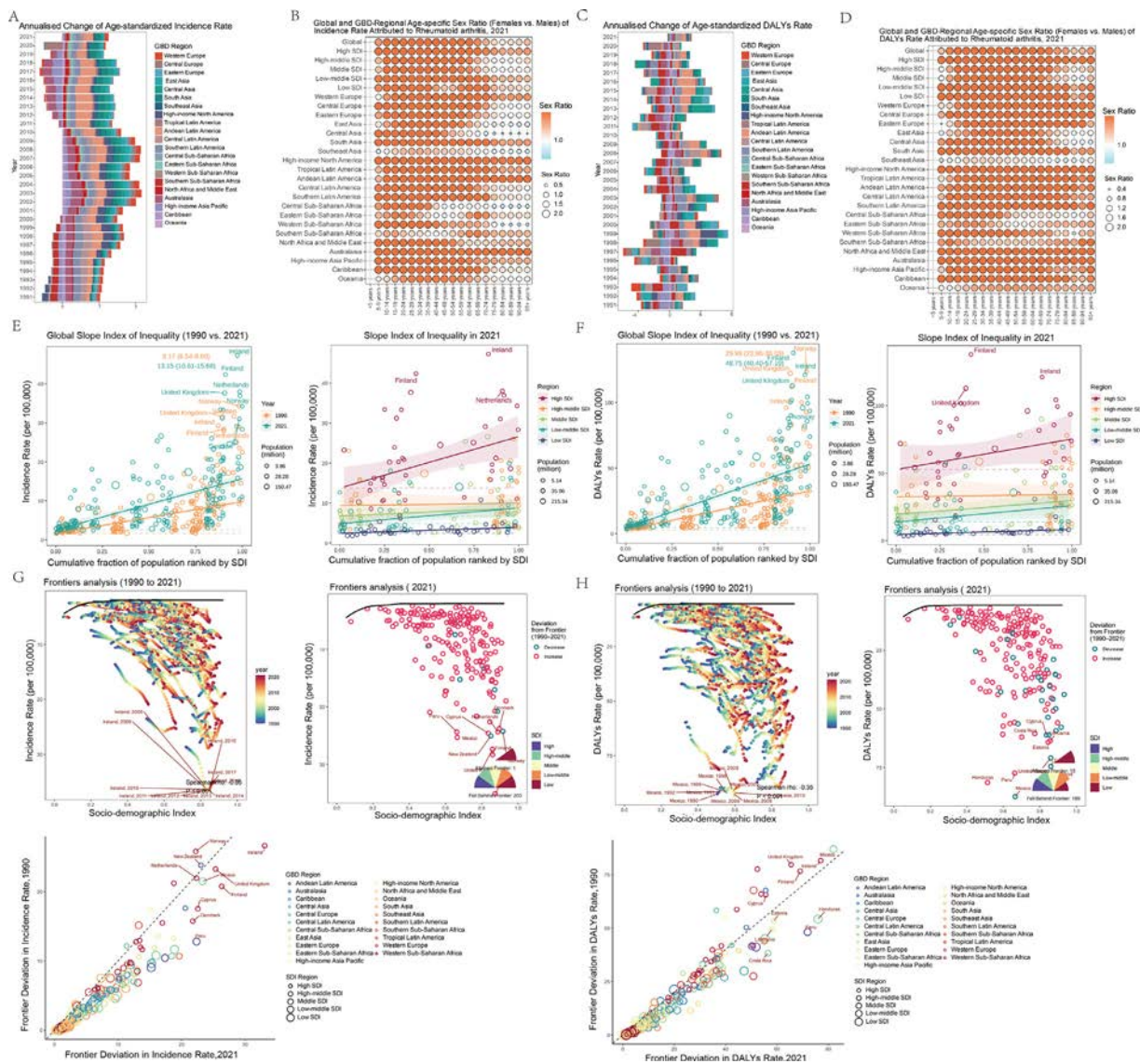


Figure 3. (A) A stacked bar plot illustrating annual changes RA incidence rates from 1990 to 2021. The total height of each bar represents the global change in RA incidence, while differently coloured segments indicate how this change is distributed among the 21 GBD regions. (B) Global and regional sex differences in RA incidence rates, defined by the ratio of female-to-male incidence. Ratios >1 indicate a female-prone pattern, and ratios <1 indicate a male-prone pattern. (C) A stacked bar plot showing annual changes in RA DALY rates from 1990 to 2021. (D) Global and regional sex differences in RA DALY rates, defined similarly by the female-to-male ratio. (E) The absolute cross-country inequality in RA incidence rates at global (left panel) and regional (right panel) levels, measured by the slope index of inequality (SII). Negative SII values imply that socioeconomically disadvantaged populations have higher burdens, whereas positive SII values imply the opposite. The left panel covers global changes from 1990 to 2021, while the right panel focuses on inequalities among 5 sociodemographic index (SDI) regions in 2021. (F) The absolute cross-country inequality in RA DALY rates at global (left panel) and regional (right panel) levels, also measured by SII. (G) Frontier analyses for RA incidence rates. The 'frontier' is the minimal incidence rate theoretically achievable at any SDI level and is shown as a solid black line in the top left and top right panels. The top left panel plots RA incidence rates for 204 countries (coloured by year) to illustrate how each country's rates with its frontier over time. The top right panel displays 204 countries' incidence rates in 2021, coloured by whether the country's gap from the frontier has increased (red) or decreased (green) since 1990. The bottom left panel compares frontier gaps for 1990 and 2021. (H) Frontier analyses for RA DALY rates, following the same approach as in (G). DALY, disability-adjusted life year; GBD, Global Burden of Disease; RA, rheumatoid arthritis.

1990. DALYs rates declined compared to 1990 (Fig 4F), with sustained EF reductions in most subregions (Fig 4E). Only 5 of 47 subregions in Japan had higher age-standardised DALY rates than Kensington and Chelsea (the lowest in the UK) in 2021. Akita displayed the lowest age-standardised DALY rate of 37.5 (95% UI: 27.4–51.5) within Japan in 2021, which was far below the average level of high SDI regions (Appendix 2). These findings indicate that economic factors alone are not the sole determinants of RA disease burden, as significant variations existed across different regions, even within the same SDI range or the same country.

To better analyse the differences between the 2 countries, we selected 2 typical cities for analysis. Greater London and Tokyo account for approximately 22% and 20% of each country's GDP, respectively, with a population of 13% (London) and 10% (Tokyo). Both cities are centred around finance, technology, and cultural and creative industries, attracting global talent. At the same time, both cities have similar population structures compared to other regions in the country, with a higher influx of young people and a relatively low degree of ageing. During the past 31 years, the incidence rate of RA in Tokyo continually decreased, especially among elderly women. The incidence rate

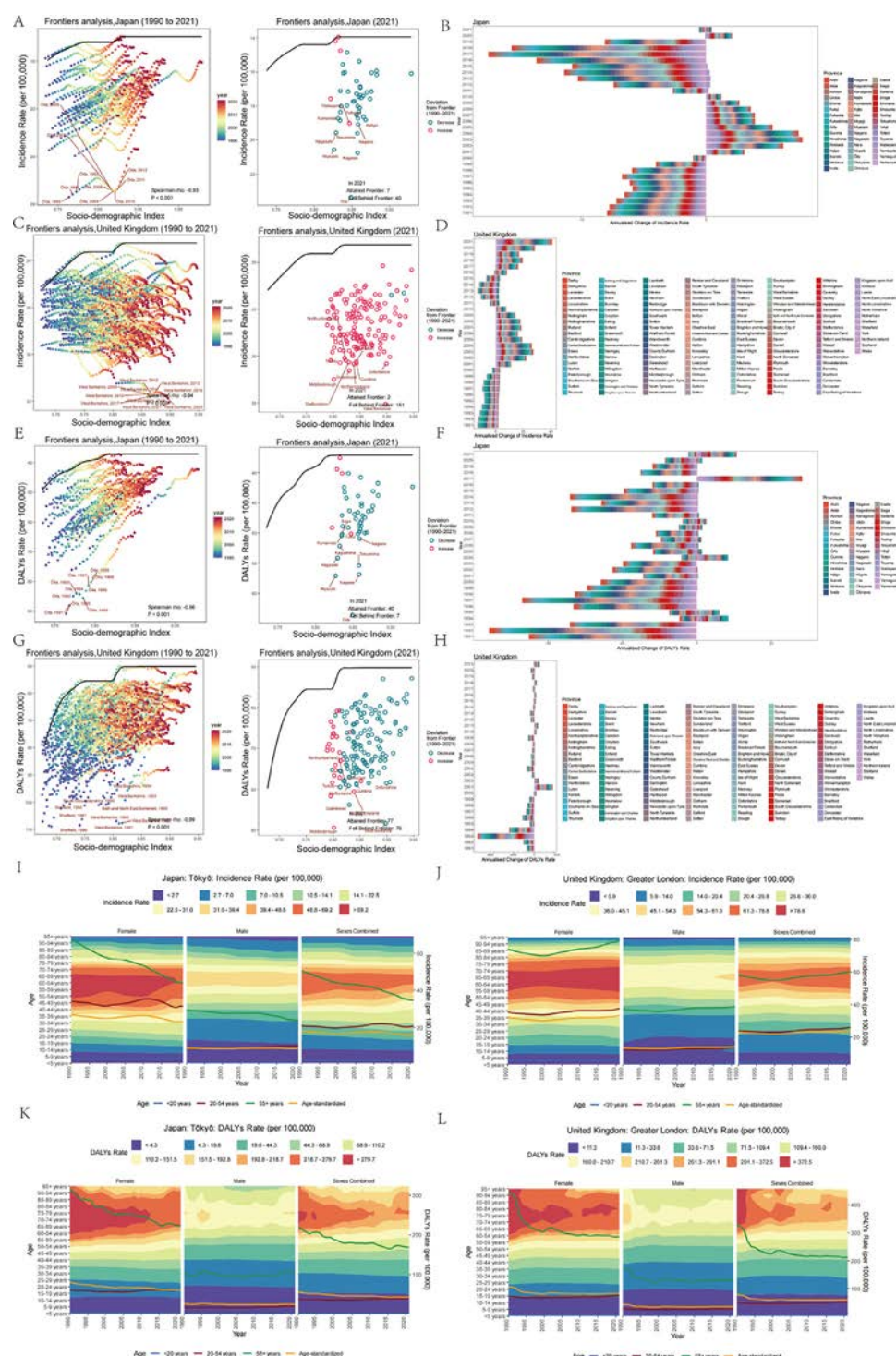


Figure 4. (A) Frontier analysis of age-standardised RA incidence rates at subnational levels in Japan from 1990 to 2021. The solid black line represents the minimal achievable incidence rate (“frontier”) for each level of socioeconomic status, serving as a benchmark against which observed rates are compared. (B) Annual changes in age-standardised RA incidence rates in Japan at subnational levels from 1990 to 2021. This panel highlights the year-to-year variations in incidence, allowing for the identification of trends and shifts over time. (C) Frontier analysis of age-standardised RA incidence rates at subnational levels in the United Kingdom from 1990 to 2021. Similar to panel (A), the frontier line delineates the theoretical minimum incidence rate achievable at each socioeconomic level, facilitating direct comparisons with observed data. (D) Annual changes in age-standardised RA incidence rates in the United Kingdom at subnational levels from 1990 to 2021. This graph depicts the temporal dynamics of RA incidence, underscoring both gradual and abrupt changes across the study period. (E) Frontier analysis of age-standardised RA DALY rates at subnational levels in Japan from 1990 to 2021. Here, the frontier line illustrates the lowest attainable DALY rate, which serves as a reference for evaluating the observed burden of disease. (F) Annual changes in age-standardised RA DALY rates in Japan at subnational levels from 1990 to 2021. This panel captures the yearly fluctuations in DALY rates, providing insights into how the overall disease burden has evolved over time. (G) Frontier analysis of age-standardised RA DALY rates at subnational levels in the United Kingdom from 1990 to 2021. As in panel (E), the black frontier line represents the minimum achievable DALY rate based on socioeconomic conditions, offering a clear standard for comparison. (H) Annual changes in age-standardised RA DALY rates in the United Kingdom at subnational levels from 1990 to 2021. This panel illustrates the annual variations in DALY rates, emphasizing temporal trends in the burden of RA across different regions. (I) Contour plot (left y-axis) showing age-specific RA incidence rates (per 100,000) in Tokyo, Japan from 1990 to 2021, where age is subdivided into 5-year intervals. The x-axis represents calendar years, and the colour scale indicates the magnitude of the incidence rate for each age-year combination. Overlaid on the same graph, the right y-axis displays line plots for 4 aggregated age

of RA in Greater London continued to increase. Although DALYs in both cities are decreasing, the age-standardised DALY rate in Greater London was 60.6 (44.0–81.2) in 2021, which was higher than 1 of 47 regions in Japan (Fig 4I–L; Appendix 2).

In addition to focusing on the differences in RA disease burden between the 2 countries, we also found that there is inequality even within the same country. The SII of the incidence rate and DALY rate in the UK was greater in 2021 than in 1990. The RA disease burden in subnational regions with relatively low SDI was more unequal (Appendix 2). Salford had an age-standardised DALY rate of 73.9 (95% UI: 55.1–97.3) in 2021. Despite being in a lower SDI level, Torbay exhibited a higher age-standardised DALYs rate of 79.4 (95% UI: 59.0–105.3) in 2021 (Appendix 2).

Drivers of RA epidemiology: population growth, ageing, and epidemiologic changes

Globally, population growth, ageing, and epidemiologic changes all contributed to increased RA incidence and prevalence (Fig 5A). Population growth and ageing significantly influenced the increase in RA-related deaths, DALYs, YLLs, and YLDs, with similar trends across SDI regions. In East and South Asia, these factors nearly accounted for the entire RA disease burden (Fig 5A; Appendix 2). At the national level, ageing had the greatest impact on DALYs in Thailand, China, Poland, Spain, and Mexico, while population growth influenced DALYs the most in Pakistan, India, Spain, and Australia (Fig 5B; Appendix 2). In the UK, both factors contributed to increasing DALYs, whereas in Japan, ageing was the primary driver (Fig 5B). Subregional analyses in these countries supported these findings (Fig 5C, D; Appendix 2). Notably, epidemiological changes helped reduce mortality rates and YLLs over the past 31 years, with Poland, Japan, and China showing the most significant declines in deaths and YLLs due to these changes (Appendix 2).

Forecasting future RA disease burden using the iTransformer model

Using an advanced deep-learning model, we forecasted RA disease burdens globally, nationally, and subregionally (Fig 6A; Appendix 2). Age-standardised incidence rates are forecasted to rise worldwide, while age-standardised DALYs rates are expected to decline, especially in high SDI regions. Nationally, the incidence rates of all age groups in high SDI countries like the UK, the USA, Ireland, and Finland will maintain an upwards trend until 2040. DALYs future trends will vary by region globally. In high-medium to low SDI regions, the predicted age-standardised DALY rates will increase until 2040 (Fig 6A). The predicted age-standardised DALYs rates in 80.4% (164/204) national regions are expected to increase until 2040 (Fig 6A; Appendix 2). Our model predicts that implementing smoking control policies could substantially reduce the RA burden, particularly in medium to high SDI regions (Fig 6B; Appendix 2). For example, in China, smoking control could reduce age-

standardised death rates by 16.8% and DALY rates by 20.6% among male RA patients (Appendix 2). Figure 6C displays the distribution of errors for each of the 5 prediction iterations—specifically, the MAPE and MSPE, which showed the favourable accuracy of our models.

DISCUSSION

Our study is the most comprehensive on RA to date, featuring the largest geographical coverage (953 global to local levels), observing for the longest time (1980–2021), and utilising extensive analyses (inequality, frontier, decomposition, and a tailored deep learning interventional forecasting algorithm). It provides robust evidence for global to local policymakers and stakeholders to formulate interventions and allocate resources effectively, mitigating RA impacts for their populations. We report 5 key findings:

1. **Global RA burden increased:** From 1980 to 2021, the global RA burden kept increasing and trended younger and broader, with UK's West Berkshire (incidence rate: 35.1/100,000) and Mexico's Zacatecas (DALY rate: 112.6/100,000) bearing the highest burdens in 2021 among 652 subnational regions.
2. **Widening inequalities:** DALY-related inequality surged 62.55% from 1990, with Finland, Ireland, and New Zealand as the most unequal countries in 2021.
3. **Failure to meet frontiers:** Only 1 country achieved the ideal frontier of incidence rates, and 19 reached DALY frontiers in 2021. As SDI increases over time, frontier deviations worsen. The burden of RA disease is severely neglected.
4. **Noneconomic disparities persisted:** Economic factors alone are not the sole determinants of RA disease burden (eg, UK vs Japan).
5. **Forecasted increases and need for positive policy:** By 2040, low-middle SDI regions may see increasing DALYs due to ageing/population growth, while DALYs in high SDI areas may decrease. Controlling smoking may reduce RA deaths by 16.8% and DALYs by 20.6% in high-smoking regions (eg, China), offering significant benefits for medium/high SDI areas.

With over 17.9 million cases worldwide and an upwards trend since 1990, RA is a growing public health concern. Higher incidence rates in Western Europe and North America may stem from better diagnostic capabilities and healthcare access [20]. The global increase in cases among individuals aged >55 aligns with the association of RA with ageing immune systems and accumulation of chronic inflammation [21]. The mortality and disability burden of RA has evolved over the past 3 decades. While age-standardised YLLs decreased, indicating improved RA management, YLDs increased by 14.1%, underscoring the chronic nature of RA and its persistent disability burden [22,23]. Sex disparities are evident, with women consistently experiencing higher RA incidence and DALYs, though regions such as Southeast Asia show higher DALYs in males, suggesting cultural and occupational influences [24]. Most countries and

groups (<20, 20 to 54, 55+, and the total age-standardised population), illustrating their annual incidence trends. (J) Using the same format as (I), this panel depicts RA incidence rates in Greater London, United Kingdom. The contour plot (left y-axis) presents age-specific rates in 5-year increments over the 1990–2021 period, while the right y-axis plots annual incidence trends for the 4 broad age categories. (K) Contour plot (left y-axis) of age-specific RA DALY rates (per 100,000) in Tokyo, Japan from 1990 to 2021, with age subdivided into 5-year intervals. The x-axis denotes calendar years, and the colour gradient reflects DALY rates. The line plots on the right y-axis show the annual DALY trends for the same 4 aggregated age groups, providing a broader age category perspective. (L) Following the same structure as (K), this panel presents RA DALY rates in Greater London, United Kingdom from 1990 to 2021. The contour plot (left y-axis) covers the finer 5-year age groupings, while the right y-axis captures the annual DALY rates for <20, 20 to 54, 55+, and the overall age-standardised population. DALY, disability-adjusted life year; RA, rheumatoid arthritis.

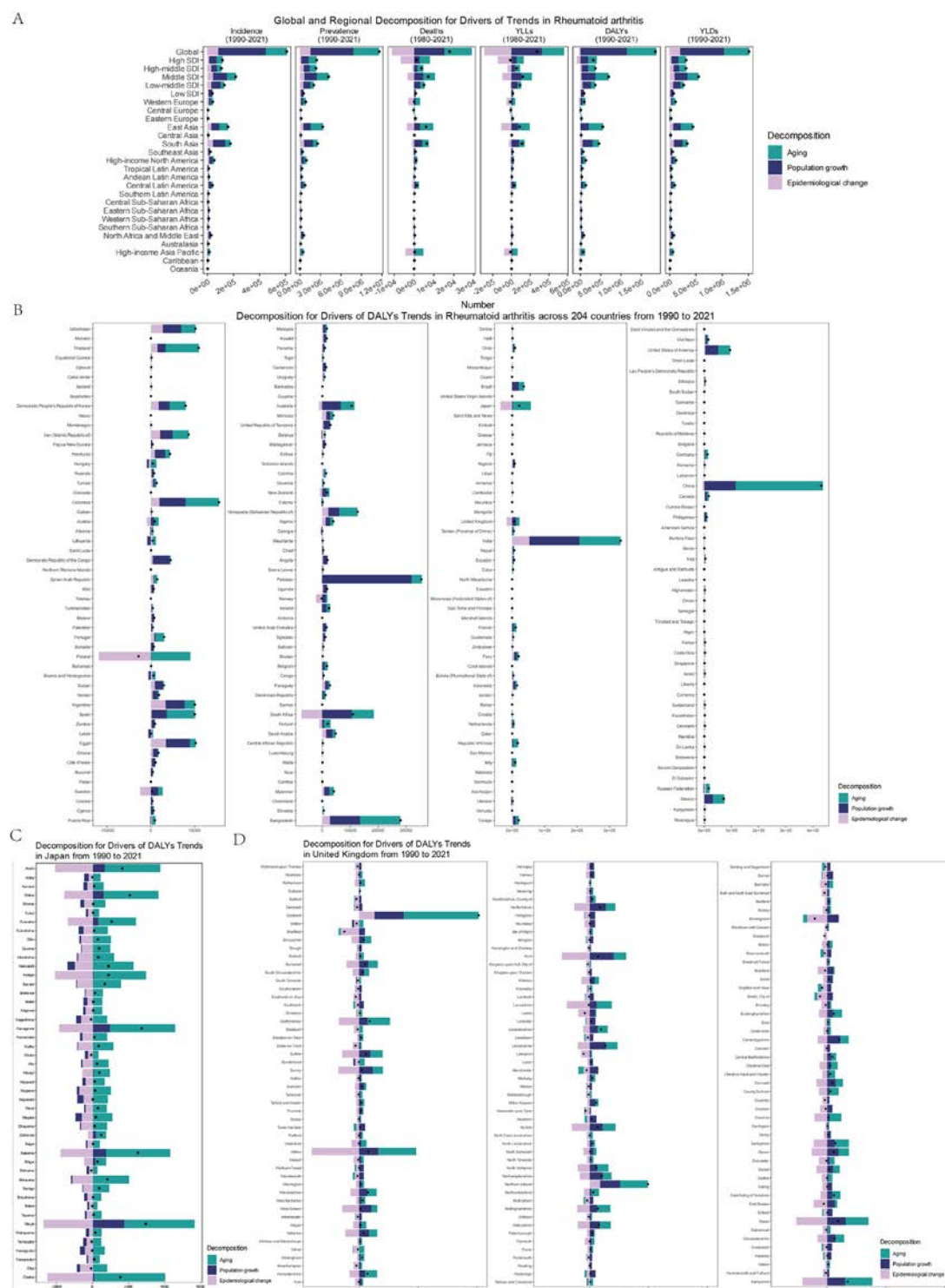


Figure 5. (A) Decomposition analysis of changes in global and regional RA burdens, including 6 estimates: incidence, prevalence, deaths, DALYs, YLLs, and YLDs. This stacked bar plot illustrates how the overall changes in these estimates are driven by 3 factors: population growth, ageing, and epidemiological changes. The black dot indicates the actual overall changes of the estimates after summing the contributions of these 3 driving factors. (B-D) Changes in RA DALYs according to population-level determinants of population growth, ageing, and epidemiological change from 1990 to 2021 at the national and subnational levels. Changes in RA according to population-level determinants of population growth, ageing, and epidemiological change from 1990 to 2021 at the global level and by SDI quintile. (B-D) Changes in RA according to population-level determinants of population growth, ageing, and epidemiological change from 1990 to 2021 at the national and subnational levels. DALY, disability-adjusted life year; RA, rheumatoid arthritis; SDI, sociodemographic index; YLL, year of life lost; YLD, year lived with disability.

regions had not reached the ideal level of RA burden in the frontiers analysis of 2021 RA disease burden. The global disease burden of RA is far underestimated. Moreover, the temporary impact of COVID-19 on RA incidence further highlights the pandemic's complex effects on chronic disease management [25,26].

Regional inequalities in RA burden are stark, with high SDI regions showing higher disease rates due to improved diagnostics and socioeconomic factors [23]. Obesity [20], active work physical activity [27], and some nutritional profiles [27,28] (increased high fat intake and reduced fruit/vegetable consumption) were reported to increase RA disease burden. European

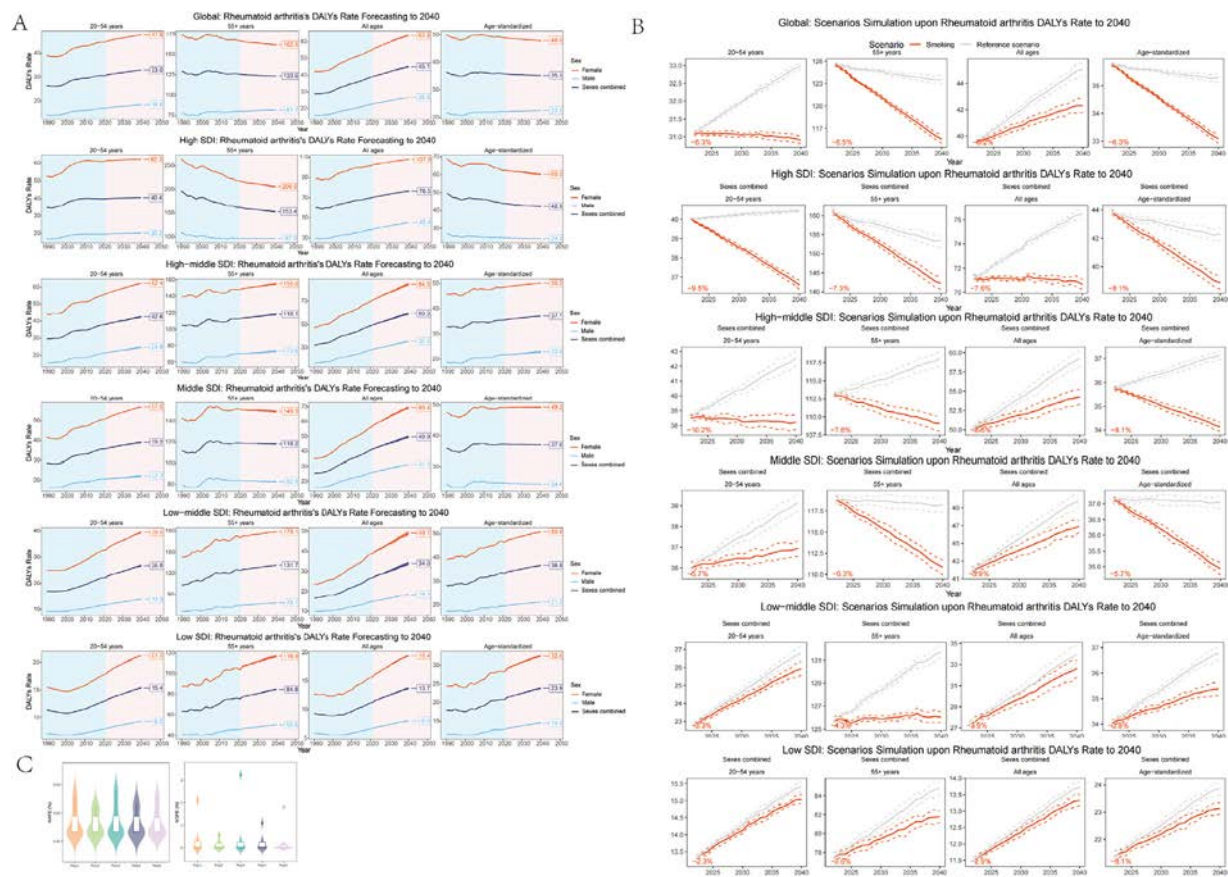


Figure 6. (A) The state-of-the-art (SOTA) attentive deep learning model predicts the temporal trend of DALYs rates of RA in individuals over 19 years old at global and different SDI-regional levels, 2022-2040. (B) SOTA attentive deep learning model predicts the temporal trend with smoking control on DALYs rates of RA beyond 19 years old at global and different SDI-regional levels, 2022-2040. (C) The two violin plots represent the performance test results. These plots display the distribution of errors for each of the five prediction iterations—specifically, the mean absolute percentage error (MAPE) and mean squared percentage error (MSPE). This approach enables a clear and intuitive comparison between the predicted values for 2003-2021 and the actual observed values over the same period. DALY, disability-adjusted life year; SDI, sociodemographic index.

countries such as Finland, Ireland, and the UK exhibit higher RA prevalence, linked to genetic markers (eg, *HLA-DRB1* alleles) [29], vitamin D deficiency [30], and advanced healthcare systems.

The overall positive correlation between SDI and RA burden aligns with prior studies [23,28], while the striking subnational inequalities (eg, high SDI regions such as the UK and Japan) highlight limitations in relying solely on economic metrics to predict disease patterns. For example, Japan’s declining DALY rates despite high SDI may reflect nationwide early diagnosis programmes and widespread use of biologic therapies [31]. Clinical trials have shown that Japanese RA patients have a higher response rate to certain biologics, including infliximab, etanercept, and tocilizumab, than patients receiving the same drug treatment in Western countries [32]. The dietary habits in Japan are rich in anti-inflammatory components, as evidenced by regional health surveys of anti-inflammatory diets [33,34]. It is worth noting that regional inequality still exists within the same country. Possible reasons for disparities in RA disease burden across UK counties may include variations in healthcare, demographic factors (ageing populations in certain regions), lifestyle differences (smoking, obesity rates), air pollution [35], and socioeconomic inequalities affecting disease management [36,37].

Population growth and ageing are key drivers of the global burden of RA, particularly in rapidly ageing regions such as East and South Asia. Countries such as Thailand and China must

prioritise geriatric care and early RA management [38], while nations such as Pakistan and India need expanded healthcare infrastructure to address population-driven increases.

Our long-term forecasts predict a continued rise in RA incidence, emphasising the need for dietary adjustments, accessible medical policies, and innovative treatments such as cell therapy. Low to medium SDI regions, forecasted to see increasing DALYs until 2040, require urgent resource allocation, early detection, affordable treatment access, and public health campaigns. Tobacco use, linked to RA onset and severity, is a cost-effective intervention target [39]. China has the world’s largest smoking population (316 million adult smokers, 27.7% prevalence in 2015) [40]. Controlling tobacco use could significantly reduce the RA burden, particularly in high-medium SDI countries such as China. Addressing the multifactorial nature of RA demands global collaboration and tailored strategies to mitigate its growing public health impact [41,42].

Strengths and limitations

The GBD 2021 dataset exhibits inherent data quality limitations, including data sparsity and regional unreliability (particularly in low SDI areas), dependence of cause-of-death accuracy on death certificate quality, and concerns regarding data completeness and coding practices [14,19,43]. These challenges are further compounded by underdiagnosis and underreporting in resource-limited regions, which may result in underestimated

RA burden. To address these issues, our methodology integrates enhanced statistical approaches: misclassified deaths are redistributed through algorithmic correction, data dispersion is managed via negative binomial regression, and advanced preprocessing techniques such as spatiotemporal smoothing with GPR and BHM are employed. These models leverage correlations across neighbouring regions and temporal periods to impute missing data and improve completeness. Despite these improvements, residual biases persist, especially in contexts of incomplete reporting or inconsistent coding, which will require ongoing refinement. Additional limitations stem from the GBD database itself: RA incidence, prevalence, DALYs, and YLDs are only available from 1990 onwards for certain countries, while the under-20 age group was excluded due to insufficient risk factor data. Furthermore, the analysis does not account for heterogeneity in healthcare infrastructure (eg, diagnostic capacity, medical resource allocation), potentially introducing confounding effects.

Ethics approval

The protocol of the GBD 2021 has been approved by the research ethics board at the University of Washington. The GBD 2021 shall be conducted in full compliance with University of Washington policies and procedures, as well as applicable federal, state, and local laws. The study was carried out in the public domain secondary database, without any nominal identity, hence, the ethical approval was exempted.

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Contributors

WY. J.*, Q.W.*, and C.J.* have contributed equally to the work. BZ.H., XT.Z. and PP.Y. contributed equally to this work and are joint corresponding authors. QR.L. is the senior author. WY. J.*, BZ.H.[#], and QR.L. conceived the study. WY. J.*, Q. W.*, and BZ.H.[#] wrote the manuscript. MY.X., LM.P., Y.Z., YB. Z., F.L., Claire. Z., YT.L., D.W., YY.W., and NN. W. calculated and corrected the data. XT.Z. and PP.Y. revised the manuscript. All authors read and approved the final manuscript and revised the manuscript with critical intellectual input.

Competing interests

All authors declare they no competing interests.

Data availability statement

The data that support the findings of this study is available in [Appendix 1](#) and [Appendix 2](#). Original data is available in Global Health Data Exchange (<https://ghdx.healthdata.org/>).

Patient consent for publication

Not Applicable.

Provenance and peer review

Peer reviewed.

Supplementary materials

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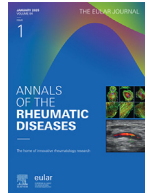
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Rheumatoid arthritis

Functional dissection of noncoding variants associated with rheumatoid arthritis

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ABSTRACT

Objectives: Noncoding variants are critical to our understanding of the genetic basis of diseases and disorders such as rheumatoid arthritis (RA). While genome-wide association studies have identified regions of the genome associated with disease, functional studies are still lagging that can identify potentially causative variants.

Methods: In order to functionally fine-map RA-associated variants, we identified variants at enhancers marked in primary activated T helper cells and conducted massively parallel reporter assay in these cells.

Results: We found that combinations of functional variant genotypes are often exclusive to patients with RA. We leveraged 3-dimensional genome architecture and expression quantitative trait loci data to identify target genes of enhancers exhibiting allelic differences in activity. We confirmed enhancer activity and target gene interactions by Clustered Regularly Intpaced Short Palindromic Repeats Cas9 (CRISPR-Cas9) deletion in primary T cells.

Conclusions: The identification of functional enhancer variants suggests possible causal variants, and their target genes reveal known and novel genes as likely drivers of RA, as well as a means for therapeutic intervention.

INTRODUCTION

Rheumatoid arthritis (RA) is a common chronic inflammatory syndrome, characterised by synovial inflammation, hyperplasia, and infiltration of joints by activated inflammatory CD4⁺ T helper (Th) cells, playing an important role in RA pathology. RA affects ~0.5% to 1% of the population and the estimated heritability is ~60%. Several genome-wide association studies (GWAS) [1–4], candidate gene approaches [1,5

–8], and meta-analysis [9] of GWAS have identified over 100 genetic loci associated with RA. GWAS identified variants and those in linkage disequilibrium (LD) occur almost exclusively in noncoding regions, potentially affecting distal transcriptional enhancer elements, as disease-associated variants are more likely to occur at transcription factor (TF)-binding sites within *cis*-regulatory elements than in coding regions [10]. Mutations in enhancer sequences can lead to disruption of DNA-binding motifs, thereby altering the binding affinity of TFs and causing

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WHAT IS ALREADY KNOWN ON THIS TOPIC

- Genome-wide association studies have identified over 100 genetic loci associated with RA, yet functional relevance of associated variants is largely unknown.

WHAT THIS STUDY ADDS

- We provide functional validation of noncoding regulatory variants in activated CD4⁺ T cells and linked them to target genes.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- The functional validation of RA-associated variants gets us one step closer to identifying causal variants and provides target genes that could serve as therapeutic targets.

variation in target gene expression. Because enhancer elements act on target genes from great distances, often bypassing nearest neighbouring genes, determining the targets of disease-associated enhancer variants remains limited. Although expression quantitative trait locus (eQTL) associate genetic variants with changes in gene expression [11], eQTL variants often do not lie in regulatory elements, and of those that do [12], most lack additional functional validation in RA-relevant cell types.

Immunogenetics of RA suggests a key role of immune-mediated provocation of Th cell responses in the initiation and continuation of disease [13]. Previously, we identified H3K4me1-marked enhancer elements in various primary human T cells and showed that RA-associated variants at enhancers can disrupt TF binding [14]. Chromatin mapping of primary immune cells, including Th cells, have identified that ~60% of autoimmune-associated variants are within immune cell enhancers [15]. However, large-scale functional testing of autoimmune disease-specific variants is still largely missing from the field, especially in disease-relevant cell types. Recent advances in massively parallel reporter assays (MPRAs) have dramatically improved the throughput of reporter assays to assess the regulatory activity of thousands of unique DNA fragments in a single experiment, including testing the effect of genetic variants [16,17]. For example, MPRA was used to determine variants associated with *cis*-eQTL in lymphoblastoid cell lines [18] and to screen variants associated with red cell blood traits, which identified variants disrupting cell type-specific transcriptional regulatory pathways [19].

In this study, we used MPRA in activated, primary human CD4⁺ T cells to screen the effects of 1765 enhancer variants in LD with 337 GWAS hits from comprehensive studies in RA. We identified 17 variants showing significant differences in allelic enhancer activity (false discovery rate [FDR] < 5%). We found that certain genotype combinations of RA-associated enhancer variants are exclusive to our RA cohort compared with healthy control subjects. We posit that the combinatorial effect of misregulating multiple genes may provide mechanistic insights on RA aetiology. We used promoter-capture Hi-C (pcHi-C) [20] and eQTL [11,12,21,22] data to determine target genes for each regulatory variant. Clustered Regularly Interspaced Short Palindromic Repeats Cas9 (CRISPR-Cas9) deletion of select enhancers in primary activated T cells confirmed the target gene identification, revealing that neither approach determines all targets. These genes may provide new therapeutic targets for RA.

METHODS*Variant prioritisation and functional analysis of single-nucleotide polymorphisms based on GWAS*

We downloaded the NHGRI GWAS Catalog (<https://www.ebi.ac.uk/gwas/docs/file-downloads>) and single-nucleotide polymorphism (SNP) coordinates are converted from hg18 to hg19 using UCSC liftOver tool. We identified 439 RA SNPs. Next, we extended the GWAS SNP sets by identifying all SNPs (proxy SNPs) in LD with a GWAS SNP using 1000 Genomes Project by using SNAP web server (<http://archive.broadinstitute.org/mpg/snap/ldsearch.php>). LD SNPs were defined as r^2 of >0.5 based on previous reports of enrichment at enhancer elements [23–25], providing a broad association that can be filtered for desired associations post testing. We overlapped the set of RA-associated SNPs with ChIP-seq mark of H3K4me1, a putative enhancer mark, and identified 1700 noncoding variants in enhancer regions of activated CD4⁺, Th1, Th2 cells.

CD4⁺ T cell culture for MPRA

All MPRA experiments were performed in activated CD4⁺ T cells. Purified human CD4⁺ T Cells were obtained from the Precision for Medicine, grown at 37 °C and 5% CO₂ in TexMACS (Miltenyi Biotech) medium with 10% human serum (Sigma) and 1% penicillin–streptomycin (Gibco). Naive CD4⁺ T cells were activated with bead-bound anti-CD3–biotin and anti-CD28–biotin and expansion of activated CD4⁺ cells was carried out by adding cytokines at 10.0 ng/mL. Interleukin-2 (Miltenyi Systems) was added to the cultures at 48 hours.

Design and synthesis of MPRA library

To multiplex enhancer SNP validation assay, we leveraged synthetic oligonucleotide array synthesis and adopted the STARR-seq MPRA plasmid [26]. An oligonucleotide library was synthesised containing the 230 nucleotide genomic regions, with 15 bp of sequence matching the Illumina primer sequence was performed by Agilent. The obtained library was then polymerase chain reaction (PCR) amplified to add Illumina primer sequence and 15 bp of sequence matching the STARR-seq backbone. The library was amplified with KAPA Hi-fi 2X (KAPA Biosystem) with the following thermal conditions: 98 °C for 2 minutes; amplification with 10 cycles of 98 °C for 20 seconds, 65 °C for 15 seconds, and 72 °C for 30 seconds; final extension at 72 °C for 2 minutes. The resulting product was purified using Ampure XP beads at 1.8 × beads: reaction ratio. The STARR-seq screening vector digested for 6 hours with SalI-HF and AgeI-HF and Linearise backbone run on the gel was purified with the Gel purification kit (Qiagen); 200.0 ng of backbone and 50.0 ng pooled insert was cloned in four 10.0-μL Infusion-HD reactions incubating at 50 °C for 15 minutes (Clontech). Resulting products were then combined purified using Ampure XP beads with a 1 × volume of beads and eluted in 8 μL of purified water and electroporated into NEB 10β electrocompetent cells at a ratio of 2 μL of reaction to 20 μL of competent cells for 4 electroporations using Bio-Rad GenePulserR II electroporator, with following electroporations conditions: 2.0 kV, 200 Ω, and 25.0 μF. Transformations were recovered for 1 hour in SOC medium while shaking (220 rpm, 37 °C) and then grown for 16 hours in 500 mL of Luria Broth while shaking (220 rpm, 37 °C). The

STARR-seq input libraries were then purified using the Qiagen Plasmid Maxiprep kit.

Output library construction

At 72 hours post activation, CD4⁺ T cells were washed in phosphate-buffered saline (PBS) and electroporated with a Neon transfection kit and device (Invitrogen) with the following transfection parameters: pulse voltage, 2100; pulse width, 20; and pulse number, 1; 3 µg of plasmid library per million cells were used. Cell pellets were rinsed once with PBS and then lysed in 2.0 mL of RLT buffer (Qiagen) with 2-mercaptoethanol (Sigma). Total RNA was prepared using the QIAGEN RNeasy kit. Poly-A RNA isolated from 50.0 µg of total RNA by µMACS mRNA isolation kit (Miltenyi Biotech). RNA was then treated with turboDNase (4 U) for 30 minutes at 37 °C (Invitrogen). DNase treated poly-A RNA was purified using the RNeasy kit. Plasmid specific cDNA was synthesised using Superscript III (Life Technologies) incubated for 1.5 hours at 55 °C and inactivated at 80 °C for 15 minutes. Following synthesis, cDNA was treated with RNaseA (Sigma) at 37 °C for 30 minutes. cDNA was purified using Ampure beads in 1.5:1 beads:cDNA ratio and then amplified and indexed for sequencing using a 2-stage PCR as described previously [26]. The cDNA sample from each replicate was used as an input into first round gene-specific PCR reaction using primers AJ10-sequence and AJ12-sequence, and KAPA Hi-fidelity polymerase (KAPA Biosystem). PCR conditions were as follows: 98 °C for 2 minutes; amplification with 15 cycles of 98 °C for 20 seconds, 65 °C for 20 seconds, and 72 °C for 60 seconds; final extension at 72 °C for 2 minutes.

Samples were then purified using Zymo PCR purification kit and eluted in 15.0 µL nuclease free water. The resulting products were used as template for the second round of PCR, which used a standard Illumina TruSeq indexing primer on the p5 end of the library and custom indexing primers to barcode the samples for multiplexing before sequencing (98 °C for 2 minutes; amplification with 8 cycles of 98 °C for 15 seconds, 65 °C for 30 seconds, and 72 °C for 30 seconds; final extension at 72 °C for 2 minutes. Final sequencing libraries were purified with Ampure XP beads (Beckman Coulter) at a 1.7 × SPRI:PCR reaction ratio. All libraries were sequenced in NextSeq 500 (Illumina) performing 1 × 75 cycles.

MPRA data normalisation and analysis

Sequencing raw reads from RNA and plasmid libraries are checked for adapter sequences and low-quality reads (*q* score < 20) using FASTQC (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and trimmed using Trim Galore package (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Trimmed reads were mapped to the amplicon library using the Bowtie2 aligner [27]. The mapped reads were quantified against all tested sequences using the ‘featureCounts’ function from custom scripts. We used the Quantitative Allele-specific Analysis of Reads (QuASAR-MPRA) method to determine statistically significant changes in allelic count data from MPRA [28]. The computed *P* values are adjusted with Benjamini–Hochberg method to control for FDR, and allelic enhancer activity was identified at an adjusted FDR of <5%.

TF binding sites

We used Perfectos-APE [29] to calculate binding scores for the reference and alternative allele in all MPRA-validated variants. We analysed SNPs with flanking sequences (25 base) using PERFECTOS-APE to predict the effect of RA SNPs on TF binding

using the predefined PWM: HOCOMOCO v11(human) [29], collection of mononucleotide PWMs, and a threshold of 0.0005 for motif *P* value and a threshold ratio of 4 for any of the alternate alleles. From this list, we filtered TFs that are expressed in activated CD4⁺ T cells. This filtered HOCOMOCO output was used to generate the variant-to-TF network using the NetworkX program in Python.

Target gene identification: pcHi-C and eQTL

pcHi-C data were collected from Javierre et al [20]. Target genes were identified based on CHiCAGO [30] scores of 5 or greater. Three sources of eQTL data were used to help determine target genes. We leveraged data from GTEx [11], DICE [21], Walsh et al [12], and the Benaroya Research Institute cohorts [22], where eQTL *P* values were calculated by linear regression analysis based on genotype and expression. All target genes were filtered for expression greater than 0.5 RPKM in activated CD4⁺ T cells.

Protein-protein interaction analysis

Protein-protein interactions (PPI) were derived using the String database [31].

GeneWalk analysis

GeneWalk analysis was performed on each MPRA significant variant target gene using the default parameters with Pathway Commons as the source of knowledge base to create the network [32].

Subjects and genotyping

This GWAS was performed in a single-stage study design involving 162 Caucasian individuals (57 males and 105 females) comprising 74 patients with RA and 88 healthy control subjects. All subjects were enrolled in the Benaroya Research Institute Disease Registry and Repository. Informed consent was obtained from all the participants of the study. The study was approved by the ethics committees of Benaroya Research Institute and carried out in accordance with the principles of Helsinki Declaration.

Peripheral blood gene expression

Peripheral whole blood was collected in Tempus Blood RNA tubes (Thermo Fisher). RNA was isolated using MagMax for Stabilised Blood Tubes RNA Isolation Kit, with globin reduction using GlobinClear Human (Thermo Fisher). Sequencing libraries were generated from total RNA using the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Takara) and NexteraXT DNA sample preparation kit (Illumina). Pooled libraries were sequenced to a target depth of 5 million reads per sample on a HiSeq2500 sequencer (Illumina) with dual-index, 58-base single-reads, using HiSeq v4 Cluster and SBS kits (Illumina). Base calls were processed to FASTQs on BaseSpace (Illumina), trimmed (Galaxy FASTQ Trimmer tool v1.0.0) [33], FastqMcf (v1.1.2), and aligned using the TopHat aligner (v1.4.1) [34] with the GRCh38 reference genome and gene annotations from Ensembl release 77. HTSeq-count (v0.4.1) [35] was used to generate gene counts. Quality metrics were compiled from PICARD (v1.134, <http://broadinstitute.github.io/picard/>), FastQC (v0.11.3, <http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>), Samtools (v1.2) [36], and HTSeq-count (v0.4.1).

Combination of SNP analysis and cell surface marker SNPs

We used a brute force search method to find all possible combinations of 2-SNP, 3-SNP, 4-SNP, and 5-SNP genotypes and searched for combinations exclusive to RA cases. None of the 2-SNP, 3-SNP, and 4-SNP combinations were exclusive to RA cases. We found several 5-SNP combinations exclusive to RA cases relative to healthy controls. We computed the statistical significance for these 5-SNP combinations by randomly permuting the disease status of the population 1000 times and computing *P* values. We filtered for combinations with at least 6 RA cases and zero healthy controls with *P* values of < 0.01. [Supplementary Table S6](#) provides the rsID and genotype for each combination of 5 SNPs and the prevalence of a given SNP across combinations. This can serve as a look-up table to know which combinations are informative.

CRISPR-Cas9 editing in activated CD4⁺ T cells

Guide RNAs

For prioritised enhancer variants, 3 gRNA were designed per enhancer variant region using Benchling (www.benchling.com) and ordered from Synthego. gRNA sequences are provided in the [Supplementary Table S9](#).

Electroporation of ribonucleoprotein (RNP) complex in activated CD4 cells: To make the RNP complex, we incubated 5.0 µL of 12.2 µM Cas9 (UC Berkeley; QB3 MacroLab) with 3.0 µL of 25.0 µM gRNA (Synthego). For all 3 gRNAs, separate RNP complexes were made and then pooled before transfection. Per transfection, we suspended 500,000 cells in 8.0 µL T buffer and mixed with 4.0 µL of pooled RNP complexes and included 0.5 µL green fluorescence protein mRNA to visualise the transfection efficiency. The pooled mixture of cells, RNP complex, and green fluorescence protein mRNA were electroporated using the following neon electroporation parameters: pulse voltage, 1400; pulse width, 10; and pulse number, 3.

CRISPR deletion experiments and qPCR analysis

Postelectroporation activated CD4 cells were incubated in fresh media with interleukin-2 for 3 days. Total RNA were isolated using Qiagen RNA isolation kit, and first-strand cDNAs were made using Superscript IV kit (Thermo). These cDNA were diluted up to 5 times and used for qPCR using predesigned Taqman probes for target genes with VIC fluorophore labels were used. For internal controls, 18s and ACTB Taqman probes with FAM fluorophore labels were used. qPCR was performed with Taqman universal PCR master mix on BioRad CFX qPCR machine. Deletion efficiency was calculated by Tracking of Indels by DEcomposition (TIDE) analysis based on Sanger sequencing of treated and nontreated samples. Efficiency ranged from 15% to 30%.

CD84 flow cytometry and human subjects

Cryopreserved peripheral blood mononuclear cells (PBMCs) were derived from the Benaroya Research Institute adult healthy volunteer biorepository or the rheumatic diseases biorepository and selected based on rs6427528 genotype. Frozen PBMCs were thawed in warm RPMI-1640 media (Cytiva HyClone) and 10% fetal bovine serum (Avantar Seradigm) at 37 °C and rested for 1 hour at 37 °C. Approximately, 5 × 10⁵ PBMCs were then plated in V-bottom plates and incubated with Fc block solution for 10 minutes at room temperature (RT) (1:40 in PBS, BD Biosciences) before surface staining was performed in PBS for 20

minutes at RT. Cells were then fixed with BD Cytofix for 5 minutes at RT and stored in fluorescence-activated cell sorting (FACS) buffer (PBS [1 ×; Cytiva HyClone], bovine serum albumin [1%; Sigma-Aldrich], sodium azide [0.1%; Sigma-Aldrich]) at 4 °C until acquisition. All data were acquired on a BD LSR II cytometer and analysed using FlowJo v10.7 (BD Life Sciences) and GraphPad Prism 10 software. All antibodies including clone, fluorophore, and source are listed in [Supplementary Table S10](#).

Statistical tests and visualisations

All other statistical tests were performed in the R environment (<https://www.r-project.org/>). Graphs were prepared using ‘ggplot2’ R packages. Figures were composed using Adobe Illustrator and biorender.com.

RESULTS

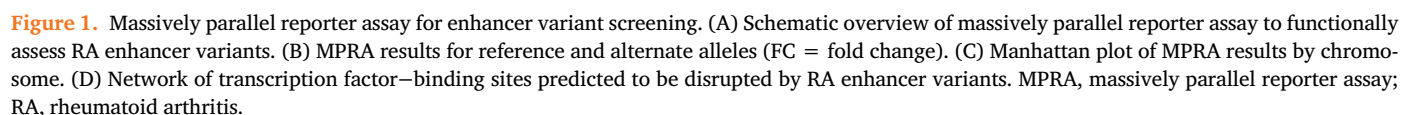
Allelic effects of RA-associated variants on enhancer function

For this study, we obtained RA-associated variants from NHGRI/EBI catalog and compiled additional variants that were in LD with lead SNPs using the 1000 Genomes data. We intersected the variants with H3K4me1-marked T cell enhancers [14], which resulted in 1765 RA-associated variants at 611 enhancers from 141 RA-associated loci ([Supplementary Table S1](#)). We tested on average 3 variants per enhancer and as many as 25 variants for a single enhancer. To determine which variants within these loci functionally alter the enhancer activity, we used MPRA. For this assay, we synthesised a long-oligo pool that contained either the reference or alternate allele centred at the SNP resulting in a library of 3530 enhancer alleles. These were cloned into a reporter vector and electroporated into primary, human activated CD4⁺ T cells ([Fig 1A](#)), which are known to be expanded in RA compared with those in osteoarthritis donors [37], followed by RNA and plasmid DNA isolation for MPRA library construction.

To determine allelic differences in enhancer activity, we calculated the log2 effect size of each allele as the ratio of RNA reads to DNA reads ([Fig 1B](#)). In total, we identified 259 variants with a greater than 2 log fold effect size (*P* < .05). After correcting for multiple testing with the Benjamini–Hochberg method, 17 variants demonstrated statistically significant differential allelic enhancer activity in activated CD4⁺ T cells at an FDR of <5% ([Fig 1B,C](#); [Table](#); [Supplementary Table S1](#)) and additional 26 variants at an FDR of <10%. While the majority of enhancer variants weaken enhancer activity, ~30% increased enhancer strength ([Fig 1B,C](#); [Table](#)).

Modulation of TF binding by enhancer variants

A primary means by which variants can affect enhancer activity is by modulating TF binding at the altered DNA sequence. To assess the effect of allelic variants on TF binding, we used the Perfectos-APE [38] database to predict weakening as well as enhancement of TF binding at enhancer variants. For each of the 17 most significant enhancer variants, at least 1 TF expressed in CD4⁺ T cells could be identified to have altered binding due to the presence of the RA-associated variant ([Fig 1D](#); [Supplementary Table S2](#)). Several of the TFs that showed alternate binding are important for regulation of immune function and proliferation. For example, the RA-associated allele for enhancer variant rs687680 is predicted to disrupt Forkhead box (FOX) family member binding. FOXO factors in particular have been



Cognate target gene identification for regulatory variants using pcHi-C and eQTL

often miss nearby neighbouring genes due to technical artefacts within 20 kb of the targeted promoter. eQTL analysis can identify candidate target genes through a statistical association and assess the association of allelic variants with the alteration in gene expression but do not necessarily identify the corresponding enhancer. To overcome these challenges, we opted for a dual analysis and used data from pcHi-C as well as from eQTL studies [11,12,21] (see Methods) to identify enhancer variant target genes expressed in activated CD4 T cells (Table; Supplementary Tables S3-S5). Using existing pcHi-C data from CD4⁺ T cells [20], we identified target genes for 9 of the 17 most significant variants (Table). Most enhancers interact with multiple genes and often skip the nearest neighbouring gene before interacting with its cognate gene, indicating that genes in the closest proximity are often poor candidates for noncoding, regulatory variants.

To determine the *cis*-eQTL association in an autoimmune relevant T cell-specific type, we used the Database of Immune Cell eQTLs, Expression, and Epigenomics (DICE) data set to determine target gene identification for enhancer variants [21]. We

Table
Enhancer MPRA allelic activity and target gene interactions for RA-associated variants

Enhancer SNP ID	Lead SNP ID	MPRA Log2(effect size)	Padj_BH	pcHi-C target genes	eQTL target genes	Reference for lead SNP (PMID)
<i>rs10484577</i>	<i>rs9372120</i>	6.0281	0.0000	PRDM1, PREP		24390342
<i>rs9994851</i>	<i>rs45475795</i>	-6.3546	0.0002			24390342
<i>rs6037959</i>	<i>rs6084875</i>	5.3989	0.0007	CDS2, PCNA, RASSF2, SNORA26, SNORA31, SNOU13, TMEM230	RASSF2, SLC23A2	24871463
<i>rs11639945</i>	<i>rs4780401</i>	6.0003	0.0008	AC009121.1, CLEC16A, DEXI, RMI2, RP11-166B2.8, RSL1D1	LITAF, SNN, TXNDC11	24390342
<i>rs67843408</i>	<i>rs1772408</i>	6.2101	0.0080		AIM2	21383967
<i>rs11076573</i>	<i>rs6500395</i>	4.1949	0.0085	C16orf87, ORC6, SIAH1, VPS35	LONP2, N4BP1, RP11-44I10.3	22491018
<i>rs8191295</i>	<i>rs4780401</i>	4.7020	0.0085	CLEC16A, DEXI, LITAF	LITAF, SNN, TXNDC11	24390342
<i>rs3825611</i>	<i>rs3783637</i>	-4.0538	0.0085		ATG14, FBXO34, GCH1	22446963
<i>rs1860823</i>	<i>rs3761847</i>	4.7513	0.0106	CNTRL, PSMD5, PSMD5-AS1, TRAF1	CNTRL, PHF19, PSMD5-AS1, RAB14, TRAF1	17804836
<i>rs9620979</i>	<i>rs1043099</i>	-5.4896	0.0119			24449572
<i>rs7173565</i>	<i>rs8032939</i>	6.0568	0.0240			24390342
<i>rs2358995</i>	<i>rs6679677</i>	3.7009	0.0435		DCLRE1B, RSBN1	17554300
<i>rs195999</i>	<i>rs7404928</i>	-4.0514	0.0344	PRKCB	PLK1, PRKCB	22446963
<i>rs2647066</i>	<i>rs660895</i>	9.8488	0.0435		HLA-DPB1, HLA-DQA1, HLA- DQA2, HLA-DQB1, HLA- DQB2, HLA-DRB1, HLA- DRB3, HLA-DRB4, HLA- DRB5, TAP2	17804836
<i>rs687680</i>	<i>rs508970</i>	-9.6308	0.0435	CD6, CCDC86, RNU6-933P, RP11-804A23.2, PRPF19, SLC15A3, TMEM109, ZP1		24390342
<i>rs1503860</i>	<i>rs6427528</i>	3.6190	0.0435	CD48, CD84, COPA, LY9, NCSTN, RP11-404F10.2, SLAMF1, SLAMF6	CD84	23555300
<i>rs1358883</i>	<i>rs3093024</i>	4.9296	0.0435		AL133458.1, RNASET2, RPS6KA2	20453841

eQTL, expression quantitative trait locus; MPRA, massively parallel reporter assay; RA, rheumatoid arthritis.

also used whole blood eQTL data from GTEx [11], eQTL data from a published RA cohort [12], and an eQTL analysis of our own RA cohort and healthy controls (Supplementary Table S4). Of the 17 most significant MPRA-validated enhancer variants, eQTL studies revealed target genes for 12 enhancer variants (Table, Supplementary Table S4). Of these, 7 enhancer variants have target genes identified in multiple eQTL analyses. For example, rs1358883 showed an association with *RNASET2* in all 4 eQTL analyses. Similarly, rs1860823 was found to be associated with *PSMD5-AS1* in DICE, GTEx, and eQTL from patients with RA, which is largely distinct from pcHi-C data that revealed chromatin interactions with *TRAF1*, *PSMD5*, and *CNTRL* (Supplementary Fig S1). In total, 14 of the 17 enhancer variants could be assigned target genes using pcHi-C and eQTL. Target genes identified using pcHi-C and eQTL analyses are often distinct. To validate interactions between enhancers with validated RA variants and distal promoters, we used CRISPR-Cas9 in activated CD4⁺ T cells. We targeted enhancers around the variant position and analysed the expression of target genes obtained from pcHi-C or eQTL analyses.

We first targeted the rs8191295 enhancer (Fig 2A-C), which is in the intron of the *SNN* gene and upstream of the lead variant rs4780401, which is located near *TXNDC11* (Fig 2C). Our eQTL analysis implicated *SNN* and *LITAF* as potential target genes (Fig 2C, bottom). Given that these are common variants no difference is seen between RA and healthy control donors, only by genotype. After CRISPR editing to create a deletion in the enhancer region in activated CD4⁺ T cells, we observed significant downregulation of pcHi-C target genes *CLEC16A* and *DEXI*,

as well as eQTL genes *SNN* and *LITAF* (Fig 2D). CRISPR editing of this enhancer also affected the expression of the next-nearest-neighbouring gene *TXNDC11* (Fig 2C,D), which is associated with the lead variant in the eQTL analysis. *CLEC16A* and *DEXI* are associated with multiple autoimmune diseases, including RA and juvenile idiopathic arthritis [41–45], and *LITAF* plays a role in inflammatory arthritis [46]. The roles of the remaining genes are understudied in the context of RA.

An intergenic enhancer on chromosome 20 near *RASSF2* harbours a set of proxy variants associated with RA (Fig 3A), of which only 1, rs6037959, has an allelic difference in MPRA activity (Fig 3B). The enhancer variant physically interacts with *RASSF2*, *TMEM230*, *PCNA*, *CDS2*, and other noncoding genes in activated CD4⁺ T cells (Fig 3C). The lead SNP, rs6084875, for the enhancer variant is associated with *RASSF2* and *SLC23A2* by eQTL analysis (Fig 3C). The expression effect is not specific to RA or healthy control donors given these are common variants. Examination of target coding genes upon CRISPR deletion in the enhancer harbouring rs6037959 shows reduced expression of target genes assigned using pcHi-C and eQTL (Fig 3D).

MPRA functionally fine-mapped rs1503860 as the only variant with a significant allelic difference (FDR < 5%) for lead variant rs6427528 near the 3' UTR of *CD84* (*SLAMF5*) (Fig 4A-C). The eQTL target gene implicated *CD84* (Supplemental Table S4). The enhancer harbouring rs1503860 physically interacts with *CD84*, *COPA*, and *SLAMF6* promoter regions in activated CD4⁺ T cells (Fig 4C). These interactions were confirmed by CRISPR deletion of the enhancer segment (Fig 4D). *VANGL2* is not expressed in activated CD4⁺ T cells and, therefore, was not

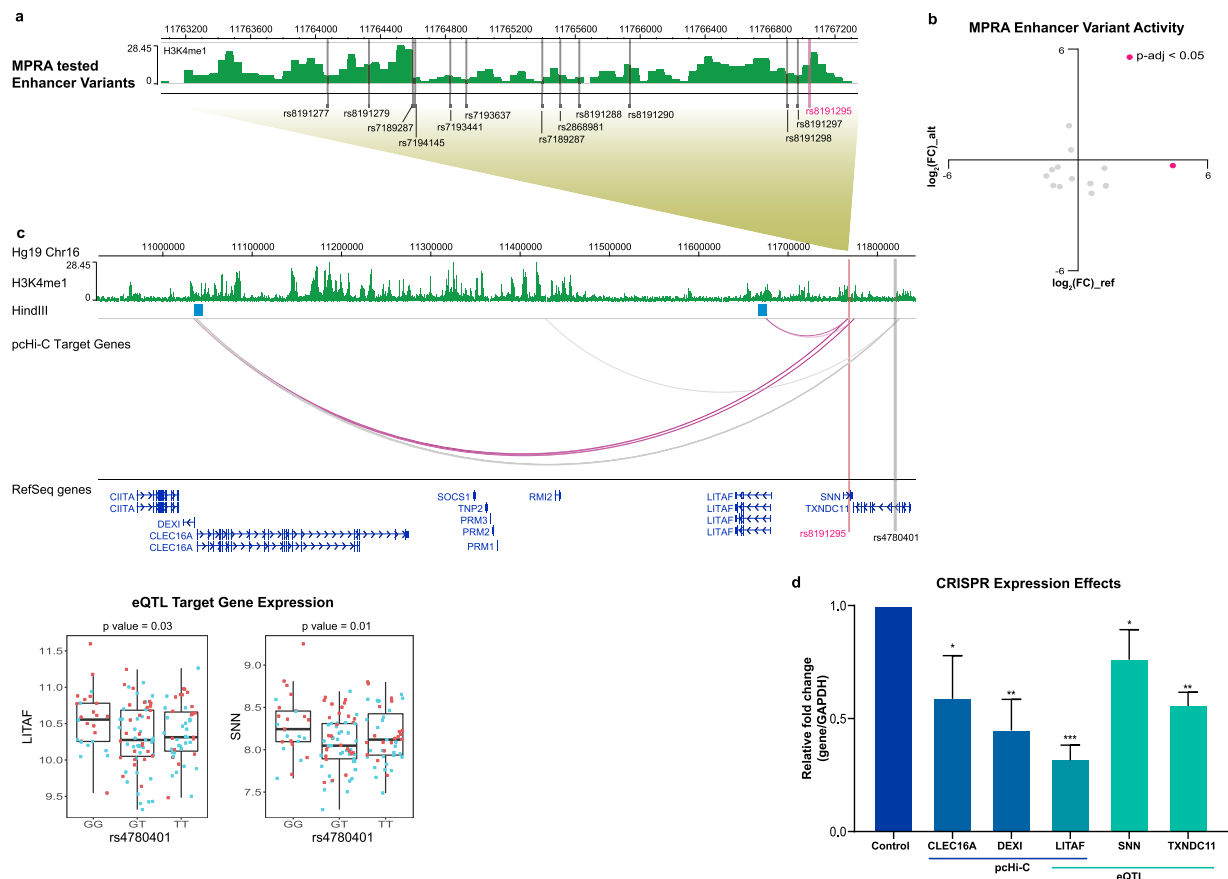


Figure 2. Enhancer activity and target gene identification for RA enhancer variant rs8191295. (A) Browser shot of RA enhancer SNPs in LD with the lead variant tested by MPRA; significant variant is shown in red. Activated CD4⁺ T cell H3K4me1 ChIP-seq data (green). (B) Scatter plot of MPRA variants for enhancer variants shown in (A). (C) Top, Browser shot of pcHi-C interactions (maroon arcs) for MPRA-validated variant rs8191295 (red line) showing interaction with *CLEC16A*, *DEXI*, and *LITAF*. RA lead variant rs4780401 location (grey line). Bottom, Significant expression changes correlated with lead variant genotypes reveal *SNN* and *LITAF* genes. Blue, healthy control donors; red, RA donors. (D) CRISPR-Cas9 enhancer deletion effects for rs8191295 enhancer target genes from pcHi-C and expression quantitative trait locus analysis. * $P < .05$, ** $P < .01$, *** $P < .001$. LD, linkage disequilibrium; MPRA, massively parallel reporter assay; RA, rheumatoid arthritis; SNP, single-nucleotide polymorphism.

tested. CD84 has been shown to limit T cell responses [47] and is expressed by a large fraction of CD4⁺CD45RA⁺ memory T cells and a smaller subset of CD4⁺CD45RA⁺ naive T cells as determined by flow cytometry (Fig 4E, left). We leveraged a cohort of healthy control donors or patients with RA and known genotypes for rs6427528 and assessed CD84 expression among total CD4⁺ T cells. We found significantly lower levels of CD84 expression among total CD4⁺ T cells, comparing AG with GG genotype carriers (Fig 4E, right). No difference in CD84 expression was observed between RA and control donors. Thus, we confirmed a regulatory role of this variant in CD84 expression using primary human T cells.

Combinatorial enhancer genotypes in RA individuals and related gene functions

For complex diseases such as RA, susceptible individuals are likely to harbour a combination of genetic variants associated with the disease as opposed to a single, disease-causing variant. A combination of RA-associated variant enhancer genotypes could explain how common variants associated with disease can be causal given that these variants have a functional effect. To test this, we assessed genotype data of the 17 highly significant MPRA enhancer variants for their presence in available case-control genotype data from our RA cohort [22]. First, we

identified the lead SNP for each of the 17 MPRA-validated SNPs. Of these, we could obtain case-control data for 16 of the enhancer variants, of which 2 enhancer variants had the same lead SNP, resulting in 15 lead SNPs in our cases and controls. We conducted a combinatorial analysis of up to 5 SNPs in case-control data and found that genotypes consisting of different combinations of 5 enhancer lead SNPs were exclusively present in cases ($P < .01$) (Fig 5A; Supplementary Table S6). We determined 72 combinations of genotypes significantly enriched in RA donors relative to case controls; however, some RA-associated SNPs were represented more frequently than others (Fig 5A). This supports our hypothesis that combinations of genotypes for RA-associated enhancer variants may explain how common variants can be associated with RA and drive disease.

Our pcHi-C and eQTL target gene analysis revealed that the functional enhancer variants control multiple genes, indicating that each enhancer variant could have a pleiotropic effect. That is, pleiotropic enhancer variants can alter the expression of multiple genes. Therefore, RA individuals with polygenic combinations of variants potentially have several genes misregulated. Given that each RA affected individual harbours a subset of enhancer variants, and thus, a subset of target genes that are misregulated, we hypothesised that there must be a relationship between genes in order for individuals with different enhancer genotypes to present with the same disease. To test this, we first

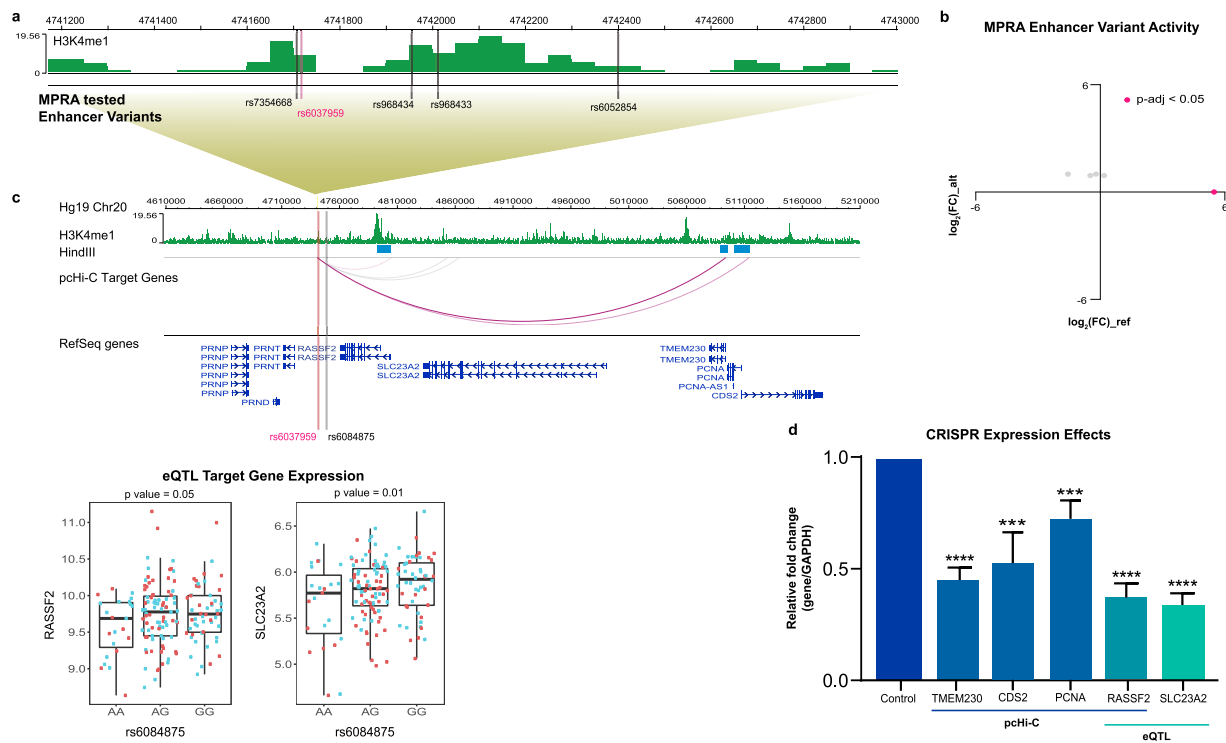


Figure 3. Enhancer activity and target gene identification for RA enhancer variant rs6037959. (A) Browser shot of RA enhancer SNPs in LD with the lead variant tested by MPRA; significant variant is shown in red. Activated CD4⁺ T cell H3K4me1 ChIP-seq data (green). (B) Scatter plot of MPRA variants for enhancer variants shown in (A). (C) Top, Browser shot of pcHi-C interactions (maroon arcs) for MPRA-validated variant rs6037959 (red line) showing interaction with *CDS2*, *PCNA*, *RASSF2*, and *TMEM230*. RA lead variant rs6084875 location (grey line). Bottom, Significant expression changes correlated with lead variant genotypes reveal *RASSF2* and *SLC23A2* genes. Blue, healthy control donors; red, RA donors. (D) CRISPR-Cas9 enhancer deletion effects for rs6037959 enhancer target genes from pcHi-C and expression quantitative trait locus (eQTL) analysis. ***P < .001, ****P < .0001. LD, linkage disequilibrium; MPRA, massively parallel reporter assay; RA, rheumatoid arthritis; SNP, single-nucleotide polymorphism.

constructed PPIs (Fig 5B). Of enhancer targets, 36 have known protein interactions with another enhancer target gene. While some coregulated genes also interact at the protein level, many genes encoding PPIs are driven by enhancers from across the genome. With a limited set of enhancer target genes, we performed a GeneWalk [32] analysis to determine enriched gene functions (Fig 5C,D; Supplementary Table S7). While expected functions such as T cell activation and proliferation were found (Supplementary Table S7), we also identified underappreciated biological contexts with respect to the aetiology of RA. For example, several genes were associated with ontology terms related to DNA damage and repair (Fig 5C) or with vesicle transport (Fig 5D).

Given the exclusive combinatorial enhancer genotypes of RA donors and the discovered relationship between enhancer target genes, this suggests common biological functions and/or pathways are disrupted in RA affected individuals. Therefore, these genes may serve as points of therapeutic intervention. To explore this further, we queried all enhancer variant target genes against the Drug Gene Interaction database (DGIdb) [48,49]. Of the 14 enhancers with identified target genes, 12 have known drug or small molecule interactions for 22 genes that can be targeted (Supplementary Table S8). Several known RA drugs were among these, including adalimumab, azathioprine, etanercept, and infliximab, suggesting other known drugs have the potential to be repurposed for RA treatment.

DISCUSSION

Lead variants from GWAS plus the variants in LD can identify thousands of variants associated with diseases like RA.

Intersecting those variants with regulatory maps provides a means to prioritise associated variants for further consideration. Functional studies of regulatory variants can reveal strong candidates for causality. In this study, we tested 1765 RA-associated variants and their reference alleles from 611 enhancers at 141 RA-associated loci using MPRA in activated, primary human CD4⁺ T cells. We identified 17 functionally significant variants, in LD with 16 lead variants, which altered enhancer activity in an allelic manner. We identified TFs that are likely to exhibit altered binding at these enhancer variants as the mechanism for the change in enhancer activity. A majority of them belong to TF families with established roles in immune function and inflammation, such as FOXO, STAT, BACH, and RUNX members. GWAS have identified the *RUNX1*, *REL*, *PRDM1*, and *BACH2* genes as associated with RA [50–52]; therefore, it is feasible that noncoding regulatory variants that alter their binding would also be potential causal candidates for RA. FOXO factors are thought to contribute to chronic inflammation through their inactivation downstream of the PI3-kinase pathway [53], downstream of T cell receptor (TCR) and CD28 ligand signalling [54]. Collectively, the disruption of TF binding by RA-associated regulatory variants provides insight into the regulatory mechanisms contributing to RA.

Enhancers can regulate distal genes through regulatory loops, often skipping the nearest neighbouring gene. Using 3-dimensional genome architecture and eQTL data analyses, we identified target genes for functional enhancer variants. For example, rs687680 resides in the genic region of *VPS37C* and interacts with multiple genes ~200 kb upstream. One such gene is *CD6* (Supplementary Fig S2). *CD6* is a surface glycoprotein expressed on T cells and plays a dual role in T cells by promoting T cell

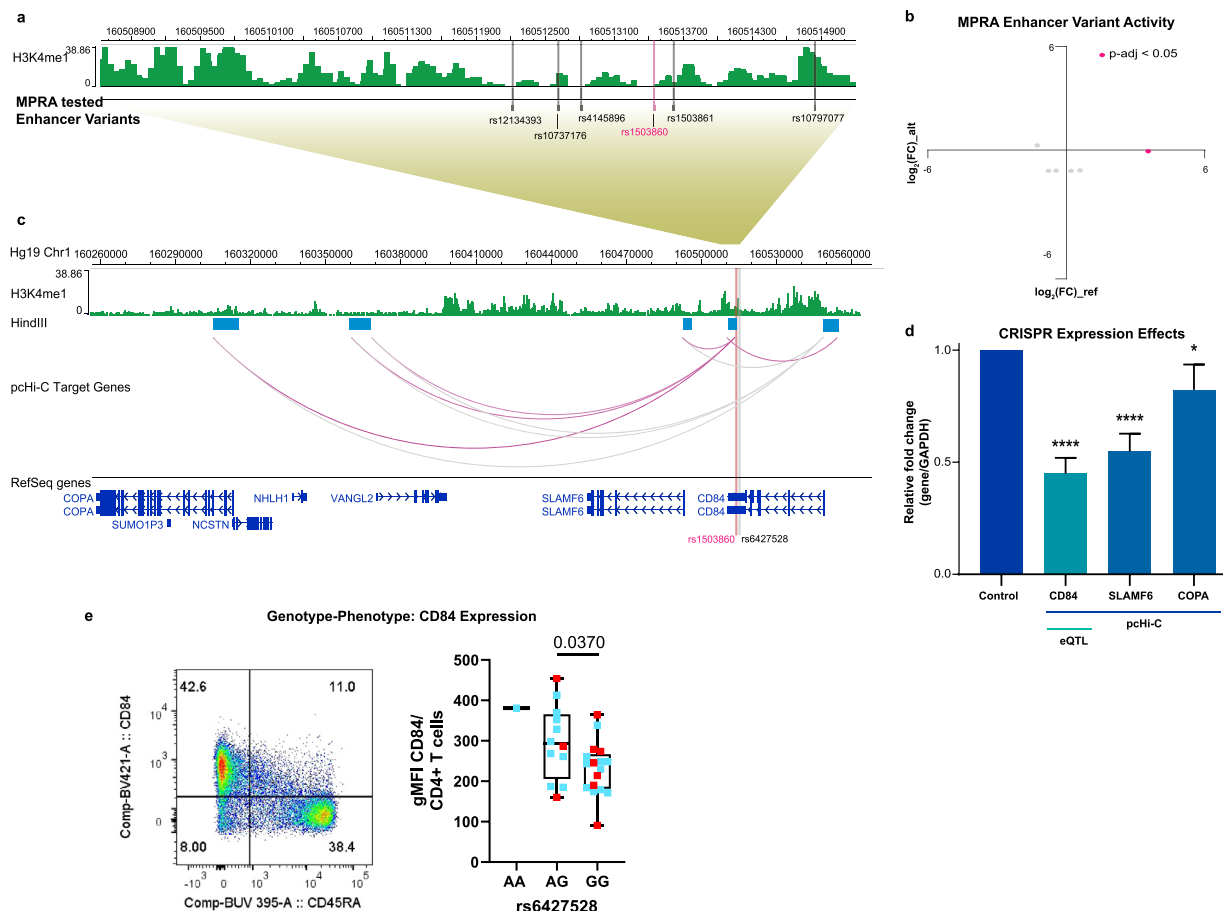
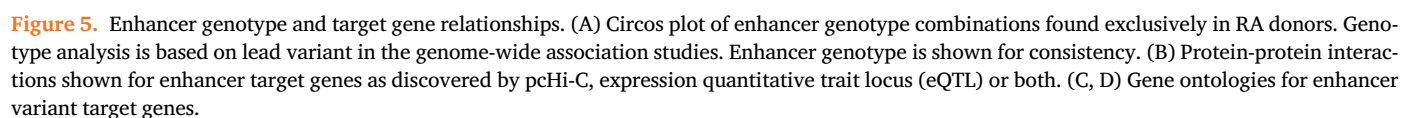


Figure 4. Enhancer activity and target gene identification for RA enhancer variant rs1503860. (A) Browser shot of RA enhancer SNPs in LD with the lead variant tested by MPRA; significant variant is shown in red. Activated CD4⁺ T cell H3K4me1 ChIP-seq data (green). (B) Scatter plot of MPRA variants for enhancer variants shown in (A). (C) Browser shot of pcHi-C interactions (maroon arcs) for MPRA-validated variant rs1503860 (red line) showing interaction with *CD84*, *SLAMF6*, and *COPA*. *VANGL2* is not expressed in activated CD4⁺ T cells. RA lead variant rs6427528 location (grey line). (D) CRISPR-Cas9 enhancer deletion effects for rs1503860 enhancer target genes from pcHi-C and expression quantitative trait locus (eQTL) analysis. *CD84* was not significant in our cohort but is an eQTL target gene in other studies (Supplementary Table S4). **P* < .05, *****P* < .0001. (E) Flow cytometric analysis of CD84 expression in CD4⁺ T cells. Representative flow plot of CD84 expression (left) and expression levels of CD84 shown as geometric mean fluorescence intensity (gMFI) in gated CD4⁺ T cells of healthy control (blue, *n* = 20) and patients with RA (red, *n* = 10) selected for rs6427528 variants (right). rs6427528 genotypes AA (*n* = 1), AG (*n* = 12), and GG (*n* = 17); Mann–Whitney test was used to compare AG and GG carriers (*P* = .0370). LD, linkage disequilibrium; MPRA, massively parallel reporter assay; RA, rheumatoid arthritis; SNP, single-nucleotide polymorphism.

activation through strong adhesion to other immune cells and inhibiting T cell receptor signalling [55]. Several studies relate CD6 with the pathogenesis of MS, Sjögren syndrome, and RA [56–58]. CD6 is also a costimulatory molecule linked to autoreactive responses, further emphasising its potential role in RA pathogenesis [59]. Another interacting gene is *SLC15A3*. *SLC15A3* is a peptide/histidine transporter expressed in the lymphatic system [60] and plays a role in Toll-like receptor–mediated inflammatory responses [61]. Toll-like receptor–induced cytokines are implicated in RA [62]. *CLEC16A* is a RA candidate risk gene [42] that interacts with rs8191295 and rs11639945, which are ~800 kb downstream. From within an intron of the *ATG5* gene, rs10484577 interacts with *PREP* and *PRDM1* (Supplementary Fig S2). *PREP* encodes for cytosolic prolyl endopeptidase, which is involved in the maturation and degradation of peptide hormones and neuropeptides. Studies have shown prolyl endopeptidase causes escalation of systemic inflammation in synovial membranes of patients with RA [63]. *PRDM1* (BLIMP1) is a repressive TF [64] and is essential for both B and T cell proliferation and homeostasis [65,66]. *PRDM1*-negative CD4⁺ T cells are hyperproliferative, which may have important implications in RA as the alternate allele of rs10484577 weakens

enhancer activity. Furthermore, conditional knockout in thymic epithelial cells results in increased autoimmunity [67], signifying the importance of *PRDM1*. As further evidence of the potentially causative role *PRDM1* may play in RA, we also found that enhancer variant rs11076573 is predicted to alter *PRDM1* binding.

We were able to validate enhancer activity and target gene interactions through enhancer perturbations using CRISPR genome editing in primary, activated CD4⁺ T cells. Deletion of 3 enhancers with functional variants showed significant decreases in target gene expression. In most instances, pcHi-C and eQTL determined different target genes, likely due to technical differences. Enhancer deletions often validated each approach, demonstrating altered expression of genes identified by each method. The validated enhancer variant target genes reveal genes with roles implicated in RA or autoimmunity, as well as others that are understudied in this context. For example, *RASSF2* encodes the Ras association domain family member 2, which has a role in apoptosis and cell cycle. *SLAMF6* encodes for the signalling lymphocytic activation molecule 6 that is highly expressed in autoimmune T cells, and variants have been reported in RA and systemic lupus erythematosus (SLE).



Other genes involved in Golgi and endoplasmic reticulum vesicular trafficking such as lysosome, phagosome, and endosome transport are also overrepresented among enhancer variant target genes. We posit that these genes may be playing a role

It is interesting to note that several target genes for the validated RA enhancer variants play a role in DNA damage response, double-stranded break repair, and nonhomologous end joining or related pathways. The role for these pathways in T cells and autoimmunity have recently been reviewed [77,78] but are still largely understudied. There are a number of mechanistic implications for DNA damage response in autoimmunity, but genotoxic stress has been linked to telomere shortening,

increased apoptosis, skewing of T cell differentiation, and possible clonal expansion.

Many studies have shown that looping interactions are largely static across cell types, so it is likely the target genes for these variants are the same in RA donor cells and not redirected to a new target gene. The enhancer variants are most likely to weaken the promoter interaction in RA individuals; which future studies may prove. Ultimately, we validated only a subset of enhancer target gene interactions. Larger screens will be required to validate all interactions, as well as the need to expand the study of regulatory variants to additional immune cells relevant to RA. At times, the eQTL data indicated a small but significant change in target gene expression, which may explain why a single variant does not cause RA. The MPRA data confirm which variants are functional, and we were able to validate the enhancer interactions, again illustrating the importance of functional studies for noncoding variants.

As demonstrated, several of the enhancer target genes have already been implicated in RA or other autoimmune disorders. Our functional validation of enhancer variants and assignment of cognate target genes suggests these are potentially causal variants of RA. Since the functional enhancer variants are common variants, a single variant is not expected to cause RA. Instead, our analysis suggests that polygenic combinations of functional enhancer variants are likely drivers of RA, which is further amplified by the fact that most functional enhancer variants interact with multiple target genes. Collectively, this may explain the complex aetiology of autoimmune diseases like RA. Future studies may reveal whether certain combinations of enhancer genotypes are associated with specific RA phenotypes, serotypes, or responses to therapy. For example, we identified functional enhancer variants controlling genes associated with seropositivity, such as *TRAF1* [79,80] and *HLA-DRB1* [81,82]. It is feasible the combinatorial genotypes that include enhancers for these genes could help stratify RA donors relative to seropositivity.

Our data demonstrate that while RA patients harbour various combinations of enhancer variants, the targets coalesce into shared pathways or PPI networks. These genes and their related pathways are potential targets for therapeutic interventions for RA. Our analysis revealed that many target genes or their pathways already have corresponding drugs that could potentially be repurposed for RA treatment or small molecule inhibitors that could serve as the basis of future screens using immune cells from RA donors. Eventually, enhancer genotypes may provide a pharmacogenomics approach to treatment and even suggest combinatorial therapies based on target gene pathways.

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Competing interests

All authors declare they have no competing interests.

CRedit authorship contribution statement

Ajay Jajodia: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis. **Arpit Mishra:** Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis. **Naresh Doni Jayavelu:** Writing – original draft, Visualization, Investigation,

Formal analysis, Data curation. **Katharina Lambert:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Nicholas Moss:** Visualization, Formal analysis. **Zongchen Yang:** Investigation. **Karen Cerosaletti:** Writing – review & editing, Writing – original draft, Supervision, Investigation. **Jane H. Buckner:** Writing – review & editing, Writing – original draft, Supervision, Resources. **R. David Hawkins:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Funding acquisition, Conceptualization.

Patient consent for publication

All subjects were enrolled in the Benaroya Research Institute Disease Registry and Repository. Informed consent was obtained from all participants of the study.

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Psoriatic arthritis

Disease-modifying antirheumatic drug–free remission in psoriatic arthritis: is it attainable and sustainable? A large longitudinal study

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ABSTRACT

Objectives: According to current management guidelines for psoriatic arthritis (PsA) tapering of disease-modifying antirheumatic drugs (DMARDs) can be considered. However, limited data are available on complete DMARD cessation, also known as disease-modifying antirheumatic drug–free remission (DFR). Therefore, our aim was to investigate whether DFR is achievable and sustainable in PsA and to evaluate possible predictors for sustained disease-modifying antirheumatic drug–free remission (SDFR).

Methods: From the Dutch southwest Early Psoriatic Arthritis cohort, all newly diagnosed patients with oligoarticular/polyarticular PsA who were treated with DMARDs were included (n = 451). Prevalence of (S)DFR and flare rates (early and late) were described. DFR was defined as the absence of clinical synovitis for ≥3 months after DMARD cessation, while for SDFR, a period of >1 year was used. Early and late flares were defined as restarting DMARD treatment ≤1 and >1 year after DMARD cessation, respectively. Subsequently, possible predictors for true SDFR, that is, SDFR without flaring were explored.

Results: After a median of 5.1 years (IQR, 3.0–7.3 years), 22% of patients with PsA had reached DFR, and after reaching DFR, 4.7% had an early flare. Thus, SDFR was achieved in 14.4% of patients with PsA; 5.3% experienced a late flare, which occurred a median of 1.7 years (IQR, 1.4–2.8 years) after DMARD cessation. Eventually, 9.1% of patients reached true SDFR. Low baseline Disease Activity Index in Psoriatic Arthritis and never using biological or targeted

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synthetic DMARDs were independent predictors for true SDFR. At the time of true SDFR, the Health Assessment Questionnaire was similar to the general population (median, 0.12; IQR, 0–0.75).

Conclusions: DFR is attainable in oligoarticular/polyarticular PsA and is sustainable in 9% of patients. However, the subgroup of patients with PsA with high disease activity at baseline and who require biological or targeted synthetic DMARDs do not achieve true SDFR. To our knowledge, this is the first study to demonstrate that chronicity can potentially be influenced in a proportion of patients with PsA.

WHAT IS ALREADY KNOWN ON THIS TOPIC

- According to current management guidelines for psoriatic arthritis (PsA) tapering of disease-modifying antirheumatic drugs (DMARDs) can be considered if patients are in sustained remission, but limited data are available on complete withdrawal of DMARDs, also known as DMARD-free remission.

WHAT THIS STUDY ADDS

- DMARD-free remission is attainable in patients with oligoarticular/polyarticular PsA and is sustainable without flare in 9% of them.
- Patients with PsA who require a biological or targeted synthetic DMARD are very unlikely to achieve sustained DMARD-free remission.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE, OR POLICY

- Discontinuation of DMARDs is possible in a proportion of patients with oligoarticular/polyarticular PsA, especially in those patients with a less severe disease who do not require a biological or targeted synthetic DMARD.

INTRODUCTION

Clinical outcomes in patients with psoriatic arthritis (PsA) have improved substantially due to an increase in therapeutic options and the implementation of a treat-to-target management approach. As a result, ~13% to 42% of patients with PsA are able to reach remission after treatment [1–3]. If patients with PsA are in sustained remission, tapering of disease-modifying antirheumatic drugs (DMARDs) can be considered, according to current management guidelines [1,4]. However, it is unclear whether complete withdrawal of DMARDs while maintaining a state of remission, that is, disease-modifying antirheumatic drug-free remission (DFR), is achievable in patients with PsA.

Previous research in rheumatoid arthritis (RA) has shown that sustained remission for ≥ 1 year after DMARD cessation, known as sustained disease-modifying antirheumatic drug-free remission (SDFR), is achievable. A systematic review has demonstrated that SDFR can be achieved in ~10% to 20% of patients with RA [5]. Noteworthy is the fact that SDFR was accompanied by a normalised functional status and low fatigue and pain levels [6]. Therefore, if there are no flares, SDFR can be considered a proxy for cure, which implies that the chronicity of the disease may be reversible.

Whether SDFR is also achievable in PsA remains unknown. Several tapering studies have been conducted in PsA, but most of them considered tapering and discontinuation of biologic disease-modifying antirheumatic drugs (bDMARDs) [7–10]. Only limited previous research has investigated complete withdrawal

of DMARDs, and most studies showed that the majority of patients with PsA will experience a disease flare quickly after DMARD discontinuation [11–13]. In contrast, 1 study has shown that SDFR is achievable in PsA, although this is an older study in which bDMARDs had only just been approved for PsA [14]. This evidence contrasts with the perception of PsA being a chronic disease that requires life-long treatment. Despite the latter study, the frequency of SDFR and flares are still unknown, because there are currently no large longitudinal studies that have studied the disease trajectory from PsA diagnosis to SDFR, including the prevalence of flares.

In the treatment of patients with PsA, it is important to consider all disease manifestations, as the ultimate goal is low disease activity in all domains [4]. Thus, not only synovitis but also psoriasis, enthesitis, and dactylitis should be taken into account. A second important goal is to optimise functional status and quality of life [4]. Therefore, the impact of the disease should be measured regularly with patient-reported outcomes (PROs). As mentioned earlier, patients with RA in SDFR had normalised PROs. Whether this is also the case in patients with PsA is still unknown.

In addition, no data are available on predictors of SDFR in PsA. From the field of RA, it is known that patients without auto-antibodies and who never required a bDMARD have a higher chance of achieving SDFR [6,15]. In PsA, polyarthritis is a known poor prognostic factor, and bDMARDs are started in patients with an inadequate response to conventional synthetic disease-modifying antirheumatic drugs (csDMARDs) and/or poor prognostic factors, such as structural damage [1,4]. Thus, patients with PsA who present with polyarthritis and those requiring bDMARDs might be less likely to achieve SDFR compared with patients with oligoarthritis or those treated with csDMARDs only. Identifying predictors for SDFR in PsA is relevant as it may guide decisions on tapering and complete withdrawal of DMARDs. Not trying to taper and eventually discontinuing DMARDs may lead to unnecessary risks of long-term adverse events due to immunosuppression (ie, increased risk of infections and the possibility of malignancy development), increased health care costs, and potentially poorer adherence [1,16,17].

In order to increase the understanding of resolution of chronicity of PsA, we first aimed to determine the ability to achieve and sustain DFR, including the prevalence of flares after achieving (S)DFR. Only those patients who achieve SDFR and do not flare thereafter can truly be considered in SDFR, that is, true SDFR. Second, we explored the presence and activity of PsA disease manifestations at the time of achieving true SDFR, which were not included in the definition of SDFR. In addition, we aimed to better understand the impact of true SDFR on patients' lives by studying PROs at the time of achieving true SDFR. Finally, we evaluated possible predictors of true SDFR, including PsA phenotype and not requiring bDMARD treatment.

METHODS

Patient selection

For this study, all patients with PsA with oligoarthritis (2–4 swollen joints) or polyarthritis (≥ 5 swollen joints) who were consecutively included in the Dutch southwest Early Psoriatic Arthritis cohort (DEPAR) between July 2013 and March 2023 and who did not participate in a clinical trial were included. Patients with an oligoarthritis or polyarthritis were selected, because patients with this phenotype are always treated with a DMARD, while this is not always the case for the other PsA phenotypes.

DEPAR is a multicentre, prospective cohort study that was approved by the medical ethics committee of the Erasmus MC, the Netherlands (MEC-2012-549). Newly diagnosed patients with PsA, with no previous DMARD treatment for joint complaints and a clinical diagnosis according to the treating rheumatologist, were eligible for inclusion. Thus, they did not have to fulfil the classification criteria for psoriatic arthritis (CASPAR) [18]. Written informed consent was obtained from all participants, according to the tenets of the Declaration of Helsinki. Patients were regularly involved in research projects from DEPAR; however, patients and/or the public were not involved in the design, conduct, reporting, or dissemination plans of this specific study. Further details on the DEPAR study are described in [Supplementary File S1 \[19–21\]](#).

Data collection

Patients were assessed every 3 months during the first year, half-yearly in the second year, and annually thereafter. At each visit, a physical examination was performed, and blood samples, medication usage, and PROs were collected.

Research nurses performed the physical examination and assessed tender joint counts (TJC68) and swollen joint counts (SJC66). They also assessed whether or not psoriasis (body surface area [BSA], 0%–100%), enthesitis (Leeds Enthesitis Index, 0–6), and dactylitis were present [22,23]. Blood samples, including C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR), were also taken. Medication data consisted of all prescribed medication for PsA, which were attained from patients' health records. In addition, PROs measuring functional ability, general health, pain, and fatigue were collected. Functional ability was measured with the Health Assessment Questionnaire – Disability Index (HAQ-DI) [24–26]. General health and pain were measured on a 0.0 to 100.0 mm visual analogue scale [27]. Fatigue was measured with a numeric rating scale, ranging from 0 to 10 [28].

In addition to the prospectively collected data in DEPAR, medical records of patients were retrospectively checked for the occurrence of (S)DFR. This was chosen, because after the second year, study visits took place annually, and thus, there were insufficient data to reliably determine (S)DFR.

Treatment

Treatment initiation and alterations were made in shared decision between the patient and treating rheumatologist. There was no specific treatment protocol, but treatment was based on (inter)national guidelines [1,4]. In general, csDMARDs were the first choice, with a preference for methotrexate especially in patients with psoriasis. If treatment failed, a bDMARD or targeted synthetic disease-modifying antirheumatic drug

(tsDMARD) was started. Tapering and eventually complete withdrawal of DMARDs could be considered in patients with PsA who were in sustained remission.

Outcomes

DFR was defined as the absence of swollen joints, measured with SJC66, on physical examination after cessation of DMARDs, including glucocorticoids, for ≥ 3 months. A minimum period of 3 months was chosen to ensure that patients who stopped taking DMARDs for a short period, because of, for example, a medication switch or blood test abnormalities, were not classified as DFR. For SDFR, a period of >1 year without swollen joints and DMARDs was used. An SJC was used to define remission, because, from a rheumatologic perspective, synovitis is the main reason to start DMARD treatment in patients with PsA with oligoarthritis/polyarthritis. This was also illustrated by the fact that other common PsA manifestations, that is, psoriasis and enthesitis, were often not reported in the patients' health records. The choice for a strict remission criterium (SJC66 = 0) was made, because treatment in PsA should be aimed at the abrogation of inflammation [1].

Early and late flares were defined as having ≥ 1 swollen joint on physical examination or restarting DMARD treatment for other reasons, including psoriasis, ≤ 1 and >1 year after DMARD cessation, respectively. The reasons for restarting DMARD treatment were obtained from patients' health records. A joint-based definition of flares was chosen instead of a PsA-specific composite disease activity measure (such as the Disease Activity Index in Psoriatic Arthritis [DAPSA]), because in clinical practice the decision to restart DMARD treatment was often not made on the basis of the DAPSA score and, therefore, not all components of the DAPSA were measured at each outpatient visit that took place outside the DEPAR study visits.

Patients with PsA who were in SDFR and did not experience a flare during the entire follow-up, were referred as true SDFR in this study. True SDFR will be used as the main outcome throughout this study, since patients who flared after achieving SDFR were not truly in persistent SDFR. Follow-up ended if patients stopped participating in the DEPAR cohort or if they were referred back to their general practitioner.

Since the definition of true SDFR was solely based on the absence of swollen joints, we explored multiple other disease characteristics at the time of achieving SDFR, including tender joint count (TJC); the presence of psoriasis, enthesitis, and dactylitis; and inflammatory blood markers. In addition, the impact of achieving true SDFR on patients' lives during follow-up was studied by plotting PROs over time of patients who did and did not achieve true SDFR.

We also investigated possible predictors for true SDFR, including demographics, disease activity, and PROs. Disease activity was measured with the DAPSA [29–31]. If CRP was missing, the clinical DAPSA was reported, which is the DAPSA without CRP. The clinical DAPSA has shown to have very good agreement with the DAPSA [32].

Statistical analysis

Descriptive statistics were used to report DFR, SDFR, true SDFR, and flare prevalences. The prevalences were calculated as the number of patients having the outcome divided by the total study population ($N = 451$). Thereafter, a Kaplan–Meier curve was used to evaluate the cumulative incidence of true SDFR. In addition, we evaluated whether patients with polyarticular PsA

and those using a bDMARD/tsDMARD were less likely to achieve true SDFR. Polyarthritis is a poor prognostic factor, and bDMARDs/tsDMARDs are given in patients with an inadequate response to csDMARDs and/or poor prognostic factors such as structural damage [1,4]. Kaplan–Meier curves were constructed with the following grouping factors: (1) phenotype (oligoarticular vs polyarticular PsA) and (2) bDMARD or tsDMARD usage (ever vs never). Differences in time-to-event functions between the groups were tested with log-rank tests.

We also aimed to find possible baseline predictors for true SDFR. First, we compared baseline characteristics between patients who did and did not reach true SDFR using logistic regression models. Included variables were age, sex, PsA symptom duration, body mass index, smoking behaviour, DAPSA and its components, presence and severity of psoriasis, presence and severity of enthesitis, presence of dactylitis, ESR, HAQ-DI, and fatigue. Univariately associated variables were entered in a multivariate logistic regression model with a backwards selection procedure to identify independent predictors of true SDFR.

All analyses were performed in Stata V18.0. *P* values ≤0.05 were considered statistically significant, except in the univariate analysis, where a *P* value of <.10 was used.

RESULTS

Patients

Until March 2023, 934 patients with PsA were included in DEPAR of whom 368 were excluded because they had a monoarthritis, enthesitis, dactylitis, or axial disease as main clinical symptom. An additional 36 and 79 patients were excluded because they did not receive a DMARD or were not treated in routine care but participated in a randomised controlled trial. Thus, 451 patients with PsA with an oligoarthritis (*n* = 265) or polyarthritis (*n* = 186) were studied (Supplementary Fig S1). Besides arthritis as their main presenting symptom, patients could also have other PsA manifestations, that is, psoriasis, enthesitis, dactylitis, and axial disease. At baseline, that is, the time of PsA diagnosis, 49% was female, the mean (SD) age was 52 (14) years, and median symptom duration (IQR) was 8.7 (4–30) months. The mean (SD) DAPSA was 21.6 (11.7). An active psoriasis and enthesitis were observed in 84% and 39% of patients, respectively (Table 1).

Prevalence of (S)DFR and flares

Included patients had a median (IQR) follow-up time in DEPAR of 5.1 (3.0–7.3) years. Of the 451 (100%) included patients with PsA, 99 (22%) achieved DFR. Subsequently, 21 (4.7%) patients had an early flare, and in 13 (2.9%) patients, we could not ascertain SDFR status due to insufficient follow-up time after achieving DFR (Supplementary Fig S1). Thus, 65 (14.4%) patients achieved SDFR. After achieving SDFR, 24 (5.3%) patients with PsA experienced a late flare. The median (IQR) time to a late flare after DMARD cessation was 1.7 (1.4–2.8) years. A total of 41 (9.1%) patients with PsA maintained in SDFR without flaring and were thus in true SDFR (Fig 1). The median (IQR) time to true SDFR was 3.2 (1.9–4.6) years, and patients subsequently had a median (IQR) follow-up time of 1.3 (0.3–2.9) years after SDFR achievement, that is, 2.3 (1.3–3.9) years after DMARD stoppage. Thus, patients remained in remission for a median of 2.3 years after DMARD cessation. Figure 2A shows the cumulative incidence of true SDFR in the total study population.

Table 1
Characteristics of total study population at PsA diagnosis

	Total study population (N = 451)
Demographic characteristics	
Age (y), mean (SD)	52.1 (13.9)
Sex (female)	220 (49)
PsA symptom duration (mo)	8.7 (4–30)
Body mass index (kg/m ²), mean (SD)	28.4 (4.9)
Current smoker	89 (23)
Disease activity	
DAPSA ^a , mean (SD)	21.6 (11.7)
DAPSA remission	6 (2)
DAPSA low disease activity	99 (26)
Swollen joint count (66)	3 (2–6)
Tender joint count (68)	5 (2–9)
Psoriasis	378 (84)
BSA (%) in case of psoriasis	3 (1–5)
Enthesitis	175 (39)
LEI in case of enthesitis	2 (1–3)
Dactylitis	72 (16)
Chronic back pain <45 y	149 (33)
CRP (mg/L)	5.0 (1–13)
ESR (mm/h)	13 (5–27)
Patient-reported outcomes	
HAQ-DI (0–3)	0.75 (0.38–1.12)
General health (VAS, 0.0–100.0 mm)	49 (27–69)
Pain (VAS, 0.0–100.0 mm)	52 (27–71)
Fatigue (NRS, 0–10)	5 (3–7)
Treatment initiated at time of diagnosis^b	
MTX monotherapy	311 (69)
HCQ/SASP/LEF/cyclosporine monotherapy	25 (6)
csDMARD combination	46 (10)
b/tsDMARD monotherapy	4 (1)
b/tsDMARD + csDMARD	18 (4)
No DMARDs	47 (10)
Glucocorticoid bridging	66 (15)
DMARD use before inclusion ^c	22 (5)

Values are shown as median (IQR) or *n* (%) unless indicated otherwise.

BSA, body surface area; b/tsDMARD, biological/targeted synthetic disease-modifying antirheumatic drug; csDMARD, conventional synthetic disease-modifying antirheumatic drug; CRP, C-reactive protein; DAPSA, Disease Activity Index in Psoriatic Arthritis; DMARD, disease-modifying antirheumatic drug; ESR, erythrocyte sedimentation rate; HAQ-DI, Health Assessment Questionnaire – Disability Index; HCQ, hydroxychloroquine; LEF, leflunomide; LEI, Leeds Enthesitis Index; MTX, methotrexate; NRS, numeric rating scale; SASP, sulphasalazine; VAS, visual analogue scale.

^a If DAPSA was missing, cDAPSA was used, which was the case in 32 patients.

^b Of the 22 patients who were prescribed a b/tsDMARD at baseline, *n* = 16 (73%) were using a tumour necrosis factor inhibitor, *n* = 1 (5%) an IL-17 inhibitor, *n* = 4 (18%) an IL-12/23 inhibitor, and *n* = 1 (5%) a PDE4 inhibitor.

^c Patients were allowed previous DMARD usage before inclusion in DEPAR for the treatment of psoriasis, but not for musculoskeletal complaints.

Treatment

In the majority of patients (69%), methotrexate monotherapy was started at diagnosis. Only 5% of patients immediately started with a b/tsDMARD, and 10% of patients were not prescribed a DMARD at diagnosis (Table 1). The treatment history of patients during follow-up in DEPAR can be found in Supplementary Table S1. Patients who achieved true SDFR were more often treated with methotrexate monotherapy and required b/tsDMARDs less frequently during follow-up compared with patients who did not achieve true SDFR.

Of the patients who achieved true SDFR (*n* = 41), 18 (44%) tapered their medication, while 23 (56%) stopped their DMARD treatment without tapering. For patients who

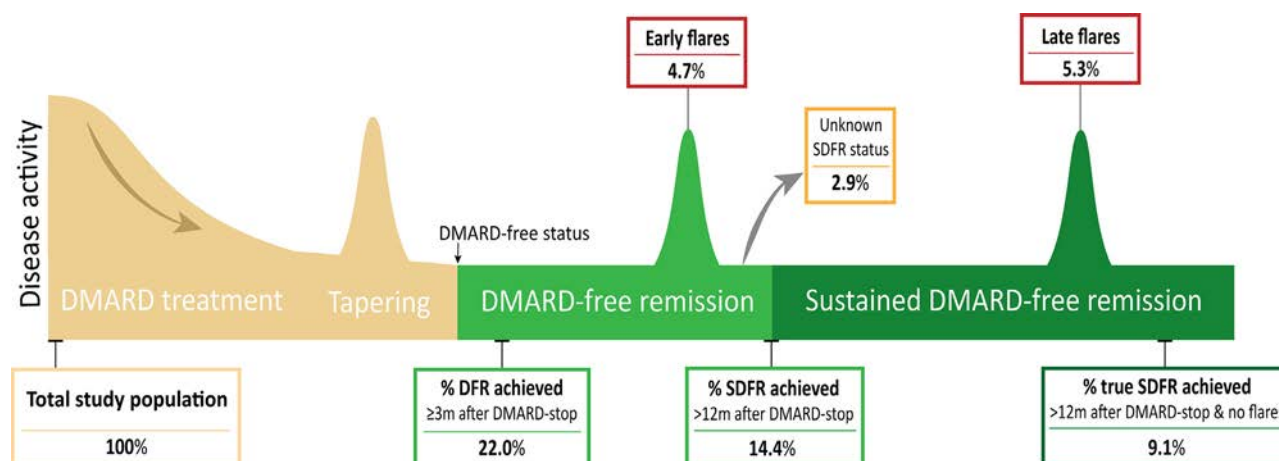


Figure 1. Schematic overview of (S)DFR and flare prevalences, in all patients with PsA with an oligoarthritis or polyarthritis who participated in the DEPAR study, depicted on a timeline. This figure shows a schematic overview of the disease trajectory of included patients with PsA. After starting DMARD treatment, disease activity decreases and a proportion of patients achieve remission, after which treatment can be tapered. During tapering, some patients will experience a flare, while others remain in remission. In DEPAR, 22% of the total study population achieved DMARD-free remission. After achieving DFR, 4.7% of the total study population experienced a flare (early flare) and in 2.9%, there was insufficient information to determine the SDFR status. A proportion of patients (14.4%) remained in remission for >1 year without DMARDs and thus achieved SDFR. After achieving SDFR, 5.3% of the total study population flared, and thus, 9.1% of the total study population was able to achieve true SDFR. Percentages were calculated as the number of patients having the outcome divided by the total study population ($n=451$). Patient had an early flare if they flared ≤ 1 year after achieving DMARD-free status, while patients had a late flare if they flared >1 year after achieving DMARD-free status. Figure was adapted from Verstappen et al. [5] with permission from the authors. DEPAR, Dutch southwest early psoriatic arthritis cohort; DFR, disease-modifying antirheumatic drug-free remission; DMARD, disease-modifying antirheumatic drug; SDFR, sustained DMARD-free remission.

tapered, the median time between inclusion and start of tapering was 10 months (IQR, 6–26 months). For the 41 patients who achieved true SDFR, the median time from inclusion to complete withdrawal of DMARDs was 26 months (IQR, 10–43 months).

Of the patients who did not achieve true SDFR ($n = 410$), 199 (49%) tapered ≥ 1 DMARDs during the study period due to remission. The median time from inclusion to start of tapering was 18 months (IQR, 10–35 months). A total of 114 (28%) patients who did not achieve true SDFR tapered for other reasons, including adverse events, and 97 (24%) did not taper their medication.

Disease symptoms and PROs of patients in true SDFR at the time of true SDFR achievement

True SDFR was defined as the absence of clinical synovitis and did not include other PsA manifestations. To ensure the validity of our definition, we explored other PsA features and PROs at the time of achieving SDFR. At the time of true SDFR achievement, patients with PsA had a median (IQR) TJC of 0 (0–1); 64% of patients had psoriasis, with a median (IQR) BSA of 2% (1%–4%), and 14% had enthesitis, compared with 80% with psoriasis and 34% with enthesitis at baseline (Tables 2 and 3). Inflammatory blood markers were at low levels (Table 2). Thus, both swollen and tender joints were absent, and there was a decrease in the number of patients with active psoriasis and enthesitis at the time of true SDFR achievement.

In addition, PRO scores had improved significantly, for example, the median (IQR) HAQ-DI was 0.62 (0.25–0.88) at diagnosis and 0.12 (0–0.75) at the time of true SDFR (Tables 2 and 3). The HAQ-DI score at true SDFR achievement was close to the estimated population mean in the same age group (50–54 years), which was 0.16 in women and 0.13 in men [33]. The median

(IQR) general health decreased from 34 (23–53) to 11 (2–35), and median pain (IQR) decreased from 30 (16–64) to 9 (2–37) (Tables 2 and 3).

PROs of patients with and without true SDFR over time

Boxplots of the HAQ-DI, general health, pain, and fatigue were used to compare aforementioned PROs over multiple years in patients who did and did not achieve true SDFR (Fig 3). The largest improvement in PROs was seen in the first year after diagnosis in all patients with PsA. Patients who achieved true SDFR had better PROs than patients who did not achieve true SDFR. The largest difference between those who did and did not achieve true SDFR was visible in the HAQ-DI (Fig 3).

Predictors of true SDFR

The following variables at PsA diagnosis were univariately associated with achieving true SDFR: DAPSA, TJC, BSA affected by psoriasis, HAQ-DI, general health, and pain (Table 3). In the multivariate analysis, only a lower DAPSA was associated with a higher chance of true SDFR (odds ratio, 0.94; 95% CI, 0.91–0.99; $P = .008$).

In addition, we evaluated whether patients with polyarticular PsA and those using bDMARDs or tsDMARDs were less likely to achieve true SDFR, which was visualised with Kaplan–Meier curves (Fig 2B, C). The estimate comparing patients with oligoarticular and polyarticular PsA indicated a 48% increased chance of achieving true SDFR in polyarticular PsA, but CIs were wide (hazards ratio, 1.48; 95% CI, 0.80–2.75; $P = .21$). Patients with PsA who ever used a bDMARD or tsDMARD had a significantly lower chance at achieving true SDFR compared with those who never used a bDMARD or tsDMARD (hazards ratio, 0.17; 95% CI, 0.08–0.39; $P < .001$). Further, 202 (45%) of

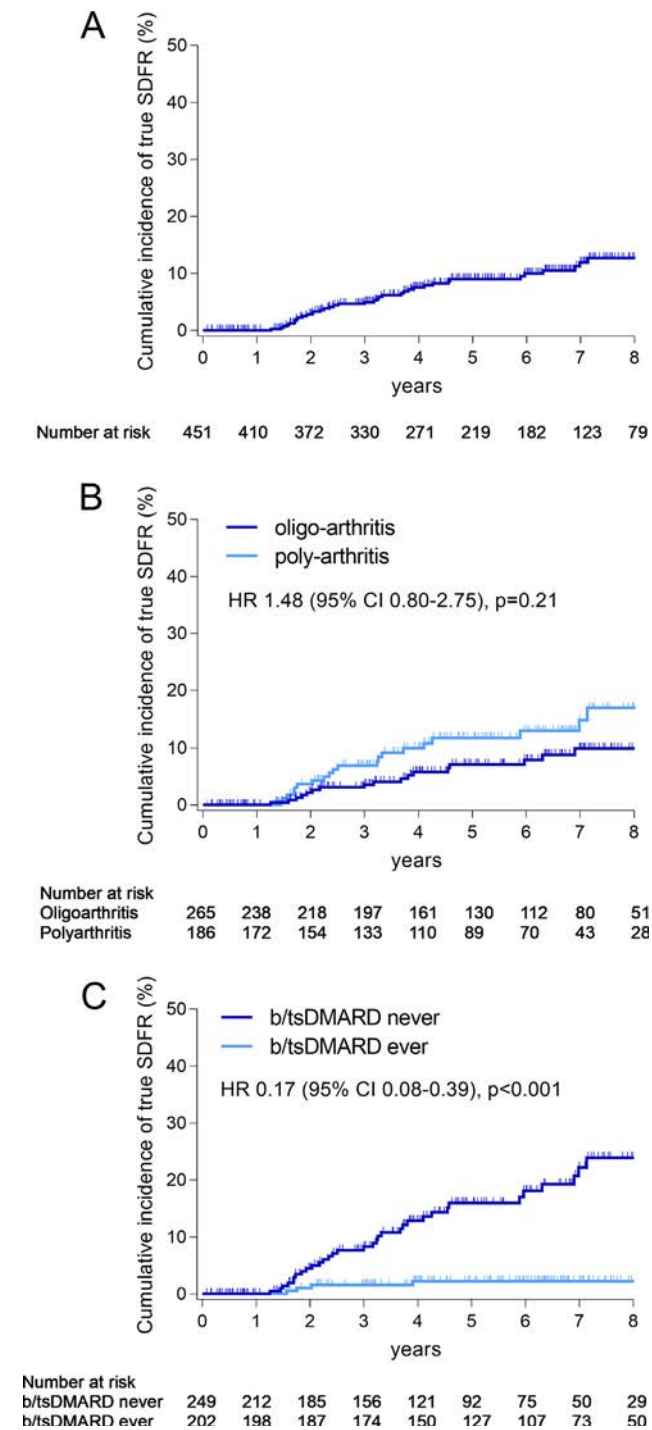


Figure 2. Kaplan–Meier curves for true SDFR development. Time-to-event was defined as time from baseline until true SDFR (yes/no), that is, the absence of clinical arthritis for a minimum of 1 year after DMARD cessation and not having a flare thereafter. Ticks shows where patients were censored. (A), Cumulative incidence of true SDFR in the total study population. (B), Cumulative incidence of true SDFR stratified for oligo- and polyarthritis patients. (C), Cumulative incidence of true SDFR stratified for b/tsDMARD use. Besides the grouping variables, diagnosis (oligoarthritis vs polyarthritis) and treatment (csDMARDs vs b/tsDMARDs) respectively, no other variables were included in the model. b/tsDMARD, biological/targeted synthetic disease-modifying antirheumatic drug; csDMARD, conventional synthetic disease-modifying antirheumatic drug; HR, hazard ratio; SDFR, sustained disease-modifying antirheumatic drug–free remission.

the included patients with PsA were prescribed a bDMARD or tsDMARD, which was started at a median (IQR) of 1 (0.5–2.3) year after inclusion in DEPAR.

Table 2
Disease characteristics of patients with PsA in true SDFR at the time of true SDFR achievement

	True SDFR (n = 30) ^a
Swollen joint count (0–66)	0 (0–0)
Tender joint count (0–68)	0 (0–1)
Psoriasis	18 (64)
BSA (%) in case of psoriasis	2 (1–4)
Enthesitis	4 (14)
LEI in case of enthesitis	3 (3–4)
Dactylitis	1 (4)
Elevated CRP ^b	4 (17)
ESR (mm/h)	7 (5–16)
HAQ-DI (0–3)	0.12 (0–0.75)
General health (VAS, 0.0–100.0 mm)	11 (2–35)
Pain (VAS, 0.0–100.0 mm)	9 (2–37)
Fatigue (NRS, 0–10)	4 (1–6)

Values are shown as median (IQR) or n (%).
BSA, Body Surface Area; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; HAQ-DI, Health Assessment Questionnaire – Disability Index; LEI, Leeds Enthesitis Index; PsA, psoriatic arthritis; SDFR, sustained disease-modifying antirheumatic drug–free remission; VAS, visual analogue scale.
^a For n = 11 patients in true SDFR, no data were available at the time of true SDFR achievement.
^b Elevated CRP was defined as a value above the cut-off level from each specific hospital laboratory.

DISCUSSION

It is currently unclear whether DFR can be achieved and sustained in patients with PsA. This study showed that 22% of patients with oligoarticular/polyarticular PsA can achieve DFR, while 14.4% attained SDFR (DFR > 1 year). Notably, both early (≤1 year) and late (>1 year) flares were common, resulting in 9.1% of patients with PsA reaching true SDFR. There was no significant difference in the prevalence of true SDFR between patients with oligoarticular PsA and those with polyarticular PsA. However, true SDFR was significantly less common in patients treated with bDMARDs or tsDMARDs compared with those treated with csDMARDs alone. In addition, higher baseline disease activity was inversely associated with true SDFR.

The 2023 guideline of the European Alliance of Associations for Rheumatology stated that tapering of DMARDs may be considered in patients with PsA who are in sustained remission, but advised against DMARD cessation [1]. Our study has shown that DFR is achievable in a subgroup of patients with oligoarticular and polyarticular PsA and can be sustained for >1 year without flare. Thus, our data are consistent with the European Alliance of Associations for Rheumatology guideline regarding the tapering of DMARDs but contradict the advice against DMARD discontinuation. This recommendation, however, is based on limited data [10,34,35]. In addition, the included studies mostly focused on tapering of bDMARDs, csDMARDs were not always completely withdrawn, the number of included patients was small, and follow-up was short. Conversely, we showed that DFR is achievable in patients with PsA with mild disease, who do not need b/tsDMARDs.

Unfortunately, it is currently unknown which patients can achieve true SDFR. Therefore, we tried to identify predictors for true SDFR. We found that patients with more severe disease, that is, those with higher DAPSA at baseline and those who required bDMARD or tDMARD treatment, were less likely to achieve true SDFR. While this provides some insights, it remains

Table 3

Characteristics of patients with PsA at diagnosis, stratified for the achievement of true SDFR, compared by univariate and multivariate analyses

	No true SDFR (n = 410)	True SDFR (n = 41)	OR (95% CI)
Demographic characteristics			
Age (y), mean (SD)	52.0 (13.8)	52.8 (15.1)	1.00 (0.98-1.03)
Sex (female)	197 (48)	23 (56)	1.38 (0.72-2.64)
Symptom duration (mo)	8.8 (4-29)	6.3 (3-34)	1.00 (1.00-1.01)
Body mass index (kg/m ²), mean (SD)	28.5 (4.9)	27.3 (5.1)	0.95 (0.89-1.02)
Current smoker	81 (23)	8 (22)	0.96 (0.42-2.19)
Disease activity			
DAPSA ^a , mean (SD)	22.1 (11.8)	16.8 (9.2)	0.95 (0.92-0.99) ^{b,c}
Swollen joint count (66)	3 (2-6)	3 (0-5)	0.93 (0.85-1.02)
Tender joint count (68)	5 (2-9)	3 (1-6)	0.93 (0.86-1.00) ^b
Psoriasis	345 (84)	33 (80)	0.77 (0.34-1.73)
BSA (%) in case of psoriasis	3 (1-5)	2 (1-4)	0.89 (0.78-1.02) ^b
Enthesitis	161 (39)	14 (34)	0.80 (0.41-1.58)
LEI in case of enthesitis	2 (1-3)	2 (1-2)	0.94 (0.61-1.45)
Dactylitis	68 (17)	4 (10)	0.54 (0.19-1.57)
CRP (mg/L)	5.1 (1-13)	3.0 (0-7)	0.98 (0.95-1.01)
ESR (mm/h)	13 (5-28)	11 (6-22)	0.99 (0.97-1.01)
Patient-reported outcomes			
HAQ-DI (0-3)	0.75 (0.38-1.12)	0.62 (0.25-0.88)	0.45 (0.22-0.93) ^b
General health (VAS, 0.0-100.0 mm)	49 (28-70)	34 (23-53)	0.98 (0.97-0.99) ^b
Pain (VAS, 0.0-100.0 mm)	52 (30-72)	30 (16-64)	0.98 (0.97-1.00) ^b
Fatigue (NRS, 0-10)	5 (3-7)	5 (3-7)	0.99 (0.87-1.12)

Values are shown as median (IQR) or n (%) unless indicated otherwise.

BSA, body surface area; CRP, C-reactive protein; DAPSA, Disease Activity Index in Psoriatic Arthritis; ESR, erythrocyte sedimentation rate; HAQ-DI, Health Assessment Questionnaire – Disability Index; LEI, Leeds Enthesitis Index; NRS, numeric rating scale; OR, odds ratio; SDFR, sustained disease-modifying antirheumatic drug-free remission; VAS, visual analogue scale.

^a If DAPSA was missing, cDAPSA was used, which was the case in 29 patients who did not reach true SDFR and in 3 patients who reached true SDFR.

^b $P < .10$ in univariate analysis.

^c Statistically significant value ($P < .05$) in multivariate analysis.

a trial-and-error process to find out in which patients DMARDs can be tapered and eventually stopped.

Contrary to PsA, DFR and its sustainability have been studied more extensively in RA. Research has shown that 5% to 24% of patients with RA can achieve DFR, while 12% to 19% can achieve SDFR without flaring during follow-up [5,6,36,37]. Notably, the prevalence of SDFR differed between patients with anticitrullinated protein antibody (ACPA)-positive RA (18%) and those with ACPA-negative (37%) RA [36]. Especially in patients with ACPA-negative RA who responded early to treatment, SDFR prevalence was high (70%) [38]. Interestingly, in our study, a higher disease activity at baseline was negatively associated with true SDFR development, which was also found in RA [5,37]. In addition, a recent study has shown that 0% of patients with RA who ever used a bDMARD, indicative of more severe disease, reached SDFR, while 15% to 37% of those who never used bDMARDs achieved SDFR [39]. Similar to patients with RA, patients with PsA who ever used a bDMARD or tsDMARD were less likely to achieve true SDFR than those who never used a bDMARD or tsDMARD. The proportion of patients with PsA using a bDMARD or tsDMARD in DEPAR (45%) is higher than that in the Dutch population with RA, which could explain the lower incidence of true SDFR in our PsA population compared with that in the RA literature [40].

In our study, SDFR was defined from a rheumatologic perspective, that is, as the absence of clinical synovitis. To verify whether true SDFR was also a relevant outcome from the patient perspective, we evaluated the disease impact at the time of SDFR achievement. At the time of true SDFR achievement, the HAQ-DI was similar to the general population [33]. In addition, general health and pain scores had improved significantly compared with those at baseline and were low (11 and 9,

respectively). This indicates that true SDFR reflects a health state with a very low disease impact, which is comparable with the general population in terms of functioning.

True SDFR is an important outcome since this state could be seen as a proxy for cure [5,6,41]. However, this is difficult to align with the current view of PsA as a chronic disease, and the mechanisms underlying true SDFR achievement are currently unknown. More research has been done in RA, and 2 hypotheses have been formulated in this regard. The first hypothesis is based on the concept that multiple biological markers cause the loss of immune tolerance, leading to RA development, and that the loss of these biological markers could reverse this process, resulting in the regaining of immune tolerance. The second hypothesis states that patients with RA who achieve SDFR have favourable disease characteristics and thus constitute an intrinsically different subgroup from those who do not achieve SDFR [42]. In autoantibody-negative RA, the subgroup hypothesis seems most likely, as patients who achieve SDFR are characterised by a strong clinical, serologic, and magnetic resonance imaging-detected response to DMARD treatment [42]. The latter scenario is also more likely to apply to PsA because there are no known autoantibodies in PsA. Moreover, most patients with PsA experience psoriasis for years, implying that they already had a chronic inflammatory disease (long) before the development of arthritis, making the first hypothesis less likely [43].

Although we defined SDFR as the absence of swollen joints, peripheral arthritis is only one of the domains affected in patients with PsA [4]. The choice for our SDFR definition was based on the fact that for rheumatologists synovitis is the main reason to start DMARD treatment in patients with PsA with oligoarthritis/polyarthritis. Needless to say, when treating patients with PsA, other affected domains, such as enthesitis and

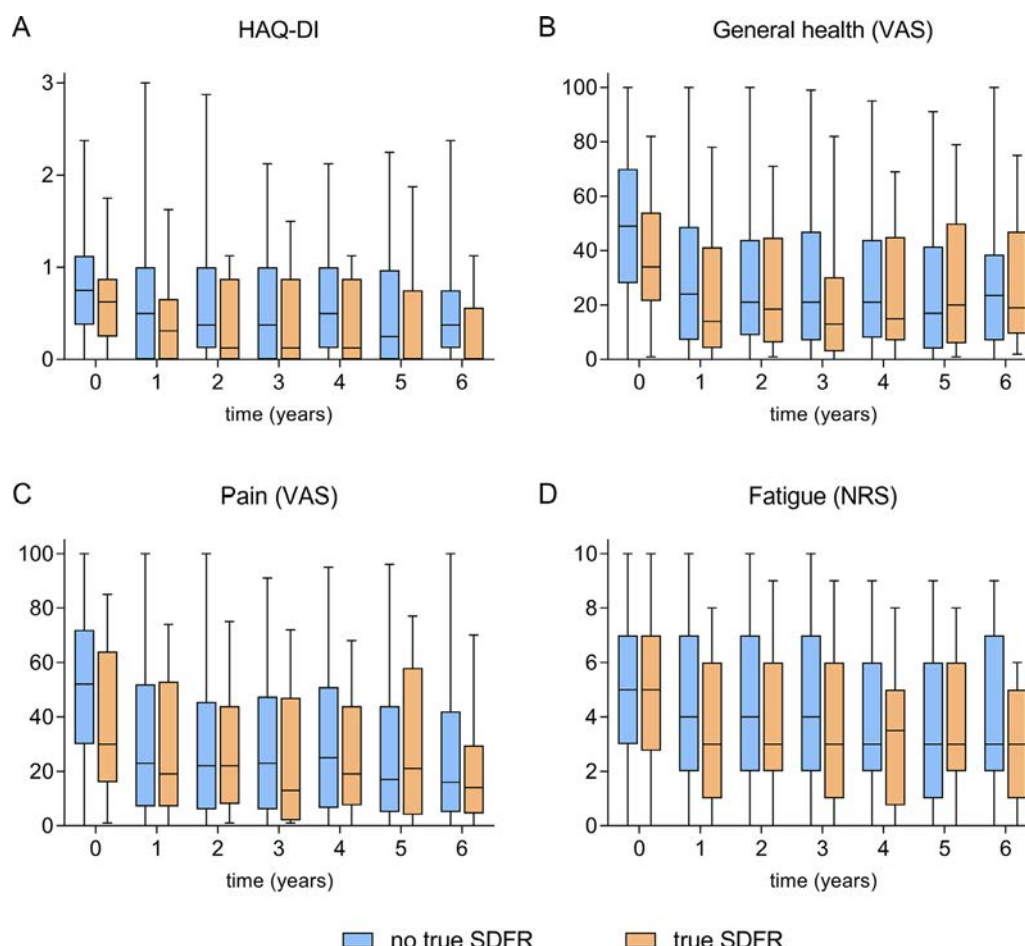


Figure 3. Patient-reported outcomes over time for patients with PsA with and without true SDFR. Boxplots of (A) HAQ-DI, (B) general health, (C) pain, and (D) fatigue in patients who did or did not achieve true SDFR are presented. HAQ-DI, Health Assessment Questionnaire – Disability Index; NRS, numeric rating scale; PsA, psoriatic arthritis; SDFR, sustained disease-modifying antirheumatic drug–free remission; VAS, visual analogue scale.

psoriasis, should also be properly treated. From the patients who achieved true SDFR, only 14% had enthesitis at the time of true SDFR achievement. Of note, enthesitis is known to be difficult to assess by physical examination [4,44,45]. On the contrary, the majority of patients still had psoriasis, but only 2% of their BSA was affected. This is less than the 3% that is used in the definition of minimal disease activity in PsA [46]. Thus, apart from the absence of swollen joints, the other PsA features were also well-controlled at the time of true SDFR achievement.

Not all patients had data available at the time of true SDFR achievement, because they declined their research visits or were referred back to their general practitioner. These patients most likely had very little or no PsA-related symptoms, leading them to refuse further participation in DEPAR. This would mean that we have reported the disease characteristics of a selection of patients who are worst off, indicating that the disease activity might even be lower at the time of true SDFR achievement.

A limitation of our study is the follow-up duration. Patients had a median follow-up time in DEPAR of 5.1 years. Patients with PsA who achieved true SDFR sustained their DFR status over multiple years (median, 2.3 years), after which most stopped participating in DEPAR. As follow-up time increases, the incidence of late flares may increase, but the incidence of true SDFR may also increase further. Although this is a DFR study with a unique follow-up duration of 5 years, future studies with longer follow-up are needed to further understand the sustainability of DFR in PsA. In addition, our results cannot be generalised to all patients with PsA, since we selected patients with

an oligoarthritis or polyarthritis as main presenting symptom for this study.

A strength of this study is the use of a large real-world PsA cohort. Treatment was not protocolised, which means that the data are representative of normal clinical practice. In addition, we used a stringent SDFR definition, in which patients had to remain in DFR for at least 1 year, and only those who did not flare during follow-up were considered to be in true SDFR.

In conclusion, about 1 in 10 patients with oligoarticular/polyarticular PsA can achieve true SDFR. Patients with PsA who achieve true SDFR have a low self-reported disease impact. The results further indicate that a subgroup of patients with PsA, especially those who do not require bDMARDs or tsDMARDs, is able to achieve drug-free remission and sustain this status over multiple years.

Competing interests

All authors declare they have no competing interests.

CRediT authorship contribution statement

Selinde V.J. Snoeck Henkemans: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Marijn Vis:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Conceptualization. **Gonul Hazal Koc:** Writing – review & editing, Data curation. **Jolanda J. Luime:** Writing – review & editing, Funding acquisition, Data curation.

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Patient consent for publication

Written informed consent was obtained from all participants according to the tenets of the Declaration of Helsinki.

Ethics approval

The study was approved by the local medical research ethics committee of Erasmus Medical Center, Rotterdam, Netherlands (MEC-2012-549).

Provenance and peer review

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Data are available from the corresponding author upon reasonable request.

Supplementary materials

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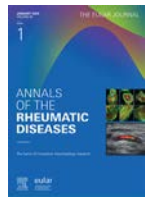
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Axial spondyloarthritis

Remote monitoring or patient-initiated care in axial spondyloarthritis: a 3-armed randomised controlled noninferiority trial

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ABSTRACT

Objectives: This study aimed to determine whether novel follow-up regimen, remote monitoring, or patient-initiated care is noninferior to usual care in maintaining low disease activity, in patients with axial spondyloarthritis (axSpA).

Methods: This is a randomised, controlled, 3-armed, parallel-group, open-label, noninferiority trial. Patients with axSpA in low disease activity on stable treatment with tumour necrosis factor inhibitor were recruited from a Norwegian outpatient clinic. Patients were randomly allocated 1:1:1 to remote monitoring, patient-initiated care, or usual care (control group), with 18 months of follow-up. Primary outcome was mean probability of axSpA Disease Activity Score (ASDAS) of <2.1, compared between groups at 6, 12, and 18 months, with 15% noninferiority margin. Secondary outcomes included other measures of disease activity, physical function, patient satisfaction, change of medication, resource use, and adverse events.

Results: Of 243 patients enrolled patients, 235 completed the study (remote monitoring = 75, patient-initiated care = 79, usual care = 81). At the 6-month, 12-month, and 18-month assessments, 90% or more patients in all 3 groups had ASDAS of <2.1. The estimated difference of probability of ASDAS < 2.1 was as follows: usual care vs remote monitoring, -4.1% (97.5% CI, -9.9% to 1.8%); usual care vs patient-initiated care, -1.1% (97.5% CI, -7.2% to 4.9%); and remote

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monitoring vs patient-initiated care, 2.9% (95% CI, -1.5% to 7.4%). Health providers' resource use was lowest in patient-initiated care; other secondary outcomes were comparable.

Conclusions: In patients with axSpA in low disease activity and on stable treatment with tumour necrosis factor inhibitor, follow-up with remote monitoring or patient-initiated care was noninferior to usual care in maintaining low disease activity, supporting the implementation of novel follow-up strategies.

WHAT IS ALREADY KNOWN ON THIS TOPIC

- Follow-up of patients with chronic diseases, traditionally offered as regular prescheduled face-to-face visits, is time-consuming for patients and resource-demanding for the health care services.
- Novel follow-up strategies such as remote monitoring or patient-initiated care may allow for targeted and efficient use of health care resources, but the knowledge is limited on the effectiveness of these follow-up regimen.

WHAT THIS STUDY ADDS

- Both remote monitoring and patient-initiated follow-up were noninferior to usual care in maintaining low disease activity in patients with axial spondyloarthritis.
- Patient-initiated care was the least resource-intensive follow-up for health care providers.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- Patients with axial spondyloarthritis in low disease activity can be managed either by remote monitoring or patient-initiated care as alternatives to usual care with prescheduled follow-up.

INTRODUCTION

Patients with chronic diseases receiving follow-up in specialist health care services usually have regular prescheduled face-to-face visits. Such follow-up is time-consuming for patients and resource-demanding for the health care services. Digital health technologies enable novel follow-up strategies such as remote monitoring of patient-reported outcomes (PROs) and sensor data [1,2], which may identify patients who need assessments or adjustments of treatment [3]. Patient-initiated care represents another alternative follow-up strategy, where patients are instructed to contact their health care provider if symptoms increase or adverse events occur [4]. Thus, both remote monitoring and patient-initiated care may represent more targeted and efficient use of health care resources, compared with regular prescheduled follow-up. However, the effectiveness and safety of such novel strategies should be evaluated before implementation [5].

Axial spondyloarthritis (axSpA) is a chronic inflammatory joint disease with a prevalence of 0.3% to 1.4% and onset typically in the third decade of life [6]. The disease is characterised by inflammation of sacroiliac joints and spine, causing back pain and stiffness and may result in structural bone changes that reduce spinal mobility [6,7]. First-line medical treatment are nonsteroidal anti-inflammatory drugs (NSAIDs). Some patients progress to disease-modifying antirheumatic drugs, usually tumour necrosis factor inhibitors (TNFi), and receive long-term follow-up in the specialist health care with assessments of treatment efficacy [7–9]. Monitoring of disease activity at least twice a year under supervision of a rheumatologist is recommended [10]. Patients are usually offered regular prescheduled visits, but with an opportunity to request additional visits if they

experience worsening. Research has shown that rheumatologists considered 34% of the prescheduled visits for patients with axSpA to be unnecessary [11] and that patients with axSpA were interested in alternative follow-up strategies [12,13]. However, only an observational study and a recently published randomised controlled trial (RCT) on remote monitoring of patients with axSpA have been conducted, indicating favourable effects of remote monitoring [14,15]. Thus, more research on the effectiveness, user satisfaction, safety, and resource utilisation of novel follow-up strategies in axSpA is needed [16].

This study assessed whether either remote monitoring or patient-initiated care were noninferior to usual care follow-up in maintaining low disease activity or inactive disease over 18 months in patients with axSpA. Secondary objectives included evaluation of other measures of disease activity, self-reported physical function, patient satisfaction, change of medication, resource use, and adverse events in the 2 novel follow-up strategies, compared with usual care over time.

METHODS

Study design and participants

The Remote Monitoring in Axial Spondyloarthritis (ReMonit) study was a single-centre, 3-armed, parallel-group, randomised, controlled, open-label trial that evaluated the noninferiority of 2 different follow-up strategies, compared with usual care follow-up over 18 months, for patients with axSpA (NCT050317) [17]. The study was conducted at a single outpatient rheumatology clinic in a Norwegian hospital and in compliance with the tenets of the Declaration of Helsinki and the International Conference on Harmonisation Guidelines for Good Clinical Practice [18].

Two patient research partners from the REMEDY patient advisory board participated as part of the study organisation (SH and TG). The patient research partners provided input on the relevance of the research question, the feasibility of the study design and study logistics from a patient perspective, and written information to patients. They participated in meetings with the study organisation throughout the study, and 1 (SH) is a coauthor of this manuscript.

The study protocol (Supplementary File S1) was approved by the Regional Committee for Medical Research Ethics South East Norway (REC south-east 229187). All patients provided a written informed consent before inclusion. The study was conducted and analysed according to the protocol and statistical analyses plan (Supplementary Files S1 and S2).

Eligible patients were adults (>18 years) with axSpA fulfilling the Assessment of SpondyloArthritis International Society criteria [19]. All patients were required to have used a stable dose of TNFi for the past 6 months and have low disease activity or inactive disease, defined as Axial Spondyloarthritis Disease Activity Score (ASDAS) of <2.1 [20–23]. Full inclusion and exclusion criteria are listed in Supplementary File S3. The patients were recruited by rheumatologists during outpatient visits. Baseline data collection included demographics, e-health

literacy [24], lifestyle, disease characteristics, measures of disease activity and function, medication used for axSpA, and patient satisfaction with care.

Randomisation

Patients were randomised 1:1:1 to 1 of the 3 groups after eligibility was confirmed. A statistician and a secretary, not involved in patient screening or enrolment, provided a computer-generated block randomisation list (random block size of 6, 9, or 12 patients) and prepared sealed, opaque envelopes containing information on group allocation. The study nurse or study coordinator informed patients about their group assignment and the designated intervention. Patients and treating clinicians were not blinded to group allocation, but the statistician programming the statistical analyses was blinded.

Interventions

The interventions were the follow-up strategy between baseline visit and study end visit at 18 months postrandomisation: either remote monitoring or patient-initiated care.

The patients in remote monitoring or patient-initiated care had no prescheduled visits between baseline and study end. In remote monitoring, patients reported the PROs monthly via a smartphone application, MyDignio [25]. The following was reported: (i) patient global assessment of disease activity (PGA) on numerical rating scale: 0 to 10 (worst), and (ii) whether they experienced a flare (significant worsening of disease-related symptoms). If patients scored PGA of ≥ 3 or reported yes or uncertain to the flare question, they were asked to additionally complete the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) questionnaire [26]. A rheumatology nurse monitored the PROs daily on the web platform, Dignio Prevent. If BASDAI score was ≥ 4 , the nurse received an alert (yellow flag), contacted the patients by phone, and assessed the need for a visit.

Patients allocated to patient-initiated care self-monitored their symptoms and could contact a rheumatology nurse if they experienced significant symptom worsening or adverse events. They also reported the same PROs as the remote monitoring group in the MyDignio application every third month, but they were informed that this reporting was for research only and would not be monitored.

The control group received usual care follow-up with pre-scheduled face-to-face visits, at the hospital every sixth month. These visits were undertaken by a rheumatology nurse under supervision by a rheumatologist.

If patients in any of the 3 groups experienced a minor disease worsening, they could initiate or intensify treatment with NSAIDs at the dosage they had used previously. All patients were encouraged to contact the rheumatology nurse if they experienced significant disease worsening or adverse events, either by phone or through a messaging system in the MyDignio app (only Remote Monitoring and Patient-initiated Care). The nurse assessed the need of a visit with a rheumatologist.

Outcomes

The primary end point was low disease activity or inactive disease, defined as ASDAS of <2.1 evaluated at 6, 12, and 18 months. ASDAS was calculated from 4 PROs (numerical rating scale, 0–10; 10 = very severe): back pain, joint pain/swelling, duration of morning stiffness, and PGA during the last week in

addition to C-reactive protein (CRP) or erythrocyte sedimentation rate. ASDAS_{CRP} was the preferred measure [23].

Most secondary end points were evaluated at 6, 12, and 18 months (Supplementary File S4). The secondary end points included measures of disease activity: ASDAS as continuous measure, ASDAS < 1.3 [21], ASDAS increase of ≥ 0.9 [27], BASDAI [26], CRP, erythrocyte sedimentation rate, PGA, general pain, joint pain, back pain, morning stiffness severity, and fatigue. Number of tender and swollen joints were evaluated at 18 months. Secondary end points included measures of function: Bath Ankylosing Spondylitis Functional Index [28], activity impairment [29], sleep disturbance due to pain [30], and overall health [31,32]. Patients also reported on satisfaction with care [33]. Medication was assessed by number of patients switching or stopping TNFi during the study (only evaluated at 18 months), use of NSAIDs, other analgesics, and corticosteroids. Number of hospital visits and brief contacts by phone between patients and health care providers were registered throughout the study as a measure of health care providers' resource use. Adverse events and serious adverse events were reported at study end at 18 months or at early study termination and were coded according to the Medical Dictionary of Regulatory Activities, version 3.0.

Populations available for analyses

All enrolled patients were included in baseline analyses. The full-analysis set included patients with at least 1 assessment of the primary outcome at 6, 12, or 18 months. The per-protocol set included patients who were adherent to the allocated follow-up regimen with no major protocol deviation (Supplementary File S5). The primary analysis was performed in the full-analysis set and repeated in the per-protocol set as a sensitivity analysis. The risk of nonadherence to study protocol was considered to be unequal between the study groups as the patients in the remote monitoring had to report PROs on a regular basis.

Statistical analyses

Using data from the ongoing NOR-DMARD study [34], we estimated the probability of a patient satisfying ASDAS of <2.1 at a single visit to be around 88% in the usual care group. We required at least 80% power to conclude that either 1 or both of remote monitoring or patient-initiated care were noninferior to usual care, using a noninferiority margin of 15%. The noninferiority margin of 15%, was considered as a clinically meaningful difference based on a combination of statistical reasoning and clinical judgement after thorough discussions between the clinicians and biostatisticians in the project group. Assuming no differences between the groups, we estimated that 74 patients in each group would suffice. Anticipating dropout, we aimed for a group size of 80 patients.

The primary analysis was performed using mixed-effect logistic regression. The model included randomisation group, baseline ASDAS, and their interactions with time (categorical) as covariates, in addition to patient-level random intercepts. The estimates of the probability of ASDAS of <2.1 for each group were formed by averaging across the time point-specific estimates of the probability that a patient-satisfied ASDAS of <2.1 . Their standard errors and that of the corresponding risk differences were estimated using subject-level bootstrapping, which were then used to form normal-based CIs. The analyses assumed that missing values of ASDAS were missing at random. Hence, missing values were not imputed.

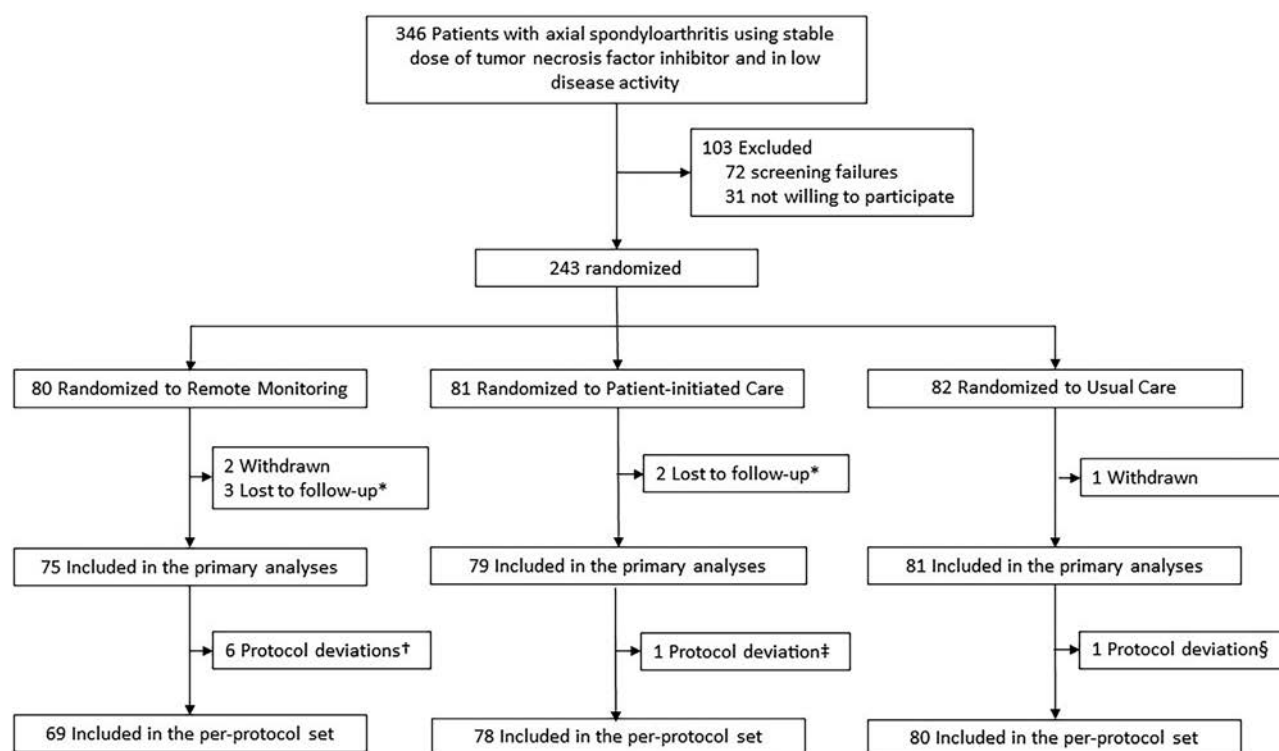


Figure 1. Flow of the participants in the ReMonit study. *Patients with no assessment of the primary end point during follow-up. †Completed <50% of the monthly patient-reported outcomes (PROs) and not completed a monthly PRO at least once between baseline and the 6-month, once between the 6-month and the 12-month, and once between the 12-month and the 18-month follow-ups; or <2 assessments of the primary end point at 6, 12, or 18 months. ‡Less than 2 assessments of the primary end point at 6, 12, or 18 months. §Less than 2 assessments of the primary end point at 6, 12, or 18 months and not attended at prescheduled visits at neither the 6-month nor the 12-month follow-up.

The noninferiority of remote monitoring vs usual care was assessed using a 2-sided 97.5% CI for the between-group difference of the probability of ASDAS of <2.1 and conducted if the CI ruled out differences in excess of 15% in favour of usual care. The same approach was used for assessing the noninferiority of patient-initiated care vs usual care. The noninferiority of patient-initiated care vs remote monitoring would only be assessed (using a 95% CI) if both these strategies were shown to be noninferior to usual care, giving a family-wise error rate of 2.5%.

Sensitivity analyses were performed in the per-protocol set; with adjustment for additional baseline characteristics (age, gender, and TNFi treatment duration); with an updated ASDAS definition [35]; and multiple imputation in combination with logistic regression estimated using the generalised estimating equation approach.

Secondary continuous and binary end points were analysed in a similar to the primary analysis. Outcomes assessed at a single time point (ASDAS increase of >0.9, tender and swollen joints) were treated likewise but without random intercepts and time interactions. Patients' satisfaction, medication, health care providers' resource use, and adverse events were summarised using descriptive statistics. All analyses, were prespecified in the statistical analysis plan (Supplementary File S1) and carried out using Stata version 16 (StataCorp) and R version 4.0.3 (R Foundation).

RESULTS

Study participants

In total, 243 of the 346 screened patients were enrolled into the study between September 2021 and June 2022, and the final visit was in January 2024. Patients were randomly allocated to

remote monitoring ($n = 80$); patient-initiated care ($n = 81$); or usual care ($n = 82$) (Fig 1). Mean age was 43 years (SD, 12 years; IQR, 35–52 years), 183 (75%) were male, and mean ASDAS was 1.0 (SD 0.5) at baseline, with balanced baseline characteristics between the groups (Table 1). All patients used TNFi, and the majority were on their first or second course of TNFi. Most of the TNFi were biosimilars at the lowest price available. Excluded patients had similar age, gender, and disease activity to the enrolled patients, but a higher proportion of the enrolled patients had education at university level and were in paid work compared with excluded patients (Supplementary Table S1). Of the 243 enrolled patients, 1 did not meet the inclusion criteria, 2 withdrew soon after inclusion, and 5 were lost to follow-up, leaving 235 patients in the full-analysis set (Fig 1). Eight patients had a major protocol deviation: 6 in remote monitoring, 1 in patient-initiated care, and 1 in usual care, leaving 227 patients in the per-protocol set.

Primary end point

In all 3 groups, and at each of the 6-month, 12-month, and 18-month assessments, $\geq 90\%$ of patients had ASDAS of <2.1, with minimal differences between the groups (Fig 2). The estimated difference in the probability of ASDAS of <2.1 between usual care and remote monitoring was -4.1% (97.5% CI, -9.9% to 1.8%) and between usual care and patient-initiated care, -1.1% (97.5% CI, -7.2% to 4.9%). Both analyses supported the conclusion of noninferiority, and hence the difference in the probability of ASDAS of <2.1 between remote monitoring and patient-initiated care was assessed (2.9%; 95% CI, -1.5% to 7.4%) (Fig 2). Sensitivity analyses showed confirmatory results (Supplementary Table S2).

Table 1
Baseline characteristics of the study population

	Remote monitoring (n = 80)	Patient-initiated care (n = 81)	Control group (n = 82)
Demographics			
Age (y), mean (SD), minimum-maximum	42 (12), 22-70	45 (10), 26-68	44 (13), 21-76
Male	62 (78)	62 (77)	59 (72)
Education at university level	69 (86)	59 (73)	68 (84)
Paid work	70 (88)	71 (88)	73 (89)
E-health literacy ^a , mean (SD)	3.5 (0.4)	3.3 (0.5)	3.4 (0.4)
Lifestyle			
Body mass index (kg/m ²), mean (SD)	24.2 (3.2)	26.3 (4.4)	25.1 (5.4)
Smoking	3 (4)	1 (1)	3 (4)
Disease characteristics			
Disease duration (y), from diagnosis, mean (SD)	13.2 (10.5)	13.4 (9.1)	14.4 (12.1)
Symptom duration (y), mean (SD)	19.5 (12.1)	19.4 (10.5)	20.2 (12.9)
Sacroiliitis	74 (93)	77 (95)	77 (94)
HLA-B27	75 (94)	77 (95)	74 (90)
Peripheral involvement ever			
Arthritis	30 (38)	43 (53)	39 (48)
Enthesitis	6 (8)	15 (19)	10 (12)
Dactylitis	4 (5)	4 (5)	2 (2)
Extramusculoskeletal manifestations ever			
Uveitis	29 (36)	26 (32)	27 (33)
Psoriasis	8 (10)	12 (15)	7 (9)
Inflammatory bowel disease	7 (9)	4 (5)	9 (11)
Disease activity			
ASDAS, mean (SD)	1.0 (0.5)	1.0 (0.5)	1.0 (0.4)
BASDAI, mean (SD)	1.3 (1.1)	1.3 (1.2)	1.3 (1.0)
CRP (mg/L), median (IQR)	1 (1-2)	1 (1-2)	1 (1-2)
ESR (mm/h), median (IQR)	5 (2-9)	5 (2-7)	5 (3-11)
PGA ^b , mean (SD)	1.7 (1.8)	1.5 (1.9)	1.4 (1.1)
General pain ^b , mean (SD)	1.3 (1.2)	1.2 (1.2)	1.3 (1.1)
Back pain ^b , mean (SD)	1.4 (1.3)	1.6 (1.7)	1.5 (1.4)
Joint pain ^b , mean (SD)	1.2 (1.2)	1.0 (1.2)	1.3 (1.2)
Morning stiffness severity ^b , mean (SD)	1.5 (1.4)	1.2 (1.3)	1.5 (1.4)
Fatigue ^b , mean (SD)	1.9 (2.1)	1.9 (2.0)	1.9 (1.8)
Peripheral disease activity			
Patients with swollen joints	0 (0)	0 (0)	0 (0)
Patients with tender joints	3 (4)	2 (3)	3 (4)
Patients with heel enthesitis	1 (1)	0 (0)	0 (0)
Functional status			
BASFI, mean (SD)	0.8 (0.9)	0.7 (1.0)	0.7 (1.0)
Activity impairment, mean (SD)	1.1 (1.5)	1.1 (1.5)	0.9 (1.0)
Sleep disturbances due to pain	17 (21)	18 (22)	10 (12)
Overall health (EQ-5D-5L), mean (SD)	80 (13)	78 (16)	80 (13)
Medication used for axSpA			
Infliximab ^c	10 (13)	8 (10)	18 (22)
Etanercept ^c	33 (41)	27 (33)	23 (28)
Adalimumab ^c	31 (39)	39 (48)	37 (45)
Golimumab	3 (4)	3 (4)	1 (1)
Certolizumab pegol	3 (4)	4 (5)	3 (4)
Previous TNFi			
0-1	72 (90)	68 (84)	75 (91)
2-4	8 (10)	13 (16)	7 (9)
DMARDs (not TNFi)	2 (3)	4 (5)	3 (4)
NSAIDs	30 (38)	27 (33)	24 (29)
Analgesics, not NSAIDs	14 (18)	21 (26)	15 (18)
Corticosteroids	2 (3)	4 (5)	0 (0)
Patient satisfaction with care			
Very satisfied	58 (73)	62 (77)	64 (78)
Satisfied	21 (26)	18 (22)	18 (22)
Both satisfied and dissatisfied	1 (1)	1 (1)	0 (0)
Dissatisfied	0 (0)	0 (0)	0 (0)
Very dissatisfied	0 (0)	0 (0)	0 (0)

ASDAS, Axial Spondyloarthritis Disease Activity Score; AxSpA, axial spondyloarthritis; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; BASFI, Bath Ankylosing Spondylitis Functional Index; CRP, C-reactive protein; DMARD, disease-modifying antirheumatic drug; EQ-5D-5L, Euro Quality of Life 5 dimensions 5 levels; ESR, erythrocyte sedimentation rate; HLA, human leucocyte antigen; NSAID, nonsteroidal anti-inflammatory drug; PGA, patient global assessment of disease activity; TNFi, tumour necrosis factor inhibitor.

Values are n (%) unless specified.

^a Range 1-4, 4 is the best score.

^b Measured on numerical rating scale 0-10 (0 = none, 10 = very severe).

^c Used as either originator or biosimilar, at the lowest price available.

Numbers and proportions of patients with ASDAS < 2.1

	6 months	12 months	18 months
Remote Monitoring	71/74 (95.9%)	70/73 (95.9%)	68/72 (94.4%)
Patient-initiated Care	68/74 (91.9%)	68/75 (90.7%)	73/79 (92.4%)
Usual Care	75/78 (96.2%)	70/75 (93.3%)	72/80 (90.0%)

Comparisons of adjusted probability of ASDAS < 2.1 between study arms

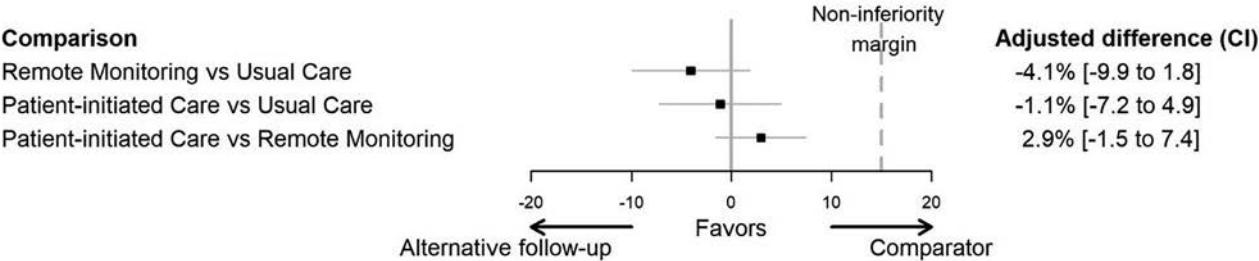


Figure 2. Probability of low disease activity with different follow-up strategies. Crude values and proportions of patient with ASDAS < 2.1 at 6-month, 12-month, and 18-month follow-ups and adjusted comparisons between the follow-up arms, analysed by mixed-effect logistic regression. The randomisation group and baseline ASDAS assessment, as well as their interactions with time (categorical), were included as covariates, in addition to patient-specific random intercepts. ASDAS, Axial Spondyloarthritis Disease Activity Score.

Secondary outcomes

Analyses of secondary end points on disease activity, including CRP, and self-reported physical function showed similar results across groups (Fig 3; Supplementary Tables S3 and S4). This was also the case for patient satisfaction, with ≥90% reporting to be satisfied or very satisfied (Fig 3; Supplementary Table S5). At 18 months, <10% had switched or stopped TNFi, with no differences between the groups. There were only small differences between the groups in the use of NSAIDs, other analgesics, and steroids throughout the study (Table 2) [36].

The number of extra visits were similar in all follow-up groups, but the total number of visits were higher in usual care due to the prescheduled visits every 6th month. The number of brief contacts by phone was higher in remote monitoring than in usual care and patient-initiated care, but a substantial proportion of these contacts were the nurse calling patients due to a PRO-related yellow flag in the remote monitoring platform (Fig 3). Number of adverse events were similar across groups (Table 3; Supplementary File S6). There were few serious adverse events, and none led to study discontinuation.

DISCUSSION

In this ReMonit study, remote monitoring and patient-initiated care were noninferior follow-up strategies in maintaining low disease activity or inactive disease compared with usual care follow-up in patients with axSpA. Furthermore, patient-initiated care was noninferior to remote monitoring. There were no differences across groups for patient satisfaction or change of TNFi medication, but there were lower number of visits and brief contacts by phone in the patient-initiated care. There were few serious adverse events, and none led to study discontinuation.

Previous research on alternative follow-up strategies of axSpA is limited. In an observational study, using a mobile health tool for axSpA was associated with lower disease activity or inactive disease and a lower need for hospital visits [14]. A recent Dutch RCT compared the combination of remote monitoring and patient-initiated care with usual care, demonstrating noninferiority in disease activity at the 12-month follow-up [15]. In this study, patients in the intervention group were remotely monitored at 6 months, while those in the control group received follow-up at the discretion of their rheumatologist. An important difference between the Dutch study and this ReMonit study is that the ReMonit study distinctly evaluated remote monitoring and patient-initiated care in separate groups. This design allowed the ReMonit study to compare the independent effects of these 2 follow-up strategies, ultimately showing that patient-initiated care was noninferior to remote monitoring. Moreover, the ReMonit study featured more frequent remote monitoring and an extended follow-up time period, both of which are crucial for implementation in clinical practice. A few RCTs among patients with other inflammatory joint diseases have compared remote monitoring of PROs or patient-initiated care to usual care, and most studies reported similar results on disease activity for remote monitoring or patient-initiated care vs usual care [37–40]. A meta-analyses indicated a favourable effect on disease activity of remote monitoring compared with usual care with regular clinic visits, but the effect-size was small [38].

The similarities between the 3 groups in primary and secondary outcomes on disease activity suggests that disease worsening is likely to be detected regardless of follow-up strategy. We found a similar low frequency of change in TNFi treatment across the groups, indicating that patients who needed change of treatment were recognised to the same extent irrespective of the follow-up strategy. Since the use of NSAIDs and analgesics did not increase in any of the follow-up groups, we can assume

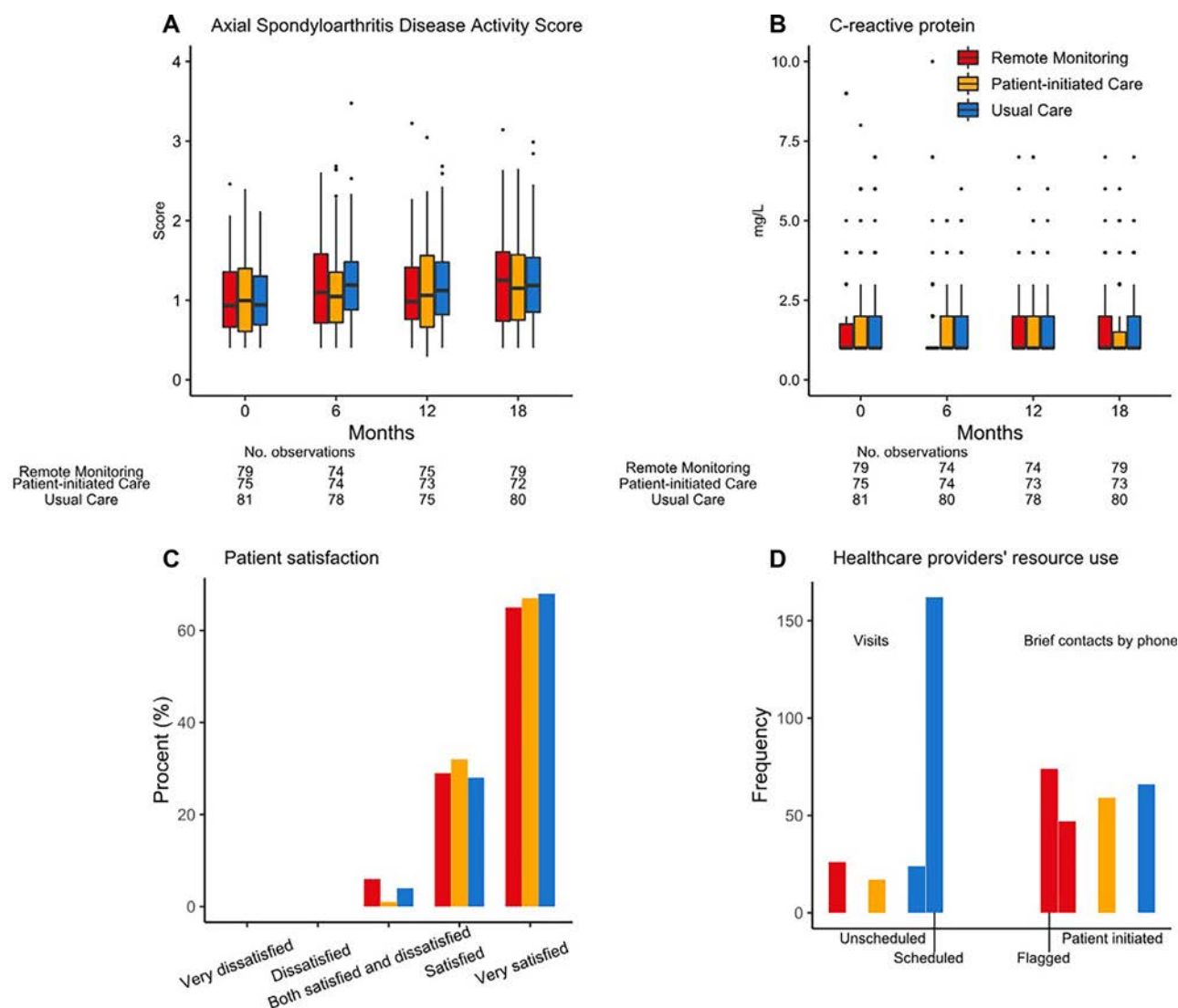


Figure 3. Secondary outcomes, disease activity, patient satisfaction, number of visits, and brief contacts by phone. A, Boxplot of disease activity over time measured by Axial Spondyloarthritis Disease Activity Score. B, Boxplot of disease activity over time measured by C-reactive protein. C, Bar graphs of patient satisfaction at study end at 18 months: 5-point response options ranging from very satisfied to very dissatisfied, presented as percentage of patients. D, Bar graphs of hospital visits and brief contacts by phone as a measures of health care providers' resource use. Unscheduled visits are extra appointments in all follow-up groups, and scheduled visits are prescheduled visits, only for the usual care group. Brief contacts by phone were patient initiated in all 3 follow-up groups, that is, the patients called the health care provider. Flagged brief contacts by phone was when the rheumatology nurse called a patient in remote monitoring due to a high patient-reported Bath Ankylosing Spondylitis Disease Activity Index score (yellow flag) indicated in the remote monitoring platform.

that patients experiencing disease worsening sought evaluation of their medical treatment at the outpatient clinic. The observed lack of difference in use or change of medication is probably also related to the low frequency of disease worsening episodes in the study sample.

Reduction of health care providers' resource use is important to achieve sustainable follow-up care. In this study, there were more visits in the usual care group, and remote monitoring had a higher number of brief contacts by phone. Fewer visits in the intervention groups is in line with the Dutch RCT on patients with axSpA [15]. Correspondingly, studies on patients with rheumatoid arthritis followed by remote monitoring or patient-initiated care showed less face-to-face visits compared with usual care [37,38]. Similar to the results of this study, 1 study found a higher number of nurse telephone contacts in the remote monitoring group compared with that of those in the usual care [41]. While monitoring of disease activity at least twice a year under supervision of a rheumatologist has been

recommended [10], the results of this indicate that monitoring could be done as infrequent as at 18-month intervals. Moreover, this study showed no additional benefit of remote monitoring over patient-initiated care.

Although there were no between-group differences in disease activity, physical function, or patient satisfaction in the ReMonit study, we cannot rule out a surplus value of face-to-face consultations. In a qualitative study among 18 patients in the intervention groups in the ReMonit study, the patients reported that the remote monitoring and patient-initiated care can meet the needs of patients in low disease activity or inactive disease. However, patients also reported that it felt safer to meet health professionals face-to-face since some of the human aspects were lost with remote monitoring [42].

Since the ReMonit study only included patients with axSpA in low disease activity or inactive disease and on stable medication, without major comorbidities, caution should be exercised when generalising the findings. However, according to the

Table 2
Use of medication for axial spondyloarthritis throughout the study

Medication	Remote monitoring (n = 75)	Patient-initiated care (n = 79)	Usual care (n = 81)
TNFi stopped or switched to another TNFi			
18 mo	2 (2)	3 (4)	4 (5)
Use of NSAIDs			
Baseline	27 (36)	27 (34)	25 (31)
6 mo	28 (38)	32 (42)	22 (27)
12 mo	26 (36)	31 (40)	28 (35)
18 mo	30 (42)	25 (33)	25 (32)
Use of analgesics, not NSAIDs ^a			
Baseline	13 (17)	21 (27)	15 (19)
6 mo	22 (29)	21 (27)	21 (26)
12 mo	20 (27)	26 (33)	20 (25)
18 mo	19 (27)	14 (18)	21 (27)
Use of corticosteroids			
Baseline	2 (3)	7 (9)	4 (5)
6 mo	3 (4)	5 (6)	7 (9)
12 mo	4 (5)	3 (4)	1 (1)
18 mo	4 (6)	0 (0)	3 (4)

NSAID, nonsteroidal anti-inflammatory drug; TNFi, tumour necrosis factor inhibitor.

Values are n (%).

^a According to the statistical analyses plan, we planned to calculate NSAIDs score [36], but the data collected on the amount of NSAIDs used were inaccurate, and this analysis was left out.

Table 3
Adverse events, reported at 18 mo

Adverse events	Remote monitoring (n = 75)	Patient-initiated care (n = 79)	Control group (n = 81)
Individuals with adverse events	26 (33)	27 (33)	25 (31)
Total No. of adverse events	38	36	33
Adverse events reported 3 times or more			
Upper respiratory tract infection	1	5	3
Lower respiratory tract infection	0	3	1
Rash	1	1	2
Uveitis	2	0	2
<i>Borrelia burgdorferi</i> infection	0	1	2
Chest pain	1	2	0
Disc herniation	2	1	0
Elevated liver enzymes	1	1	1
Essential hypertension	2	0	1
Serious adverse events ^a			
Individuals with serious adverse events	5 (6)	2 (3)	1 (1)
Total No. of serious adverse events ^{b,c}	6	2	1

Values are n (%).

^a The serious adverse event term included any untoward medical occurrence that resulted in death, was immediately life-threatening, required in-patient hospitalisation or prolongation of existing hospitalisation, resulted in persistent or significant disability/incapacity, or resulted in congenital birth anomaly/defect or as significant medical events that may jeopardise the participant or may require medical or surgical intervention to prevent 1 of the other aforementioned outcomes.

^b The serious adverse events were as follows: 1 case each of pericarditis, endometritis postpartum, palpitations, femur fracture, myocardial infarction, gastrointestinal bleeding, neutropenia after initiation of sulfasalazine, and hypomagnesaemia.

^c None of the serious adverse events led to study discontinuation or death.

European registries in the EuroSpA collaboration, a majority of patients with axSpA treated with TNFis have ASDAS of <2.1 [43]. Thus, the patients in the ReMonit study sample may represent a large group of patients with axSpA. Future studies are needed to evaluate if these alternative follow-up strategies are feasible in patients that have recently initiated new medication or in patients who do not reach low disease activity or inactive disease despite optimal treatment.

The ReMonit study was conducted in a country with a high standard of living and a well-developed and highly accessible health care system. In countries with limited health care budgets, there may be a lack of health care personnel, and the remote monitoring or patient-initiated care could be attractive. Remote monitoring and patient-initiated care would also be convenient in countries with less infrastructure and long travel distances to specialist health care. However, remote monitoring requires

availability of digital resources and digital literacy, but patient-initiated care is feasible with less digital resources.

Implementation of less resource-intensive, effective, and safe follow-up strategies would be beneficial for the society, health care sector, and patients. The lower resource use in patient-initiated care can liberate time for health care providers to care for patients with high disease activity or more complex diseases and thus facilitate more targeted use of health care resources in rheumatology. This may also be relevant for other fields of medicine.

From an environmental perspective, less resource-intensive follow-up is favourable, by reducing the carbon footprint. When RCTs demonstrate equivalent clinical outcomes, we should recommend the low-carbon alternative [44]. Reducing patient travelling through remote health care services can significantly decrease emissions [45], but use of technology may increase

energy use related to collection, storage, and processing data [46]. Patient-initiated care reduces patient travelling and additionally diminishes need for technology and staff resources, making it likely to be the follow-up in our trial with lowest environmental impact. By adopting this follow-up regime, health care systems enhance efficiency and contribute to environmental sustainability.

Strengths and limitations

The strengths of this study include the RCT design with comparison of 2 novel follow-up strategies to conventional follow-up over a long period, as well as a comprehensive data collection. This study has some limitations. First, there may be a selection bias as patients motivated for an alternative follow-up would be more likely to participate. Hence, the results regarding patient satisfaction may be overestimated in the intervention groups. However, very few patients declined to participate, reducing the effect of a possible selection bias. Second, this study was open-labelled and most of the outcomes were PROs, which may have been affected by patients' enthusiasm for their allocated follow-up and bias their reporting of the outcomes. In addition, the main benchmark that signalled for action in the remote monitoring was a PRO, which may influence the interpretation of the outcomes in this noninferiority trial. In order to reduce the bias of the outcomes, we included a CRP as an objective marker of inflammation, the primary outcome ASDAS includes CRP, and treatment discontinuation of TNFi, which can be regarded as a semiobjective outcome. Furthermore, the main outcome, ASDAS, was far below the noninferiority margin, indicating robustness of the results. Third, reporting of adverse events at the ultimate study visit was susceptible to recall bias, especially in the intervention groups. However, serious adverse events were reported in all 3 groups, indicating that we managed to capture information about serious adverse events also from patients in groups without regular visits.

Conclusions

Among patients with axSpA in low disease activity or inactive disease and on stable medication, follow-up with remote monitoring or patient-initiated care were noninferior to usual care in maintaining low disease activity, supporting implementation of remote monitoring or patient-initiated care. Patient-initiated care was the least resource-intensive follow-up for health care providers. Further research on alternative follow-up strategies for patients with higher disease activity is warranted.

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Contributors

All authors conceived the study and designed the methodology. IJB, NO, and JS curated the data and wrote the original draft. JS performed formal analyses. IJB and NO were responsible for funding acquisition. IJB, NO, EEKT, EM, EEK, and SAP performed investigations. NO was responsible for project administration. IJB, NO, EM, EKK, SAP, and JS were responsible for resources. EAH and SL supervised the study. All authors

reviewed and approved the final version of the manuscript. IJB, NO, and JS had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. IJB and NO are responsible for the overall content as guarantors. The corresponding author attests that all listed authors meet authorship criteria and that no others meeting the criteria have been omitted.

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Competing interests

IJB reported financial support provided by South-Eastern Norway Regional Health Authority. GB has reported speakers fee Johnson & Johnson. LG has reported research grants from AbbVie, Biogen, Lilly, Novartis, and UCB; consulting fees from AbbVie, Amgen, BMS, Celltrion, Janssen, Lilly, MSD, Novartis, Pfizer, Stada, and UCB; and nonfinancial support from AbbVie, Amgen, Biogen, Janssen, MSD, Pfizer, and UCB, all disclosures outside the submitted work. The other authors declare they have no competing interests.

Patient and public involvement

Two patient research partners from the REMEDY patient advisory board participated as part of the study organisation (Sarah Hakim and Tale Gjøvik). The patient research partners provided input on the relevance of the research question, the feasibility of the study design and study logistics from a patient perspective, and written information to patients. They participated in meetings with the study organisation throughout the study, and one (SH) is a coauthor of this manuscript.

Patient consent for publication

All patients provided an informed written consent before participation.

Ethics approval

The study involves human participants and was conducted in compliance with the tenets of the Declaration of Helsinki and the International Conference on Harmonisation Guidelines for Good Clinical Practice. The study protocol was approved by the Regional Committee for Medical Research Ethics South East Norway (REC south-east 229187).

Provenance and peer review

Not commissioned; externally peer reviewed.

Data availability statement

A deidentified patient data set can be made available to researchers upon reasonable request. The data will only be made available after submission of a project plan outlining the

reason for the request and any proposed analyses. The data sharing will have to be approved by the ReMonit project group and may require a data access agreement to be signed.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ard.2025.04.019.

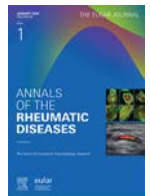
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Axial spondyloarthritis

CD4 + tissue-resident memory Th17 cells are a major source of IL-17A in Spondyloarthritis synovial tissue

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ABSTRACT

Objectives: Interleukin (IL)-17A is a key driver of spondyloarthritis (SpA) joint pathology. We aimed to identify its cellular source in synovial tissue from patients with 2 forms of SpA, namely axial SpA (AxSpA) and psoriatic arthritis (PsA).

Methods: Synovial tissue from patients with SpA was profiled using single-cell RNA sequencing (scRNA-seq; AxSpA, n = 5; PsA, n = 6) or spatial RNA profiling (PsA, n = 4). CellPhoneDB was used to infer cell–cell communication. Tissue-resident memory Th17 (TRM17)-like cells were generated *in vitro* using blood memory CD4+ T cells from SpA patients. An epigenetic inhibitor library, siRNA, and clustered regularly interspaced short palindromic repeats (CRISPR) were used to identify epigenetic regulator(s) for TRM17.

Results: scRNA-seq showed that CD4+ CXCR6+ TRM17 cells are the predominant spontaneous IL17A producers in SpA synovium. Cell–cell communication and single-cell spatial analysis support the interaction between TRM17 and CLEC10A+ dendritic cells, which were activated in SpA. Both sublining and lining fibroblasts in SpA synovium showed evidence of interleukin (IL)-17A activation. *In vitro*-generated CD4+ TRM17-like cells phenocopied joint tissue TRM17, producing IL-17A/F upon T cell-receptor (TCR) stimulation, which was further enhanced by cytokines. Perturbation of BRD1 inhibited the generation of TRM17-like cells.

Conclusions: CD4+ TRM17 cells are the predominant source of IL-17A in SpA synovial tissue. TCR stimulation is essential for the secretion of IL-17A by CD4+ TRM17-like cells. The epigenetic regulator bromodomain-containing protein 1 (BRD1) contributes to the generation of CD4+ TRM17. Depleting CD4+ TRM17 cells in SpA is thus a therapeutic strategy with potential to induce long-term remission.

INTRODUCTION

The spondyloarthritides (SpA) are a group of common inflammatory arthritides that include axial SpA (AxSpA) and psoriatic arthritis (PsA). The AxSpA and PsA share pathological drivers including interleukin 17 (IL-17) and tumour necrosis factor (TNF). Drugs targeting IL-17 are effective in over 50% of SpA patients but require continued long-term administration, and therapies to target the cells producing IL-17 have not yet been developed.

IL-17A is known to promote tissue inflammation, at least in part through fibroblast activation. Among the different forms of IL-17 (A–F), IL-17A is the key driver in joint inflammation whereas a major role for IL-17F in skin inflammation has been supported by recent trials [1]. IL-17A can be produced by a variety of immune cells, including conventional T cells, innate-like lymphocytes, and innate lymphoid cells (ILCs). Whether a specific cell type dominates IL-17A production in SpA is debated [2]. Different studies have implicated all 3 subsets of innate-like lymphocytes ($\gamma\delta$ T cells, invariant natural killer T [iNKT] cells, and mucosal associated invariant T [MAIT] cells) and CD8+ tissue-resident memory T cells as the sources of IL-17A in SpA joints [3–9]. Increasing evidence, however, is highlighting a role for CD4+ tissue-resident memory Th17 (TRM17) cells as key IL-17A producers in tissues such as the skin, lung and kidney [10–12]. Furthermore, CD4+ Th17 cells expressing CXCR6, an established marker for tissue residency, have been linked to brain inflammation in a murine model of multiple sclerosis [13]. It is therefore possible that a role for CD4+ TRM17 cells in SpA may have been missed in previous studies focused on

synovial fluid samples and/or utilising potent *in vitro* stimulation to determine the source of IL-17.

Notably, CD4+ CXCR6+ Th17 cells in inflamed brain tissue have been found to exhibit a distinct epigenetic profile that is different from homeostatic Th17 cells with stem-like features [13]. Considering the established role of epigenetic modifiers in the regulation of T cell differentiation and function [14,15], it is plausible to predict that epigenetic regulators could be involved in the generation of CD4+ TRM17 cells.

Through profiling unstimulated synovial tissue using single-cell RNA sequencing (scRNA-seq) and spatial RNA profiling, we here show that CD4+ CXCR6+ TRM17 cells are the predominant source of IL-17A in SpA synovial tissue. We also establish a model to generate CD4+ TRM17-like cells *in vitro* and show that T-cell receptor (TCR) engagement, rather than cytokines, is essential for these cells to produce IL-17A and IL-17F. Lastly, we show that the epigenetic regulator bromodomain-containing protein 1 (BRD1) contributes to the generation of CD4+ TRM17-like cells.

METHODS

Patient recruitment

Fresh synovial tissue was obtained at arthroplasty or synovial biopsy was collected from patients attending the Oxford University Hospitals, Queen Elizabeth Hospital, and Shanghai Sixth People's Hospital with appropriate consent and ethical approvals. All patients included in this study met the criteria of the Assessment of Spondyloarthritis International Society for AxSpA

WHAT IS ALREADY KNOWN ON THIS TOPIC

- Interleukin (IL)-17A plays a key role in the immunopathogenesis of spondyloarthritis (SpA), but its cellular source in joint tissue has not been determined previously. The induction and accumulation of CD4+ tissue-resident memory Th17 (TRM17) cells following the clearance of pathogens has been described in skin, lung, and kidney. Whether CD4+ TRM17 cells also accumulate in the joint and contribute to the pathology of SpA is not clear.

WHAT THIS STUDY ADDS

- CD4+ TRM17 cells are present in SpA synovial tissue and are the predominant source of *IL17A*.
- CD4+ TRM17 cells in SpA joints express *IL17A* without any *in vitro* exogenous stimulation.
- T-cell receptor (TCR) rather than cytokine stimulation is essential for IL-17A production by CD4+ TRM17-like cells.
- The epigenetic regulator BRD1 contributes to the generation of CD4+ TRM17-like cells.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- Our findings identify CD4+ TRM17 cells as the primary source of IL-17A in SpA synovium, a previously unrecognised role for these cells. Key questions remain: How do CD4+ TRM17 cells relate to IL-17A producers in synovial fluid? What mechanisms induce and maintain them in the joint? How do they interact with other cells to promote arthritis? These questions warrant further investigation. In addition, our data suggest that targeting CD4+ TRM17 cells, the ‘factory’ of IL-17A in SpA synovial tissue, has the potential to induce long-term remission, encouraging future efforts to develop new therapies to deplete CD4+ TRM17 cells in SpA.

or Classification criteria for Psoriatic Arthritis (CASPAR) criteria for PsA. Patients currently or previously receiving interleukin 17 inhibitor (IL17i) therapy were excluded from the study population. The demographics of AxSpA and PsA patients recruited for this study are shown in [Supplementary Table S1](#).

Computational analysis of scRNA-seq data

The paired reads obtained were mapped to the hg38 reference genome to generate gene expression matrices using Cell Ranger. For PsA samples (from the Cartography Consortium), the full dataset was analysed in Panpipes [16], and the T cell compartment was partitioned out for downstream analyses along with the AxSpA data. T cells of PsA samples and full dataset of AxSpA samples were then merged and analysed using the Seurat R package (v4.0.5). Cells exhibiting low-quality transcriptional profiles were excluded based on the application of the following exclusion criteria: a number of detected genes below 500, a proportion of mitochondrial RNA exceeding 12% of the total unique molecular identifiers (UMIs), and a total UMI count below 700. These parameters for quality control were selected in accordance with current widely adopted practices in the single-cell genomics field [17–19]. The raw read counts were normalised using the `NormalizeData` function, and variable genes were identified for each sample. To identify cell types, we mapped our cells into a synovium reference map built by Fan Zhang et al. [20] using the Symphony algorithm. For the reclustering analysis of cellular subsets, a certain population were extracted and data from different sample were subject to FastMNN algorithms for integration. We then used `FindNeighbors` and `FindCluster` functions to cluster cells based on global

transcriptional profile. For differential expression analysis, the `FindMarkers` function was used to test the normalised data with the default Wald test method. Unless specified, default parameters were used for each function.

For cell–cell interaction inference, normalised expression matrix and cluster annotations were exported from the Seurat object as input for CellPhoneDB. CellPhoneDB v5.0.0 was used as a ligand–receptor interaction database. ‘statistical analysis’ method from CellPhoneDB tools was used to assess the strength and significance of interactions.

CosMx spatial analysis

Synovial biopsy tissue sections from 8 ultrasound-guided biopsies were distributed across 2 flow cells and run on the CosMx Spatial Molecular Imager. Cell segmentation and initial quality control were completed in the AtoMx Spatial Informatics Platform before exporting and using R (v4.4.0) and Seurat (v5.1.0) for further quality control, clustering, and cluster annotation. Data were processed through iterations of clustering, annotation, identification, and removal of multiplets/poorly segmented cells based on gene expression profiles, number of genes detected, and number of transcripts detected. Subclustering was performed until low quality/poorly segmented cells were minimised in the dataset.

Patient and public involvement

We have actively engaged the patients with SpA through the Oxford Patient Engagement Network for Arthritis and Musculoskeletal Conditions (OPEN ARMS, <https://www.ndorms.ox.ac.uk/get-involved/open-arms-1/open-arms>) and the Botnar AxSpA day. We will discuss with patients about the impact of findings from this study and codevelop a dissemination plan to maximise the potential benefits of our findings to patients.

Additional methods

Information for Th17 expansion assay for the screen of epigenetic inhibitor library, siRNA screen of the targets of OF-1, intracellular cytokine staining (ICS) and enzyme-linked immunosorbent assays (ELISA) are available in Supplementary Methods.

RESULTS

Tissue-resident memory CD4+ T cells are the main source of IL17A in SpA joint tissue

To investigate the cellular origin of IL-17A in the inflamed joints of SpA patients, we performed single-cell transcriptomic profiling on synovial tissue from 5 AxSpA and 6 PsA patients (demographics shown in [Supplementary Table S1A and B](#)). After removing low-quality cells, we obtained 85,307 cells ([Supplementary Fig S1](#)). These cells were categorised into T cells, natural killer (NK), mast and ILCs, B and plasma cells, myeloid cells, stromal cells, and endothelial cells ([Fig 1A](#)). Within this cellular milieu, we found almost all *IL17A*+ cells are localised within the *CD4*+ T cell subset ([Fig 1B](#)). Notably, the vast majority of the *IL17A*+ cells did not express *CD8A* (encoding the CD8 protein), *TRAV1-2* (encoding the TCR α -variable 1-2 protein, abundant in MAIT cells), or *TRDC* (encoding the delta constant region of the TCR, characteristic of $\gamma\delta$ T cells) ([Fig 1C](#)). As ILCs, especially ILC3 cells, are known to be capable of producing IL-17A, we investigated further into ILCs via the reclustering of the

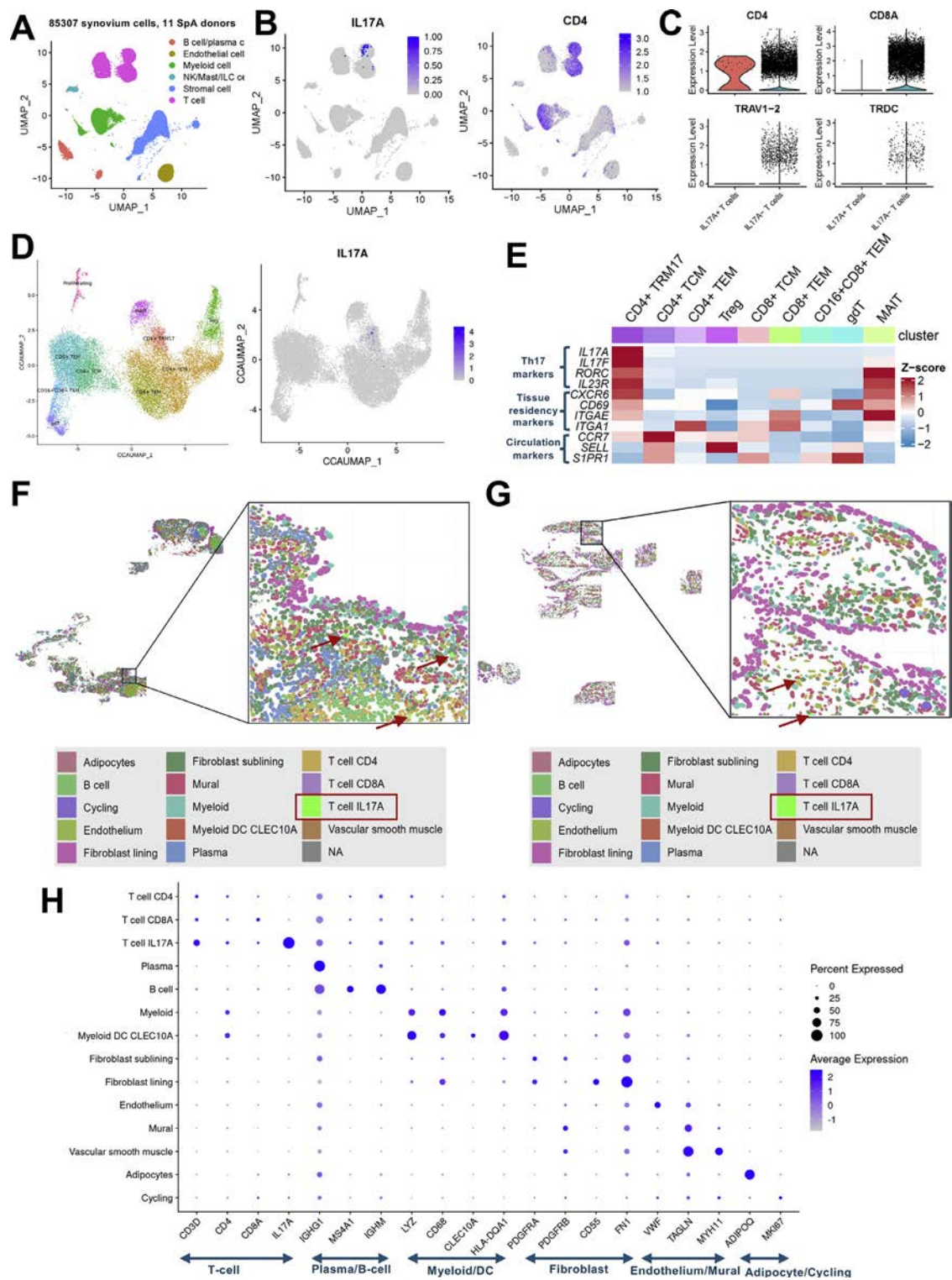


Figure 1. Tissue-resident memory CD4 + T cells are the main source of IL17A in SpA joint tissue. (A) UMAP visualisation of major cell types of cells from SpA synovial tissue. (B) UMAP visualisation of normalised expression of *IL17A* and *CD4*. (C) Violin plot of normalised expression of *CD4*, *CD8A*, *TRAV1-2*, *TRDC* in *IL-17A*⁺ and *IL-17A*⁻ T cells. (D) UMAP visualisation of subsets of T cells from SpA synovial tissues and normalised expression of *IL17A*. (E) Heatmap of normalised and scaled expression of Th17, tissue residency, and circulation signature markers in different subsets of T cells. CosMx was used for spatial profiling of synovial tissue from 4 PsA patients. (F) and (G) Representative images from 2 PsA patients. (H) Expression of cell type marker genes by IL17A + T cells and other cell populations identified from CosMx data. The ‘IL17A + T’ subset is excluded from the ‘CD4 + T’ and ‘CD8 + T’ subsets. IL17A, interleukin 17A; PsA, psoriatic arthritis; SpA, spondyloarthritis; UMAP, uniform manifold approximation and map.

cluster that contains NK, mast, and ILCs (Supplementary Fig S2A). Despite expressing high levels of *RORC* and *IL23R*, *IL7R* + *KIT* + ILCs did not express detectable *IL17A* (Supplementary Fig S2B). These results identify $\alpha\beta$ CD4 + T cells, not $\gamma\delta$ T, MAIT, ILCs, or CD8 + T cells, as the primary source of *IL17A* in

SpA synovial tissue. In line with previous findings [21], the expression of *IL17F* is not limited to T cells and extends to stroma and endothelial cells (Supplementary Fig S3). *TNF*, a key driver of SpA pathology, is predominantly expressed by myeloid cells (Supplementary Fig S4).

To pinpoint the *IL17A*-expressing T cell population, we performed subclustering analysis for all T cells from SpA synovial tissue. Ten distinct clusters were identified (Fig 1D and Supplementary Fig S5A). *IL17A*-expressing T cells are largely present in the *CD4* + *TRM17* population, exhibiting both Th17 (*IL17A*, *IL17F*, *RORC*, *IL23R*) and TRM (*CXCR6*, *CD69*, *ITGAE*) characteristics (Fig 1E and Supplementary Fig S5B). Separate analysis of AxSpA and PsA samples confirmed that *CD4* + *TRM17* cells are the primary source of *IL17A* in both disease states (Supplementary Fig S6 A–C). Analysis of the Accelerating Medicines Partnership rheumatoid arthritis (RA) dataset showed that in RA synovial tissue *IL17A* was also predominantly expressed by a *CD69* + *CXCR6* + *CD4* + *TRM17* population (Supplementary Fig S7 A–C). Notably, *CXCR6* + *CD4* + *TRM17* cells have recently been identified to drive the pathology in skin, kidney and brain inflammatory diseases [10,12,13].

To investigate the cellular niche of *IL17A*-expressing *CD4* + *TRM17* cells, we carried out spatial transcriptome analysis on joint synovial tissue from 4 PsA patients using CosMx Spatial Molecular Imaging (Fig 1F and G). We scanned 80 fields of view with a multiplex panel of 1000 genes (CosMx™ Human Universal Cell Characterization RNA Panel). A limitation of this approach was the absence or low expression of many *CD4* + *TRM* cell feature genes identified in our previous scRNA-seq data. However, *IL17A* was successfully detected, enabling the definition of *IL17A*-expressing T cells which were predominantly localised in the sublining regions (24 of 27 *IL17A* + T cells) (Figure 1F and G). We annotated cells based on canonical markers, and identified adipocyte, B, endothelial, sublining fibroblast, lining fibroblast, myeloid, *CLEC10A* + myeloid, plasma, *IL17A* + T, *CD4* + T, *CD8* + T, and mural cells (Fig 1F–H, Supplementary Fig S1C and D). Notably, in the analysis shown in Figure 1H, the '*IL17A* + T' subset is excluded from the '*CD4* + T' and '*CD8* + T' subsets.

Both sublining and lining fibroblasts in SpA joint tissue exhibit an enhanced IL-17 response signature

The induction of a proinflammatory state in fibroblasts has been postulated as a critical mechanism underlying IL-17-mediated chronic inflammation. Within the synovium, sublining and lining fibroblasts are 2 distinct subsets that together constitute synovial fibroblasts. We then investigated whether sublining and lining fibroblasts from SpA synovium have been influenced by *CD4* + *TRM17*. To this end, we integrated a publicly available dataset of fibroblasts from healthy synovium [22] with our dataset of SpA synovial fibroblasts, resulting in a combined dataset of 11,173 healthy and 27,975 SpA fibroblasts (Fig 2A). Consistent with previous studies [23,24], we identified *THY1* + *PRG4*- sublining and *THY1-PRG4* + lining fibroblasts as 2 major subsets, with *MMP1* and *MMP3* enriched in the lining fibroblasts (Fig 2A, proinflammatory S9A and B). An IL-17 signalling score (indicative of response to IL-17) was then calculated using a set of genes induced by IL-17 in human synovial fibroblasts [25]. We found both sublining and lining fibroblast subsets in SpA exhibited an enhanced IL-17 response signature compared to their healthy counterparts (Fig 2B). Notably, established IL-17-induced effector cytokines and chemokines, such as *CXCL1*, *CXCL8*, *IL6*, and *CCL20*, were consistently upregulated in both sublining and lining fibroblast subsets within the SpA synovium. Thus, despite enrichment of *CD4* + *TRM17* in sublining synovium, both sublining and lining fibroblasts show evidence of IL-17-induced activation.

We then asked if the enhanced IL-17 signature in SpA synovium fibroblasts is contributed by certain subset(s) within the sublining and lining fibroblast populations. To this end, we conducted a detailed analysis of cell states within these 2 populations separately (Fig 2C and F). In the sublining fibroblasts, among 8 identified subsets, a cluster (S1) marked by the expression of *CD74* and *CHI3L2* exhibited the highest IL-17 signalling score (Fig 2D). This subset was also characterised by the upregulation of proangiogenic genes, including *SEMA3A* and *VEGFC* (Fig 2E). In the lining fibroblast compartment, the L4 cluster displayed the strongest IL-17 signalling signature (Fig 2G) and was enriched in the expression of genes associated with oxidative stress and inflammatory responses, such as *SOD2*, *MT2A*, *RCAN1*, and *TNFAIP6* (Fig 2H).

CLEC10A + dendritic cells are potential antigen-presenting cells for *CD4* + *TRM17* cells

Tissue-resident memory T cells typically require antigen-presenting cells (APCs) for their generation and effector function. It has been well established that myeloid cells, especially dendritic cells (DCs), are the key APCs to support T cell function. To identify the myeloid cellular subset that serves as APC for *CD4* + *TRM17* cells, we extracted and reclustered the myeloid cells from SpA tissue (Fig 1A) and identified 10 transcriptionally distinct myeloid subsets: plasmacytoid dendritic cells (pDCs), *CLEC9A* + DCs, *CLEC10A* + DCs, *TNF* + macrophage cluster 1 (M1), *TNIP3* + macrophage cluster 2 (M2), *EGR2* + macrophage cluster 3 (M3), *GIMAP* + macrophage cluster 4 (M4), *C9* + macrophage cluster 5 (M5), *PRODH2* + macrophage cluster 6 (M6), and *LYVE1* + macrophage cluster 7 (M7) (Fig 3A). We then searched for potential APCs for *CD4* + *TRM17* using cell–cell communication analysis for all 10 myeloid subsets and *IL17A* + T cells (Figure 3B). The strongest interaction with *IL17A* + cells was observed with *CLEC10A* + DCs, which expressed high levels of ligands for *CD28* (*CD80* and *CD86*), produced a chemokine (*CCL20*) known to be chemotactic for Th17 cells through *CCR6*, and were enriched for the key cytokine for the generation of tissue-resident memory T cells (*TGFB1*, encoding TGF-β).

Activated T cells have been shown to act on myeloid cells and induce their activation [26,27]. Thus, if *CLEC10A* + DC cells have served as the APC for *CD4* + *TRM17* cells, they should have received reciprocal signals from these cells and show signs of activation. To look for evidence of *CLEC10A* + DC activation in SpA synovium, we carried out a differential gene expression analysis that revealed 541 upregulated and 90 downregulated genes in *CLEC10A* + DCs in SpA versus healthy synovium (Fig 3C). Notably, genes associated with an inflammatory response (*RELB*, *IRF4*, and *NFKB1*) were significantly increased. Pathway enrichment analysis of these upregulated genes using the Molecular Signatures database (MSigDB) revealed significant enrichment in several proinflammatory pathways, including *TNFα* signalling, IL-2/STAT5 signalling, IFNγ response, and IL-6/JAK/STAT3 signalling (Fig 3D). However, proinflammatory pathways were also elevated in *TNF* + M1 and *LYVE1* + M7 cells from SpA patients (Supplementary Fig S10). Therefore, we cannot determine whether the enrichment of proinflammatory pathways in SpA *CLEC10A* + DCs is due to T cell interactions or the joint's inflammatory environment. Based on the hypothesis that APCs for *IL17A* + T cells would have encountered IL-17, we analysed the expression of genes known to be induced by IL-17A in monocytes or macrophages (*MMP9*, *CCL4*, *CCL5*, *CSF2*, *IL3*, *IL9*) [28,29]. We observed increased expression of *MMP9* and

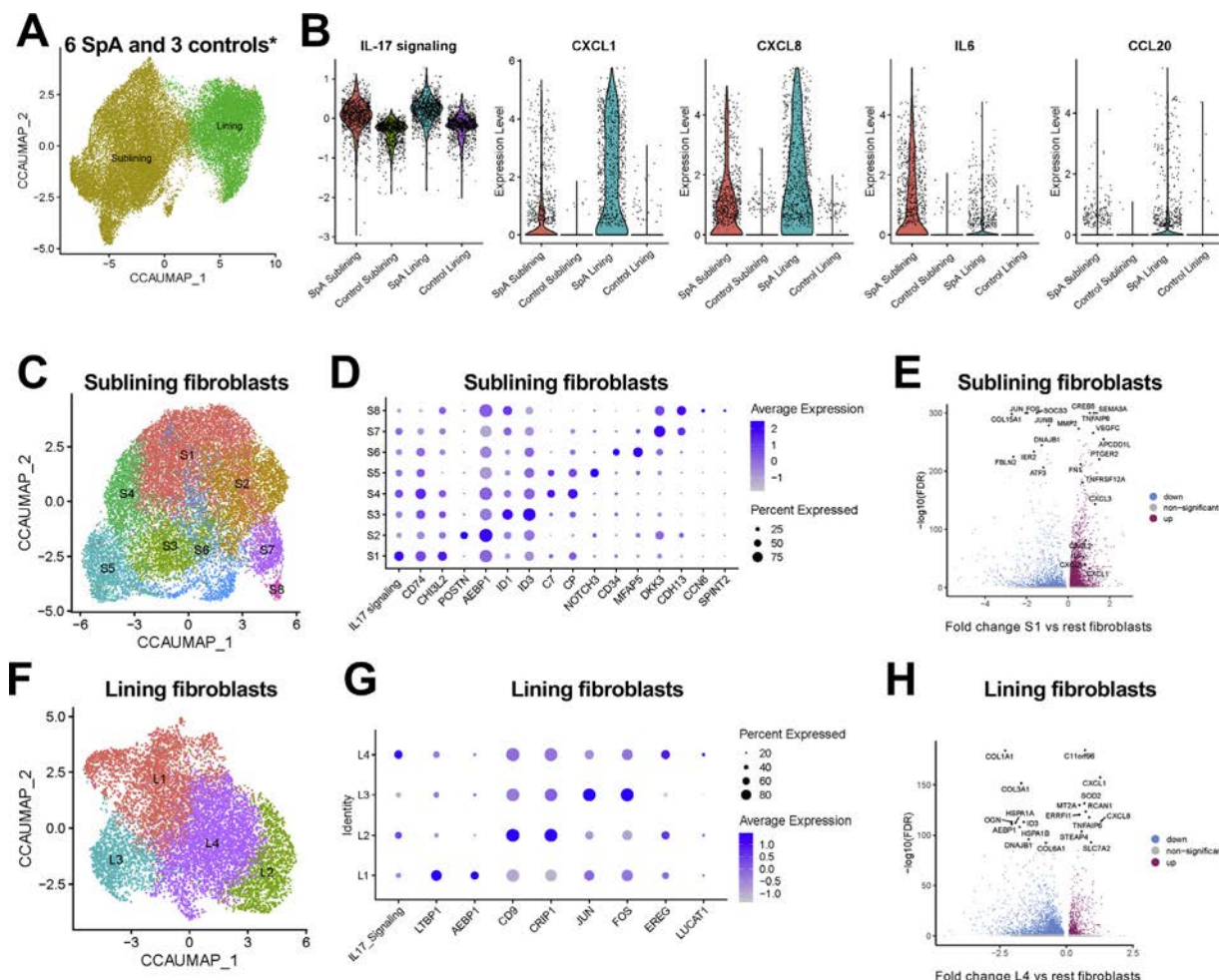


Figure 2. An enhanced IL-17 response signature is present in both sublining and lining fibroblasts from SpA synovium. (A) UMAP visualisation of fibroblast subsets of combined cells from SpA and healthy synovial tissues. (B) Violin plot of IL-17 signalling score and normalised expression of 4 selected IL-17 signalling genes in different fibroblast subsets in SpA and healthy synovium. The data for control synovium ($n = 3$) were sourced from GSE216651. (C) UMAP visualisation of subsets of sublining fibroblast cells from SpA synovium. (D) Relative expression of marker genes of different sublining fibroblast subsets from SpA synovium. (E) Volcano plot of differentially expressed genes in S1 sublining fibroblast cells compared to other sublining fibroblast cells in SpA synovium ($FDR < 0.05$). (F) UMAP visualisation of subsets of lining fibroblast cells from SpA synovium. (G) Relative expression of marker genes of different lining fibroblast subsets from SpA synovium. (H) Volcano plot of differentially expressed gene in L4 lining fibroblast cells compared to other lining fibroblast cells in SpA synovium ($FDR < 0.05$). FDR, false discovery rate; IL17A, interleukin 17A; PsA, psoriatic arthritis; SpA, spondyloarthritis; UMAP, uniform manifold approximation and map.

CSF2 in *CLEC10A* + dendritic cells (DCs), but a greater number of genes were enriched in *TNF* + M1 and *TNIP3* + M2 macrophages (Supplementary Fig S11). Lastly, we sought evidence of direct physical cell interactions between *CLEC10A* + dendritic cells (DCs) and *IL17A* + T cells in our spatial transcriptomic datasets. Owing to the restricted number of genes profiled in the spatial transcriptomic data, we were unable to resolve all myeloid cell subsets identified in Figure 3A. However, we were able to identify *CLEC10A* + DCs within the spatial transcriptomic data based on their unique marker expression profile. Quantification of cell proximity revealed that *CLEC10A* + DCs were one of the top 2 subsets within myeloid cells significantly enriched in close proximity to *IL17A* + T cells (Figure 3E). Representative examples of *CLEC10A* + DC and *IL17A* + T cell interactions are shown in Figure 3F. We also observed that *IL17A* + T cells are more frequently in proximity to sublining fibroblasts than lining fibroblasts, and to B cells and CD4+ cells than CD8+ cells. Taken together, these data suggest that *CLEC10A* + dendritic cells are activated in SpA joints and are the potential APCs for CD4+ TRM17.

TCR engagement and cytokine stimulations codrive the production of IL-17A/F by *in vitro* generated CD4+ TRM17-like cells

We then asked what signals induce the production of IL-17A by CD4+ TRM17 cells. Due to the difficulties in obtaining adequate number of CD4+ TRM17 from synovial tissue for functional studies, we developed an *in vitro* model to induce CD4+ TRM17-like cells. It has been shown that following immunisation, effector Th17 cells can give rise to lung CD4+ TRM17 cells, which are maintained in the tissue by the survival cytokine IL-7 following the clearance of antigens [11]. Accordingly, we generated a two-phase *in vitro* model to generate CD4+ TRM17-like cells (Fig 4A). In phase I, circulating blood memory CD4+ T cells from patients with SpA were stimulated with anti-CD3 antibody, anti-CD28 antibody, and Th17 expansion cytokines (IL-1 β and IL-23) to generate effector Th17 cells. These cells were then cultured with TGF- β (to model the tissue niche) and IL-7 (to provide the survival signal) in the absence of TCR stimulation in phase II. Using this model, we were able to induce IL-

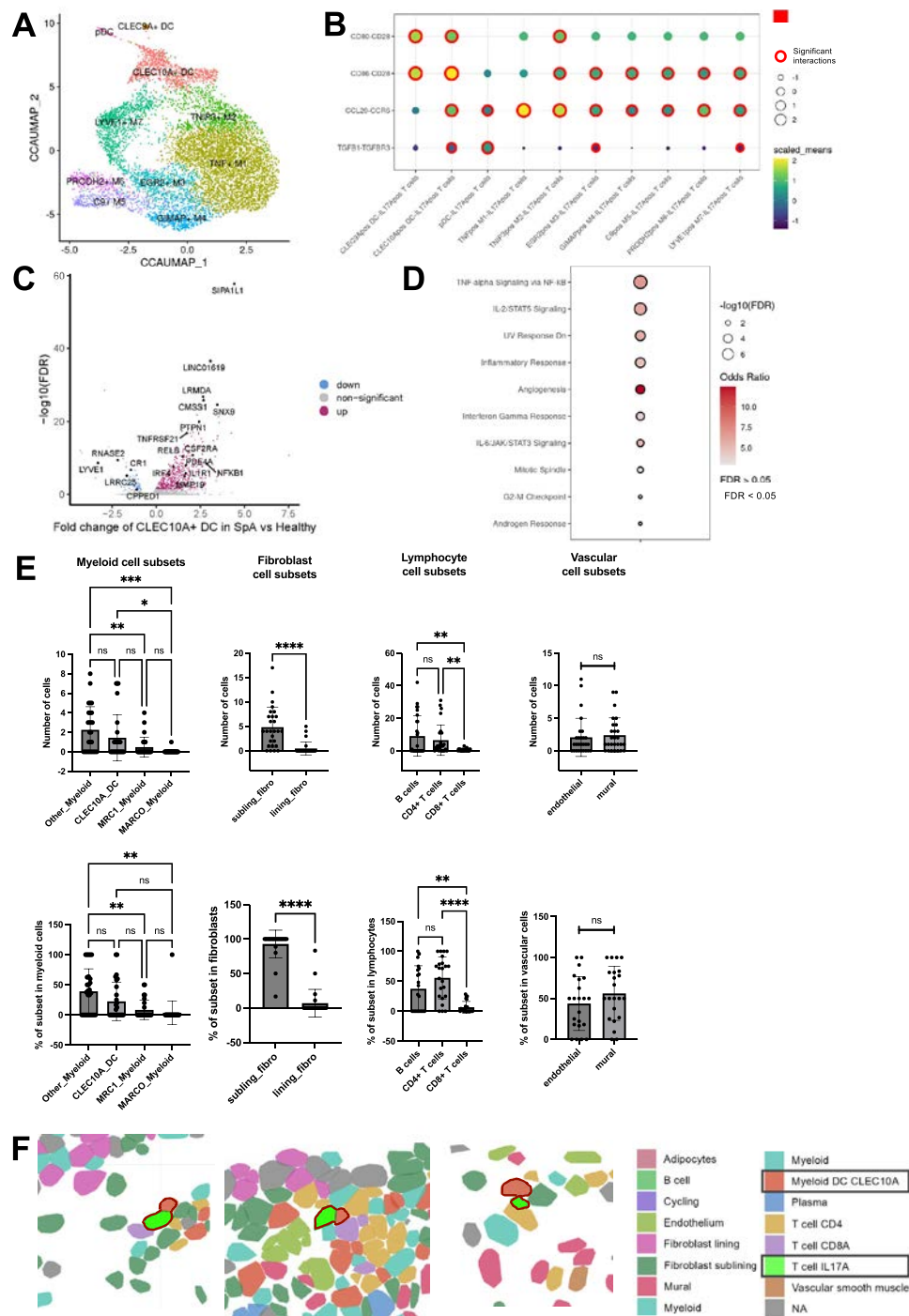


Figure 3. Cell–cell communication and single-cell spatial analysis support the interaction between CLEC10A + dendritic cells and TRM17. (A) UMAP visualisation of subsets of myeloid cells from SpA synovial tissues. (B) Cell–cell interactions between different myeloid subsets and IL17A + T cells in SpA synovium inferred by CellPhoneDB. (C) Volcano plot of differentially expressed gene in CLEC10A + DCs in SpA synovium compared to healthy synovium (FDR < 0.05). (D) Enriched pathway in CLEC10A + DCs (all upregulated genes were used for enrichment analysis using MSigDB database). (E) The number and proportion of myeloid, fibroblast, lymphocyte, and vascular cell subsets located within 50 μ m of IL17A + T cells was quantified. (F) Three representative CosMx images of SpA synovium showing interaction between CLEC10A + DCs and IL17A + cells (highlighted in red outline). Statistical significance was assessed using one-way ANOVA (for myeloid and lymphocyte subsets) or the paired two-tailed Student’s t test (for fibroblast and vascular subsets) (* $P \leq .05$, ** $P \leq .01$, *** $P \leq .001$, **** $P \leq .0001$). Median $\pm$ SEM is shown in (E). ANOVA, analysis of variance; DC, dendritic cells; IL17A, interleukin 17A; MSigDB, Molecular Signatures database; PsA, psoriatic arthritis; SEM, standard error of the mean; SpA, spondyloarthritis; UMAP, uniform manifold approximation and map.

17A + CXCR6 + CD4 + TRM17-like cells which were largely absent in the blood memory compartment (Fig 4B and C). We then assessed the expression of TRM markers using our assay in comparison to a 6-day Th17 expansion assay (anti-CD3/CD28, IL-1 β , IL-23). Although the CD4 + TRM17 assay slightly improved IL-17A + cell induction, it significantly increased the

percentage of Th17 cells expressing CXCR6, CD49a, CD103, and CD69 (Supplementary Fig S12A, B). This was confirmed at the RNA level by quantitative polymerase chain reaction (qPCR) (Supplementary Fig S12C). Taken together, these data suggest that our assay successfully induces Th17 cells with TRM-like features *in vitro*.

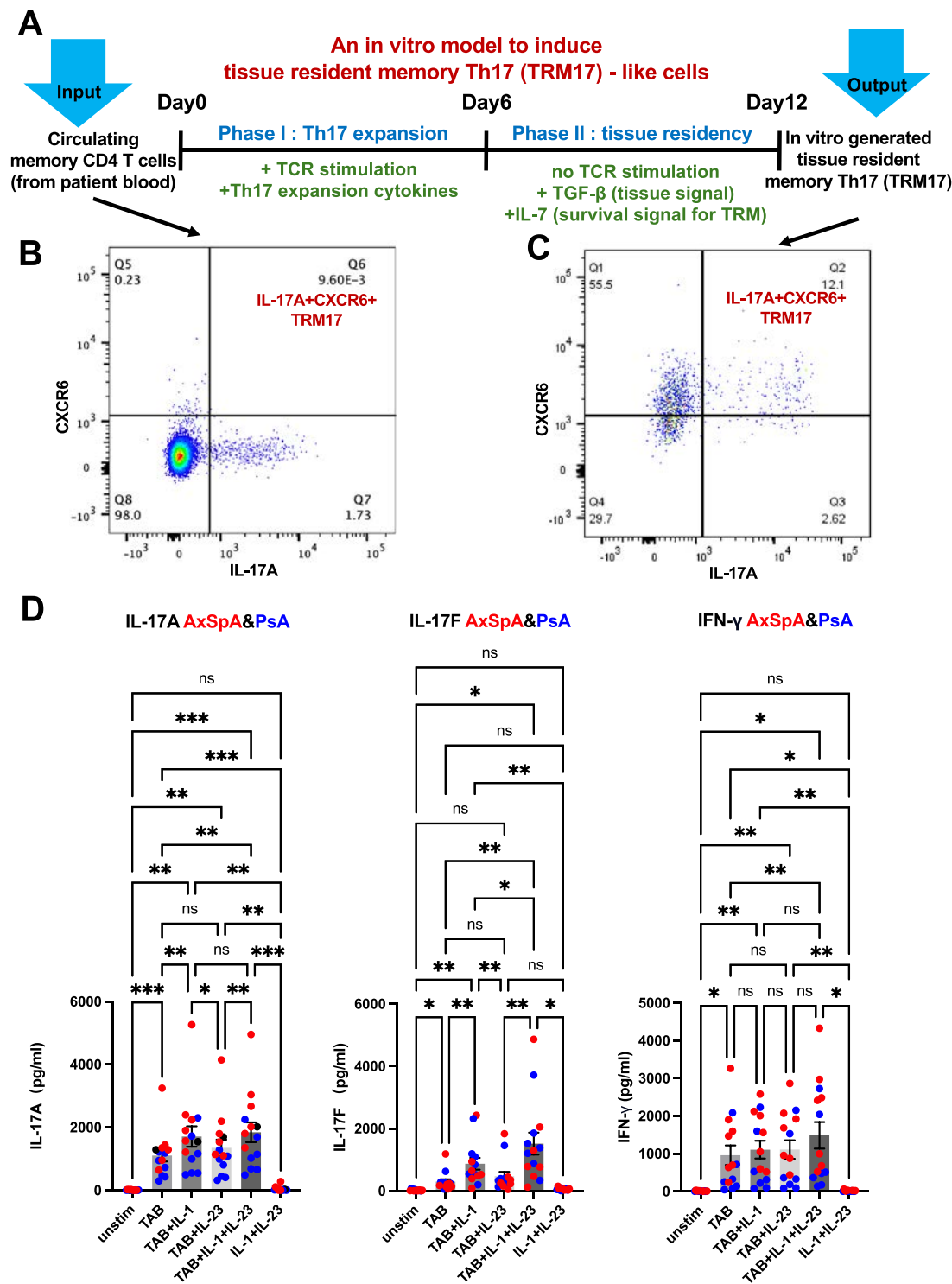


Figure 4. T-cell receptor engagement and cytokine stimulations codrive the production of IL-17A/F by in vitro generated TRM17-like cells. A, Schematic representation of the in vitro TRM17 model. Assessment of IL-17A + CXCR6 + TRM17 in circulating memory CD4 + T cells (B) and induced TMR17 cells (C). D, The secretion of IL-17A, IFN- γ , and IL-17F by TRM-like cells stimulated with vehicle control, cytokines (IL-1 β and IL-23), TABs, or the combinations of cytokine and TABs. Data from 7 AxSpA (red) and 7 PsA (blue) patients are shown. Statistical significance was assessed using one-way ANOVA (* $P \leq .05$, ** $P \leq .01$, *** $P \leq .001$). Median $\pm$ SEM is shown in (D). ANOVA, analysis of variance; AxSpA, axial spondyloarthritis; IFN, interferon; IL, interleukin; PsA, psoriatic arthritis; SEM, standard error of the mean; SpA, spondyloarthritis; TAB, T-cell activation beads.

While IL-23 blockade has proven highly efficacious for skin psoriasis, the effects on AxSpA and psoriatic arthritis have been less impressive [30–33]. This led us to investigate the role of IL-23 in IL-17A production by CD4 + TRM17-like cells. Figure 4D shows that TCR engagement using anti-CD3 and anti-CD28 T cell activation beads (TAB), rather than cytokine stimulation (IL-1 β and IL-23), induces IL-17A production in restimulated CD4 + TRM17-like cells from SpA patients. Furthermore, the

addition of IL-23 to either TAB or TAB + IL-1 β conditions did not significantly enhance IL-17A production. Interestingly, in the presence of TAB and IL-1 β , IL-23 significantly increased the secretion of IL-17F. This suggests that the lack of effect of IL-23 on IL-17A production is not due to the pre-existence of IL-23 in the culture media. Consistent with this, IL-23 blockade did not reduce IL-17A secretion in either the TAB alone or TAB + IL-1 β conditions (Supplementary Fig S13). IL-1 β blockade has been

tested in AxSpA and PsA with mixed results. We observed an IL-17-specific enhancement by IL-1 β , but not IFN- γ , either in TAB or TAB + IL-23 conditions. A similar pattern was observed when AxSpA and PsA samples were analysed separately (Supplementary Fig S14), as well as in CD4+ TRM17-like cells generated from healthy controls (Supplementary Fig S15). We also performed a similar experiment using cells generated from a 6-day Th17 expansion assay (anti-CD3/CD28, IL-1 β , IL-23) (Supplementary Fig S16). Like the CD4+ TRM17 assay, IL-17A secretion was predominantly driven by TAB and IL-1 β . Surprisingly, IL-17F was only induced when both TAB and cytokines (IL-1 β or IL-23) were present. Taken together, these data suggest that both TCR engagement and cytokine stimulation codrive the production of IL-17A/F by *in vitro* generated CD4+ TRM17-like cells.

Identification of BRD1 as a novel epigenetic regulator of Th17/CD4+ TRM17 cells

The unique epigenetic profile of CXCR6+ Th17 cells reported in the murine multiple sclerosis model [13] drove us to hypothesise that epigenetic regulators could contribute to the generation of CD4+ TRM17 cells. To test this hypothesis, we screened a library of 38 epigenetic inhibitors using cells from SpA patients. Due to the low-throughput nature of the CD4+ TRM17 model, we performed the initial screen using a Th17 expansion assay and discovered 3 inhibitors (bromosporine, PFI-1 and OF-1) that suppress Th17 responses in both ICS and IL-17 enzyme-linked immunosorbent assays (ELISAs) (Figure 5A). We deprioritised the first 2 inhibitors as bromosporine is a pan-bromodomain inhibitor with multiple targets and the target of PFI-1, the bromodomain of bromodomain and extraterminal domain family, has already been shown to regulate Th17 cells [34,35]. In contrast OF-1, an inhibitor for the bromodomain and PHD finger-containing protein (BRPF) family [36], has not been implicated previously in Th17 response and thus was chosen for further investigation. To this end, we first used siRNA to screen all 3 members in the BRPF family and found BRD1 silencing inhibited IL-17A production (Fig 5B). A similar trend was observed in BRPF1-silenced cells. Silencing efficiency was greater than 50% for all 3 genes (Fig S17A), a level generally considered acceptable for primary immune cell experiments. Further transcriptome analysis showed that knockdown of BRD1, but not BRPF1, reduced both Th17 marker genes (*IL17A*, *IL17F*, *RORC*, and *IL23R*) and TRM markers (*CXCR6* and *ITGA1*) (Fig 5C). Taken together these data suggest a potential role of BRD1 in CD4+ TRM17 cell generation.

We then used our CD4+ TRM17 *in vitro* model to investigate the role of BRD1 in the generation of TRM17-like cells. As the transient nature of siRNA-mediated gene suppression is not suitable for the 12 days CD4+ TRM17 model, we deployed clustered regularly interspaced short palindromic repeats (CRISPR) technology to stably knockout BRD1 (Fig 5D) and achieved over 50% knockout efficiency (Figure S17B). The BRD1-knock out (KO) CD4+ T cells were rested for 3 days (allowing the degradation of endogenous BRD1 protein) before being used as the input for CD4+ TRM17 generation model. Figure 5E shows that, in comparison to AAVS control guide RNA, BRD1 knockout significantly inhibited the generation of CXCR6+ IL-17A+ TRM17-like cells (Fig 5E). No changes in cell viability or proliferation were observed. Additionally, the restimulation of TRM17-like cells induced a lower level of IL-17A secretion in BRD1-KO cells than the adeno-associated virus integration site 1 control cells (Fig 5F). Similar results were observed for IL-17F (Supplementary Fig S17). In contrast to IL-17A and IL-17F, the percentage of IFN-

γ +CXCR6+ cells and the secretion IFN- γ were not affected by BRD1 KO (Fig 5E and F).

DISCUSSION

In this study, we show that CXCR6+ tissue-resident memory CD4+ T cells are the main source of IL-17A in SpA joint tissue. Despite the predominant localisation of CD4+ TRM17 cells in the sublining regions, both sublining and lining fibroblasts in SpA joints exhibit an enhanced IL-17 response signature suggesting the diffusion of IL-17A within the tissue. We also show that TCR engagement and cytokine stimulations codrive the production of IL-17A/F by *in vitro* generated TRM17-like cells. Lastly, we identify BRD1 as a novel Th17 regulator that contributes to the generation of CD4+ TRM17-like cells *in vitro*.

Previous studies in SpA synovial fluid (SF) identified $\gamma\delta$ T cells, MAIT cells, iNKT and CD8+ tissue-resident memory T cells as key sources of IL-17 [3–9]. Our findings indicate CD4+ TRM17 cells as the primary source of IL-17 in SpA synovial tissue (ST). This discrepancy suggests that distinct inflammatory drivers are present in SF versus ST. The relationship between SF and ST inflammation within IL-17 pathology and warrants further investigation. Our SpA ST dataset makes a significant contribution to existing knowledge. In addition to demonstrating CD4+ TRM IL-17A and IL-17F production, we also highlight the activation and function of various non-T cell populations within the SpA synovium. Specifically, we observe TNF production primarily by myeloid cells (mainly macrophages). Furthermore, we detect the activation of fibroblasts and *CLEC10A*+ DCs in these AxSpA patients compared to healthy controls using infrapatellar fat pad and synovium tissues studied elsewhere.

Importantly, we found the *in vitro* generated CD4+ TRM17-like cells produced IL-17A mainly in response to TCR engagement rather than cytokine stimulation. In the presence of IL-1 β , IL-23 enhanced the TCR stimulation induced production of IL-17F but not IL-17A, suggesting that cytokine stimulation has a greater influence on IL-17F production. In line with this, IL-17F has been shown to be the dominant IL-17 isoform produced by cytokine-activated innate lymphocytes (MAIT cells, $\gamma\delta$ T cells and ILC3s) [37,38], indicating a closer association of IL-17F with the innate response in lymphocytes. We also observed that IL-1 β enhanced IL-17A/F production in the presence of TCR engagement, and this enhancement occurred both with and without IL-23.

We acknowledge the inherent limitations of this study. Our sample size is relatively small, largely due to the costs of scRNA-seq and the availability of tissue. More than half of the samples came from patients with long disease duration (>10 years). While we cannot be certain that our findings fully represent early-stage SpA, we did detect CD4 TRM17 IL-17 production in samples from patients with a shorter disease duration (<5 years). A larger cohort, stratified by disease duration (and therapy), would be necessary to further confirm and expand upon our findings. In addition, we used human infrapatellar fat pad and STs as controls for the study of fibroblasts and myeloid cells. A comparison with synovium from healthy knee, hip, or spine should be conducted in the future to confirm our findings.

In summary, we demonstrate for the first time that the CD4+ TRM17 population is the main source of IL-17A in SpA ST. In addition, our data suggest that targeting CD4+ TRM17, the ‘factory’ of IL-17A, has the potential to induce long-term remission, encouraging future efforts to develop new therapies to deplete CD4+ TRM17 cells in SpA.

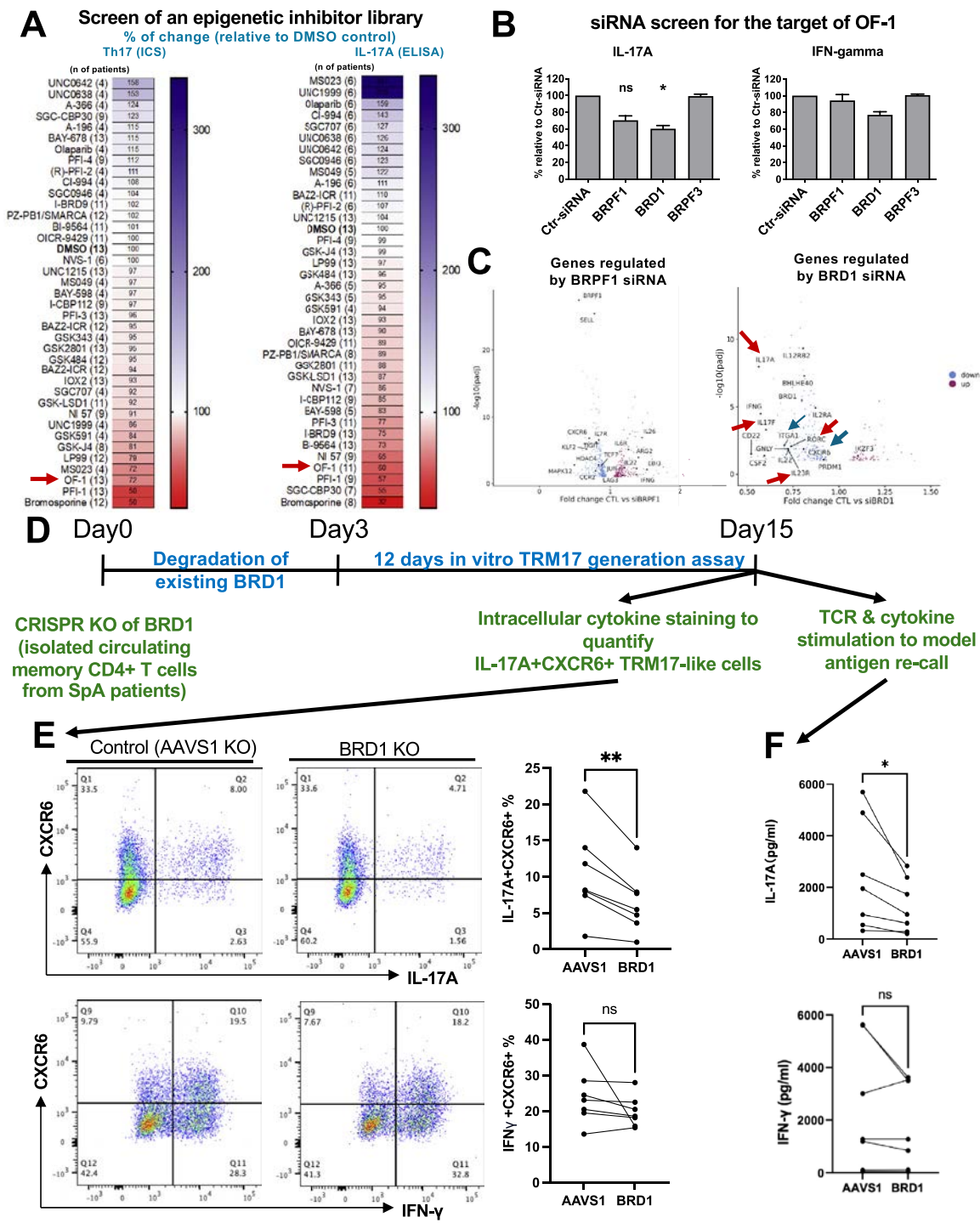


Figure 5. The epigenetic regulator BRD1 plays a role in TRM17 generation. A library of epigenetic inhibitors was screened using CD4+ T cells from SpA patients. Depending on the amount of blood obtained, a range of 4–13 donors were recruited for different inhibitors. The changes of Th17 frequency (based on IL-17A intracellular cytokine staining) and the level of IL-17A secretion (ELISA) relative to vehicle control (DMSO) are shown (A). (B) The respective influence of siRNAs for BRPF1, BRD1, and BRPF3 on IL-17A secretion by CD4+ T cells stimulated with TABs and Th17-promoting cytokines (IL-1 β and IL-23) (data from 3 SpA patients). (C) The transcriptome changes induced by the siRNA silencing of BRPF1 and BRD1 (data from 6 SpA patients). D, Schematic representation of BRD1 knockout for the in vitro TRM17 model. E and F, Effect of BRD1 knockout on the generation of TRM17-like cells and their IL-17A production upon antigen recall (data from 7 SpA patients). The *P* value was assessed using the Friedman test for (B) and paired Student's *t* test for (E and F) (**P* $\leq$.05; ***P* $\leq$.01). Median $\pm$ SEM is shown in (B). AxSpA, axial spondyloarthritis; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; SEM, standard error of the mean; SpA, spondyloarthritis; TAB, T-cell activation beads.

Competing interests

This work has been partially funded by J&J to the Cartography Consortium. LC has received research support from Novartis. PB has received research support from Regeneron,

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relationships that could have appeared to influence the work reported in this paper.

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Patient consent for publication

Not applicable.

Ethics approval

Venous blood was obtained under protocols approved by the Oxford Research Ethics committee (ethics reference number 06/Q1606/139). Synovial tissue were obtained under protocols approved by the Oxford Research Ethics committee (ethics reference number 06/Q1606/139), South Birmingham Research Ethics committee (ethics reference number 14/WM/1109) and Ethics Committee of Shanghai Sixth People's Hospital (2024-KY-132).

Provenance and peer review

Not commissioned; externally peer reviewed.

Data availability statement

Data has been deposited at GEO (GSE290921) and will be released following the publication of the work.

Cartography Consortium

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Supplementary materials

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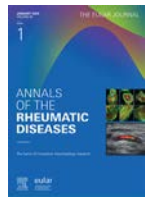
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Systemic lupus erythematosus

Novel IgG and IgA autoantibodies validated in two independent cohorts are associated with disease activity and determine organ manifestations in systemic lupus erythematosus: implications for anti-LIN28A, anti-HMGN5, anti-IRF5, and anti-TGIF1

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ABSTRACT

Objectives: This study aimed to identify and validate novel autoantibodies that reflect global and organ-specific disease activity in systemic lupus erythematosus (SLE).

Methods: Plasma samples were screened for IgG and IgA seroreactivity against 1609 protein autoantigens using a microarray (i-Ome Discovery; Sengenics). We determined differentially abundant autoantibodies (daAABs) in patients with SLE vs healthy controls within a discovery (n = 196 vs n = 110; NCT02890121) and an independent validation cohort (n = 30 vs n = 83; NCT02890134) from the European PRECISESADS project. Validated daAABs were analysed in relation to global and organ-specific disease activity using linear and logistic regression, along with daAAB target pathway enrichment analysis.

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Results: We validated 89 IgG and 66 IgA daAAbs. IgG anti-LIN28A, IgG anti-HMG5, and both isotypes for anti-IRF5 and anti-TGIF1 were associated with a SLE disease activity index 2000 score of ≥ 10 , negatively associated with lupus low disease activity state, and highly prevalent in subgroups with active disease across organ manifestations. IgG anti-LIN28A levels exceeded the cutoff for positivity in 53% of patients with central nervous system (CNS) involvement, a prevalence higher than that observed for anti-double-stranded DNA (20%) and 47% of patients with renal activity. A cluster of IgG and IgA daAAbs against RNA-binding proteins, including anti-LIN28A, was linked to CNS involvement. IgA anti-FOSL2 was elevated uniquely in patients with musculoskeletal activity. Enriched pathways involving DNA binding and repair showed considerable overlap across manifestations.

Conclusions: Novel IgG and IgA autoantibodies, including IgG anti-LIN28A, IgG anti-HMG5, IgG and IgA anti-IRF5, and IgG and IgA anti-TGIF1 were associated with SLE disease activity and highly abundant across organ manifestations.

WHAT IS ALREADY KNOWN ON THIS TOPIC

- Autoantibodies are a hallmark of systemic lupus erythematosus (SLE), but among a multitude of autoantigen specificities, few are mapped and used in routine clinical practice.
- Autoantibodies currently used for surveillance, such as anti-double-stranded DNA (anti-dsDNA), show only modest associations with SLE disease activity.

WHAT THIS STUDY ADDS

- IgG anti-LIN28A, IgG anti-HMG5, IgG and IgA anti-IRF5, and IgG and IgA anti-TGIF1 were associated with high disease activity and negatively associated with lupus low disease activity state and were highly prevalent in subgroups of patients across organ manifestations.
- Extensive profiling of IgA autoantibodies in SLE is novel and suggests a potential role for mucosal immunity in SLE pathogenesis.
- IgG anti-LIN28A levels exceeded the cutoff for positivity in more than half of patients with central nervous system (CNS) involvement, whereas only a fifth of patients with CNS activity were anti-dsDNA positive.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- This study demonstrated associations between novel IgG and IgA autoantibodies, identified in a large discovery SLE cohort and validated in an independent inception cohort, and SLE disease activity, as well as high abundance of several of these autoantibodies across organ manifestations; autoantibody profiling using these novel autoantibodies may better characterise patients with SLE, predict impending flares, and guide personalised therapies.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a prototypical autoimmune disease, characterised by the presence of multiple autoantibodies that have been associated with its pathogenesis and clinical manifestations. These autoantibodies target self-antigens exposed during cell death or tissue damage, forming immune complexes that either get deposited from circulation or form locally in tissues and trigger inflammatory responses, ultimately resulting in organ damage [1,2]. Additionally, autoantibodies contribute to SLE pathogenesis by interacting with cell surface receptors and penetrating cells, thereby altering cellular processes and contributing to the phenotypical heterogeneity of SLE [3,4].

Antinuclear antibodies (ANAs) are the most frequently tested in clinical practice, with specific antibodies such as anti-double-stranded DNA (dsDNA) being both diagnostic and correlating, although modestly, with disease activity [5]. However, many other autoantibodies have been identified, expanding our understanding of the complexity of SLE [6]. Notably, autoantibody testing in clinical practice and research has predominantly focused on the immunoglobulin (Ig)G isotype. However, other isotypes, such as IgA and IgM, may also have relevance in SLE. For example, while non-IgG antiphospholipid antibodies are not universally accepted for their relevance in the classification of antiphospholipid syndrome, studies suggest they may contribute to disease mechanisms [7]. This underscores the potential value of expanding autoantibody profiling to obtain a more comprehensive understanding of the immunologic landscape of SLE.

Understanding the role of autoantibodies in SLE is essential for improving diagnostics, monitoring, prognostication, and the development of novel therapies, particularly in the era of personalised medicine. The chronic nature of SLE, characterised by alternating episodes of flares and remission, necessitates regular monitoring [8]. Identifying an increase in SLE disease activity can be challenging in routine clinical practice due to the heterogeneity of the disease and the subtlety of early signs of flares or unspecific symptoms. Early identification of increasing disease activity and evolution towards a flare is crucial for rapid treatment intervention, as high disease activity is associated with poor outcomes, including accrual of organ damage, which is linked to increased morbidity and mortality [9]. The 2023 European Alliance of Associations for Rheumatology recommendations state that the aim of SLE treatment is remission or low disease activity when remission is not achievable [10]. These goals are of clinical [11–13] and biological [14] relevance in SLE, and optimising surveillance and treatment evaluation is of great importance towards their induction and maintenance, especially in the light of new targeted therapies for SLE [15] and the growing awareness of the long-term adverse effects of glucocorticoids (GCs) [1]. Hence, to maximise the benefits of advanced treatments, identifying novel and reliable biomarkers of disease activity and impending flares remains an urgent unmet need.

To address this need, we aimed to uncover novel biomarkers that could enhance our ability to monitor SLE disease activity more accurately and tailor treatments more effectively using the i-Ome Discovery protein microarray (Sengenics Corporation) that encompasses 1609 protein autoantigens. We specifically screened for IgG and IgA specificities against these autoantigens. This high-throughput immunoassay platform uses KoRectly EXpressed (KREX) technology, which preserves the structure of

correctly folded full-length proteins, ensuring specific and biologically meaningful binding. Our primary goal was to identify novel autoantibodies that reflect global and organ-specific SLE disease activity.

METHODS

Study population

We used plasma samples from 2 independent multicentre cohorts of patients with SLE and healthy controls (HCs) collected within the frame of the 5-year European PRECISESADS (Molecular reclassification to find clinically useful biomarkers for systemic autoimmune diseases) project [16]. Specifically, 199 patients with SLE from a cross-sectional cohort and 111 HCs formed a discovery cohort (NTC02890121), and 30 patients with SLE from an inception cohort, enrolled within 1 year from diagnosis, along with 84 HCs formed a validation cohort (NCT02890134). Patients with SLE in the validation cohort underwent longitudinal sampling at baseline and at 6 and 14 months. All patients with SLE met the revised 1997 American College of Rheumatology criteria for SLE [17]. Patients were excluded if they had received high doses of prednisone equivalents (>15.0 mg/d), intravenous GCs, or high doses of immunosuppressants within 3 months preceding recruitment. Additionally, treatment with cyclophosphamide or belimumab in the past 6 months and rituximab in the past year was not permitted. The complete set of inclusion and exclusion criteria is available online in [Supplementary Table S1](#).

Before recruitment in PRECISESADS, all patients and HCs provided written informed consent. The PRECISESADS protocol was approved by local ethics review boards at all participating centres ([Supplementary Material, page 3](#), for a list of local investigators). This study was approved by the Swedish Ethical Review Authority (registration number: 2022-03907-01).

Clinical definitions

The Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2K) [18] and physician's global assessment (PGA; on a scale 0–100) were used to evaluate global SLE disease activity, with a SLEDAI-2K score ≥ 10 indicating high disease activity, as previously suggested [9].

Low disease activity was defined according to the Lupus Low Disease Activity State (LLDAS) criteria and required a SLEDAI-2K score of ≤ 4 , excluding major organ activity and fever, no new activity since the previous assessment, and a Safety of Estrogens in Lupus Erythematosus National Assessment (SELENA)-SLEDAI PGA score of ≤ 1 (on a scale 0–3), allowing for a prednisone or equivalent dose ≤ 7.5 mg/d and immunosuppressive or approved biological agents at standard and tolerable doses [19].

Remission was defined according to the Definition Of Remission In Systemic lupus erythematosus (DORIS) criteria and required a clinical SLEDAI-2K score of 0 and a SELENA-SLEDAI PGA score of <0.5 (on a scale 0–3), allowing for serological activity and use of low-dose GCs (prednisone or equivalent ≤ 5.0 mg daily) and immunosuppressive or biological agents at standard and tolerable doses [20].

Active disease within an organ system was defined as a score above zero in at least 1 descriptor within the respective SLEDAI-2K organ system. Based on the central nervous system (CNS) organ system of SLEDAI-2K, we subgrouped the patients with active CNS lupus into patients with focal and patients with

diffuse syndromes [21]. In cases where both focal and diffuse syndromes were present, major involvement was given priority over minor, as previously defined by Ainala et al. [22]. We defined current or past involvement of a particular organ system using information from SLEDAI-2K organ system scores, indicating active disease, and report of previous involvement as per the case report form of the PRECISESADS study protocol ([Supplementary Material, pages 4–5](#)).

Autoantibody measurement and gene expression analysis

Plasma samples were screened for IgG and IgA autoantibody specificities against a panel of 1609 human proteins using the i-Ome Discovery protein microarray (Sengenics Corporation; [Supplementary Material, sheet 1](#)) [23]. This high-throughput immunoassay platform uses KREX technology, which preserves the native 3-dimensional conformation of full-length proteins, thereby preserving *in vivo*-relevant epitopes and ensuring specific and biologically meaningful binding of autoantibodies to their targets. A detailed description is provided in [Supplementary Material, page 6](#).

Comparisons with conventional autoantibody specificities were enabled by measurements of IgG anti-dsDNA, IgG anti-Smith (anti-Sm), and IgG anti-U1RNP levels using an automated chemiluminescent immunoassay (IDS-iSYS; Immunodiagnostic Systems Holdings). IgG antichromatin levels were measured using an anti-dsDNA–NcX ELISA assay (EUROIMMUN Medizinische Labordiagnostika AG). Anti-dsDNA and antichromatin positivity were defined as levels ≥ 40.0 and ≥ 100.0 units/mL, respectively, as per assay protocols [24]. Genome-wide RNA sequencing of peripheral whole-blood samples was performed using Illumina assays (Illumina), as previously detailed [16].

Bioinformatics and statistical analysis

We conducted an analysis of differentially abundant autoantibodies (daAABs) using the limma R package [25], adjusting for polyspecific antibody (PSA) status, recruiting centre, age, and batch, all of which significantly impacted data composition. PSA status was determined based on antigens known to be targeted by the PSA phenotype ([Supplementary Material, sheet 2](#)). We first analysed daAABs in patients with SLE vs HC from the discovery and validation cohorts separately. daAABs in the discovery cohort with a false discovery rate–corrected *P* value of <0.05 that were also found among daAABs in the validation cohort with a *P* value of <0.05 were considered validated. Validated daAABs qualified for subsequent analysis in relation to SLEDAI-2K using multivariable linear regression and in relation to a SLEDAI-2K score of ≥ 10 , LLDAS, and DORIS remission using multivariable logistic regression in the discovery cohort, adjusting for age and sex. Participant sex was self-reported. Validated daAABs that were both positively associated with a SLEDAI-2K score of ≥ 10 and negatively associated with LLDAS qualified for analysis of autoantibody positivity, defined as levels higher than the median plus 2 IQRs of the distribution of levels of the autoantibody in the HC population of the discovery cohort. A comparison of autoantibody positivity between subgroups of patients with focal and diffuse CNS lupus was performed using the Fisher exact test. These daAABs also qualified for longitudinal assessments in relation to PGA in the validation cohort and for correlation analysis between levels of daAABs and conventional autoantibody specificities currently used in clinical practice in the discovery cohort. In addition, gene expression of the genes encoding the daAAB protein targets was analysed in relation to

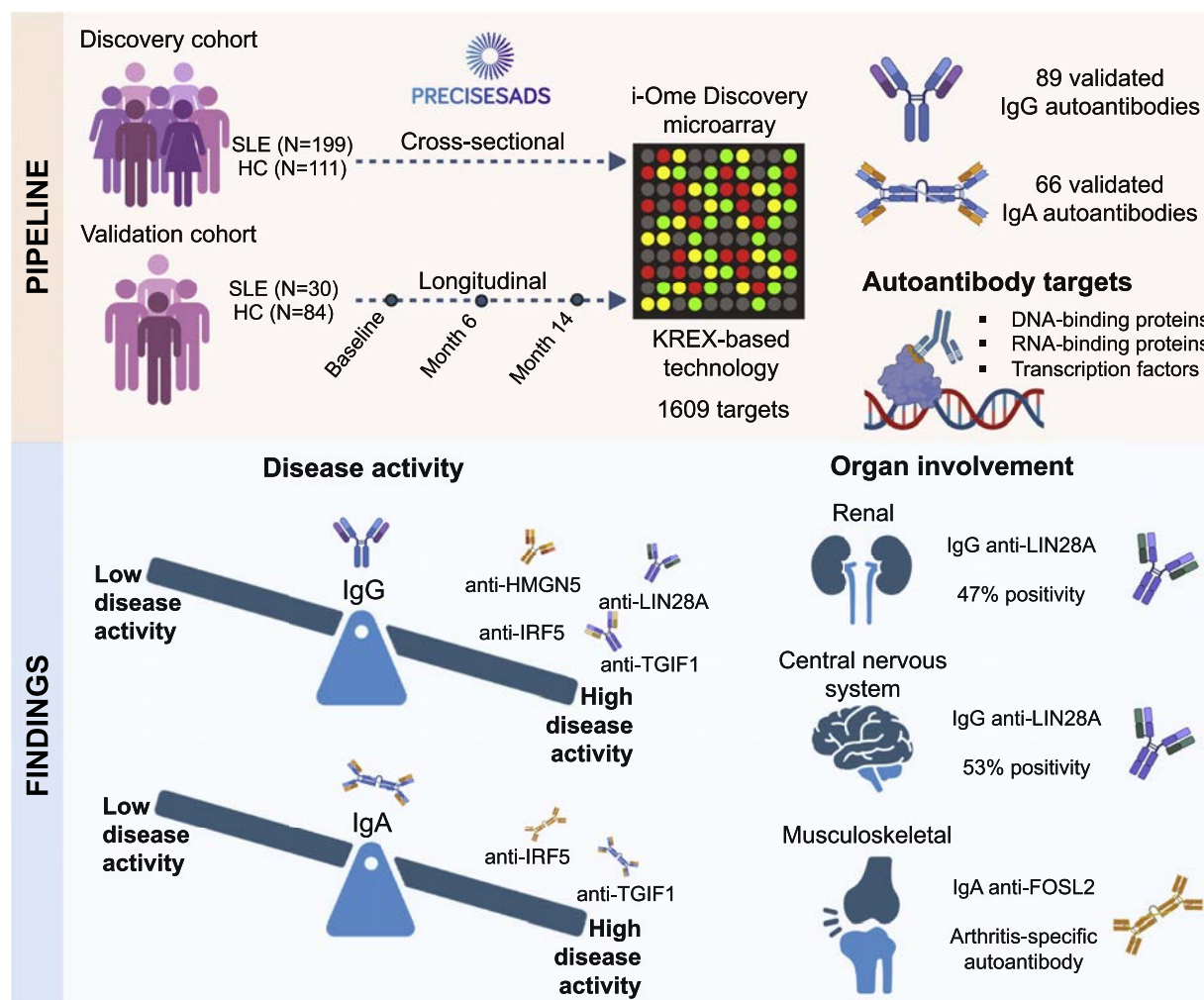


Figure 1. Schematic overview of the analytical pipeline as well as validated autoantibodies and their association with global disease activity and organ involvement. Plasma from patients with SLE in 2 independent cohorts was screened for IgG and IgA seroreactivity against 1609 protein autoantigens using the i-Ome Discovery microarray (Sengenics), with 3 longitudinal assessments conducted in the smaller validation cohort. The analysed autoantibody targets included DNA-binding and RNA-binding proteins as well as transcription factors. Among autoantibodies associated with high disease activity, IgG anti-LIN28A positivity was observed in about half of the patients with active renal and central nervous system involvement. HC, healthy control; KREX, KoRectly EXpressed; SLE, systemic lupus erythematosus.

SLEDAI-2K using multivariable linear regression and in relation to SLEDAI-2K of ≥ 10 , LLDAS, and DORIS remission using multivariable logistic regression in the discovery cohort, adjusting for age and sex.

Validated daAAbs also qualified for subsequent analysis of daAAbs across SLE manifestations. A detailed description of the analytical pipeline is found in [Supplementary Material, pages 6–8](#). The analytical pipeline as well as key findings are summarised in [Figure 1](#).

Patient and public involvement

The patient research partners Yvonne Enman and Karin Blomkvist Sporre were involved in the design and dissemination plans of this research. The public was not involved in the design, conduct, reporting, or dissemination plans of this research.

RESULTS

Characteristics of the 196 patients with SLE and 110 HCs in the discovery cohort as well as the 30 patients with SLE and 83 HCs in the validation cohort are presented in [Table](#).

Differentially abundant autoantibodies associated with SLE

We identified 109 IgG daAAbs in patients with SLE ($n = 195$) vs HC ($n = 108$) in the discovery cohort, of which 89 were validated in the independent inception cohort ([Fig 2A,B](#); [Supplementary Material, sheets 2–3](#)). We identified 115 IgA daAAbs in patients with SLE ($n = 196$) vs HC ($n = 110$) in the discovery cohort, of which 66 were validated in the independent inception cohort ([Fig 2C,D](#); [Supplementary Material, sheets 3–6](#)).

Differentially abundant autoantibodies associated with SLE disease activity

To explore potential associations between validated daAAbs and SLE disease activity, we assessed IgG and IgA daAAb levels in relation to several key clinical measures: SLEDAI-2K, high disease activity (SLEDAI-2K ≥ 10), LLDAS, and DORIS remission ([Supplementary Material, sheets 7–14](#)). Among 89 validated IgG daAAbs, 17 IgG daAAbs were associated with SLEDAI-2K ([Fig 3A](#)), with anti-ACPP and anti-PABPC1 exhibiting the lowest and highest coefficients, respectively. Additionally, 21 IgG daAAbs were associated with SLEDAI-2K of ≥ 10 ([Fig 3B](#)), including anti-ACPP and anti-PABPC1; 56 IgG daAAbs were

Table

Baseline characteristics of patients with SLE and healthy controls in the discovery and validation cohorts

	Discovery cohort		Validation cohort	
	SLE (n = 199)	HC (n = 111)	SLE (n = 30)	HC (n = 84)
Demographics				
Age (y)	46.9 ± 14.2	48.8 ± 15.0	35.2 ± 11.9	48.6 ± 13.2
Female sex	188 (94.5)	105 (94.6)	24 (80.0)	79 (94.0)
Ancestry				
Black/African American	0 (0.0)	0 (0.0)	3 (10.0)	0 (0.0)
Caucasian/White	199 (100.0)	111 (100.0)	25 (83.3)	84 (100.0)
Other	0 (0.0)	0 (0.0)	2 (6.7)	0 (0.0)
Clinical data				
Disease duration (y)	14.7 ± 10.2	NA	NA	NA
SLEDAI-2K score	6.9 ± 6.2; n = 193	NA	NA	NA
CNS (vs active non-CNS SLE)	18 (39.1); n = 46	NA	NA	NA
Vascular (vs active nonvascular SLE)	12 (25.5); n = 47	NA	NA	NA
Musculoskeletal (vs active nonmusculoskeletal SLE)	31 (67.4); n = 46	NA	NA	NA
Renal (vs active nonrenal SLE)	45 (52.9); n = 85	NA	NA	NA
Dermal (vs active nondermal SLE)	103 (90.4); n = 114	NA	NA	NA
Serosal (vs active nonserosal SLE)	5 (4.4); n = 113	NA	NA	NA
Immunologic (vs active nonimmunologic SLE)	128 (84.8); n = 151	NA	NA	NA
Constitutional (vs active nonconstitutional SLE)	3 (2.2); n = 139	NA	NA	NA
Haematologic (vs active nonhaematologic SLE)	39 (57.4); n = 68	NA	NA	NA
PGA score (0-100)	NA	NA	28.8 (22.6)	NA
LLDAS	73 (38.6); n = 189	NA	NA	NA
DORIS remission	29 (15.2); n = 191	NA	NA	NA
SLE manifestations (ever)	n = 193			
Neuropsychiatric	32 (16.6)	NA	NA	NA
Vascular	16 (8.3)	NA	NA	NA
Musculoskeletal	164 (85.0)	NA	NA	NA
Renal	71 (36.8)	NA	NA	NA
Mucocutaneous	172 (89.1)	NA	NA	NA
Serosal	43 (22.3)	NA	NA	NA
Immunologic	163 (84.5)	NA	NA	NA
Constitutional	12 (6.2)	NA	NA	NA
Haematologic	144 (74.6)	NA	NA	NA
Medications (current use)				
Glucocorticoids	102 (51.3)	NA	6 (20.0)	NA
Prednisone equivalent dose during follow-up (mg/d); median (IQR)	1.3 (0.0-5.0)	NA	NA	NA
Antimalarial agents	156 (78.4)	NA	16 (53.3)	NA
Immunosuppressants				
Azathioprine	60 (30.3); n = 198	NA	0 (0.0)	NA
Calcineurin inhibitors	2 (1.0)	NA	0 (0.0)	NA
Leflunomide	0 (0.0); n = 198	NA	0 (0.0)	NA
Methotrexate	16 (8.1); n = 198	NA	0 (0.0)	NA
Mycophenolic acid	25 (12.6)	NA	0 (0.0)	NA

ANA, antinuclear antibody; CNS, central nervous system; DORIS, definition of remission in SLE; HC, healthy control; LLDAS, lupus low disease activity state; NA, not applicable; PGA, physician's global assessment; SLE, systemic lupus erythematosus; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000.

Data are presented as numbers (%) or means ± standard deviation. In case of nonnormal distributions, the medians (IQR) are indicated. In case of missing values, the total number of patients with available data is indicated.

associated with LLDAS (Fig 3C), including anti-NHLH1 and anti-PRSS50; and 28 IgG daAAs were associated with DORIS remission (Fig 3D), including anti-RPL5 and anti-PRKACB.

Among 115 validated IgA daAAs, we found anti-RNF7 and anti-FEN1 to be associated with SLEDAI-2K (Fig 3E), and 6 with a SLEDAI-2K score of ≥10 (Fig 3F), including anti-RNF7 and anti-TSC22D4. We also identified 34 IgA daAAs that were associated with LLDAS (Fig 3G), including anti-ATF2 and anti-RNF7. IgA anti-E2F6, IgA anti-APOBEC3G, and IgA anti-MAPK12 were associated with DORIS remission (Fig 3H).

Autoantibody positivity across SLE manifestations

To investigate potential associations between validated daAAs and SLE manifestations, we calculated the proportions of autoantibody-positive patients stratified by organ system and compared them with the proportion of autoantibody-positive

patients in DORIS remission as well as HCs. To qualify for this investigation, validated daAAs were required to be both positively associated with high disease activity (a SLEDAI-2K score of ≥10) and negatively associated with LLDAS. Nine IgG daAAs fulfilled this requirement; ranked in decreasing order of log₂ fold change (FC) in patients with SLE vs HCs in the discovery cohort, those were anti-LIN28A, anti-HMG5, anti-IRF5, anti-TGIF1, anti-NHLH1, anti-ZNF276, anti-PABPC1, anti-IGF2BP3, and anti-RARA (Fig 4A-I; [Supplementary Material, sheet 15](#)). Notably, 53% (9/17) of patients with CNS activity and 47% (20/43) of patients with renal activity exhibited positive levels of IgG anti-LIN28A, in comparison with 21% (6/29) of patients in DORIS remission. In a subgroup analysis of patients with active CNS lupus, no difference was observed in the proportion of patients with positive levels of IgG anti-LIN28A between those with diffuse (3/4; 75%) and those with focal syndromes (3/6; 50%; *P* = .571). Additionally, 29% (9/31) of patients with

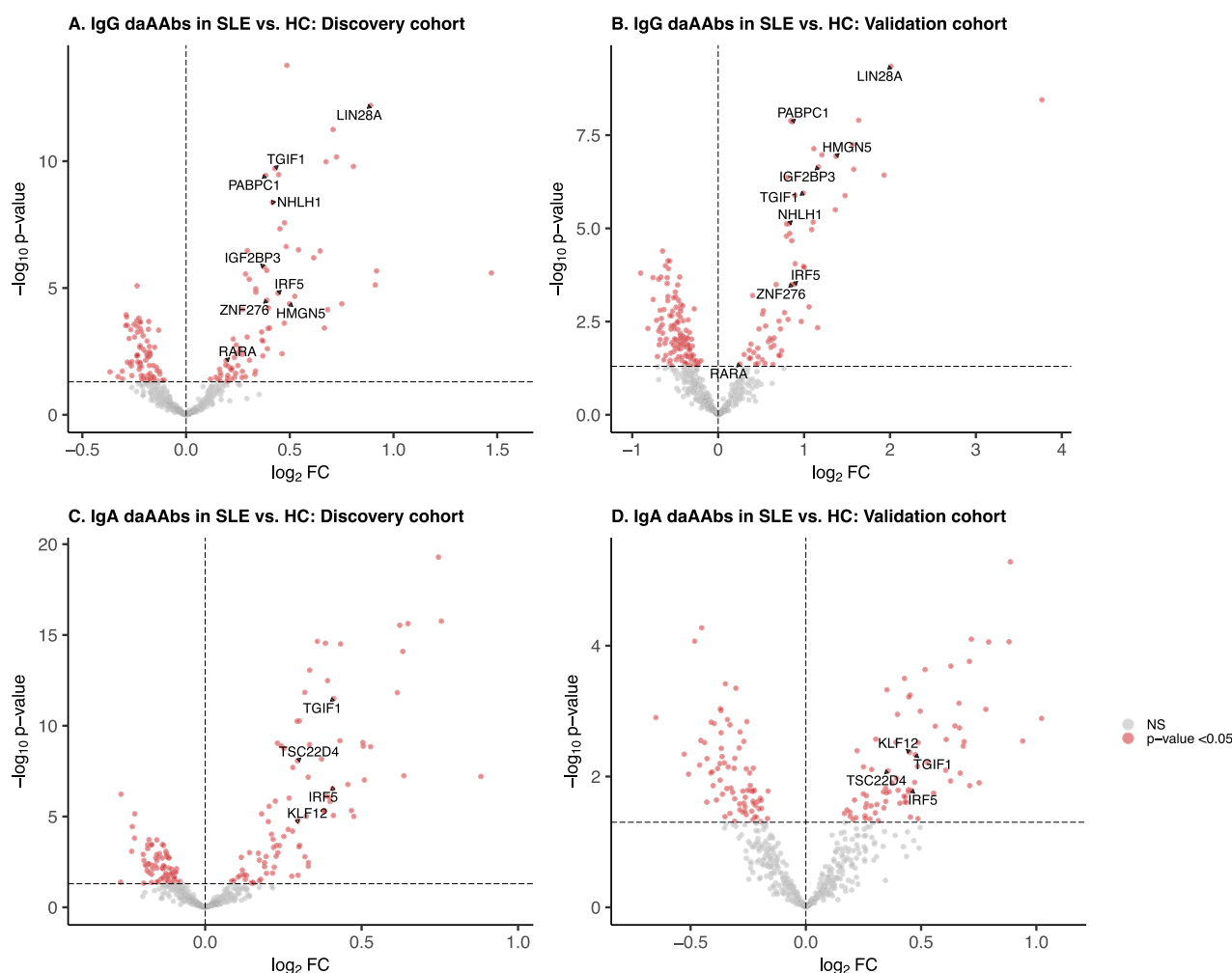


Figure 2. IgG and IgA daAAbs in patients with SLE vs HC. The volcano plots display IgG daAAbs in the (A) discovery and (B) validation cohorts, as well as IgA daAAbs in the (C) discovery and (D) validation cohorts. daAAbs with a $P < .05$ are highlighted in red, while nonsignificant daAAbs are in grey. Selected daAAbs are labelled, if associated with SLEDAI-2K of ≥ 10 and negatively associated with LLDAS, with the full list of daAAbs provided in [Supplemental Material](#), sheets 3-6. daAAb, differentially abundant autoantibody; FC, fold change; HC, healthy control; LLDAS, lupus low disease activity state; NS, nonsignificant; SLE, systemic lupus erythematosus; SLEDAI-2K, systemic lupus erythematosus disease activity index 2000.

musculoskeletal activity were IgG anti-HMGN5 positive, and the proportions were even higher for the other SLE manifestations (30%–67%) except for vascular activity (1/11; 9%), in comparison with only 3% (1/29) in patients with DORIS remission.

Similarly, 4 validated IgA daAAbs fulfilled the requirement of associations with high disease activity and LLDAS; ranked in decreasing order of log₂ FC in patients with SLE vs HC in the discovery cohort, those were anti-TGIF1, anti-IRF5, anti-KLF12, and anti-TSC22D4 (Fig 4J–M; [Supplementary Material](#), sheet 16). The proportion of patients with positive levels of IgA anti-TGIF1 was 33% (6/18) in patients with CNS activity and ranged between 41% and 80% across the other organ systems except for vascular disease (2/11; 18%), in comparison with 28% (8/29) in patients with DORIS remission. A similar pattern was observed for IgG anti-TGIF1. The proportion of patients with positive levels of IgA anti-IRF5 were 28% (5/18) in patients with CNS activity and ranged between 33% and 60% across the other organ systems except for vascular disease (2/11; 18%), in comparison with 17% (5/29) in patients with DORIS remission.

The proportion of patients with positive levels of anti-dsDNA ranged between 20% and 100% across SLE manifestations. Among patients with CNS activity, 20% (3/15) were anti-dsDNA positive. The proportion of patients with positive levels of

antichromatin ranged between 57% and 100% across SLE manifestations ([Supplementary Table S2](#)).

Longitudinal variation in disease activity and levels of differentially abundant autoantibodies in SLE

PGA scores and levels of all validated IgG and IgA daAAbs that were associated with high disease activity and negatively associated with LLDAS generally decreased over the 14-month follow-up in the validation cohort ([Supplementary Fig S1](#); [Supplementary Material](#), sheet 17). Autoantibody levels showed mild reductions, with the possible exception for IgG RARA.

Correlations between levels of differentially abundant autoantibodies and conventional autoantibody specificities

Levels of validated IgG daAAbs that met the criteria for associations with high disease activity and LLDAS correlated most strongly with anti-dsDNA and antichromatin, with modest correlations observed for IgG Sjögren's-syndrome-related antigen B (SSB) (Fig 5A; [Supplementary Table S3](#)). Levels of validated IgA daAAbs that met the same criteria correlated most strongly with

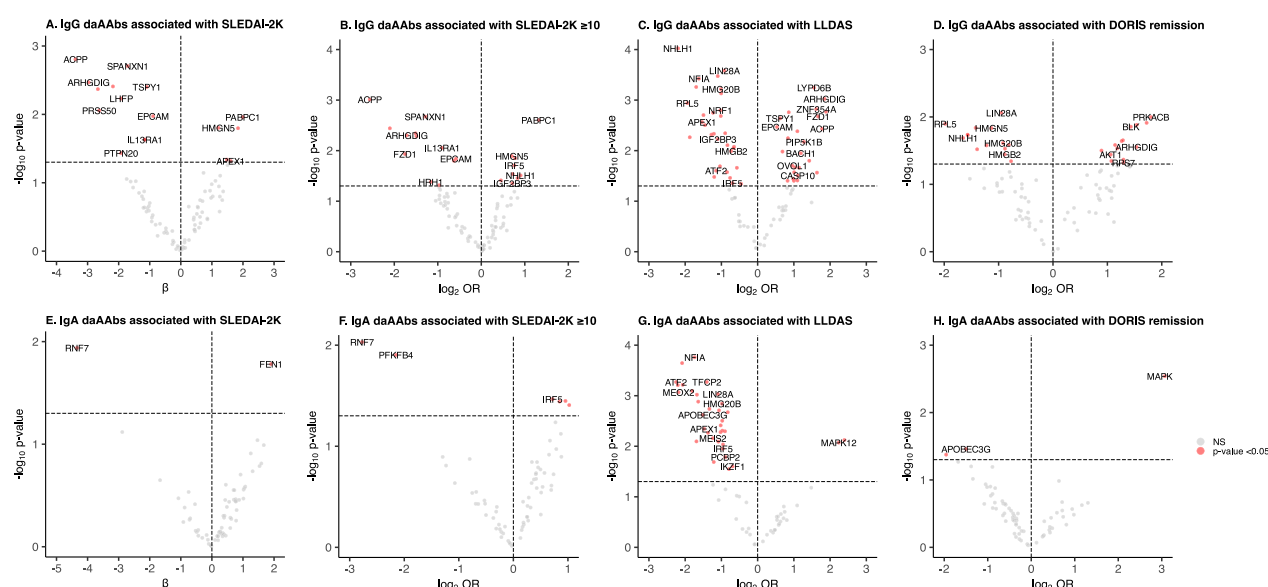


Figure 3. IgG and IgA daAAb targets associated with SLE disease activity. The volcano plots illustrate IgG daAAb targets in patients with SLE vs HC in the discovery cohort, associated with (A) SLEDAI-2K using multivariable linear regression, and (B) SLEDAI-2K ≥ 10 , (C) LLDAS, and (D) DORIS remission using multivariable logistic regression. The volcano plots show IgA daAAb targets in patients with SLE vs HC in the discovery cohort, associated with (E) SLEDAI-2K using multivariable linear regression and (F) SLEDAI-2K ≥ 10 , (G) LLDAS, and (H) DORIS remission using multivariable logistic regression. daAAb targets with a P value < 0.05 are highlighted in red, while nonsignificant daAAb targets are in grey. The labelling of daAAb targets is stochastic, and a full list of daAAb targets are provided in [Supplemental Material, sheets 7–14](#). daAAb, differentially abundant autoantibody; DORIS, definition of remission in SLE; HC, healthy control; LLDAS, lupus low disease activity state; NS, nonsignificant; SLE, systemic lupus erythematosus; SLEDAI-2K, systemic lupus erythematosus disease activity index 2000.

IgA anti-SSB, while modest correlations were seen with anti-dsDNA and antichromatin ([Fig 5B](#); [Supplementary Table S3](#)).

Gene expression of differentially abundant autoantibody targets associated with SLE disease activity

To investigate potential associations between the blood transcripts of daAAb targets linked to high disease activity and LLDAS, we assessed gene expression in relation to clinical measures, following a similar approach to that used for the validated daAAb targets ([Supplementary Material, sheets 18–21](#)). Among the 11 genes corresponding to the IgG and IgA daAAb targets, 6 were associated with SLEDAI-2K ([Supplementary Fig S2A](#)), with *PABPC1* and *RARA* showing the lowest and highest coefficients, respectively. Additionally, 4 genes were associated with SLEDAI-2K of ≥ 10 ([Supplementary Fig S2B](#)), including *PABPC1* and *NHLH1*, 7 genes were associated with LLDAS ([Supplementary Fig S2C](#)), including *RARA* and *PABPC1*, and 4 genes were associated with DORIS remission ([Supplementary Fig S2D](#)), including *NHLH1* and *TGIF1*.

Differentially abundant autoantibodies associated with SLE manifestations

To also examine organ-specificity for validated daAAb targets in an exploratory manner, we performed subgroup daAAb analysis stratified by organ system vs HC in the discovery cohort ([Fig 6A, B](#); [Supplementary Material, sheets 22–23](#)). IgG anti-*RARA* and IgG anti-*SOX15* were solely elevated in patients with renal and immunologic activity, IgG anti-*ELF1* and IgG anti-*ERG* were exclusively elevated in patients with dermal and immunologic activity, and IgG anti-*USF2* was uniquely elevated in patients with serosal and immunologic activity. IgA anti-*E2F6* and IgA anti-*SRPK1* were solely elevated in patients with renal and

immunological activity, IgA anti-*FOSL2* was exclusively elevated in patients with musculoskeletal activity, and IgA anti-*FEN1* was uniquely elevated in patients with dermal and immunologic activity.

Among IgG and IgA daAAb targets in patients with current or past activity in one or more organ systems compared with HCs in the discovery cohort ([Supplementary Fig S3A,B](#); [Supplementary Material, sheets 24–25](#)), IgG anti-*SOX15* was solely elevated in patients with current or past musculoskeletal and immunologic activity. IgA anti-*FOSL2* was uniquely elevated in patients with current or past serosal activity, and IgA anti-*UBE2I* was exclusively elevated in patients with current or past musculoskeletal and immunologic activity.

Clusters of differentially abundant autoantibodies across SLE manifestations

We further performed cluster analysis for elevated IgG and IgA daAAb targets in patients with active disease and identified 3 autoantibody clusters for each isotype ([Fig 6C,D](#)). For IgG daAAb targets, cluster 1 comprised RNA-binding proteins such as *SSB* and *LIN28A* and was predominantly represented in patients with CNS, vascular, and musculoskeletal activity. Among the other 2 clusters, which were represented in patients with varying activity patterns, IgG cluster 2 included transcription factors like *IRF5* and *ATF2*, while IgG cluster 3 encompassed DNA repair proteins such as *APEX1* and *FEN1*. For IgA daAAb targets, clusters 1 and 2 were represented in patients with varying activity patterns, whereas cluster 3 was predominantly represented in patients with CNS and vascular activity. IgA cluster 1 comprised DNA-binding proteins such as *HMGN5* and *ZNF276*, cluster 2 included the DNA repair proteins *APOBEC3G* and *FEN1*, while cluster 3 comprised RNA-binding proteins such as *SSB* and *LIN28A* as well as transcription factors such as *IRF5* and *ATF2*.

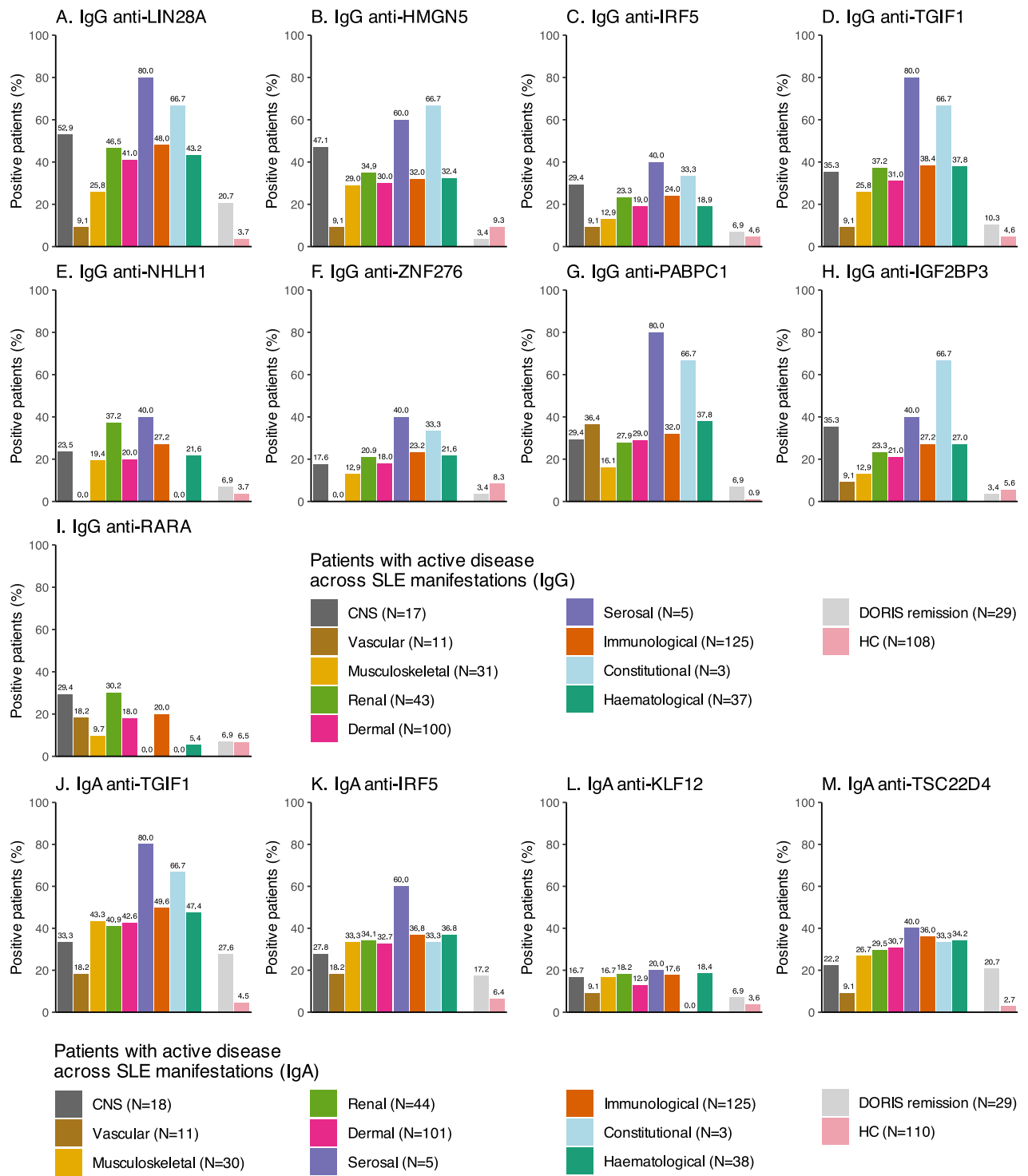
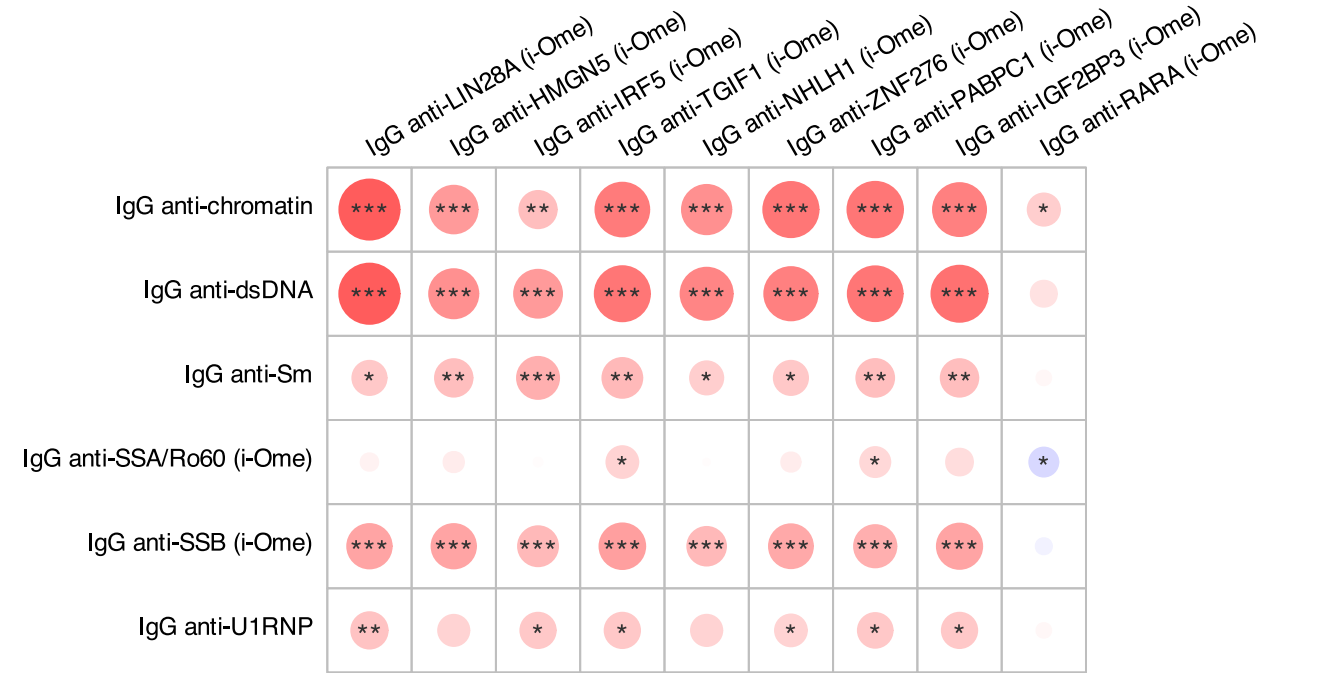


Figure 4. Proportions of patients with IgG and IgA autoantibody-positive SLE stratified by organ system and in comparison with the proportion of patients in DORIS remission as well as HC. The bar plots display the proportions of patients with autoantibody-positive SLE in the discovery cohort stratified by organ system and in comparison with the proportion of patients in DORIS remission for (A) IgG anti-LIN28A, (B) IgG anti-HMG5, (C) IgG anti-IRF5, (D) IgG anti-TGIF1, (E) IgG anti-NHLH1, (F) IgG anti-ZNF276, (G) IgG anti-PABPC1, (H) IgG anti-IGF2BP3, (I) IgG anti-RARA, (J) IgA anti-TGIF1, (K) IgA anti-IRF5, (L) IgA anti-KLF12, and (M) IgA anti-TSC22D4. The IgG and IgA daAabs are ranked in decreasing order of log₂ fold change in patients with SLE vs healthy controls. CNS, central nervous system; daAab, differentially abundant autoantibody; DORIS, definition of remission in SLE; HC, healthy control; SLE, systemic lupus erythematosus.

Similarly, we identified 3 autoantibody clusters of elevated IgG and IgA daAabs associated with current or past involvement of the different organ systems (Supplementary Fig S3C,D). We identified a cluster with RNA-binding proteins such as SSB and LIN28A,

which was predominantly represented in patients with current or past neuropsychiatric, vascular, and constitutional activity in the analysis of both isotypes, as well as in patients with current or past musculoskeletal activity in the analysis of IgA daAabs.

A. Correlations between levels of IgG daAAbs and conventional autoantibody specificities



B. Correlations between levels of IgA daAAbs and conventional autoantibody specificities

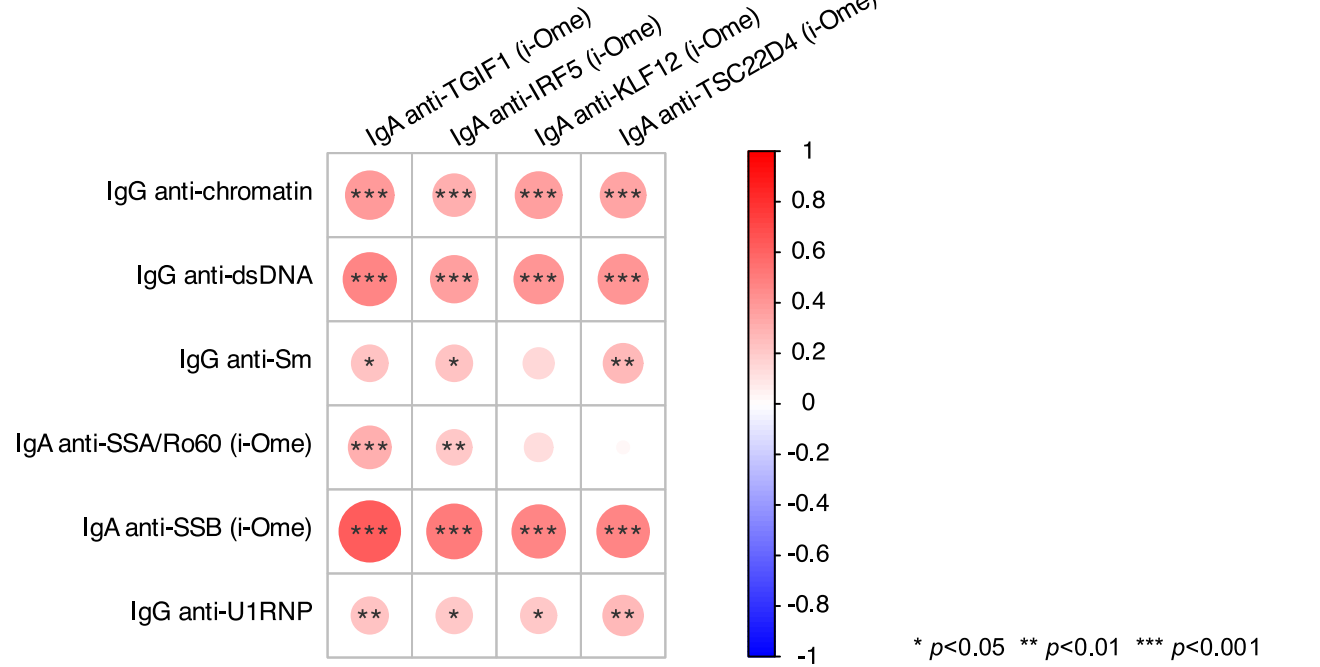


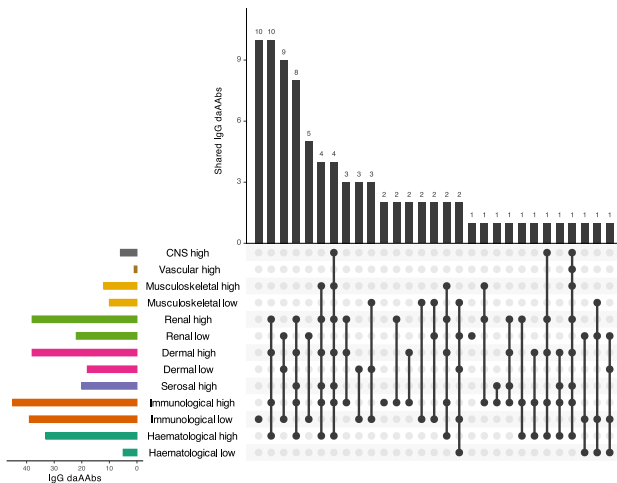
Figure 5. Correlations between levels of daAAbs and conventional autoantibody specificities. The correlation heatmaps shows Spearman rank correlation coefficients for correlations between levels of (A) IgG and (B) IgA daAAbs (measured using the i-Ome Discovery protein microarray) and levels of

Enriched pathways associated with differentially abundant autoantibody targets across SLE manifestations

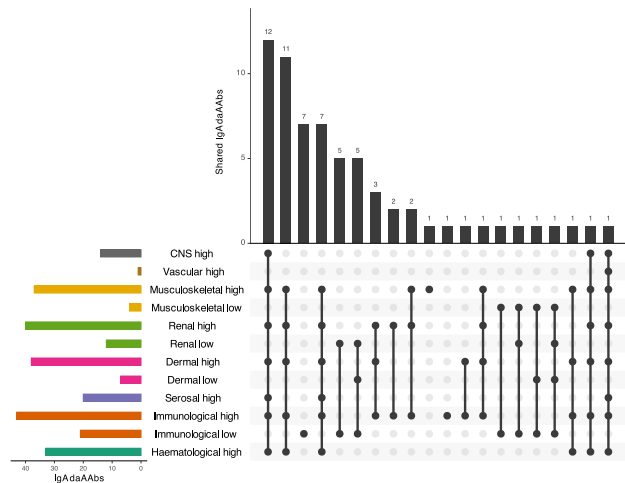
Figure 7A illustrates the enriched gene ontology (GO) terms associated with IgG daAAb targets across SLE manifestations (Supplementary Material, sheet 26). Several SLE manifestations exhibited common enriched GO terms, which formed 3 pathway

clusters. Cluster 1 was linked to DNA repair, cluster 2 was related to cartilage development, RNA regulation, and serine/threonine kinase activity, and cluster 3 was linked to DNA binding, neuronal differentiation, and ubiquitin-protein ligase activity. Notably, LIN28A was included among the autoantigens within the enriched ‘positive regulation of neuron differentiation’ GO term and FOSL2 was included among the

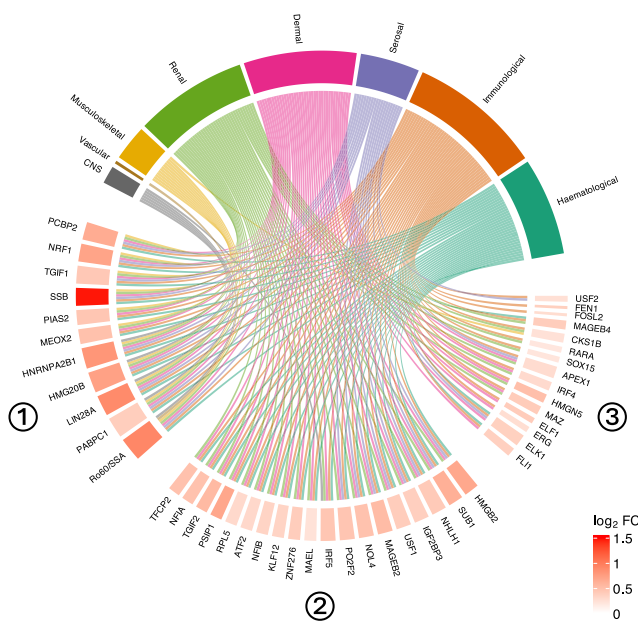
A. IgG daAABs associated with activity across SLE manifestations



B. IgA daAABs associated with activity across SLE manifestations



C. Elevated IgG daAABs associated with activity across SLE manifestations



D. Elevated IgA daAABs associated with activity across SLE manifestations

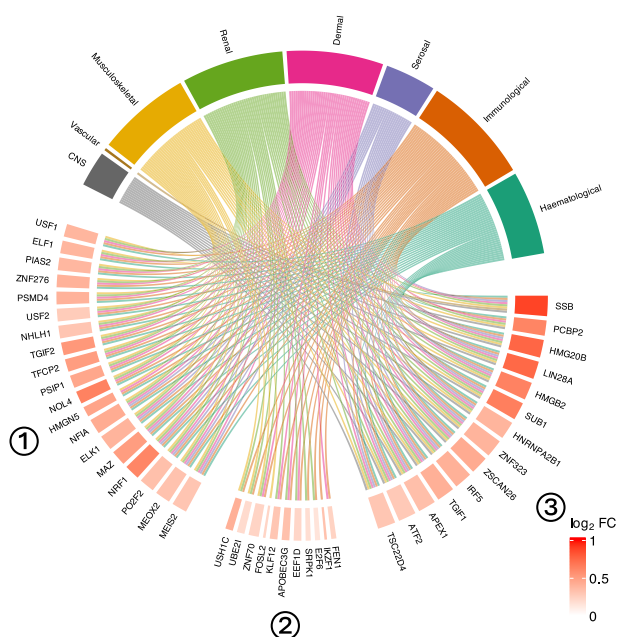


Figure 6. IgG and IgA daAABs in patients with active disease across SLE manifestations. The UpSet plots display (A) IgG and (B) IgA daAABs from subgroup daAAb analysis in patients with active SLE stratified by organ system with current activity vs healthy controls in the discovery cohort. The chord diagrams illustrate the log₂ FC of elevated (C) IgG and (D) IgA daAABs from the subgroup daAAb analysis. CNS, central nervous system; daAAb, differentially abundant autoantibody; FC, fold change; SLE, systemic lupus erythematosus.

autoantigens within the enriched ‘growth plate cartilage development’ GO term. No enriched KEGG or Reactome pathways were identified.

Similarly, SLE manifestations shared enriched GO terms associated with IgA daAAb targets, forming 3 pathway clusters (Fig 7B; Supplementary Material, sheet 27). Cluster 1 was related to DNA binding, cluster 2 was linked to RNA–protein interactions, and cluster 3 was related to nuclease activity. No enriched KEGG or Reactome pathways were identified.

DISCUSSION

Autoantibodies are a hallmark of autoimmunity, SLE in particular. In this study, we screened plasma samples from 2 independent cohorts of patients with SLE and HC for IgG and IgA seroreactivity against 1609 protein autoantigens. We identified and validated several novel IgG and IgA autoantibodies in

patients with SLE. Of particular interest were anti-LIN28A, anti-HMG5, anti-IRF5, and anti-TGIF1 antibodies, ranked in decreasing order of elevation in comparison with HC, whose IgG levels were associated with high disease activity and negatively associated with LLDAS. More than half of patients with CNS involvement had IgG LIN28A levels that exceeded the cutoff for positivity, whereas only a fifth of patients in DORIS remission were positive. Additionally, IgG and IgA LIN28A formed autoantibody clusters predominantly represented in patients with CNS activity. Upon further validation, these novel autoantibodies offer promise for early identification of active SLE or evolving flares, thus timely intervention and prevention of organ damage. Importantly, the IgA isotype of autoantibodies has not been sufficiently explored in previous literature of SLE and represents a significant novelty of this study, highlighting a potential role for mucosal immunity in SLE pathogenesis.

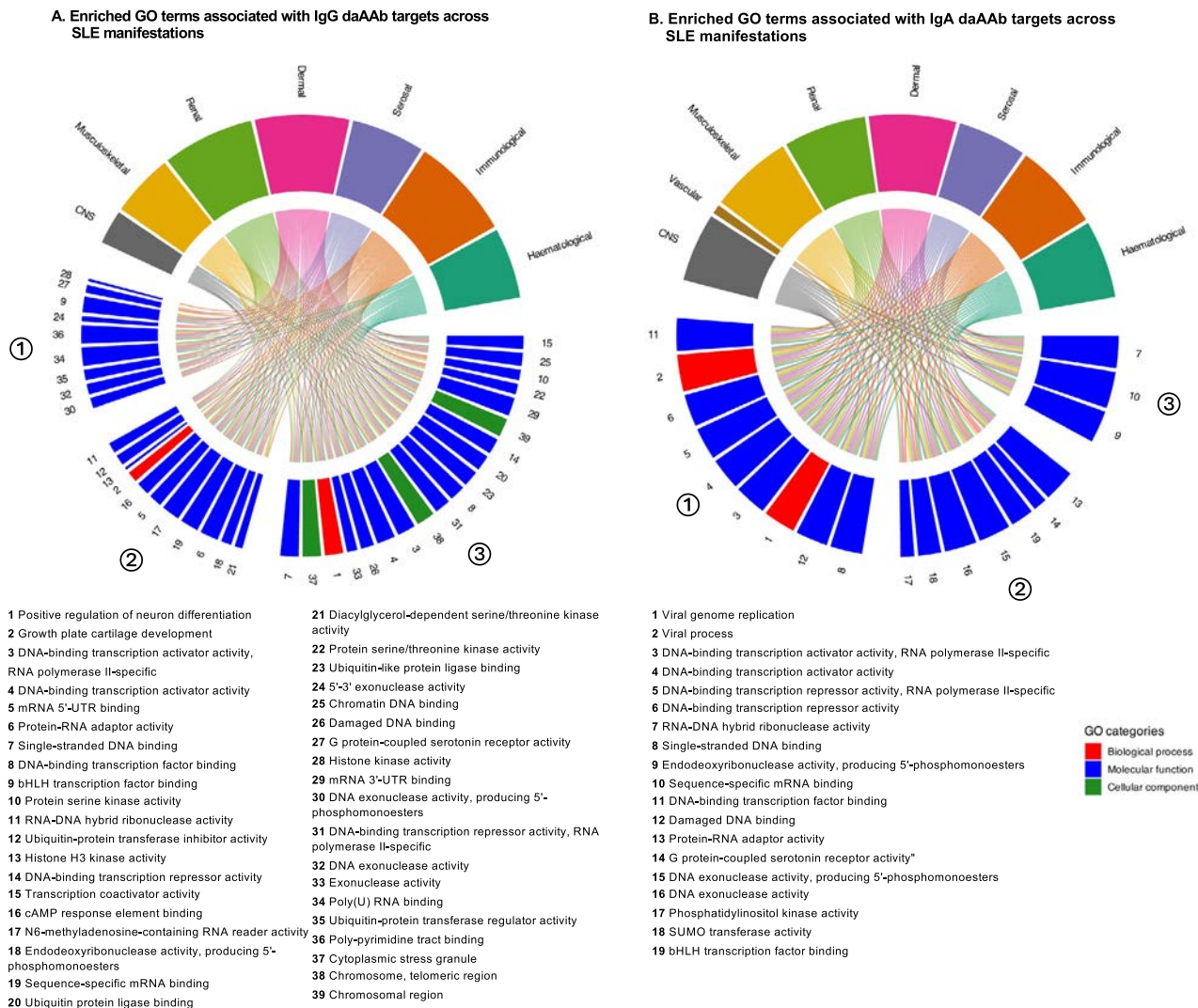


Figure 7. Enriched GO terms associated with IgG and IgA daAAb targets across SLE manifestations. The chord diagrams illustrate enriched GO terms from the enrichment analysis of (A) IgG and (B) IgA daAAb targets from subgroup daAAb analysis in patients with active SLE stratified by organ system with current activity vs healthy controls in the discovery cohort. CNS, central nervous system; daAAb, differentially abundant autoantibodies; GO, gene ontology; SLE, systemic lupus erythematosus.

Anti-LIN28A was associated with high disease activity (IgG) and negatively associated with LLDAS (IgG and IgA) and DORIS remission (IgG). Furthermore, in subgroup analysis across organ manifestations, IgG anti-LIN28A levels exceeded the cutoff for positivity in approximately half of the patients with CNS and renal activity, whereas only 21% of patients in DORIS remission were positive. Additionally, the IgG and IgA clusters that included anti-LIN28A were predominantly represented in patients with CNS activity, and LIN28A was found in an enriched pathway involving neuronal differentiation. In a recent analysis of the same cohorts, we found high specificity for SLE for IgG and IgA LIN28A compared with primary Sjögren's disease, systemic sclerosis, and HCs combined [26]. Interestingly, IgG anti-LIN28A was identified as one of the most prevalent novel autoantibodies in a previous study by Lewis et al. [27] using a protein microarray by Sengenics to screen for 1543 IgG autoantibodies in 2 independent cohorts of patients with SLE [27]. Similar to our findings, Lewis et al. [27] also identified IgG anti-IRF5, IgG anti-TGIF1, IgG anti-PABPC1, IgG anti-IGF2BP3, and IgG anti-RARA as autoantigens in SLE, whereas IgG anti-NHLH1 did not reach statistical significance, and IgG anti-HMG5 and anti-ZNF276 were not included in their panel

[27]. That study reported IgG LIN28A to be present in 22% of patients with SLE, defining positivity as levels higher than the mean distribution plus 2 SDs in the HC population. Despite using a more stringent cutoff to define autoantibody positivity in our study, requiring levels higher than the median plus 2 IQRs in the HC population, we found a higher prevalence of IgG anti-LIN28A than that reported by Lewis et al. [27] across all SLE manifestations but not vascular disease. It is worth noting that anti-dsDNA has been reported in up to 50% of patients with active neuropsychiatric SLE [28] and 20% of patients in our study population, which anti-LIN28A exceeded. This is particularly interesting in light of LIN28A being an RNA-binding protein involved in embryonic development and the promotion of CNS axon regeneration [29]. LIN28A has also been associated with numerous cancers, including brain tumours [30]. Targeting the LIN28A and tumour suppressor lethal-7 (*let-7*) pathway has been implicated as a potential therapy in certain paediatric CNS tumours [30,31]. Interestingly, high expression of LIN28A in mouse nephrons was recently shown to lead to inflammatory kidney injury rather than oncogenesis [32]. In addition to anti-LIN28A, we also identified autoantibodies against the RNA-binding protein IGF2BP3 among IgG autoantibodies associated

with high disease activity and negatively associated with LLDAS. This is of particular interest in light of that LIN28A forms an RNA-binding complex with IGF2BP3 to promote DNA repair in response to oxidative stress [33]. Whether these mechanisms hold true for SLE specifically and targeting the LIN28A protein or pathway has merit for drug development for SLE warrants further study.

Similarly, IgG anti-HMG5 was associated with high disease activity, and a considerable proportion of patients exhibited positive levels across organ manifestations, notably 47% in patients with CNS activity. HMG5 is a nucleosome-binding protein that interacts with histone H1 to unfold chromatin, thereby influencing transcription and DNA repair [34]. Besides HMG5, targets of validated IgG and IgA daAABs included the chromatin proteins HMGB2 and HMG20B. Reassuringly, a high proportion of patients had positive levels of antichromatin in subgroups of patients stratified by organ-specific disease activity. HMG5 has been implicated in development and oncogenesis [35], but its role in SLE remains unclear. To the authors' knowledge, this is the first report of IgG anti-HMG5 in SLE.

IgG and IgA anti-IRF5 were associated with high disease activity, and at least 28% of patients had positive IgA levels across all but vascular SLE manifestations, whereas 17% of patients in DORIS remission were positive. In addition, IgA anti-IRF5 was included in an autoantibody cluster that was predominantly represented in patients with CNS and vascular activity. IRF5 is a transcription factor that regulates type I IFN production as well as IgG switch in B cells, and genetic variants of IRF5 constitute a known risk factor for SLE [36,37]. In addition to the implicated role of IRF5 in SLE pathogenesis, Lewis et al. [27] identified IgG IRF5 as a novel autoantigen in 2 independent cohorts of patients with SLE [27]. Besides corroborating the presence of IgG anti-IRF5 in SLE, to our knowledge, we, in this study, for the first time revealed the presence of IgA anti-IRF5 in patients with SLE with active disease. IgA anti-IRF5 levels exhibited a strong correlation with IgA autoantibodies against the RNA-binding protein SSB, which is typically present in interferonopathies such as SLE and Sjögren's disease [16]. However, as expected, we did not observe an association between IgG SSB or IgG SSA and global disease activity, as these autoantibodies are primarily considered diagnostic markers and have been linked to specific disease manifestations [38]. This reinforces the significance of our findings and the autoantibodies identified herein, which demonstrated associations with disease activity. Notably, IRF5 has been shown to distinguish patients with SLE from population-based non-SLE controls, and IRF5-positive microparticles have been associated with higher disease activity in patients with SLE [39]. Epstein–Barr virus triggers antibody responses towards IRF5 epitopes, and the levels of those antibodies have been linked to systemic inflammation in patients with rheumatoid arthritis [40]. Moreover, a weak immune response to an IRF5 peptide was reported among patients with SLE [41]. It is therefore unclear whether anti-IRF5 antibodies are driving inflammatory processes in SLE in a disease-specific manner or reflect a consequence of a generic autoimmune response. In light of interferon pathways being important in SLE pathogenesis and precipitation [42,43], linking anti-IRF5 to activity positions this autoantibody as an emerging biomarker of disease activity and IRF5 as a potential target for therapeutic interventions.

IgG and IgA anti-TGIF1 were associated with high disease activity and a substantial proportion of patients had positive levels across all but vascular manifestations. In contrast, *TGIF1* gene expression was negatively associated with increasing disease

activity and was associated with LLDAS and DORIS remission. The opposing trends observed between anti-TGIF1 levels and *TGIF1* gene expression may be explained by a counterregulatory mechanism, potentially involving a negative feedback loop that downregulates *TGIF1* expression during active SLE. Furthermore, in daAAB cluster analysis, IgG and IgA anti-TGIF1 were predominantly represented in patients with CNS activity. Transforming growth factor (TGF)- β 1 possesses a wide repertoire of anti-inflammatory and immunosuppressive effects and has been associated with disease activity in SLE [44]. TGIF1 acts as a transcriptional repressor capable of inhibiting TGF- β 1 signalling by repressing SMAD transcriptional activity [45]. *TGIF1* has been linked to a common gene expression signature in patients with SLE and patients with multiple sclerosis [46], and our finding of IgG and novel IgA anti-TGIF1 complements the previously reported IgG autoantibodies targeting SMAD pathways in SLE [27].

We found IgA anti-MAPK12 to be associated with LLDAS and anti-IgA MAPK associated with DORIS remission. Notably, similar associations were not found for the corresponding IgG autoantibodies, which could potentially reflect the phase of the autoimmune response at the time of sampling. The p21ras/MAPK pathway is crucial for T cell signalling [47], and its upregulation has been linked to increased disease activity in SLE [48]. The c-Jun N-terminal kinase (JNK) pathway-associated phosphatase (JKAP) activates the MAPK JNK, and downregulation of JKAP in T cells has been correlated with SLEDAI scores and in patients with active lupus nephritis (LN), it has been associated with poor renal outcome [49]. In light of these reports, the observed associations between IgA anti-MAPK12 and anti-MAPK with low activity states in our study suggest that these autoantibodies may signify protective or restorative states in the immune dysregulation of SLE.

Among the few antibodies displaying organ specificity, we found IgA anti-FOSL2 to be elevated exclusively in patients with SLE with musculoskeletal activity. Additionally, FOSL2 was identified in an enriched pathway involving growth plate cartilage development. FOSL2 is an oncogenic transcription factor implicated in T cell malignancies [50]. It has been reported to be increased in the kidney tissue of patients with LN, and it was specifically localised in the glomerular podocytes in a murine model of lupus [51]. Interferon gamma (IFN- γ) significantly increases FOSL2 expression, and knockdown of FOSL2 was shown to attenuate podocyte damage induced by IFN- γ treatment *in vitro* in the same study. Interestingly, reduced binding of FOSL2 to the single variant rs7034653, using an anti-FOSL2 antibody, was shown to reduce TRAF1 expression and increase tumour necrosis factor production in patients with juvenile idiopathic arthritis [52]. Whether a similar mechanism applies to SLE requires further investigation, yet the organ-specific association of IgA anti-FOSL2 with musculoskeletal activity supports this notion.

Several SLE manifestations exhibited common enriched pathways associated with IgG and IgA daAAB targets. These pathways included those linked to DNA-binding, RNA regulation, serine/threonine kinase activity, and ubiquitin-protein ligase activity, all of which are known to be relevant to SLE pathogenesis [53,54]. The binding of ANAs to DNA and RNA to form immune complexes leading to tissue injury and, ultimately, damage is central to SLE pathogenesis [55], and study of immune complex formation engaging the IgG and IgA daAABs discovered in this study would be intriguing. The serine/threonine kinase ρ -associated, coiled-coil-containing protein kinase 2 (ROCK2) has been shown to activate IRF4, an essential transcription factor for the production of interleukins 17 and 21,

and inhibition of ROCK dampened inflammatory and autoantibody responses in mouse models of autoimmunity [56]. Notably, IgG anti-IRF4 was implicated in 1 of the 3 autoantibody clusters in our study, with representation across various SLE manifestations. Furthermore, we recently identified a dysregulated ubiquitination gene module in the peripheral blood of patients with SLE, which, again, was not manifestation specific [57]. In the same study, druggability suggested bortezomib, an inhibitor of the ubiquitin-proteasome system, as a potential treatment for SLE, which has also been supported by results from a phase II clinical trial and real-world experience [57–60].

The exclusion criteria set by the PRECISESADS study protocol may have introduced selection bias, as patients with severe SLE manifestations requiring high doses of GCs or other potent immunomodulatory treatments were excluded. However, this approach minimised the potential confounding effects of such treatments on the molecular aberrations investigated within the PRECISESADS project, including autoantibody levels and gene expression in this study. The small sample size of the validation cohort may have limited us from uncovering autoantibodies of relevance in SLE pathogenesis yet with a smaller magnitude of differentiation. Furthermore, it restricted our ability to assess correlations between longitudinal autoantibody levels and disease activity. Nevertheless, both autoantibody levels and disease activity exhibited a downwards trend, with the possible exception of IgG RARA, which may suggest differential cellular origin for these autoantibodies, warranting further investigation. While the associations between daAAs and disease activity need further validation, the use of a validation cohort to determine which autoantibodies would qualify for downstream analysis enhanced the robustness of our pipeline and the reliability of our findings. While validation using a solid-phase quantitative assay remains a future objective due to several factors, including logistical and financial constraints, the validation performed in independent cohorts in this study, along with the corroboration of findings consistent with previous investigations [27,61], strengthens our results. Moreover, methodological crossvalidation presents inherent challenges, including the lack of commercial kits for the novel autoantibodies identified. Additionally, such crossvalidation would overlook the advantage of the KREX platform used in this study, which offers high sensitivity and preserves protein conformation. Future translational studies dedicated to assay development and validation in larger, more ethnically diverse SLE populations will be essential to further advance our findings. Importantly, the comprehensive exploration of IgA autoantibodies in SLE is novel. This adds a new dimension to our understanding of the immunologic landscape in SLE, revealing potential roles of IgA autoantibodies in disease activity and organ involvement, and a potential role of mucosal immunity in SLE pathogenesis. Investigation of autoantibody isotypes beyond IgG and IgA, as well as subclass profiling might provide further insight.

In conclusion, this study identified and validated novel IgG and IgA autoantibodies. Among these, IgG anti-LIN28A, IgG anti-HMGN5, and both isotypes for anti-IRF5 and anti-TGIF1 were associated with high disease activity and were highly prevalent in subgroups of patients with active disease across organ manifestations. IgG anti-LIN28A levels exceeded the cutoff for positivity in more than half of the patients with CNS involvement, a prevalence higher than that observed for the routine clinical marker anti-dsDNA, and almost half of the patients with renal activity. Additionally, IgG and IgA LIN28A formed autoantibody clusters predominantly represented in patients with CNS activity. These findings indicate a potential role of mucosal

immunity in SLE pathogenesis and have direct implications for the early identification of patients with active or evolving disease and for timely and informed therapeutic intervention.

Competing interests

IP has received research funding and/or honoraria from Amgen, AstraZeneca, Aurinia, BMS, Elli Lilly, Gilead, GSK, Janssen, Novartis, Otsuka, and Roche. The other authors declare no conflicts of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

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Contributors

JL, DN, HI, P-JJ, MEA-R, and IP were involved in the conceptualisation of the study. JL, DN, DL, LB, MOB, GB, MEA-R, and IP were involved in data curation. JL, DN, DL, and IP were involved in the visualisation of the findings. JL, DN, DL, HI, LB, GB, P-JJ, MEA-R, NS, and IP were involved in the interpretation of results. JL and IP were involved in the manuscript drafting. All authors were involved in data analysis, critical review of manuscript drafts, and approval of the final draft before submission. IP acts as the guarantor of the study.

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Patient consent for publication

All patients and healthy controls provided written informed consent before recruitment.

Ethics approval

This study involved human participants. It was reviewed and approved by local ethics committees at all recruiting centres: Comitato Etico Area 2, Fondazione IRCCS Ca Granda Ospedale Maggiore Policlinico di Milano and University of Milan (approval no. 425 bis 19 November 2014 and no. 671_2018 19 September 2018); Klinikum der Universität zu Köln, Cologne, Germany; Comité d'Éthique Hospitalo-Facultaire, Pôle de pathologies rhumatismales systémiques et inflammatoires, Institut de Recherche Expérimentale et Clinique, Université catholique de Louvain, Brussels, Belgium; Csongrad Megyei Kormányhivatal, University of Szeged, Szeged, Hungary; Comité

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Data availability statement

Data are available upon reasonable request. Raw data remain the property of the PRECISESADS consortium and are protected under the European General Data Protection Regulation (GDPR). Metadata and aggregated processed data are available upon reasonable request from the corresponding author and from the European Genome-Phenome Archive (EGA).

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Supplementary materials

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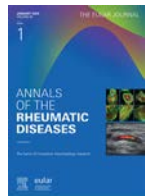
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Systemic lupus erythematosus

New IgG and IgA autoantibody specificities against DNA-binding and RNA-binding proteins discriminate systemic lupus erythematosus from health and non-lupus autoimmunity—could anti-LIN28A enhance precision in diagnostics?

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ABSTRACT

Objectives: In response to the urgent unmet needs of heterogeneity, unpredictability, and diagnostic delay in systemic lupus erythematosus (SLE), we aimed to identify and validate new immunoglobulin (Ig)G and IgA autoantibody specificities.

Methods: Using a KoRectly EXpressed technology-based microarrays (i-Ome Discovery; Sengenics), we screened for circulating IgG and IgA autoantibodies against 1609 proteins in 2 independent cohorts (discovery: NTC02890121; validation: NCT02890134) comprising patients with SLE (n = 199 and n = 30), primary Sjögren's disease (n = 115 and n = 31), and systemic sclerosis (n = 115 and n = 24), and healthy controls (HCs; n = 111 and n = 84), respectively.

Results: We identified and validated 5 IgG (anti-Lin-28 homolog A [LIN28A], anti-HNRNPA2B1, anti-HMG20B, anti-HMGB2, and anti-alpha-globin transcription factor CP2 [TFCP2]) and 4 IgA

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(anti-LIN28A, anti-HMG20B, anti-SUB1, and anti-TFCP2) autoantibodies that demonstrated high specificity for SLE (0.91–0.94), along with consistent and robust positivity (0.22–0.69) in differentially abundant autoantibody (daAAb) analysis between SLE and comparator groups. IgG and IgA anti-LIN28A levels varied over a 14-month follow-up in the validation cohort of newly diagnosed patients with SLE and exhibited metrics that outperformed those of traditional autoantibody markers such as anti-double-stranded DNA. Clustering of patients with SLE based on autoantibody positivity (levels above the HC median plus 2 IQRs in the discovery cohort) status revealed 1 subgroup demonstrating seroreactivity against multiple antigens, 3 exhibiting varying reactivity, and 1 showing no reactivity. In pathway analysis, daAAb targets pointed to DNA-binding and RNA-binding and transcription functions.

Conclusions: Novel autoantibodies validated in this study may enhance diagnostics and molecular characterisation in SLE. The prominent IgA seroreactivity implicates important roles of mucosal tissues in SLE autoimmunity, warranting further investigation.

WHAT IS ALREADY KNOWN ON THIS TOPIC

- Systemic lupus erythematosus (SLE) is characterised by prominent autoantibody production, yet the clinical utility of many remains unclear, and several specificities have yet to be fully characterised; IgA seroreactivity is largely unexplored.
- Diagnosis of SLE is supported by the presence of certain autoantibodies, but currently used autoantibody specificities have either high sensitivity across autoimmune diseases or high specificity yet low sensitivity (eg, anti-double-stranded DNA).

WHAT THIS STUDY ADDS

- We identified and validated novel IgG and IgA autoantibody specificities against DNA-binding and RNA-binding proteins, which distinguish patients with SLE from healthy individuals and from patients with other autoimmune conditions; IgA seroreactivity implicates important roles of mucosal immunity in SLE.
- Among validated autoantibodies, 5 IgG (anti-Lin-28 homolog A [LIN28A], anti-HNRNPA2B1, anti-HMG20B, anti-HMGB2, and anti-alpha-globin transcription factor CP2 [TFCP2]) and 4 IgA (anti-LIN28A, anti-HMG20B, anti-SUB1, and anti-TFCP2) autoantibodies were highly specific for SLE.
- IgG and IgA anti-LIN28A levels varied over time in patients with SLE and exhibited metrics that outperformed those of anti-double-stranded DNA.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- This study revealed novel autoantibodies that may enhance SLE diagnostics and molecular characterisation of patients; IgG and IgA anti-LIN28A showed particular promise.

INTRODUCTION

Precision medicine tailors treatments to individual patient profiles, relying on biomarkers to understand diseases and predict treatment responses. In this context, measuring humoral antibodies, especially autoantibodies, is particularly important for diagnosing autoimmune diseases, guiding patient stratification, and monitoring therapeutic outcomes [1]. Systemic lupus erythematosus (SLE) is a complex autoimmune disease characterised by significant clinical and molecular heterogeneity [2,3]. The hallmark of SLE is the production of a multitude of autoantibodies, yet their contribution to disease pathogenesis remains poorly understood [2]. Despite the identification of a multitude of autoantibodies [4], only a select few are routinely used in clinical practice,

highlighting the need for biomarkers with greater diagnostic and prognostic values [5]. Notably, there are several autoantibodies present in patients with SLE whose specificities remain unknown, highlighting the need for further research to understand their potential diagnostic implications [1].

Diagnostic uncertainty is a frequent challenge in the field of rheumatology, particularly in SLE. Systemic autoimmune diseases commonly share clinical features and serological markers, complicating the differentiation across distinct diseases, especially in the early phases of their course [6,7]. Importantly, only a limited number of clinical features and serologic markers exhibit adequate discriminative accuracy [2], which is further complicated by the lack of diagnostic criteria in most of these conditions [8]. This results in significant delays before a patient's clinical profile evolves into what can be recognised as a definitive diagnosis [8].

The diagnostic procedures for SLE remain largely clinical but are accompanied by serologic features suggestive of SLE [8]. Classification criteria are usually used to support the diagnosis; however, despite their high sensitivity, they fail to classify up to 10% of patients in the early stages of the disease [9]. In addition, currently used autoantibodies vary in their diagnostic utility, with some, such as antinuclear antibodies (ANAs), exhibiting high sensitivity across systemic autoimmune diseases but lacking specificity, while others, like those against double-stranded DNA (anti-dsDNA) and the Smith antigen (anti-Sm), provide high specificity for SLE but low sensitivity [1]. Although autoantibodies tend to be present before the onset of SLE, almost half of the patients are seronegative for currently known disease-specific antibodies at the time of the first disease manifestation [10]. Furthermore, some patients with SLE test negative for all traditional autoantibody specificities currently used in routine clinical praxis despite being positive for ANA; these patients represent a challenging subgroup for early diagnosis [11]. Despite significant efforts to improve the diagnostic procedures [8], diagnosing SLE early and accurately remains a challenge, with a diagnostic delay from the onset of the first symptom until a definite diagnosis of SLE being approximately 2 years [12].

To identify novel autoantibodies that could help differentiate SLE from healthy individuals and other autoimmune diseases, we used KoRectly EXpressed (KREX) technology-based protein immunome microarrays. These microarrays enabled a broad screening of plasma samples for the presence of immunoglobulin (Ig)G and IgA autoantibodies targeting 1609 protein antigens in patients with SLE, primary Sjögren's disease (SjD), systemic sclerosis (SSc), and healthy controls (HCs). By displaying only full-length, correctly folded proteins, the microarrays preserve the conformational epitopes recognised by 90% of humoral

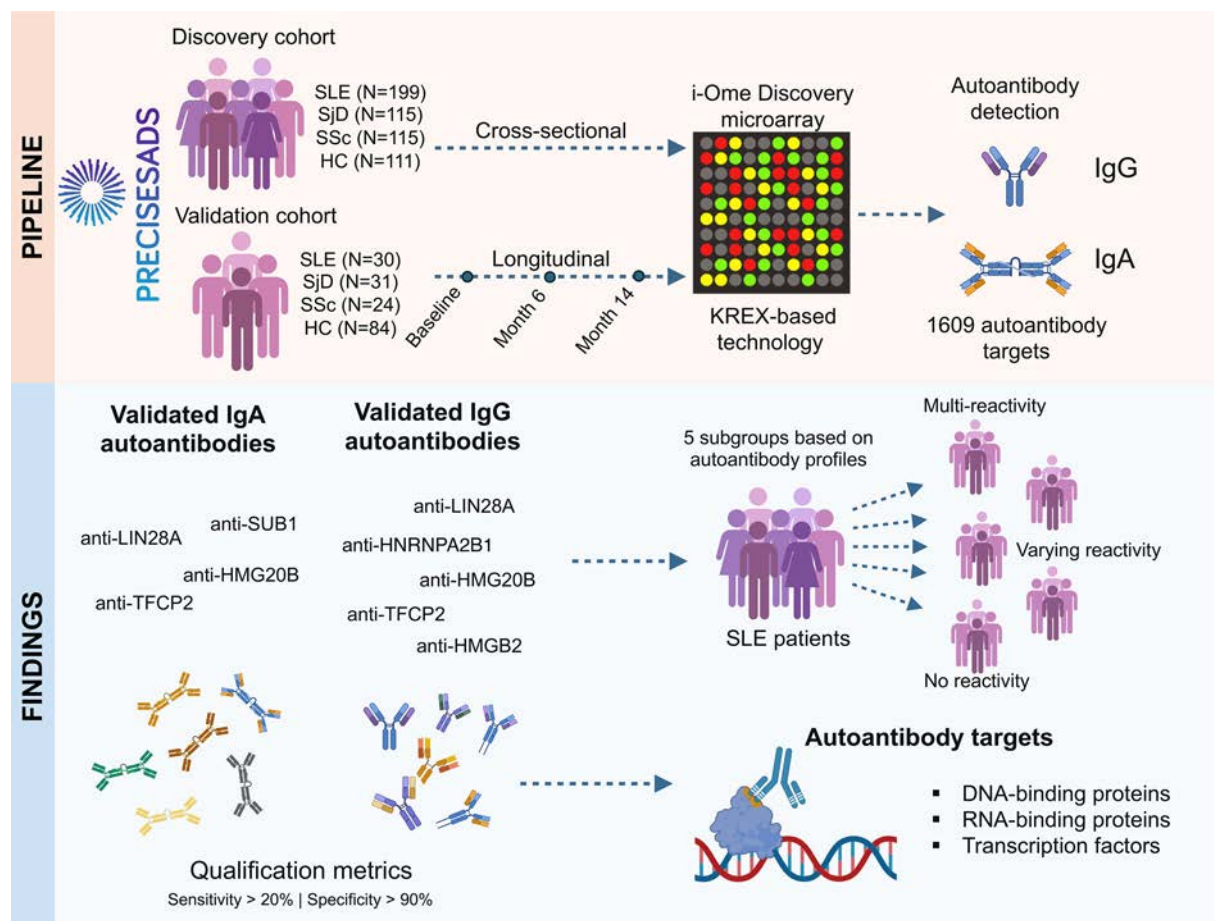


Figure 1. Graphical illustration of the analytical pipeline and main findings of the study. daAAb, differentially abundant autoantibody; HC, healthy control; KREX, KoRectly EXpressed; SjD, Sjögren's disease; SLE, systemic lupus erythematosus; SSc, systemic sclerosis.

antibodies, ensuring *in vivo* relevant antibody–antigen binding. This approach has previously facilitated the discovery of autoantibodies in rheumatoid arthritis (RA) [13] and in neurologic and malignant diseases [14,15].

METHODS

Study population

Plasma samples from patients with SLE, SjD, and SSc and HCs were obtained from 2 independent cohorts (discovery and validation) within the European PRECISESADS (Molecular reclassification to find clinically useful biomarkers for systemic autoimmune diseases) project [7]. Specifically, EDTA-anticoagulated plasma was used. All samples were collected between February 1, 2014, and January 31, 2019, and stored at -80°C until the day of analysis to ensure sample integrity. The discovery cohort comprised 199 patients with SLE, 115 patients with SjD, 115 patients with SSc, and 111 HCs from a cross-sectional cohort (NTC02890121; [Supplementary Table S1](#)). The validation cohort included 30 patients with SLE, 31 patients with SjD, 24 patients with SSc, and 84 HCs from an independent inception cohort (NCT02890134; [Supplementary Table S2](#)). Patients with SLE from the validation cohort were longitudinally sampled at baseline (within 1 year from diagnosis) and after 6 and 14 months. All individuals diagnosed with SLE fulfilled the revised 1997 American College of Rheumatology classification criteria [16]. Patients diagnosed with SjD fulfilled the revised 2002 classification criteria [17]. Patients diagnosed with SSc fulfilled the 2013 classification criteria [18]. None of the patients fulfilled

international classification criteria for more than 1 rheumatic disease. Exclusion criteria included high doses of prednisone equivalents ($>15\text{ mg/d}$), intravenous glucocorticoids or high doses of immunosuppressants within the preceding 3 months, cyclophosphamide or belimumab within the preceding 6 months, and rituximab within the preceding 12 months. Detailed inclusion and exclusion criteria can be found in [Supplementary Table S3](#). Patients from the inception (validation) cohort were not on immunosuppressants at baseline but were allowed to be receiving standard of care therapy at months 6 and 14. The analytical pipeline is illustrated in [Figure 1](#).

Before participation in the PRECISESADS project, all patients and HCs provided written informed consent. Ethical approval for the PRECISESADS protocol was obtained from local ethics review boards across all participating centres, while this study was reviewed and approved by the Swedish Ethical Review Authority (application number: 2022-03907-01).

Autoantibody measurement

Plasma samples were analysed for IgG and IgA autoantibody specificities against a comprehensive panel of 1609 human proteins, using the i-Ome Discovery protein microarray ([Supplementary File S1](#)). This high-throughput assay platform uses KREX technology, which preserves the native 3-dimensional structure of full-length proteins, thus maintaining epitopes that are relevant to *in vivo* conditions, ensuring that the binding of autoantibodies is specific and biologically meaningful [15]. In brief, the samples were randomised, diluted 1:200 in serum assay buffer, and incubated on the microarrays for 1 hour at

room temperature (20 °C). Following this, the microarrays were washed and treated with Cy3-labelled anti-IgG and Cy5-labelled anti-IgA antibodies and then washed again. Once dried, the microarrays were scanned using a constant gain setting and 10 µm resolution on an Agilent G4600AD microarray laser scanner. Data extraction was performed using GenePix Pro 7 software (Molecular Devices) from 16-bit TIFF image files.

Four SLE samples from the discovery cohort and 1 from the validation cohort, 1 SjD sample in the discovery cohort, 1 SSc sample in the discovery cohort, and 3 HC samples from the discovery cohort were excluded from further IgG differentially abundant autoantibody (daAAb) analysis due to more than 1% of antigens in these samples exhibiting antibody signals with a coefficient of variation greater than 20%, a result that was confirmed upon reanalysis. For the same reasons, 3 SLE samples in the discovery cohort, 2 SSc samples in the validation cohort, and 1 HC sample in the discovery and 1 in the validation cohort were excluded from subsequent IgA daAAb analysis.

Conventional autoantibodies

ANA were determined by indirect immunofluorescence on HEp-2 cells. Conventional IgG anti-dsDNA, IgG anti-Smith, IgG anti-U1RNP, IgG and IgM anticardiolipin, and IgG and IgM anti-β2GPI antibodies were measured using an automated chemiluminescent immunoanalyser (IDS-iSYS; Immunodiagnostic Systems Holdings Ltd) [7]. The IDS-iSYS platform uses bacterial plasmid dsDNA, bovine Smith antigen and cardiolipin, and human U1RNP and β2-GPI.

Antichromatin/nucleosome antibody determination was performed using the anti-dsDNA-NcX enzyme-linked immunosorbent assay (ELISA) kit from EUROIMMUN Medizinische Labordiagnostika AG [19], following the protocol described by Biesen et al [20]. This assay uses nucleosomes thought to retain histone proteins, thereby presumably preserving the native chromatin structure. This may enable more accurate detection of autoantibodies targeting specific epitopes influenced by histone arrangement.

Bioinformatic and statistical analysis

Log₂-transformed mean net signal intensities for each IgG and IgA autoantibody were median normalised and batch corrected using the ComBat function of the sva R package [21] and then filtered to exclude antigens with signals highly correlated with those of on-array nonspecific binding controls. Median-normalised batch-corrected autoantibody data underwent daAAb analysis with the limma R package [22], adjusting for polypeptide antibody (PSA) status, recruiting centre, age, and batch. PSA status was determined based on antigens known to be targeted by the PSA phenotype (Supplementary File S2). Autoantibody levels were compared pairwise in patients with SLE vs patients with SjD, patients with SSc, and HCs in the discovery and validation cohorts separately. daAABs with a false discovery rate (FDR)-corrected *P* value of <.05 and |log₂ fold change (FC)| >0.38 in the discovery cohort that also differed in the baseline samples of the validation cohort with a *P* value of <.05 were considered validated. Results of the daAAb analysis were plotted using the Enhanced Volcano R package.

We defined positive levels of autoantibodies as those exceeding the median plus 2 IQRs in the distribution of the respective autoantibody in the HC population of the discovery cohort. The χ^2 tests was used to compare positivity percentages; differences

were deemed significant upon application of Bonferroni correction.

Diagnostic performance metrics including sensitivity, specificity, negative and positive predictive values, and accuracy were computed for IgG and IgA daAABs for SLE vs HC, SjD, and SSc combined. Sensitivity and specificity thresholds of 0.2 and 0.8, respectively, were applied to select daAABs for subsequent analysis. Metrics for those daAABs were plotted using the ComplexHeatmap R package. Correlation analyses were performed using the Spearman rank correlation coefficient with the R base correlation test function, and *P* values were adjusted with Bonferroni correction.

Variation of daAAb levels over time in longitudinal series of samples was assessed with repeated-measurements Analysis of Variance (ANOVA). Based on cutoffs for positivity as described above, we transformed continuous daAAb data to binary (positive/negative) and clustered both autoantibody specificities and patients with SLE based on Euclidean distances. Clustered data were visualised with the ComplexHeatmap R package.

Enriched Gene ontology (GO) terms were determined by daAAb analysis between SLE and HC, SjD, and SSc combined in pooled datasets deriving from the discovery and the validation cohorts. Corresponding gene names, FDR-corrected *P* values, and log₂ FC values from the limma output were used as input for the enrichGO function of the clusterProfiler R package. Obtained enriched GO terms and genes were sorted based on FDR-corrected *P* values and then selected daAABs yielding FDR-corrected *P* value of <.05 were filtered and plotted in chord plots using the circlize R package, grouped by daAAb clusters. The ggplot2 R package was used for additional visualisation not detailed above. Statistical analyses not detailed above were performed using R base functions and the FSA R package using the R software version 4.4.1 (R Foundation for Statistical Computing).

Patient and public involvement

Patient research partners Yvonne Enman and Karin Blomkvist Sporre contributed to the design and dissemination strategies of this study. The public was not engaged in the study design, execution, reporting, or dissemination processes.

RESULTS

Demographics and characteristics of study participants are presented in [Supplementary Tables S1 and S2](#).

Differentially abundant IgG and IgA autoantibodies in SLE across pairwise comparisons with HC, SjD, and SSc

We identified 27 elevated IgG daAABs in SLE vs HC in the discovery cohort (Fig 2A; [Supplementary File S3](#), sheet 1), among which 25 were confirmed in the validation cohort (Fig 2D,G; [Supplementary File S3](#), sheet 2), 18 elevated IgG daAABs in SLE vs SjD (Fig 2B; [Supplementary File S3](#), sheet 3), among which 16 were validated (Fig 2E,G; [Supplementary File S3](#), sheet 4), and 18 elevated IgG daAABs in SLE vs SSc (Fig 2C; [Supplementary File S3](#), sheet 5), among which 17 were validated (Fig 2F,G; [Supplementary File S3](#), sheet 6).

Similarly, we identified 21 elevated IgA daAABs in SLE vs HC in the discovery cohort (Fig 3A; [Supplementary File S4](#), sheet 1), among which 18 were validated (Fig 3D,G; [Supplementary File S4](#), sheet 2), 14 elevated and 1 reduced IgA daAABs in SLE vs SjD (Fig 3B; [Supplementary File S4](#), sheet 3), among which 8 elevated IgG daAABs were validated (Fig 3E,G; [Supplementary](#)

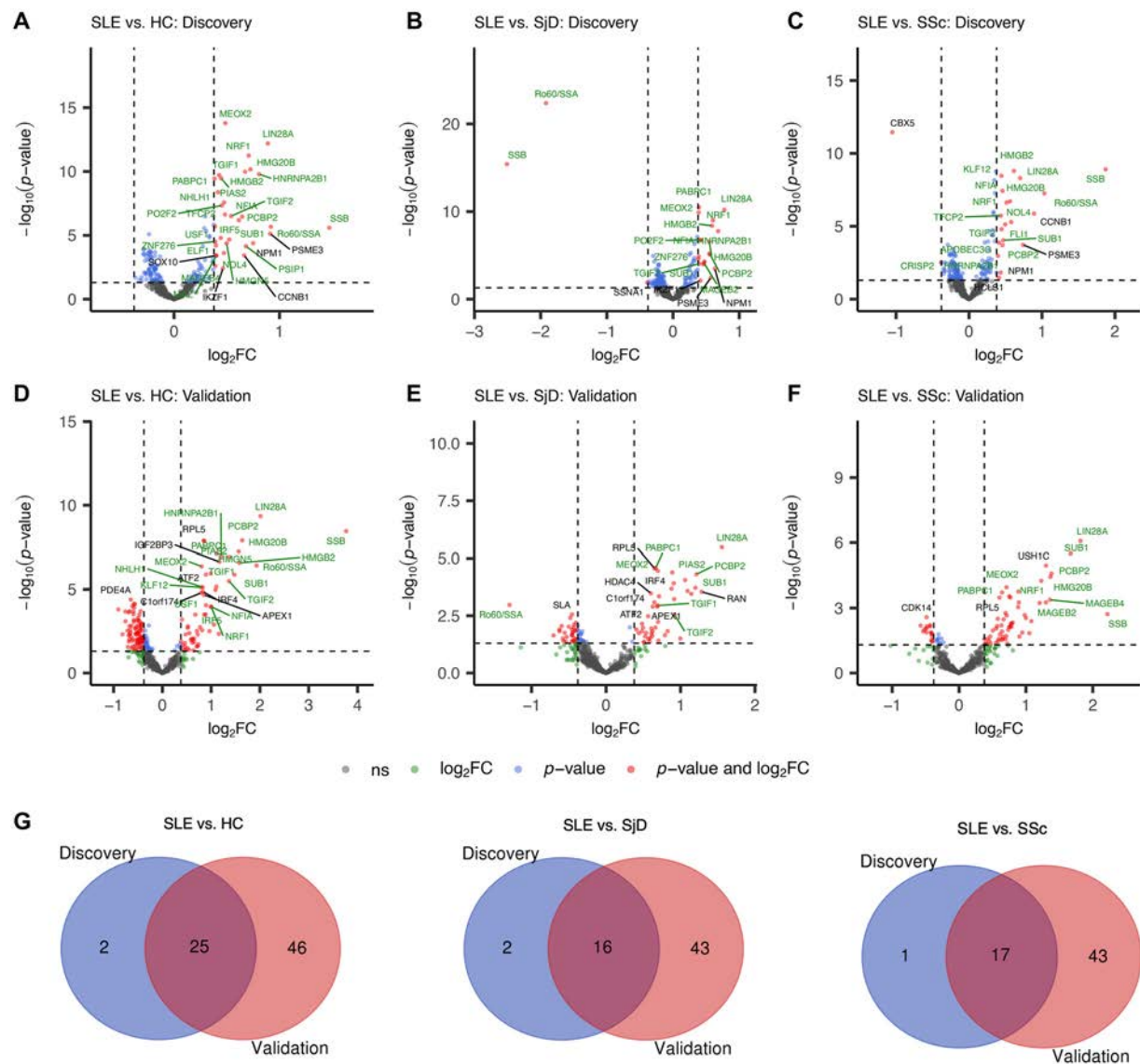


Figure 2. IgG daAABs in SLE vs HC, SJD, and SSc. The volcano plots display IgG daAABs in the (A–C) discovery and (D–F) validation cohorts, comparing SLE vs HC (A,D), SLE vs SJD (B,E), and SLE vs SSc (C,F). daAABs with $|\log_2FC| > 0.38$ and a $P < .05$ are denoted with red dots, while nonsignificant daAABs are denoted with grey dots. Selected daAABs are labelled. Green labels represent daAABs that are significantly abundant in both discovery and validation cohorts. Venn diagrams (G) show the number of validated (purple) and nonvalidated (blue) daAABs across the same comparisons. The full list of daAABs is provided in [Supplementary File S3](#). daAAb, differentially abundant autoantibody; FC, fold change; HC, healthy control; SJD, Sjögren’s disease; SLE, systemic lupus erythematosus; SSc, systemic sclerosis.

File S4, sheet 4), and 19 elevated IgG daAABs in SLE vs SSc (Fig 3C; [Supplementary File S4](#), sheet 5), among which 18 were validated (Fig 3F,G; [Supplementary File S4](#), sheet 6).

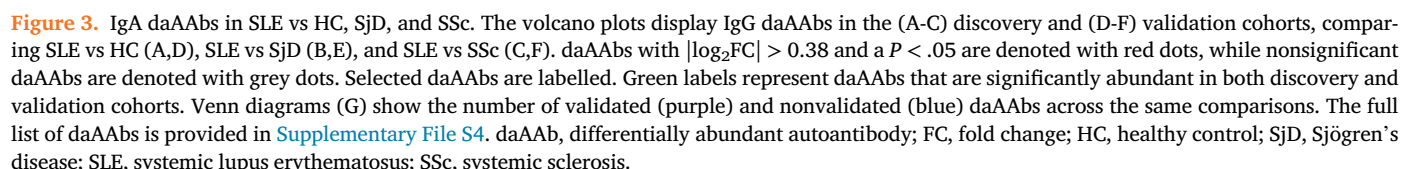
We next visualised the distributions of levels of autoantibodies that were consistently significantly elevated in SLE across all 3 pairwise comparisons—SLE vs HC, SLE vs SJD, and SLE vs SSc. Levels of 7 IgG daAABs (anti-Lin-28 homolog A [LIN28A], anti-HNRNPA2B1, anti-HMG20B, anti-HMGB2, anti-TGIF2, anti-NFIA, and anti-alpha-globin transcription factor CP2 [TFCP2]) were greater in SLE compared with each 1 of the 3 comparator groups in the discovery cohort (Fig 4A). Among those daAABs, levels of all but anti-NFIA were also significantly greater in SLE across all comparisons in the validation cohort (Fig 4B).

Accordingly, levels of 14 IgA daAABs (anti-LIN28A, anti-HMG20B, anti-HMGB2, anti-SUB1, anti-PCBP2, anti-NRF1, anti-TFCP2, anti-MAZ, anti-ZSCAN26, anti-TGIF2, anti-HMGN5, anti-NFIA, anti-NOL4, and anti-PSIP1) were greater in SLE than

in HC, SJD, and SSc in the discovery cohort (Fig 4C). Of these, levels of IgA anti-LIN28A, anti-HMG20B, anti-SUB1, and anti-TFCP2 were consistently greater across all comparisons in the validation cohort (Fig 4D).

Diagnostic metrics of IgG and IgA autoantibodies in SLE

Diagnostic metrics of IgG daAABs against all comparator groups are summarised in Figure 5A and [Supplementary File S5](#). Among those, 17 daAABs demonstrated sensitivity greater than 20% and accuracy greater than 65%, as well as high specificity ranging from 87% to 98% in both the discovery and validation cohorts. IgG anti-LIN28A showed the best metrics with a specificity of 93% and 89% and a sensitivity of 37% and 69% in the discovery (accuracy: 73%) and the validation cohort (accuracy: 86%), respectively, followed by IgG anti-HNRNPA2B1 that demonstrated a specificity of 91% in both cohorts and a sensitivity of 33% in the discovery cohort (accuracy: 70%) and 55% in the validation cohort



Diagnostic metrics of traditional autoantibodies are shown in **Figure 5C** (detailed in **Supplementary File S5**, sheet 5); IgG anti-dsDNA yielded 99% specificity, 31% sensitivity, and 75%

Correlation analysis revealed moderate to strong correlations between IgG antichromatin levels determined by ELISA and levels of IgG (Fig 5D; [Supplementary File S5](#), sheet 6) or IgA (Fig 5E; [Supplementary File S6](#), sheet 5) autoantibodies identified by KREX technology against chromatin-associated HMG proteins, that is, HMG20B, HMGB2, and HMGN5, as well as with against HNRNPA2B1, PSIP1, TGIF1, LIN28A, TGIF2, and TFCP2, which are not directly associated with chromatin. IgG anti-LIN28A exhibited the strongest correlation ($R = 0.63$; $P < .001$) with IgG antichromatin, followed by IgG anti-PABPC1, anti-ZNF276, anti-HMG20B, and IgG anti-HMGB2, suggesting distinct patterns of chromatin-related autoantibody responses.

Percentages of patients with positive levels of SLE-specific daAABs (Fig 4) are shown in Figure 6 (discovery cohort) and in

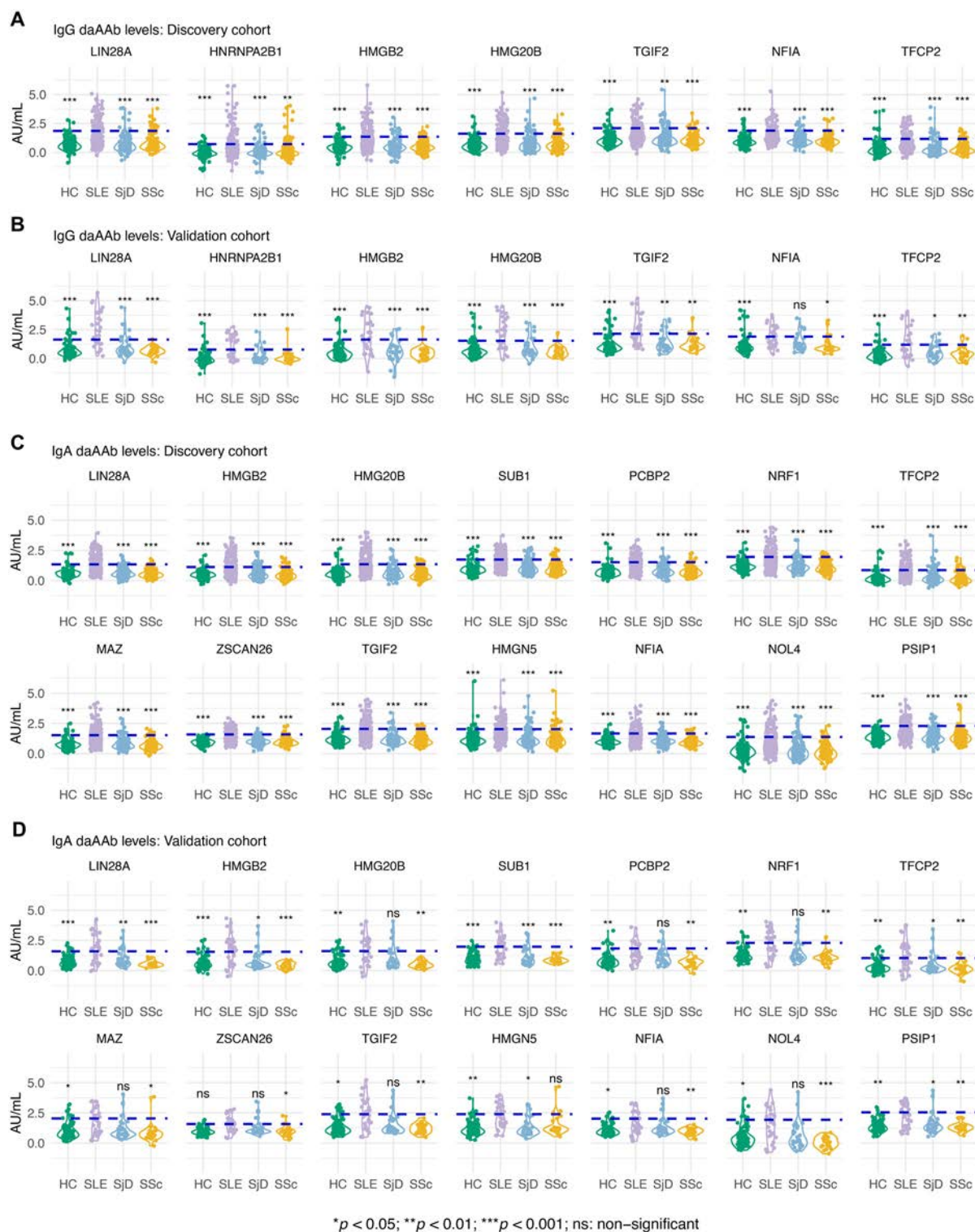


Figure 4. IgG and IgA daAAb levels in SLE vs HC, SjD, and SSc. The violin plots display IgG (A,B) and IgA (C,D) daAAb levels in the discovery (A,C) and validation cohorts (B,D). Positivity cutoff was defined as the median plus 2 IQRs in the HC distribution (dotted line). daAAb, differentially abundant autoantibody; HC, healthy control; SjD, Sjögren's disease; SLE, systemic lupus erythematosus; SSc, systemic sclerosis.

Supplementary Fig S1 (validation cohort). Positivity frequencies for IgG anti-LIN28A, IgG anti-HNRNPA2B1, IgG anti-HMG20B, IgG anti-HMGB2, and IgG anti-TFCEP2 were significantly greater in SLE across all comparisons (**Fig 6A**). Positivity frequencies for IgG anti-TGIF2 and IgG anti-NFIA were significantly greater in SLE vs HC and vs SSc but not vs SjD (**Fig 6A**; **Supplementary Table S3**). These results were replicated in the validation cohort (**Supplementary Fig S1A**; **Supplementary File S7**).

Among SLE-specific IgA daAAb (anti-LIN28A, anti-HMG20B, anti-HMGB2, anti-SUB1, anti-PCBP2, anti-NRF1, anti-TFCEP2, anti-MAZ, anti-ZSCAN26, anti-TGIF2, anti-HMGN5, anti-NFIA, anti-NOL4, and anti-PSIP1), all 14 exhibited significantly greater positivity frequencies across all comparisons in the discovery cohort (**Fig 6B**; **Supplementary File S8**). These results were replicated in the validation cohort for anti-LIN28A, anti-HMG20B, anti-HMGB2, anti-SUB1, anti-NRF1, anti-TFCEP2, anti-MAZ, anti-

A. IgG daAAbs

	Discovery					Validation				
	Sensitivity	Specificity	PPV	NPV	ACC	Sensitivity	Specificity	PPV	NPV	ACC
LIN28A	0.37	0.93	0.76	0.72	0.73	0.69	0.89	0.57	0.93	0.86
HNRNPA2B1	0.33	0.91	0.69	0.70	0.70	0.55	0.91	0.55	0.91	0.85
MEOX2	0.32	0.95	0.78	0.71	0.72	0.48	0.96	0.74	0.90	0.88
HMGB2	0.32	0.91	0.67	0.70	0.69	0.55	0.94	0.64	0.91	0.87
HMG20B	0.32	0.91	0.67	0.70	0.69	0.62	0.89	0.55	0.92	0.85
PIAS2	0.32	0.91	0.67	0.70	0.69	0.55	0.94	0.67	0.91	0.88
TGIF1	0.30	0.94	0.73	0.70	0.70	0.55	0.94	0.67	0.91	0.88
PO2F2	0.29	0.90	0.62	0.68	0.67	0.55	0.90	0.53	0.91	0.84
HMG5	0.26	0.88	0.55	0.67	0.65	0.48	0.94	0.61	0.90	0.86
PABPC1	0.25	0.98	0.88	0.69	0.71	0.62	0.96	0.78	0.92	0.90
TGIF2	0.25	0.90	0.60	0.67	0.66	0.55	0.88	0.50	0.90	0.83
USF1	0.23	0.91	0.61	0.67	0.66	0.34	0.94	0.53	0.87	0.83
PSIP1	0.23	0.90	0.56	0.67	0.65	0.48	0.92	0.56	0.90	0.85
NFIA	0.22	0.95	0.71	0.68	0.68	0.48	0.88	0.47	0.89	0.82
TFCP2	0.22	0.91	0.60	0.67	0.66	0.55	0.92	0.59	0.91	0.86
NHLH1	0.21	0.96	0.74	0.68	0.68	0.62	0.87	0.50	0.92	0.83
FLI1	0.21	0.91	0.57	0.66	0.65	0.34	0.91	0.43	0.87	0.81

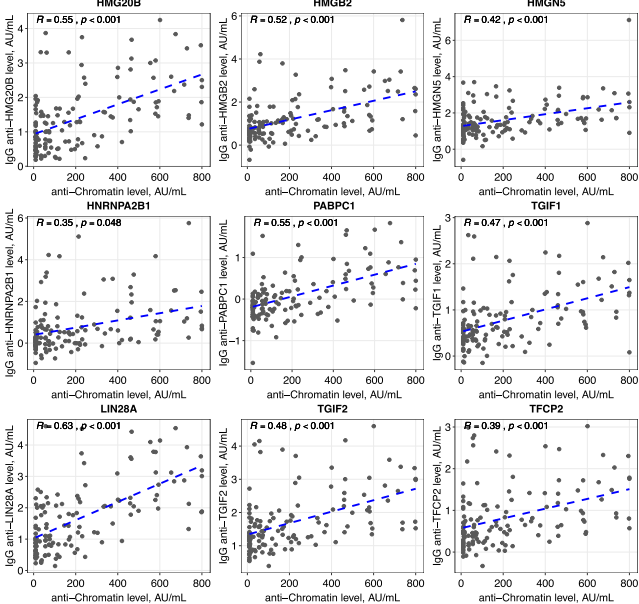
B. IgA daAAbs

	Discovery					Validation				
	Sensitivity	Specificity	PPV	NPV	ACC	Sensitivity	Specificity	PPV	NPV	ACC
LIN28A	0.45	0.94	0.80	0.75	0.76	0.60	0.93	0.64	0.91	0.87
HMGB2	0.41	0.91	0.72	0.73	0.73	0.57	0.94	0.68	0.91	0.87
HMG20B	0.40	0.92	0.75	0.73	0.73	0.53	0.89	0.52	0.90	0.83
TGIF1	0.40	0.91	0.71	0.72	0.72	0.47	0.90	0.52	0.88	0.83
SUB1	0.37	0.91	0.71	0.72	0.71	0.53	0.95	0.70	0.90	0.87
PCBP2	0.36	0.94	0.77	0.72	0.73	0.40	0.93	0.57	0.88	0.84
ZNF323	0.36	0.94	0.79	0.72	0.73	0.50	0.92	0.58	0.89	0.84
NRF1	0.35	0.93	0.74	0.71	0.72	0.43	0.93	0.57	0.88	0.84
TFCP2	0.35	0.91	0.68	0.71	0.70	0.53	0.90	0.53	0.90	0.83
USF1	0.34	0.91	0.68	0.70	0.70	0.47	0.94	0.64	0.89	0.86
MAZ	0.32	0.93	0.73	0.70	0.71	0.40	0.93	0.55	0.88	0.83
ZNF276	0.32	0.93	0.71	0.70	0.70	0.30	0.96	0.64	0.86	0.84
PSMD4	0.32	0.91	0.66	0.70	0.69	0.53	0.88	0.50	0.90	0.82
IRF5	0.31	0.90	0.64	0.69	0.68	0.53	0.88	0.50	0.90	0.82
ZSCAN26	0.30	0.96	0.81	0.70	0.72	0.47	0.93	0.58	0.89	0.84
TGIF2	0.30	0.94	0.73	0.70	0.70	0.43	0.93	0.57	0.88	0.84
HMG5	0.30	0.90	0.64	0.69	0.68	0.37	0.92	0.50	0.87	0.82
NFIA	0.29	0.92	0.69	0.69	0.69	0.40	0.95	0.63	0.88	0.85
ELK1	0.27	0.93	0.68	0.69	0.69	0.50	0.91	0.56	0.89	0.84
NOL4	0.26	0.92	0.65	0.68	0.68	0.43	0.93	0.59	0.88	0.84
PSIP1	0.22	0.96	0.75	0.68	0.69	0.37	0.96	0.69	0.87	0.86

C. Traditional autoantibodies

	Sensitivity	Specificity	PPV	NPV	ACC
IgG anti-dsDNA	0.31	0.99	0.92	0.73	0.75
IgG anti-Sm	0.04	1.00	1.00	0.68	0.68
IgG anti-b2GPI	0.11	1.00	1.00	0.69	0.70
IgM anti-b2GPI	0.04	0.97	0.46	0.66	0.66
IgG anti-cardiolipin	0.11	1.00	1.00	0.68	0.70
IgM anti-cardiolipin	0.05	0.98	0.54	0.66	0.66
IgG anti-U1RNP	0.20	0.96	0.72	0.71	0.71
IgG anti-SSA	0.32	0.77	0.42	0.69	0.62
IgG anti-SSB	0.08	0.94	0.38	0.67	0.65
IgG anti-chromatin	0.52	0.95	0.87	0.76	0.79

D. Correlations between IgG daAAbs and IgG anti-chromatin



E. Correlations between IgA daAAbs and IgG anti-chromatin

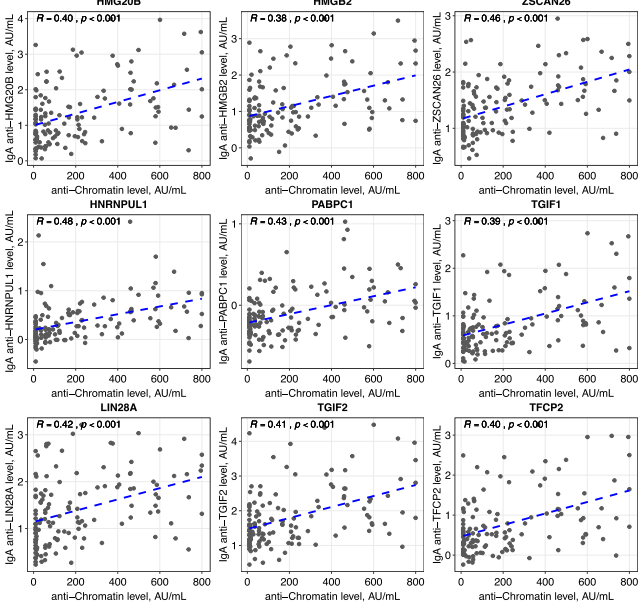


Figure 5. Diagnostic performance of IgG and IgA daAAbs and conventional autoantibodies in SLE against HC, SjD, and SSc combined and correlations with IgG antichromatin. Heatmaps show the diagnostic metrics including sensitivity, specificity, PPV, NPV, and accuracy for IgG (A) and IgA (B) daAAbs in the discovery (left) and validation (right) cohorts, and for conventional autoantibodies in the discovery cohort (C). daAAbs with significantly greater levels in SLE across all comparisons in the discovery cohort are in bold. Plotted in D-E are correlations between levels of selected IgG (D) and IgA (E) daAAbs and ELISA-determined levels of IgG antichromatin. Metrics of the full list of IgG and IgA daAAbs and detailed results from correlation analyses with IgG antichromatin are provided in [Supplementary Files S5 and S6](#). ACC: accuracy; daAAb, differentially abundant autoantibody; ELISA, enzyme-linked immunosorbent assay; HC, healthy control; NPV, negative predictive value; PPV, positive predictive value; SjD, Sjögren's disease; SLE, systemic lupus erythematosus; SSc, systemic sclerosis.

ZSCAN26, and anti-TGIF2 ([Supplementary Fig S1B](#)). To aid comparison, positivity frequencies for conventional autoantibodies in the discovery cohort are shown in [Supplementary Fig S2A](#).

Variation of IgG and IgA autoantibody levels over time in SLE

Among SLE-specific IgG daAAbs, levels of anti-LIN28A, anti-HMG20B, and anti-TFCP2 decreased over a course of 14 months, while levels of anti-HNRNPA2B1, anti-HMGB2, and anti-NFIA showed no statistically significant variation ([Fig 7A](#)). Positivity frequencies for IgG daAAbs over time are presented in [Supplementary Figure S2B](#).

Among SLE-specific IgA daAAbs, levels of anti-LIN28A, anti-SUB1, anti-PCBP2, and anti-TGIF2 decreased over time, while levels of anti-HMG20B, anti-HMGB2, anti-NRF1, anti-MAZ, anti-HMG5, anti-NFIA, anti-NOL4, and anti-PSIP1 showed no statistically significant variation ([Fig 7B](#)). Positivity frequencies for IgA daAAbs over time are presented in [Supplementary Figure S2C](#).

IgG and IgA multireactivity against protein antigens in SLE and pathway enrichment analysis

To investigate multiple IgG and IgA reactivity against protein antigens, we clustered patients with SLE and daAAbs based on

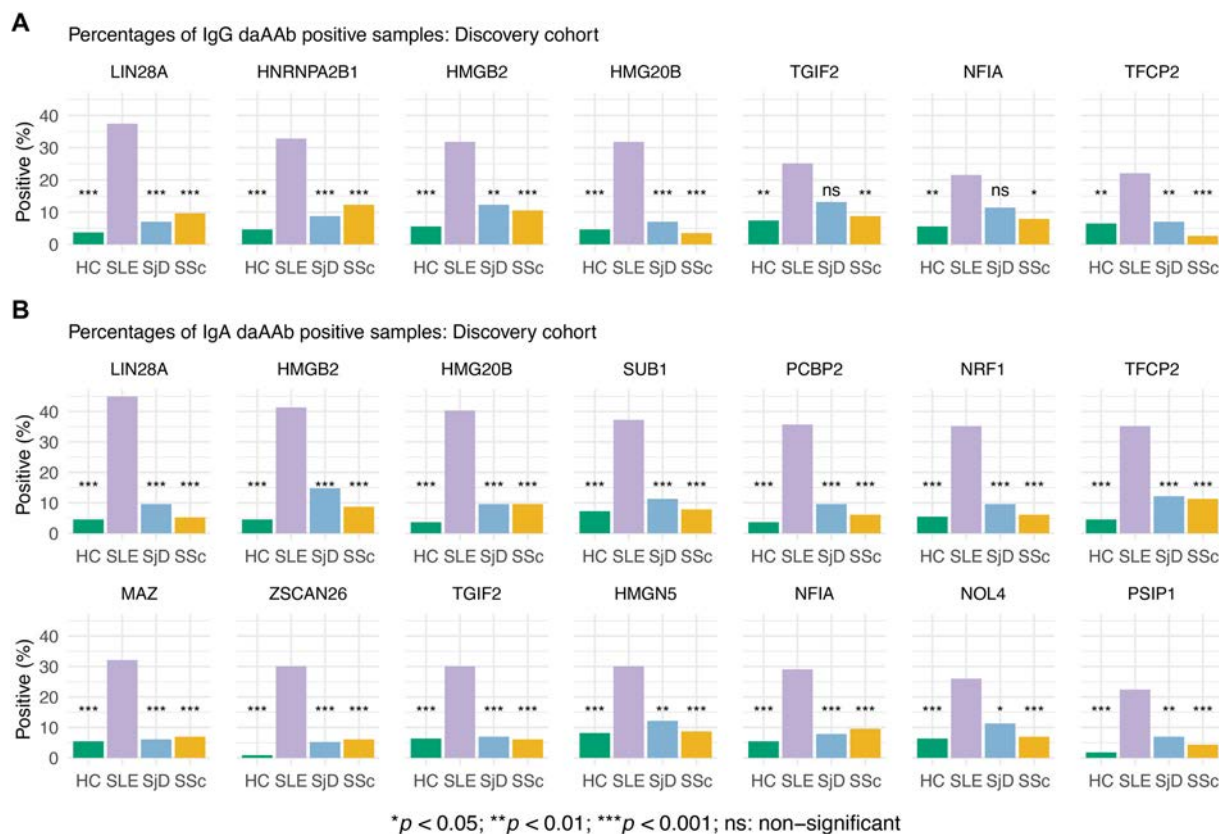


Figure 6. Positivity frequencies of IgG and IgA daAAbs in SLE vs HC, SjD, and SSc. Bar plots display percentages of patients with positive levels of IgG (A) and IgA (B) daAAbs in the discovery cohort. Positivity cutoff was defined as the median plus 2 IQRs in the HC distribution. daAAbs with significantly greater levels in SLE across all comparisons in the discovery cohort are illustrated. Positivity frequencies of the full list of IgG and IgA daAAbs are provided in [Supplementary Files S7 and S8](#). daAAb, differentially abundant autoantibody; HC, healthy control; SjD, Sjögren's disease; SLE, systemic lupus erythematosus; SSc, systemic sclerosis.



Figure 7. Variation in IgG and IgA daAAb levels over the course of SLE. Box plots illustrate the levels of IgG (A) and IgA (B) daAAbs in an inception cohort of SLE patients, measured at 3 time points (BL, M6, and M14) over a 14-month observational period. daAAbs with significantly greater levels in SLE across all comparisons in the discovery cohort are illustrated. All P values are derived from repeated-measurement Analysis of Variance (ANOVA). daAAb, differentially abundant autoantibody; BL, baseline; M6, month 6; M14, month 14; SLE, systemic lupus erythematosus.

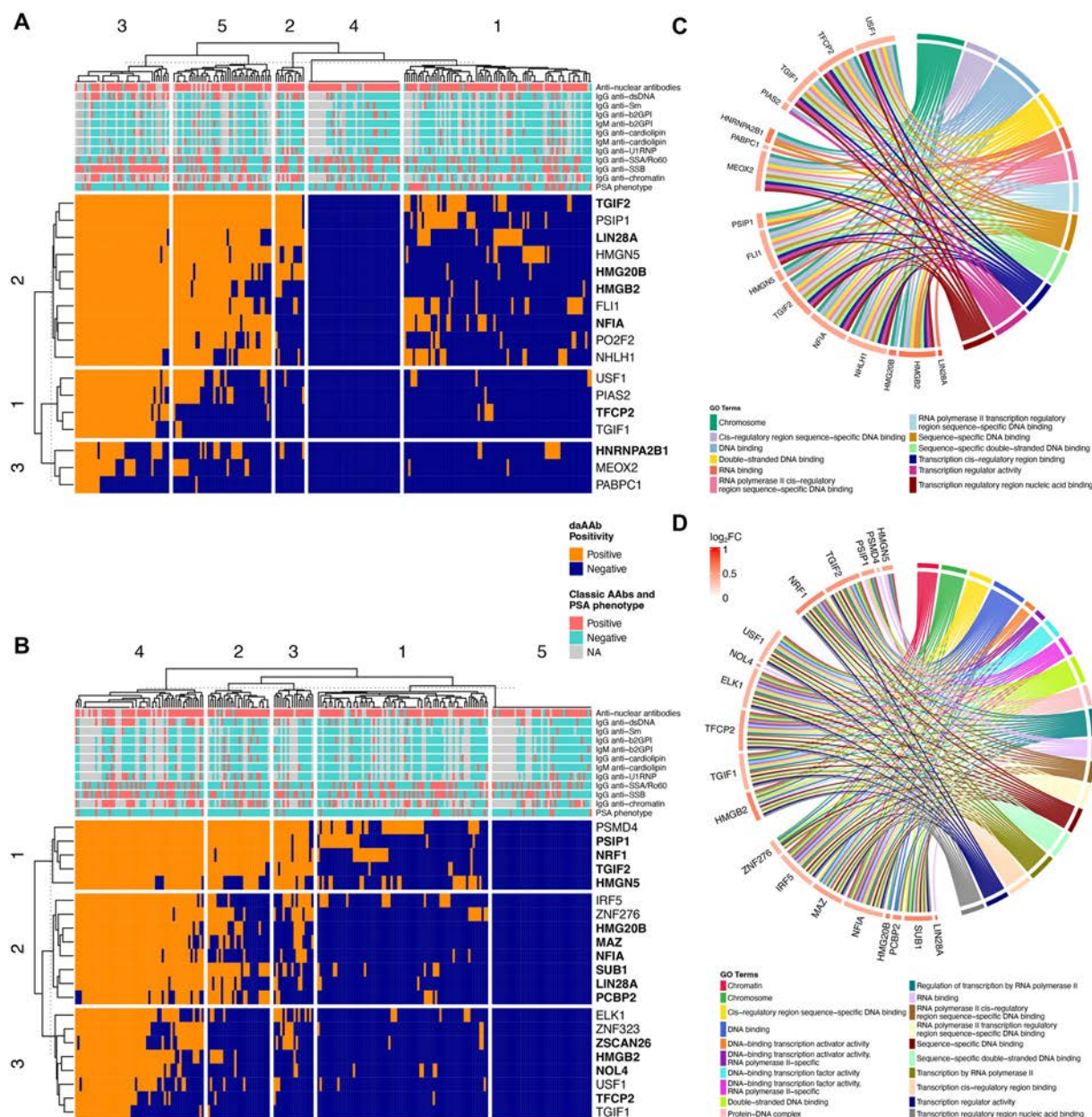


Figure 8. Hierarchical clustering based on daAAb positivity status and enriched GO terms associated with SLE-specific IgG and IgA daAAb targets. The heatmaps show subgroups of patients with SLE positive (orange) or negative (blue) results for IgG (A) or IgA (B) daAAb in the discovery and validation cohorts combined. Columns denote patients with SLE, and rows denote daAAb. Annotations on the top of the heatmap denote positivity status for conventional autoantibodies, including antinuclear antibodies (ANA; indirect immunofluorescence on HEp-2 cells), IgG anti-dsDNA (chemiluminescence), IgG anti-Smith (chemiluminescence), IgG and IgM anti-b2GPI (chemiluminescence), IgG and IgM anticardiolipin (chemiluminescence), IgG anti-SSA/Ro60 (KREX), and IgG anti-SSB (KREX), IgG antichromatin (ELISA), as well as PSA with red (positive) and turquoise (negative). Grey colour indicates missing data for conventional autoantibodies. The chord diagrams illustrate enriched GO terms from the pathway enrichment analysis of IgG (C) and IgA (D) daAAb targets; daAAb clusters derived from the cluster analysis in the heatmaps (A,B). daAAb, differentially abundant autoantibody; dsDNA, double-stranded DNA; ELISA, enzyme-linked immunosorbent assay; GO, gene ontology; KREX, KoRectly EXpressed; PSA, polyspecific antibody; SLE, systemic lupus erythematosus; SSA, Sjögren's syndrome-related antigen A; SSB, Sjögren's syndrome-related antigen B.

positivity status. As shown in [Figure 8A](#), 1 subgroup of patients with SLE demonstrated IgG reactivity against multiple antigens, 3 subgroups of patients exhibited more varying reactivity, and 1 subgroup showed no reactivity. IgG daAAb were divided into 3 different clusters. Similarly, 1 subgroup of patients with SLE showed IgA reactivity against multiple antigens, 3 subgroups of patients exhibited more varying reactivity, and 1 subgroup displayed no reactivity ([Fig 8B](#)). IgA daAAb were divided into 3 different clusters. Conventional autoantibodies were present across patient subgroups both by IgG and IgA daAAb. Notably, antichromatin antibodies were highly abundant in the

multireactive subgroups. For completeness, we also performed clustering analyses based on the levels of IgG ([Supplementary Fig S3A](#)) and IgA ([Supplementary Fig S3B](#)) daAAb.

Finally, we performed pathway enrichment analysis of IgG and IgA daAAb target. [Figure 8C](#) summarises the enriched GO terms associated with IgG daAAb targets, in their majority pertaining to DNA and RNA binding, linking to the regulation of transcription and translation functions. There was a considerable overlap of daAAb target clusters across enriched GO terms, with no functional specificity for any of the 3 clusters. Similar GO terms and associated functions were derived for IgA daAAb

targets, with an apparent functional overlap across clusters (Fig 8D).

DISCUSSION

Beyond germline-encoded and constitutively present autoantibodies, a multitude of autoantibodies constitute defining features of autoimmunity for their potential roles in disease pathogenesis—whether causal, consequential, or protective—and their utility in diagnosis and monitoring. Their stable structure and high production, even when autoantigen levels are low, make autoantibodies well-suited as biomarkers [23]. Some autoantibodies appear years before clinical disease, making them valuable for early detection and diagnosis. However, their personalised nature adds complexity to the development of predictive algorithms [10]. Furthermore, antibodies persist due to their long half-lives, limited proteolysis, and slow clearance from circulation [24]. In this study, we screened plasma from a large discovery and an independent validation cohort of patients with SLE and healthy individuals to determine IgG and IgA seroreactivity against 1609 protein autoantigens. Our primary goal was to identify novel autoantibodies as potential biomarkers for SLE diagnosis. To investigate disease specificity, we also included patients with primary SjD and SSc, given their clinical and immunologic overlap with SLE, especially in early stages [6,7]. We identified and validated 5 IgG (anti-LIN28A, anti-HNRNPA2B1, anti-HMG20B, anti-HMGB2, and anti-TFCP2) and 4 IgA (anti-LIN28A, anti-HMG20B, anti-SUB1, and anti-TFCP2) autoantibodies that demonstrated high specificity for SLE, along with consistent and robust positivity frequencies. Interestingly, among patients with ANA-negative SLE, only 1 in the IgG analysis and 3 in the IgA analysis were negative for both conventional and newly identified autoantibodies, supporting the potential complementary diagnostic utility of the novel autoantibodies identified in this study. Known autoantibody specificities did not show distinct patterns of accumulation within any particular cluster of the newly identified IgG or IgA autoantibodies, suggesting that the specificities identified in this study may provide complementary insights into SLE autoimmunity. Upon further validation and standardisation, these autoantibodies may support SLE diagnosis in clinical practice.

Two previous studies have used the Sengenics i-Ome high-throughput proteomic microarray to identify IgG autoantibodies in geographically distinct SLE populations from Singapore [25] and the UK [5]. Among the top 20 autoantibodies identified in the Singapore cohort, anti-HMG20B and anti-HMGB2 were also significant in our study. The UK-based study included a larger sample size with a discovery ($n = 192$) and a validation cohort ($n = 91$), comprising individuals of European, African, and Hispanic backgrounds. Several autoantibodies overlapped between the study by Lewis et al [5] and ours, including LIN28A, HMGB2, HMG20B, HNRNPA2B1, and PCBP2. Positivity frequencies for these IgG autoantibodies ranged from 22.4% for anti-LIN28A to 14.8% for anti-HMG20B. IgG anti-HNRNPA2B1 (anti-RA33) is well-documented in autoimmunity, including in SLE and RA [26–29]. Thus, while differences in RA33 epitopes between RA and SLE have been reported [30], the specificity of this autoantibody for SLE may be reduced when assessed alongside RA populations. This underscores the importance of evaluating disease-specific reactivity patterns across autoimmune conditions and partially supports the notion of interindividual differences in genetic backgrounds differentially impacting immune functions, alongside each individual's unique immune exposure history related to infections, vaccines, injuries, among

other factors, all of which may play a major role in the evolution of immune subtypes within the healthy population and may be hypothesised to influence autoantibody response patterns in people with autoimmune disease [7]. However, the limited sample size in the Singapore study underscores the need for validation in larger, ethnically diverse cohorts [25]. While most SLE autoantibody studies focus on the IgG isotype, including the 2 previous studies that used the Sengenics i-Ome microarray, our study also examined IgA autoantibodies.

Research has primarily focused on IgG antibodies due to their pathogenic role, ability to activate complement, and abundance as the main circulating immunoglobulin [31]. However, in recent decades, IgM and IgA have also been recognised as contributors to disease pathogenesis and as potential biomarkers [32]. For instance, antiphospholipid antibodies (aPL) were initially limited to IgG in clinical practice, with IgM aPL only later being included in diagnostic protocols for antiphospholipid syndrome [33]. More recently, IgA aPL were detected in patients with thrombotic events negative for IgG and IgM aPL [34]. Overall, accumulating evidence supports IgA autoantibodies as valuable biomarkers in autoimmune conditions [34]. Beyond their diagnostic potential, IgA autoantibodies are implicated in disease pathogenesis, as evidenced by autoimmune manifestations in IgA multiple myeloma [35]. Studies have also identified pathogenic roles for IgA antibodies in conditions such as IgA nephropathy, IgA vasculitis, dermatitis herpetiformis, inflammatory bowel disease, linear IgA bullous dermatosis, and RA [36].

Approximately 30% of patients with SLE have detectable IgA anti-dsDNA antibodies, linked to disease activity and severity [37]. Elevated IgA ANA levels have also been observed in saliva and stool samples from patients with SLE [38]. IgA deposits, alongside IgG, IgM, C3, and C1q, are commonly found in the glomeruli of lupus nephritis patients and aid histopathological classification [39]. Additionally, a higher prevalence of IgA B cell clones has been reported in SLE compared with healthy state and other autoimmune conditions [40]. However, from a mechanistic perspective, the pathogenic role and antigen specificities of IgA autoantibodies in SLE remain largely unexplored.

A recent study demonstrated that IgA1 autoantibodies in SLE target several of the same self-antigens as IgG autoantibodies, including Smith ribonuclear proteins [40]. Notably, it also demonstrated that plasmacytoid dendritic cells express IgA1-binding Fc- α receptors and that IgA1-containing immune complexes can stimulate interferon α production [40]. This response requires both IgA1 and IgG, suggesting synergistic effects between isotypes. However, many specific IgA targets remain undefined. In our study, we identified and validated 18 autoantigens targeted by both IgG and IgA autoantibodies, with IgA against LIN28A and TFCP2 showing robust and consistent reactivity. Overall, the multiple IgA daAabs identified and validated in this study imply potential important roles for mucosal immunity in SLE.

LIN28A, also known as CSDD1, FLJ12457, LIN-28, and ZCCHC1, is an RNA-binding protein found in the cytosol and nucleolus, regulating stem cell renewal by inhibiting *let-7* microRNA maturation [41]. Mechanistically, the LIN28–*let-7* axis is partly regulated by the Sjögren's syndrome–related antigen B (SSB) protein [41]. LIN28A has been implicated in autoimmune diseases, with research indicating that it can activate inflammatory pathways by elevating interleukin 6 and tumour necrosis factor α levels [42]. Overexpression of LIN28A has been observed in kidneys with severe inflammation mediated by the AKT–NF- κ B–CXCL1 signalling pathway [43], while *let-7* has been implicated in lupus nephritis pathogenesis through modulation of NF- κ B signalling pathways [44]. Moreover, LIN28A has

shown potential regenerative effects on cartilage in osteoarthritis models [45], contributes to cardiovascular disease by stimulating vascular smooth muscle cell proliferation [46], and influences liver fibrosis by modulating the balance between fibrotic processes and tissue regeneration [47]. Collectively, LIN28A emerges as a promising autoantigen in SLE, warranting further investigation into its role in disease mechanisms.

TFCP2, also known as late SV40 factor, is a DNA-binding protein that regulates transcription by modulating erythroid cell gene expression and activating various cellular and viral gene promoters in the nucleoplasm and cytosol [48]. TFCP2 has been implicated in modulating therapeutic interferon responses in multiple sclerosis and associated with several autoimmune conditions [49]. A variant in the *TFCP2L1* gene (rs17006292) has been linked to Adamantiades-Behçet disease in the Han Chinese population [50]. Elevated TFCP2 mRNA levels have been reported in peripheral blood of patients with RA compared with those in patients with osteoarthritis and linked to innate immune responses [51], although the precise mechanisms remain unclear. Additionally, TFCP2 has been proposed to repress class switch recombination by histone deacetylation of chromatin within S regions, thereby limiting accessibility to the switch recombination machinery [52].

Several studies have categorised patients with SLE based on conventional autoantibody profiles, yielding consistent clustering patterns [11,53,54]. Typically, 4 subgroups are identified: first subgroup marked by anti-Sjögren's syndrome-related antigen A (SSA)/SSB, linked to skin involvement and Sjögren-like symptoms; second characterised by antinucleosome, anti-SmRNP, anti-dsDNA, and anti-RNP antibodies, often associated with lupus nephritis; third marked by IgG/IgM anti- β 2GPI and IgG/IgM anticardiolipin autoantibodies, correlating with vascular and thrombotic events; and fourth subgroup characterised by ANA positive but lacking conventional autoantibodies [11]. In our study, 5 patient subgroups emerged based on IgG autoantibodies and 5 based on IgA autoantibodies. One subgroup showed broad reactivity, 3 showed varied patterns, and 1 was seronegative for the screened specificities. Importantly, conventional autoantibodies such as anti-dsDNA, anti-Sm, anti-U1RNP, antiphospholipid antibodies, anti-SSA/Ro60, and anti-SSB were evenly distributed across clusters. The seronegative subgroup included individuals with positive result for several conventional autoantibodies, while some patients in the multireactive subgroup lacked them. Notably, among the few patients with ANA-negative SLE, only 1 in the IgG analysis and 3 in the IgA analysis were negative for both conventional and newly identified autoantibodies. These novel autoantibodies could complement conventional markers in clinical practice to improve diagnostics and monitoring.

Given that several identified autoantigens are nuclear or associated with chromatin, we annotated patient subgroups for antichromatin antibodies and conducted correlation analyses for selected specificities. Antichromatin antibodies have reported specificity over 90% and sensitivity of around 50% in SLE [55]. The multireactive subgroup showed a high prevalence of antichromatin antibodies, and moderate to strong correlations were observed between antichromatin antibodies detected by ELISA and both chromatin-associated and nonchromatin-associated autoantibodies identified by KREX. These findings underscore the complexity of SLE serology and suggest that some newly identified specificities may overlap with targets recognised by conventional antichromatin antibodies.

In addition to patient subgroups, autoantibody clusters revealed shared functional properties of the target antigens

based on GO terms, with most proteins involved in DNA or RNA binding and the regulation of transcription and translation [56]. These findings are consistent with long-established paradigms in SLE, further supporting the reliability of our results and carrying significant implications for disease pathogenesis, particularly in light of the increased and rapid proliferation of immune cells during active phases, impaired DNA damage repair mechanisms, and defective clearance of apoptotic material [56,57]. Whether our findings reflect a consequence of the immunologic aberrancies in SLE or the autoantibodies possess intrinsic pathogenic properties requires further investigation.

A major strength of the study was the longitudinal determination of autoantibody levels in the inception cohort at 3 time points. This revealed variability in certain autoantibodies, with levels decreasing from active disease shortly after diagnosis over a 14-month period, while others remained stable. These patterns suggest that, beyond diagnostic use, some autoantibodies may have value in monitoring disease activity, as explored in a follow-up study [58]. This also has implications regarding the cellular origin of these autoantibodies, possibly involving nonterminally differentiated B cells, warranting further investigation. However, certain limitations should be noted. Firstly, all study participants were of European origin, limiting the direct applicability of the findings to other ethnic groups [59]. Nevertheless, overlap with previous studies suggests shared mechanisms across populations and supports the relevance and reliability of our findings [5,25]. Secondly, our validation cohort was relatively small. Despite this, major autoantibody hits distinguishing SLE from other diseases and healthy individuals were consistent in both cohorts, reinforcing the robustness of our results. Thirdly, while validation using ELISA or similar solid-phase assays may appear a logical next step, several factors placed such validation beyond the scope of this exploratory study, including logistical and financial considerations. Moreover, ELISA may not provide a fully orthogonal comparison with KREX-based findings, given inherent differences in assay sensitivity and the preservation of protein conformation. To this end, biological validation in independent cohorts, as performed in this study, offers robust corroboration. Future translational studies specifically focused on assay development, as well as validation in larger and ethnically more diverse SLE populations, will be required to further advance our findings. Lastly, a detailed characterisation of ANA patterns in relation to the newly identified autoantibodies was beyond the scope of this study but remains an important area for future investigation.

In summary, we identified and validated previously unrecognised IgG and IgA autoantibody reactivity in SLE. Several autoantibody hits showed high specificity in distinguishing SLE from healthy individuals and other autoimmune diseases (SjD and SSc), some outperforming traditional markers such as anti-dsDNA and anti-Sm. Notably, anti-LIN28A appeared among both IgG and IgA hits, corroborating previous smaller studies. The exploration of IgA autoantibodies is novel, and findings from this analysis provide incentive for a better characterisation of IgA cell responses in SLE and investigation of potential roles of mucosal tissues in disease priming and precipitation. Upon further validation, our results hold strong potential to advance autoantibody-based diagnostics and molecular characterisation of SLE, supporting informed and personalised therapeutic strategies.

Competing interests

IP has received research funding and/or honoraria from Amgen, AstraZeneca, Aurinia, BMS, Elli Lilly, Gilead, GSK,

Janssen, Novartis, Otsuka, and Roche. The other authors declare no conflicts of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

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Collaborators

PRECISESADS Clinical Consortium: Jacques-Olivier Pers, Alain Saraux, Valérie Devauchelle-Pensec, and Sandrine Jousse-Joulin (Centre Hospitalier Universitaire de Brest, Hospital de la Cavale Blanche, Brest, France); Bernard Lauwerys, Julie Ducreux, and Anne-Lise Maudoux (Pôle de pathologies rhumatismales systémiques et inflammatoires, Institut de Recherche Expérimentale et Clinique, Université catholique de Louvain, Brussels, Belgium); Ana Tavares and Isabel Almeida (Centro Hospitalar do Porto, Portugal); Miguel Angel Gonzalez-Gay Mantecón, Ricardo Blanco Alonso, and Alfonso Corrales Martínez (Servicio Cantabro de Salud, Hospital Universitario Marqués de Valdecilla, Santander, Spain); Ignasi Rodríguez-Pintó and Gerard Espinosa (Department of Autoimmune Diseases, Hospital Clínic, Institut d'Investigacions Biomèdiques August Pi i Sunyer, Barcelona, Catalonia, Spain); Rik Lories (Katholieke Universiteit Leuven, Belgium); Nicolas Hunzelmann and Doreen Belz (Klinikum der Universitaet zu Koeln, Cologne, Germany); Niklas Baerlecken (Medizinische Hochschule Hannover, Germany); Michael Zauner and Michaela Lehner (Medical University Vienna, Vienna, Austria); Eduardo Collantes, Ma Angeles Aguirre-Zamorano, Alejandro Escudero-Contreras, and Ma Carmen Castro-Villegas (Servicio Andaluz de Salud, Hospital Universitario Reina Sofía Córdoba, Spain); Norberto Ortego and María Concepción Fernández Roldán (Servicio Andaluz de Salud, Complejo hospitalario Universitario de Granada (Hospital Universitario San Cecilio), Spain); Enrique Raya and Inmaculada Jiménez Moleón (Servicio Andaluz de Salud, Complejo hospitalario Universitario de Granada (Hospital Virgen de las Nieves), Spain); Enrique de Ramon and Isabel Díaz Quintero (Servicio Andaluz de Salud, Hospital Regional Universitario de Málaga, Spain); Pier Luigi Meroni, Tommaso Schioppo, and Carolina Artusi (Università degli studi di Milano, Milan, Italy); Carlo Chizzolini, Aleksandra Zuber, and Donatienne Wynar (Hospitaux Universitaires de Genève, Switzerland); Attila Balog, Magdolna Deák, Márta Bocskai, Sonja Dulic, and Gabriella Kádár (University of Szeged, Szeged, Hungary); Falk Hiepe and Silvia Thiel (Charité, Berlin, Germany); Manuel Rodriguez Maresca, Antonio López-Berrio, Rocío Aguilar-Quesada, and Héctor Navarro-Linares (Andalusian Public Health System Biobank, Granada, Spain).

Contributors

IP, JL, P-JJ, MEA-R, HI, and DN were involved in the conceptualisation of the study. IP, DL, JL, LB, MOB, GB, MEA-R, and

DN were involved in data curation. IP, DL, JL, and DN were involved in the visualisation of the findings. IP, DN, JL, NS, LB, GB, P-JJ, MEA-R, HI, and DN were involved in the interpretation of results. IP and DN were involved in the manuscript drafting. All authors were involved in data analysis, critical review of manuscript drafts, and approval of the final draft before submission. IP acts as the guarantor of the study.

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Patient consent for publication

Before participation in the PRECISESADS project, all patients and healthy controls provided written informed consent.

Ethics approval

This study involved human participants. It was reviewed and approved by local ethics committees at all recruiting centres: Comitato Etico Area 2, Fondazione IRCCS Ca Granda Ospedale Maggiore Policlinico di Milano and University of Milan (approval no. 425 bis 19 November 2014 and no. 671_2018 19 September 2018); Klinikum der Universitaet zu Koeln, Cologne, Germany; Comité d'Ethique Hospitalo-Facultaire, Pôle de pathologies rhumatismales systémiques et inflammatoires, Institut de Recherche Expérimentale et Clinique, Université catholique de Louvain, Brussels, Belgium; Csongrad Megyei Kormányhivatal, University of Szeged, Szeged, Hungary; Comité Etica de Investigación Clínica del Hospital Clínic de Barcelona, Hospital Clínic I Provincia, Institut d'Investigacions Biomèdiques August Pi i Sunyer, Barcelona, Spain; Comité de Ética e la Investigación de Centro de Granada (CEI—Granada), Servicio Andaluz de Salud, Hospital Universitario Reina Sofía Córdoba, Spain; Comissão de ética para a Saude—CES do CHP, Centro Hospitalar do Porto, Portugal; Comité de Protection des Personnes Ouest VI, Centre Hospitalier Universitaire de Brest, Hospital de la Cavale Blanche, Avenue Tanguy Prigent 29609, Brest, France; DEAS—Commission Cantonale d'éthique de la recherche Hopitaux universitaires de Geneve, Hospitaux Universitaires de Genève, Switzerland; Andalusian Public Health System Biobank,

Granada, Spain; Commissie Medische Ethiek UZ KU Leuven/Onderzoek, Katholieke Universiteit Leuven, Belgium; Ethikkommission, Charite, Berlin, Germany; Ethikkommission, Medizinische Hochschule Hannover, Germany. Participants gave informed consent to participate in the study before taking part. The study protocol for the present analysis was reviewed and approved by the Swedish Ethical Review Authority (approval no. 2022-03907-01).

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Data availability statement

Data are available upon reasonable request. Raw data remain the property of the PRECISESADS consortium and are protected under the European General Data Protection Regulation. Meta-data and aggregated processed data are available upon reasonable request from the corresponding author and from the European Genome-Phenome Archive.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ard.2025.04.003.

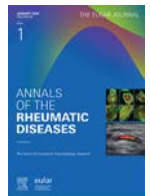
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Sjögren's syndrome

Development of salivary gland organoids derived from patient biopsies: a functional model of Sjögren's disease

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ABSTRACT

Objectives: Salivary gland epithelial cells (SGECs) play a key role in Sjögren's disease (SjD) as an active contributor to the pathogenesis. Current models lack clear epithelial readouts. Our aim was to establish a more advanced model by developing salivary gland organoids (SGOs) from labial salivary gland biopsies (LSGBs) of SjD patients and sicca controls.

Methods: We included SjD patients fulfilling the American College of Rheumatology/European League Against Rheumatism 2016 criteria and sicca controls. LSGBs were dissociated, encapsulated in extracellular matrix, and submerged in growth expansion medium for long-term culture. SGOs were primarily cultured in differentiation medium and then transferred to a low attachment plate to form differentiated SGOs (DIF-SGOs).

Results: We included 13 SjD and 15 controls. In both groups, SGOs were formed, demonstrating long-term culture viability and comparable self-renewal capacity (3.3 ± 1.7 months). DIF-SGOs comparably exhibited mature acinar (aquaporin 5, amylase), ductal (cytokeratins 5 and 7) and myoepithelial (α -smooth muscle actin) markers in both groups. DIF-SGOs were responsive to inflammation by expressing *BAFF*, *CXCL10* and *IL7* upon stimulation with poly(I:C) and interferon- α . DIF-SGOs also demonstrated a swelling response to cholinergic stimulation by pilocarpine. We observed significant differences between SjD- and control-derived SGOs. Notably, SjD-derived DIF-SGOs consistently maintained a persistent interferon signature throughout long-term culture. In addition, the swelling capacity was reduced in SjD-derived DIF-SGOs compared to control. However, treatment with tofacitinib enhanced the swelling ability, suggesting a potential effect on saliva production.

Conclusions: We successfully developed SGOs from LSGBs of SjD patients, allowing long-term culture and faithfully recapitulating the disease phenotype. This model holds promise as a valuable tool for drug screening.

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WHAT IS ALREADY KNOWN ON THIS TOPIC

- Salivary gland epithelial cells (SGECs) play a key role in Sjögren's disease (SjD) as an active contributor to the inflammation.
- Animal models do not recapitulate the complexity of the disease phenotype.
- Organoids are promising tools for disease modelling and drug testing.

WHAT THIS STUDY ADDS

- Salivary gland organoids (SGOs) from SjD patients can proliferate in long-term culture and be frozen, allowing biobanking.
- SjD-derived SGOs display altered morphology despite comparable SGEC markers.
- SjD-derived SGOs have a persistent interferon signature.
- The cholinergic response of SjD-derived SGOs is altered compared to that of controls.
- Tofacitinib enhances swelling capacity of SGOs.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- SGECs from SjD patients are altered despite the absence of immune infiltrate, highlighting their role in the pathogenesis.
- SjD-derived SGOs recapitulate the disease phenotype and could be an emerging tool for preclinical studies and high-throughput drug testing.

INTRODUCTION

Sjögren's disease (SjD) is a systemic autoimmune epithelitis that leads to dryness, fatigue, and joint pain [1]. The clinical presentation of SjD patients is heterogeneous, with 30% to 40% of patients experiencing systemic manifestations [1]. Advances in describing and understanding this heterogeneity have been made through the identification of distinct patient clusters [2]. This heterogeneity highlights the necessity for personalised medicine in the management of SjD. Therefore, there is a need to develop tools that support this tailored approach.

Glandular infiltration by immune cells is an important characteristic of SjD and is central to the classification criteria, which involve assessing sialadenitis in labial salivary gland biopsy (LSGB) [3]. Moreover, salivary gland epithelial cells (SGECs) play a key role in the pathogenesis of SjD. On one hand, their function is impaired, leading to reduced salivary production by acinar cells and disruption of the hydroelectrolytic balance by ductal cells [4]. On the other hand, SGECs maintain inflammation through interactions with immune cells, promoting the survival and activation of B and T cells [4,5]. These interactions perpetuate a cycle of inflammation.

So far, research on the role of SGECs in SjD has been predominantly performed on 2D culture models, such as cell lines or primary SGECs derived from LSGBs. These models have enabled molecular biology studies and cocultures with immune cells [5,6]. However, they do not recapitulate the complexity of tissue architecture and have several limitations, including short culture durations, absence of complex organisation and lack of epithelial functionality.

By the addition of complex cell-cell and cell-matrix interactions, the 3-dimensional (3D) culture of primary cells enables the organotypic organisation, resulting in enhanced differentiation and functionality of the cellular construct [7]. Additionally,

organoids derived from adult stem cells (aSCs) can preserve disease characteristics, making them valuable models for studying pathologies such as tumours or inflammatory diseases such as inflammatory bowel disease [8–12]. Furthermore, these organoids hold promise for personalised medicine by allowing for *in vitro* assessment of the therapeutic response, a potential already being explored in oncology [13,14]. Despite the promising advancements in organoid development, very few organoid systems for autoimmune disorders have been developed.

Human salivary gland organoids (SGOs) have already been established, primarily for regenerative medicine purposes [15–17]. However, creating organoids from SjD is challenging due to the reduced proliferation and limited differentiation capacity of aSCs previously described in SjD [18,19].

Thus, our aim was to develop differentiated and functional SGOs from LSGBs of SjD patients that accurately replicate the disease, enabling disease modelling and drug testing.

METHODS

Patient samples

LSGBs were obtained for diagnostic purposes from SjD patients fulfilling the 2016 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) criteria and controls with sicca syndrome who were referred to the Department of Rheumatology of Bicêtre Hospital, Université Paris-Saclay, France. Patients with incomplete SjD, characterised by the presence of some hallmarks of SjD without meeting the ACR/EULAR criteria, and patients affected by autoimmune diseases other than SjD were excluded from the study. Sicca controls could have objective xerostomia and/or xerophthalmia but did not have autoimmune abnormalities. Clinical data, histology (focus score), immunological status (anti-SSA antibodies, rheumatoid factor), disease activity (EULAR Sjögren's syndrome disease activity index [ESSDAI]) and patient-reported outcomes (EULAR Sjögren's syndrome patient reported index [ESSPRI]) were collected on the day of sampling. All the samples were collected between December 2022 and June 2024.

Organoid establishment and long-term culture

LSGBs were collected and initially maintained in epithelial medium as previously described [5]. LSGBs were then sliced with scissors and dissociated using a magnetic stirring bar at 37°C in a medium composed of RPMI, 10% fetal bovine serum (FBS), 1.5 mg/mL collagenase P (Sigma, 11213857001), 100 µg/mL DNase I (Sigma, DN25), HEPES, 10 µM Y-27632 (Abcam, 129830-38-2), and 1% penicillin/streptomycin (Pen/Strep) for 25 minutes. The resulting cell suspension was subsequently washed with complete medium (RPMI, 10% FBS, 1% Pen/Strep) and filtered through a 100 µm sieve. It was then encapsulated in Growth Factor Reduced Matrigel (Dutcher, 356231) and seeded into 6-well plates. After 20 minutes at 37°C to allow Matrigel to polymerize, growth expansion medium (GEM) was added to promote organoid forming and self-renewal. The composition of GEM was adapted from a previous publication [16]: AdDMEM/F12 containing 100 ng/mL noggin (Miltenyi, 130-103-456), 5 ng/mL FGF-2 (Miltenyi, 130-093-840), 10 ng/mL FGF-10 (Miltenyi, 130-127-856), 5 µM A83-01 (Biotechne, 2939), 10 ng/mL NRG1 (Peprotech, 100-03), 1.25 mM N-acetyl-cysteine (Sigma, A9165), 10 mM nicotinamide (Sigma, N0636), 3 µM prostaglandin E2 (Biotechne, 2296), 1% R-spondin 1 homemade conditioned medium (CM), 10 µM Y-27632, 1× B-27 (ThermoFisher,

17504044), 1× Glutamax, 1× HEPES, and 10 µg/mL Primocin (Invivogen, ant-pm-05). The medium was changed every 2 days to ensure a continuous supply of fresh nutrients for organoid growth and development.

Organoids were passaged every 7 to 10 days using 0.05% trypsin-EDTA (ThermoFisher, 25300054) at 37°C for 10 minutes and then seeded at a density of 2000 cells/µL of Matrigel. An organoid lineage of expanded SGOs (EXP-SGOs) was considered established after 2 passages. The proliferation ratio was calculated by dividing the number of viable cells after passaging by the number of cells initially seeded. Cell viability was assessed using Trypan blue staining (Sigma-Aldrich, 17-942E) after each passage.

Organoid differentiation

EXP-SGOs were first transferred to be cultured in differentiation medium (DM) embedded in Matrigel. The composition of DM was as follows: AddMEM/F12 containing 5 ng/mL FGF-2, 10 ng/mL FGF-10, 0.5µM A83-01, 10 ng/mL NRG1, 1.25 mM N-acetylcysteine, concentration for DAPT (Sigma, D5942), 1% R-spondin 1 CM, 1× B-27, 1× Glutamax, 1× HEPES, and 10 µg/mL Primocin. After 7 days of culture in Matrigel in DM, SGOs were dissociated into single-cell suspension and seeded at a density of 2000 cells per well in ultra-low attachment 96-well plates (Corning, 7007). They were further cultivated for 7 to 10 days in DM supplemented with 3% Matrigel to obtain morphologically mature SGOs, as assessed by brightfield microscopy. Physical force was applied through repeated centrifugations of the plate after seeding to achieve a homogeneous population of differentiated organoids (DIF-SGOs). The medium was replaced twice a week to maintain a continuous supply of fresh media, supporting organoid development and differentiation.

Swelling assay

DIF-SGOs were cultured in ultra-low attachment 96-well plates as previously described. After 7 to 10 days of culture, DIF-SGOs were exposed to cholinergic stimulation with pilocarpine (Sigma, P6503-5G) at doses of 25 µM and 50 µM for 24 hours. Live cell images were captured using Incucyte SX5 microscope every 30 minutes during the stimulation period. Each condition was tested in 6 replicates. Swelling was quantified by calculating the ratio of the object area measured by Incucyte SX5 software to the initial object area at each time point.

In a subsequent experiment, 30 nM tofacitinib was added to DIF-SGOs 24 hours prior to cholinergic stimulation. The *in vitro* concentration was chosen based on prior report [20].

Statistics

Statistical analysis was conducted using GraphPad Prism 10 software. Mann–Whitney and Friedman test were used for quantitative unpaired and paired analysis, respectively, and Fisher’s exact test for qualitative analysis. A *P* value < .05 was considered statistically significant.

RESULTS

Establishment of EXP-SGOs for long-term culture

We successfully established 39 SGO lines from LSGBs obtained from patients for diagnostic purposes, achieving a high success rate of 94.9%. Nine SGO lines were excluded from the

Table
Characteristics of the patients

	SjD, n = 13	Sicca controls, n = 15
Age, y	50.5 ± 13.9	59.2 ± 13
Women	12 (92.3)	15 (100)
Dryness duration, y	10.4 ± 13.4	3.4 ± 2.9
Pathologic Schirmer test	9/10 (90)	10/13 (76.9)
Altered salivary flow	6/12 (50)	5/14 (35.7)
Focus score ≥1	8 (61.5)	0
Anti-Ro60 positivity	11 (84.6)	0
Rheumatoid factor positivity	8 (61.5)	1 (7.1)
ESSDAI	3.5 ± 3.9	NA
ESSPRI	5.8 ± 1.8	NA

Data are expressed as n (%) or mean ± SD.
ESSDAI, EULAR Sjögren’s syndrome disease activity index; ESSPRI, EULAR Sjögren’s syndrome patient reported index; NA, not applicable.

analysis, mainly due to incomplete SjD (n = 3) and associated autoimmune disorder (n = 4). This left 13 SGO lines from SjD patients and 15 from controls for analysis. The study flowchart is presented in [Supplementary Figure S1](#).

The characteristics of the SjD patients and controls are summarised in the [Table](#). The mean age was 50.5 ± 13.9 years in SjD and 59.2 ± 13 years in controls, with a comparable proportion of women (92.3% and 100%, respectively) and similar duration of dryness. SjD patients exhibited low systemic activity (mean ESSDAI: 3.5 ± 3.9) and high patient-reported outcomes (mean ESSPRI: 5.8 ± 1.8), and 84.6% were positive for anti-Ro60/SSA antibodies.

LSGBs were dissociated on the day of sampling, and the resulting cell suspension was cultured in extracellular matrix (ECM) using GEM for long-term culture ([Fig 1A](#)). The dissociation protocol preserved SGEC clusters, which initially formed highly cystic EXP-SGOs ([Fig 1B](#)). After the first passage, the EXP-SGOs transitioned to a more tissue-like appearance, meaning less cystic and more solid composition. Both SjD- and control-derived EXP-SGOs demonstrated high proliferation capacity, with sustained SGEC proliferation over time ([Fig 1B, C](#); [Video S1](#)). SjD-derived EXP-SGOs could be cultured for an average of 3.7 ± 2.4 months and control-derived EXP-SGOs for 2.9 ± 0.6 months. The proliferation ratio for each passage was similar between the 2 groups ([Fig 1C](#); [Supplementary Fig S2A](#)), and there was no significant difference in viability or culture duration between passages ([Supplementary Fig S2B, C](#)). The duration of symptoms and the focus score did not affect the expansion ability of EXP-SGOs ([Supplementary Fig S2D, E](#)). Additionally, live cell microscopy (Incucyte SX5) revealed comparable numbers and areas of EXP-SGOs between the 2 groups ([Fig 1D](#); [Supplementary Fig S2F, G](#) and [Table S1](#)). Thus, the organoid-forming capacity during culture was comparable between the 2 groups.

The histological examination of EXP-SGOs showed similar characteristics between SjD and control, with both displaying a round and poorly differentiated morphology ([Fig 1E](#)). Notably, active proliferation was observed at the periphery of the organoids, as indicated by the peripheral expression of the proliferation markers Ki67 and p63 ([Fig 1F](#)). No CD45+ immune cells were detected in EXP-SGOs ([Supplementary Fig S3](#)).

All established EXP-SGOs lines were cryopreserved for biobanking. Upon thawing, SGOs demonstrated sustained proliferation and differentiation capabilities, with high viability observed in the 2 groups ([Supplementary Fig S4](#)).

Thus, we have developed a robust protocol for the generation of EXP-SGOs from LSGBs of SjD patients and sicca controls, achieving a high success rate, enabling long-term culture and

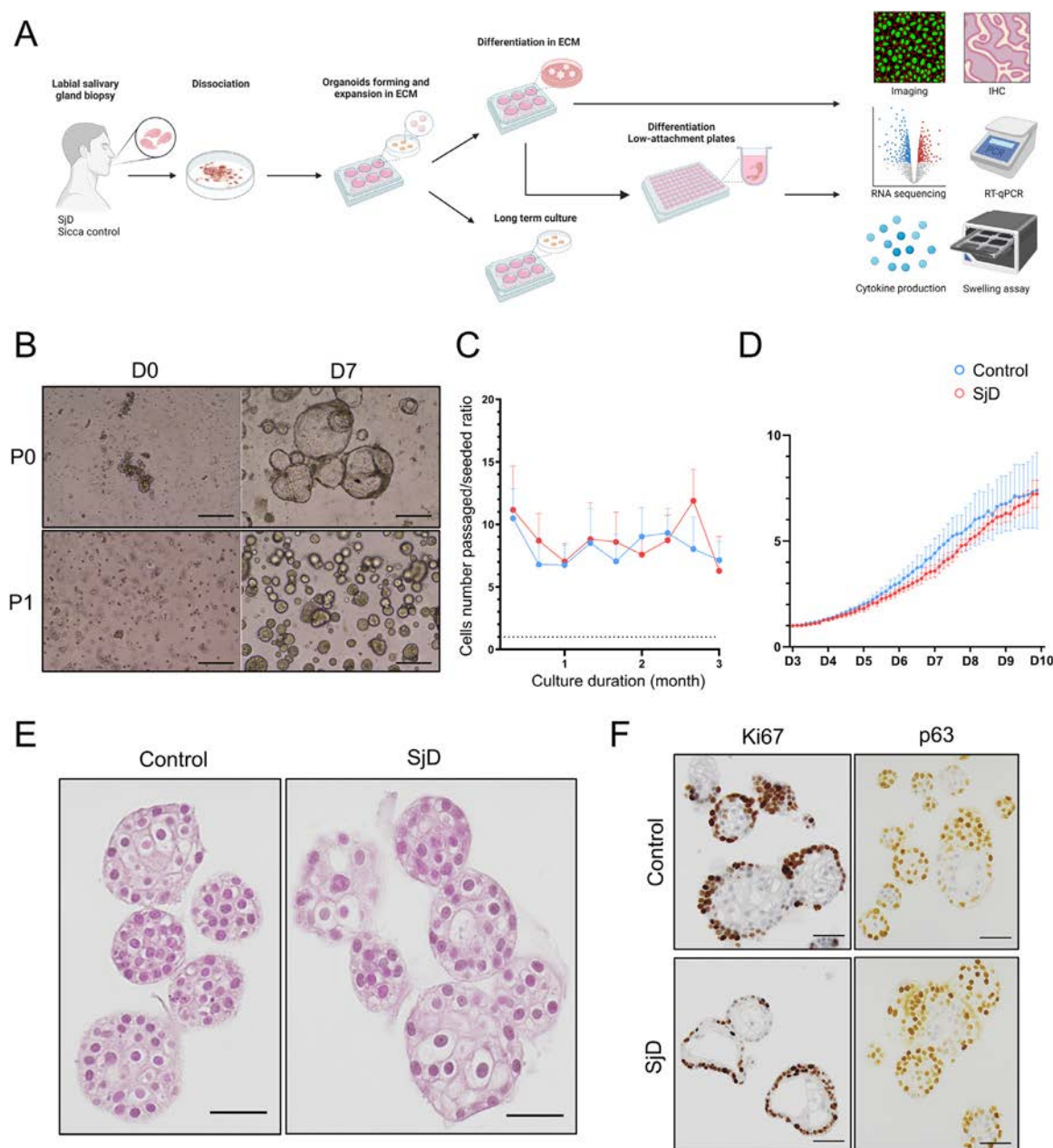


Figure 1. Establishment of long-term culture and proliferation of EXP-SGOs from SjD and sicca controls. (A) Schematic representation of the different steps of SGO culture from LSGBs to characterisation. (B) Brightfield microscopy images showing the morphology of SjD-derived EXP-SGOs cultured in ECM at day 0 and day 7 postdissociation (passage 0) and after passage 1. Scale bar = 200 µm. (C) Proliferation ratio of EXP-SGOs from SjD patients (n = 13) and sicca controls (n = 14) over a 3-month period. The ratio, calculated as the number of cells at passage relative to the number of cells seeded, indicates the level of proliferation; a ratio of 1 represents no proliferation. Data are presented as mean ± SEM. (D) Expansion of EXP-SGOs from SjD (n = 4) and controls (n = 3) from passages 6 to 11 during culture, assessed by Incucyte SX5. The object average is defined as the total organoid area divided by the number of organoids, expressed as a ratio from the day of organoid formation. Data are shown as mean ± SD. (E) Hematoxylin and eosin staining of EXP-SGOs from SjD and control groups, demonstrating similar appearances of undifferentiated organoids. Scale bar = 50 µm. (F) IHC staining of EXP-SGOs from SjD and control subjects revealing marginal expression of Ki67 and p63 markers, which highlight proliferation primarily in the peripheral regions of SGOs. Scale bar = 25 µm. ECM, extracellular matrix; EXP, expansive; IHC, immunohistochemistry; RT-qPCR, reverse transcription quantitative polymerase chain reaction; SGO, salivary gland organoid; SjD, Sjögren's disease.

biobanking. EXP-SGOs from SjD patients displayed comparable characteristics to those from controls in terms of morphology and proliferation.

SjD-derived DIF-SGOs exhibit comparable epithelial markers but display morphological alterations

Once the SGO lines were established, differentiation was initiated by embedding SGOs in ECM and submerging them in DM. The chemical differentiation was promoted by the inhibition of the Notch pathway using DAPT and the withdrawal of several

proliferative components. Subsequently, DIF-SGOs were transferred to ultra-low attachment plates containing DM supplemented with 3% Matrigel for 7 to 10 days, allowing enhancement of their differentiation through the application of physical and mechanical forces to facilitate their uniformity and positioning.

DIF-SGOs demonstrated an organotypic morphology, developing branching and acinar-like structures similar to those of the native gland (Fig 2A, B). Cystic-like structures were frequently observed and became enhanced throughout the differentiation process. Interestingly, the morphology of DIF-SGOs

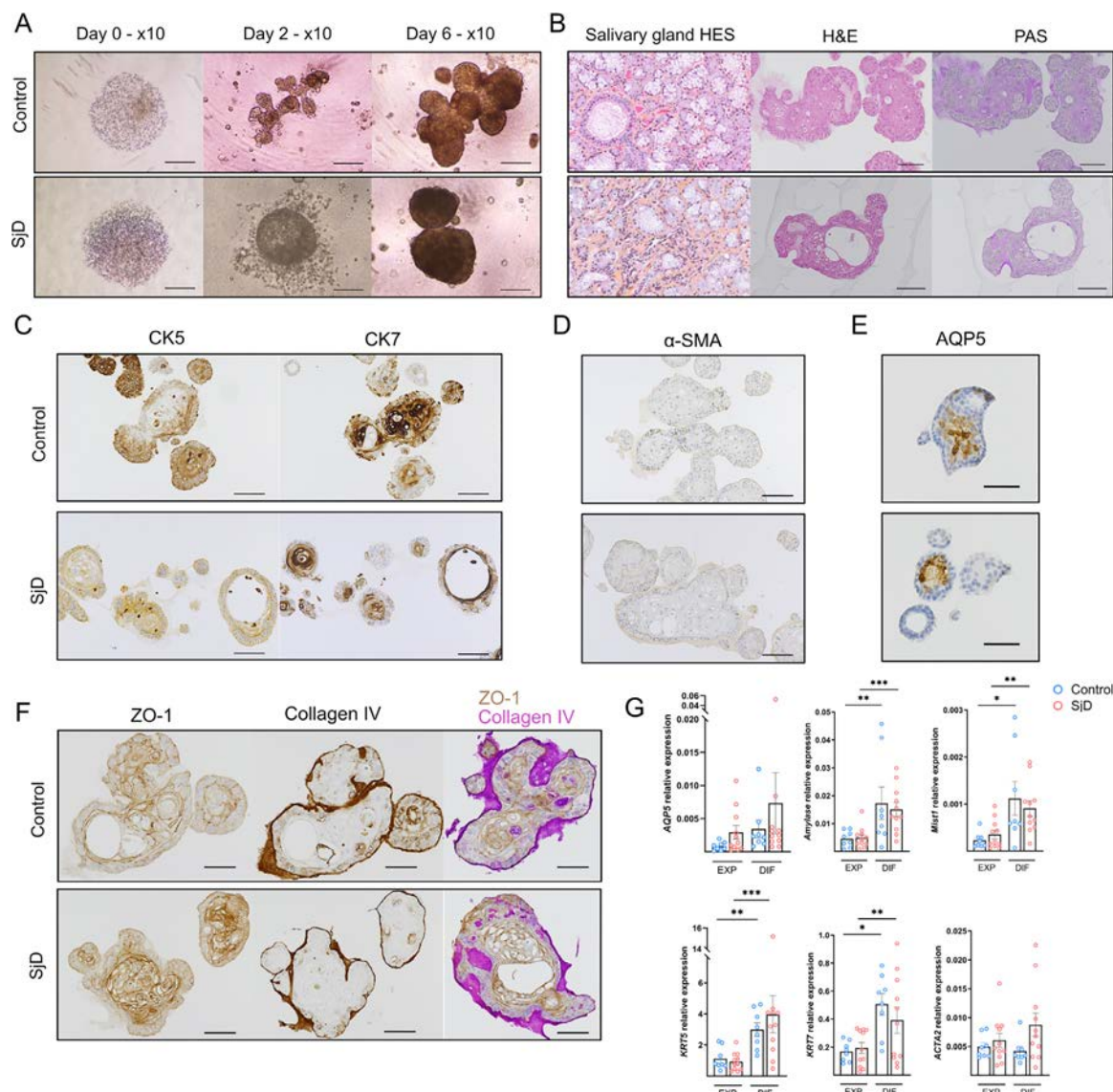


Figure 2. SjD-derived SGOs display comparable epithelial markers but altered morphology. To differentiate SGOs, cells were cultured in differentiation medium after passage, initially in extracellular matrix and subsequently in low attachment plates. (A) Brightfield microscopy images of DIF-SGOs from SjD and control groups (passage 7) at days 0, 4 and 8. Scale bars = 200 μm. (B) H&E and PAS staining of DIF-SGOs derived from SjD and control subjects, along with HES staining of paired salivary glands. Scale bars = 100 μm. (C–F) Immunohistochemical staining of DIF-SGOs for markers AQP5, CK5, CK7, α-SMA, ZO-1, and collagen-IV in both SjD and control samples. (G) Relative mRNA expression levels of AQP5, α-amylase, *Mist1*, CK5, CK7, and *ACTA2/α-SMA* in SjD-derived (n = 11) and control-derived (n = 8) EXP-SGOs and DIF-SGOs from passages 2 to 11. Expression levels are normalised to *GAPDH* and *ACTB* and expressed as mean ± SEM. Wilcoxon test was used. $P < 0.05$ considered significant. ACTB, actin beta; AQP5, aquaporin 5; CK, cytokeratin; DIF, differentiated; EXP, expansive; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; H&E, haematoxylin and eosin; HES, haematoxylin, eosin, and natural saffron; PAS, periodic acid–Schiff; SGO, salivary gland organoid; SjD, Sjögren's disease; α-SMA, alpha-smooth muscle actin; ZO-1, zonula occludens-1. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

derived from SjD patients differed from those derived from controls. Specifically, SjD-derived DIF-SGOs showed less branching, resulting in diminished organotypic differentiation (Fig 2A). Positive periodic acid–Schiff staining was found within DIF-SGOs in the 2 groups, indicating active glycoprotein production (Fig 2B).

To further explore the epithelial characteristics of DIF-SGOs, we investigated the expression of various epithelial markers in both groups. The ductal markers cytokeratin (CK) 5 and CK7, the myoepithelial marker alpha-smooth muscle actin (α-SMA) and the acinar marker aquaporin 5 (AQP5) were all expressed in both SjD- and control-derived DIF-SGOs (Fig 2C–E). The basal ductal marker CK5 was widely distributed within the organoids, whereas the luminal ductal marker CK7 was predominantly located in the core. The cells positive for acinar markers were

organised into clusters near cystic structures. Tight junctions were also present in DIF-SGOs, as highlighted by intercellular zonula occludens-1 staining (Fig 2F). Polarisation was further examined through collagen-IV staining, which displayed peripheral expression, indicating an ‘in-out’ polarisation of DIF-SGOs (Fig 2F). Furthermore, DIF-SGOs exhibited reduced expression of the proliferative markers Ki67 and p63 compared to EXP-SGOs (Supplementary Fig S5), signifying a lower proliferative state. This distribution suggests a centripetal differentiation pattern in DIF-SGOs.

We next examined the 2 groups for expression of SGEC markers that have been previously validated as differentiation markers (Fig 2G) [16,18]. The mature acinar markers α-amylase ($P < .01$) and *Mist1* ($P < .05$), as well as the ductal markers CK5 ($P < .01$) and CK7 ($P < .05$), were significantly induced by

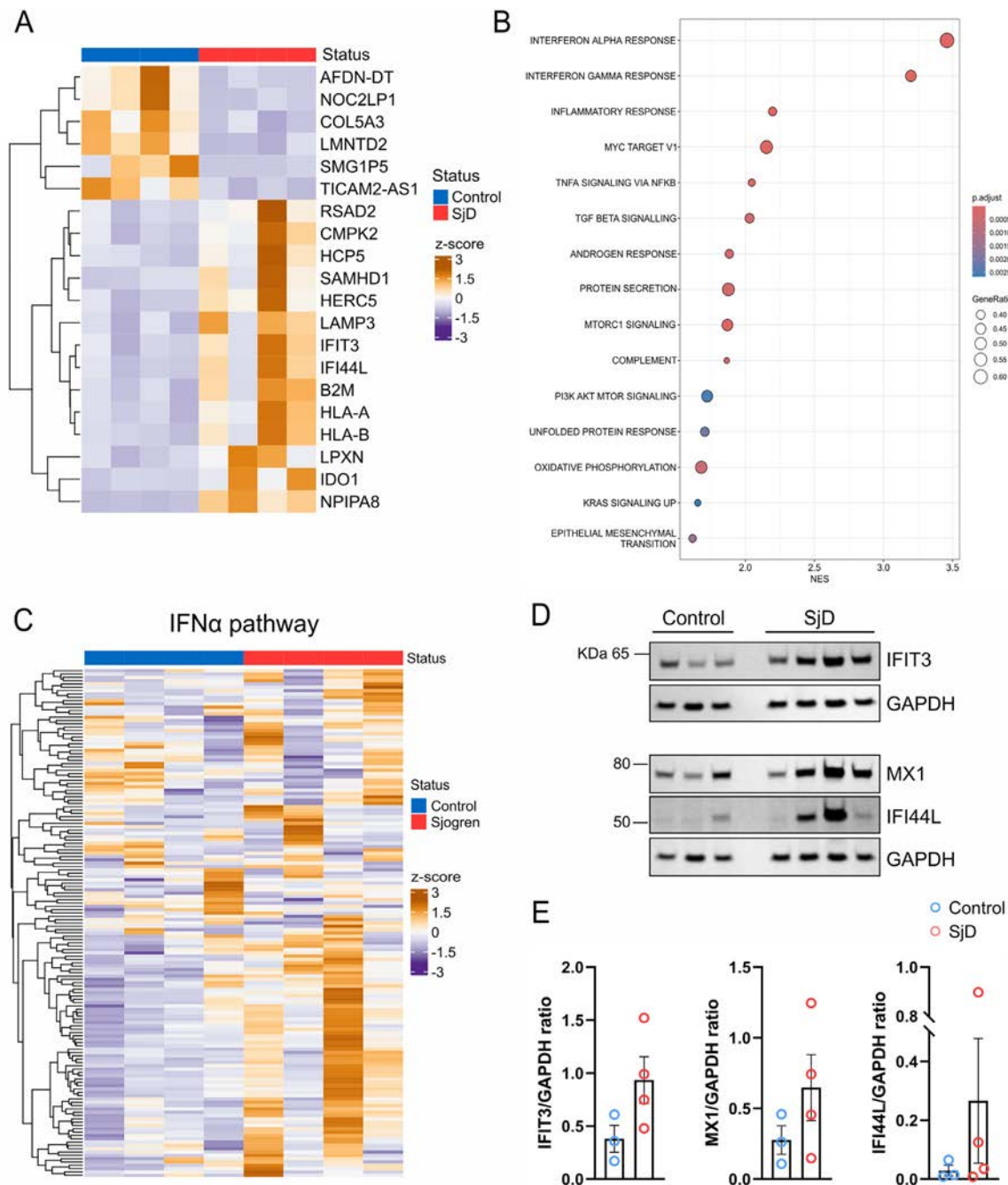


Figure 3. Persistent interferon signature in SjD-derived DIF-SGOs. SjD-derived (n = 4) and control-derived (n = 4) DIF-SGOs from passages 2 to 9 were cultured in differentiation medium for 7 to 9 days, and total mRNA expression was analysed by bulk RNA sequencing. SjD-derived DIF-SGOs showed a sustained enrichment in the IFN pathway despite prolonged culture. (A) Heatmap depicting differentially expressed genes between control and SjD-derived DIF-SGOs. (B) Enriched pathways in SjD-derived DIF-SGOs compared to controls. (C) Heatmap depicting the relative expression levels of IFN α -related genes in DIF-SGOs derived from SjD patients and controls. (D) Western blot analysis of IFIT3, MIX1 and IFI44L expression in control- (n = 3) and SjD-derived (n = 4) DIF-SGOs, with GAPDH as a loading control. (E) Normalised expression of IFIT3, MIX1 and IFI44L in control- (n = 3) and SjD-derived (n = 4) DIF-SGOs, with GAPDH normalisation. Statistical analysis was performed using the Mann–Whitney U test. DIF-SGO, differentiated salivary gland organoid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFI44L, interferon-induced protein 44 like; IFIT3, interferon-induced protein with tetratricopeptide repeats 3; IFN, interferon; MIX1, interferon-induced GTP-binding protein; SjD, Sjögren’s disease.

differentiation in both groups. *AQP5* was also induced by differentiation, but induction did not reach statistical significance. The myoepithelial marker *ACTA2*/ α -SMA was expressed in both EXP- and DIF-SGOs but not induced. Importantly, the expression levels of acinar, ductal and myoepithelial markers were comparable between DIF-SGOs derived from SjD patients and controls (Fig 2G). To further investigate, we performed a deconvolution analysis of the RNA sequencing (RNA-seq) data using a previously published salivary gland epithelial dataset [21] to assess

SGEC subset composition in DIF-SGOs (Supplementary Fig S6). DIF-SGO cell subset composition was found to be heterogeneous. However, no striking difference was found regarding ductal, acinar, myoepithelial, and progenitor cell-associated marker gene expression between SjD- and control-derived DIF-SGOs.

Thus, SjD-derived SGOs can differentiate as assessed by expression of epithelial markers, but they display altered morphology.

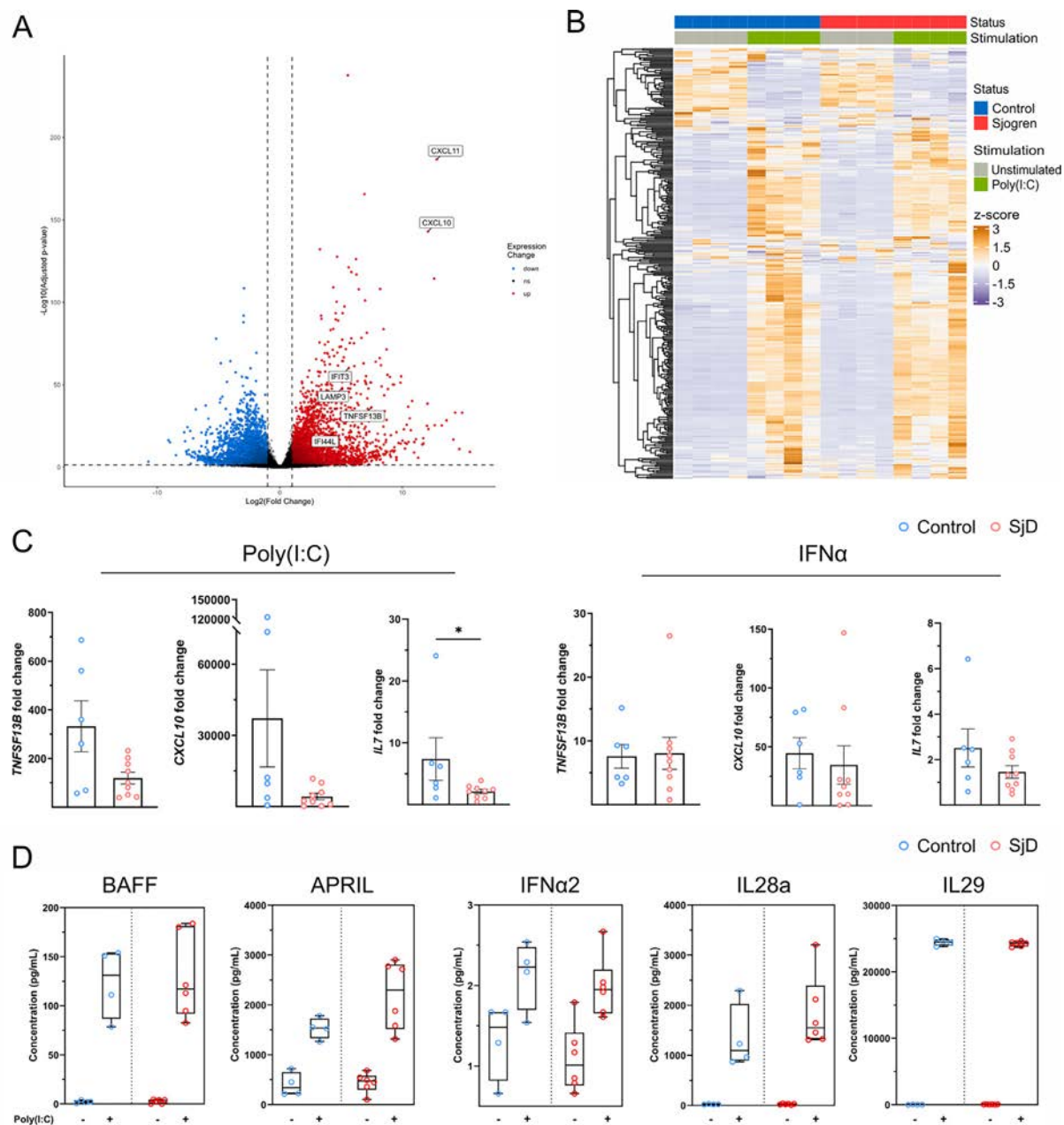


Figure 4. DIF-SGO responsiveness to inflammatory stimuli. Sjd- and control-derived DIF-SGOs from passages 2 to 9 were exposed to 10 μ g/mL poly(I:C), 2400 UI/mL IFN α or dimethyl sulfoxide for 24 hours after differentiation. Bulk RNA sequencing (n = 4 Sjd, n = 4 control) and RT-qPCR analyses were performed. (A) Volcano plot showing differentially expressed genes in Sjd-derived DIF-SGOs exposed to poly(I:C) compared to unstimulated condition. (B) Heatmap illustrating the modulation of IFN-stimulated genes by poly(I:C) in Sjd- and control-derived DIF-SGOs. (C) mRNA expression levels of *TNFSF13B*/*BAFF*, *CXCL10* and *IL-7* after exposure to poly(I:C) or IFN α in Sjd- and control-derived DIF-SGOs. (D) Protein concentrations of BAFF, APRIL, IFN α 2, IL28a and IL29 in the supernatant of control-derived (n = 4), Sjd-derived (n = 6) DIF-SGOs assessed by multiplex following poly(I:C) stimulation. ACTB, actin beta; BAFF, B-cell activating factor; DIF-SGO, differentiated salivary gland organoid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN, interferon; IL, interleukin; RT-qPCR, reverse transcription quantitative polymerase chain reaction; Sjd, Sjögren's disease. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Sjd-derived DIF-SGOs display a persistent interferon signature

To further investigate the differences between DIF-SGOs derived from Sjd patients and controls, we conducted a transcriptome analysis using bulk RNA-seq. We compared DIF-SGOs from 4 Sjd patients and 4 controls that were cultured from EXP-SGOs for 2.3 ± 1 and 1.8 ± 0.8 months respectively (Supplementary Table S3).

We identified 20 differentially expressed genes, of which 8 (40%) were interferon (IFN) stimulated genes (ISGs), including *IFI44L*, *IFIT3* and *LAMP3* (Fig 3A). Notably, we observed an

enrichment in pathways related to inflammation, despite the prolonged culture duration without inflammatory cytokine exposition. The IFN type I and II pathways were the most significantly enriched in Sjd-derived DIF-SGOs (Fig 3B). Most ISGs enriched in Sjd-derived DIF-SGOs (52/91, 57%) were commonly associated with both IFN α and IFN γ pathways (Supplementary Fig S7). The enriched ISGs were heterogeneous among Sjd patients, notably IFN α -induced genes that were highly expressed in Sjd-derived DIF-SGOs (Fig 3C). We confirmed the higher expression of ISGs at the protein level (Fig 3D, E), observing increased expression of IFIT3, MX1 and IFI44L in Sjd-derived DIF-SGOs than in controls

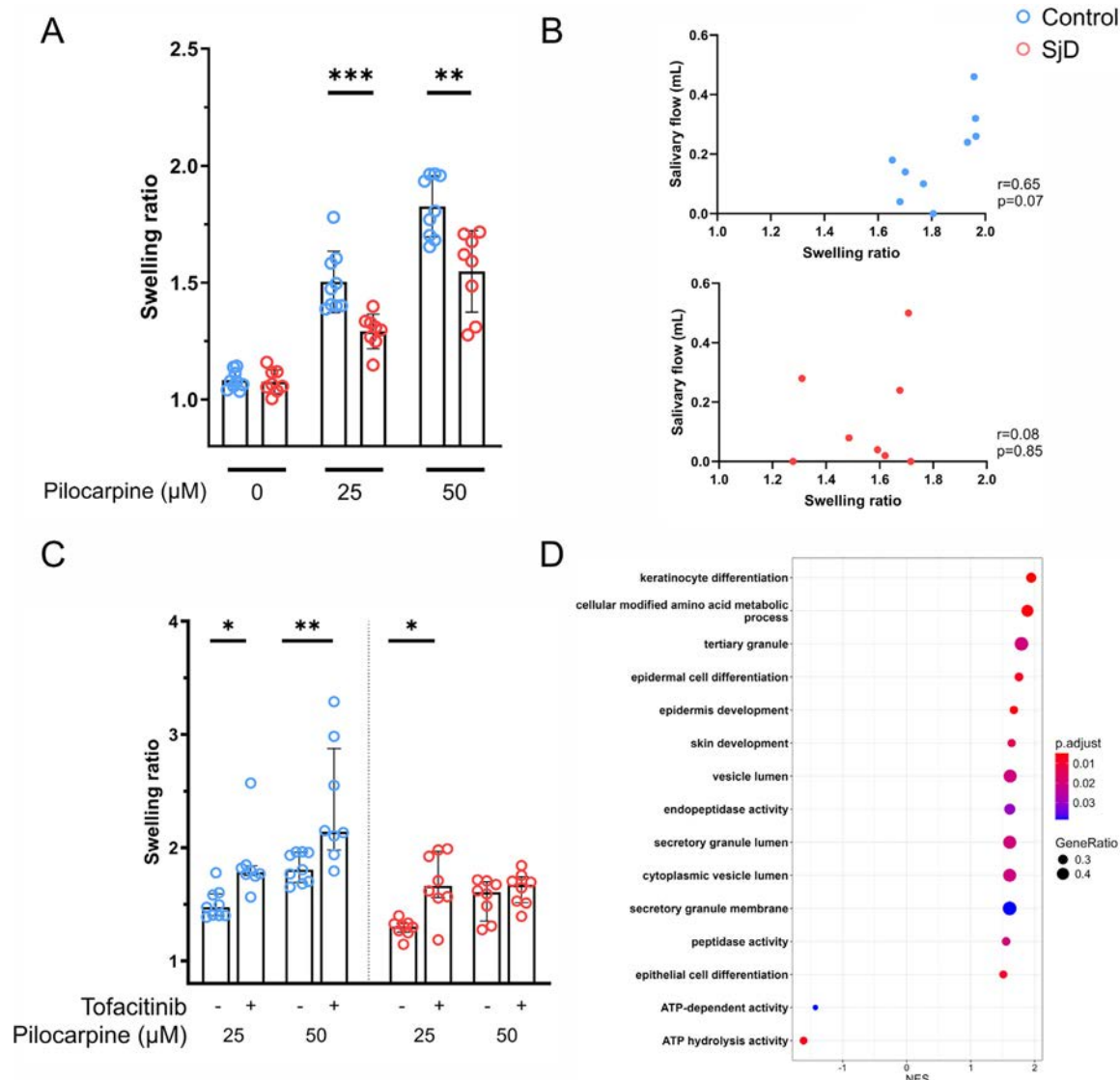


Figure 5. Reduced swelling capacity of SjD-derived DIF-SGOs. DIF-SGOs from passages 2 to 9 were assessed for swelling capacity over 24 hours after exposure to 25 or 50 μM pilocarpine, measured as the ratio of organoid area before and after exposure. Part of the experiments included pretreatment with tofacitinib for 24 hours. (A) Swelling ratio of DIF-SGOs derived from SjD ($n = 8$) and control ($n = 9$) groups after exposure to 25 or 50 μM of pilocarpine. (B) Swelling capacity of DIF-SGOs from SjD ($n = 8$) and controls ($n = 8$) after 24 hours exposure to tofacitinib. Wilcoxon test was used. $P < .05$ was considered significant. (C) Correlations between swelling ratios and patients' unstimulated salivary flow were analysed using the Spearman test. P and r values are indicated on the graph, with $P < .05$ considered statistically significant. (D) Enriched pathways in SjD-derived DIF-SGOs exposed to tofacitinib compared to unexposed. SjD-derived DIF-SGOs ($n = 4$) were initially cultured in differentiation medium, exposed to or left unexposed to tofacitinib (30 nM), then all were treated with pilocarpine 25 μM . Total mRNA expression was analysed by bulk RNA sequencing. DIF-SGO, differentiated salivary gland organoid; SjD, Sjögren's disease. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

(mean culture duration: 2.62 ± 0.67 and 2.73 ± 1.7 months, respectively). Pathways related to transforming growth factor beta ($\text{TGF}\beta$) signalling and epithelial-mesenchymal transition were also enriched, highlighting a profibrotic pattern in SjD.

Thus, SjD-derived DIF-SGOs revealed a persistent IFN imprint despite the absence of inflammation, underscoring a unique and enduring alteration of SGEs in SjD.

DIF-SGOs are responsive to inflammatory stimulation

We then evaluated the functionality of DIF-SGOs by examining their ability to respond to inflammatory stimuli. DIF-SGOs derived from SjD and controls were exposed to poly(I:C), a double-stranded RNA that activates toll-like receptor 3, mimicking viral infection, as well as $\text{IFN}\alpha 2$, which plays a crucial role in SjD and antiviral responses.

After poly(I:C) exposure, 10,302 and 10,841 genes were differentially expressed in SjD and control DIF-SGOs, respectively, compared to the unstimulated condition (Fig 4A). Among them, ISGs such as *CXCL11*, *TNFAIP3* and *IFI44* were significantly upregulated (Fig 4A). The pathways enriched in response to poly(I:C) were comparable between the 2 groups, predominantly driven by the IFN pathways (Supplementary Fig S8), with similar upregulation of type I and II ISGs in SjD- and control-derived SGOs (Fig 4B).

We then confirmed the induction of *TNFSF13/BAFF*, *CXCL10* and *IL7*, ISGs that have been shown to play a key role in SjD pathogenesis, by poly(I:C) and $\text{IFN}\alpha 2$ (Fig 4C). There was a trend towards a reduced response to poly(I:C) in SjD-derived DIF-SGOs, which only reached significance for IL-7 ($P < .05$). Furthermore, we confirmed the inflammatory response of DIF-SGOs at the protein level (Fig 4D), as evidenced by the secretion

of ISGs (BAFF, APRIL), IFN α 2 and IFN λ (IL28a, IL29) in response to poly(I:C). The induction of these inflammatory mediators was similar in the 2 groups.

In summary, DIF-SGOs from SjD and controls can respond to inflammation.

SjD-derived DIF-SGOs exhibit reduced swelling capacity

To further assess the function of DIF-SGOs, we evaluated their swelling capacity after exposure to pilocarpine, a cholinergic agonist approved for the treatment of SjD-related xerostomia [22]. DIF-SGOs exhibited swelling within hours of pilocarpine exposure, reaching peak levels at 24 hours, with a clear dose-dependent effect (Fig 5A). However, SjD-derived DIF-SGOs showed a significantly reduced swelling capacity at both doses (25 μ M, $P < .001$ and 50 μ M, $P < .01$) compared to controls (Fig 5A; Videos S2, S3). Interestingly, comparing DIF-SGOs and clinical data in patients, we observed a trend ($P = .07$) towards a correlation between the swelling ratio of DIF-SGOs and the unstimulated salivary flow in controls. This was not the case in SjD patients (Fig 5B). No variation in swelling assay based on the focus score in DIF-SGOs derived from SjD patients was observed, suggesting that the alteration observed could be partly independent of the immune infiltrate (Supplementary Fig S9). This finding might suggest an impaired response to cholinergic stimulation in SjD-derived DIF-SGOs. Thus, the swelling assay seems to be a good proxy for saliva secretion capacity, and DIF-SGOs could represent a promising tool to investigate the modulation of the cholinergic pathway in SjD patients.

As a proof of concept, we assessed the potential of the swelling assay for screening therapeutics. Given the IFN imprint observed in SjD-derived DIF-SGOs, we investigated tofacitinib, a Janus kinase inhibitor (JAKi) with a broad spectrum of inhibition of the JAK/STAT pathway that has activity against IFN signalling. Interestingly, tofacitinib exposure led to an increased swelling capacity in DIF-SGOs derived from controls and SjD patients (Fig 5C). While the effect was observed with both doses in controls, the improvement was only observed with 25 μ M pilocarpine in SjD, suggesting saturation of the effect. These results might support the involvement of the JAK/STAT pathway in the cholinergic response and highlight the potency of JAKis to partially restore salivary flow. To elucidate the mode of action of tofacitinib in the swelling assay, we conducted RNA-seq on DIF-SGOs exposed to tofacitinib for 24 hours. We did not observe any modulation of IFN pathways (data not shown). Instead, we observed alterations in pathways associated with differentiation and vesicular trafficking (Fig 5D). This suggests that tofacitinib might exert its effects through mechanisms beyond the immunological pathways typically associated with its activity.

In summary, these findings suggest that SjD-derived DIF-SGOs exhibit an altered response to cholinergic agonists and that evaluating this response could be a reliable method for assessing drug efficacy on salivary gland secretory function.

DISCUSSION

In this study, we established a robust protocol for long-term culture of SGOs derived from LSGBs of SjD patients and sicca controls, allowing organotypic differentiation and preserving functional capacities. SjD-derived SGOs exhibited several key features recapitulating the disease, including a sustained IFN imprint and reduced swelling capacity.

The establishment of human SGOs derived from various glandular tissues, such as parotid or submandibular glands, has

previously been reported [16–18]. A notable issue has been the reduced proliferation capacity of aSCs in long-term cultures described in SjD, leading to reduced organoid formation from parotid and lachrymal glands [18,19]. The main hypothesis was an increased senescence of aSCs related to chronic inflammation [18]. More recently, functional SjD-derived SGOs exhibiting swelling ability in response to forskolin stimulation have been reported, but this study did not extend to long-term culture [23].

Here, we report that SGECS from LSGBs of SjD have persistent proliferation capacity. The originality of our medium is linked to the use of NRG1 instead of Wnt3a CM, commonly used in organoid culture [17,18,24,25]. NRG1 has been shown to induce various endogenous paracrine Wnt ligands (Wnt3a, Wnt5a, Wnt10b) in SGOs, thereby allowing the substitution of Wnt3a CM [16]. Thus, endogenous Wnt ligands could be more favourable for establishing SjD-derived SGOs. Additionally, the dissociation protocol we used is highly protective of the cells, leading to increased viability and a high success rate of SGO formation, and could be more suitable to preserve aSCs from SjD.

We characterised and compared SGOs derived from SjD patients and controls. We observed similar expression of SGECS markers in SjD and controls but reduced branching. Importantly, branching has been described to be associated with function [26]. This might suggest establishment of a glandular structure rather than altered differentiation in SjD. One hypothesis could be a profibrotic profile of SGECS from SjD patients. It has been shown that SGECS from SjD patients are more likely to undergo epithelial-mesenchymal transition [27], a pathway that was enriched in SjD-derived DIF-SGOs. The profibrotic TGF β pathway was also enriched in SjD-derived DIF-SGOs, supporting this hypothesis. The persistent IFN imprint observed could also be involved: various ISGs could interfere, directly or indirectly, with several pathways involved in the differentiation process (eg, Notch, FGF). This point needs to be further investigated.

The IFN signature is characteristic of SjD and has been extensively described in immune cells [28,29] and SGECS [30–32]. The long-term culture enabled us to show that DIF-SGOs display a persistent IFN imprint despite the absence of inflammatory stimuli. We can hypothesise that epigenetic modifications might sustain this IFN imprint. Modifications of DNA methylation that regulate the access of transcription factors to ISG promoters, such as IFI44L, have been described in SjD patients [33,34]. Posttranscriptional modifications may also be involved. N6-methyladenosine (m⁶A) mRNA modification has recently been demonstrated to regulate SGECS function and to act as a negative feedback mechanism on IFN signalling in SjD [35]. Finally, while the impact of the IFN signature on morphogenesis and function remains speculative at this stage, our findings highlight that SGECS are directly affected in SjD, regardless of immune infiltration.

The ability to mimic the function of the tissue is one of the key features of organoids. We report for the first time a reduced swelling ability of SjD-derived DIF-SGOs upon exposure to pilocarpine—a clinically relevant cholinergic agonist approved in the treatment of SjD. Some hypotheses can be raised. First, the 3D organisation of acini is required for their proper function [36], thus the reduced branching observed in SjD-derived DIF-SGOs could lead to altered function. Second, the IFN imprint may interfere with the downstream M3 muscarinic acetylcholine receptor (M3R) pathway, contributing to the reduced swelling ability. The specific role of IFN on fluid secretion has not been investigated to date; however, some genes in the M3R pathway are modulated by IFN. In addition, we did not find a correlation between the unstimulated salivary flow and the

swelling ratio in SjD-derived DIF-SGOs. It would be interesting to determine whether this lack of response to cholinergic stimulation in SjD-derived DIF-SGOs translates in patients to a lack of efficacy of pilocarpine treatment. Unfortunately, we do not have the clinical data needed to answer this question, which would merit evaluation in a future study.

Altered salivary flow is one of the main symptoms in SjD. The altered swelling capacity observed in SjD-derived DIF-SGOs paves the way for drug testing specifically designed for the treatment of xerostomia, particularly for emerging therapeutics or repurposing drugs [37,38]. As a proof of concept, we explored the effect of tofacitinib, a JAKi with a global inhibitory effect on all JAKs that notably target the IFN pathway [39]. Interestingly, JAKis are under investigation in SjD [40–42]. Surprisingly, tofacitinib enhanced the swelling ability of both SjD and control SGOs. Its effect could be mediated through the regulation of JAK-dependent cytokine produced by SGEs, such as IFN or IL-7 [43], or by the regulation of mediators of the cholinergic pathways, such as protein kinase C. It could also represent a nonimmunological impact of the JAK/STAT pathway, potentially involving vesicle trafficking, cell junction or cytoskeleton, which are crucial for proper exocrine function [36]. Our RNA-seq results comparing tofacitinib exposed versus nonexposed SjD-derived DIF-SGOs support this point. We also observed different responses between SjD and control SGOs. This result is intriguing, and at this point, only hypotheses can be made, including a saturation of the cholinergic receptor M3R, an alteration in the nonimmunological effect of JAK/STAT or a specific toxicity of tofacitinib in SjD-derived SGOs. To understand this finding, we will need to specifically investigate the cholinergic pathway in SGOs and the potential differences between SjD and control SGOs.

Therefore, this study paves the way for future research. One of the key aspects to be addressed is the ability to include immune cells in SGOs to better mimic SjD sialadenitis. These developments are currently underway.

In conclusion, we established a reproducible protocol to produce SjD-derived SGOs from LSGBs for long-term culture. These SGOs are functional and recapitulate the SjD phenotype with reduced swelling capacity and persistent IFN imprint. This approach not only enhances our understanding of SjD pathophysiology but also sets a foundation for employing organoids in modelling systemic autoimmune diseases. It also holds promise as a valuable tool for drug screening.

Competing interests

GN received honoraria from Biogen, Pfizer, Novartis, Lilly, and Amgen. XM received honoraria from Bristol Myers Squibb, GSK, Novartis, Otsuka, and Pfizer. The remaining authors declare that they have no known competing financial interests.

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Contributors

GN was the guarantor of the study. LM and NG performed the experiments and the statistical analysis. NG performed multiplex analysis. QP and TL performed the Hematoxylin and Eosin and Immunohistochemistry analysis (Supplementary Table S2). SESS performed enrichment and deconvolution analysis. JP performed western blot analysis. GN, XM and RB were involved in the conceptualization of the project. LM, GN, NG, and XM wrote the manuscript. All authors reviewed and approved the manuscript.

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Not applicable.

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The study was approved by the local ethics committee, and written informed consent was obtained from all participants (Comité d'éthique de la recherche, CER Paris-Saclay, PP14-033).

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Data availability statement

Data are available on reasonable request.

Supplementary materials

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Myositis

The role of multicriteria decision analysis in the development of candidate classification criteria for antisynthetase syndrome: analysis from the CLASS project

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ABSTRACT

Objectives: To develop and evaluate the performance of multicriteria decision analysis (MCDA)-driven candidate classification criteria for antisynthetase syndrome (ASSD).

Methods: A list of variables associated with ASSD was developed using a systematic literature review and then refined into an ASSD key domains and variables list by myositis and interstitial lung disease (ILD) experts. This list was used to create preferences surveys in which experts were presented with pairwise comparisons of clinical vignettes and asked to select the case that was more likely to represent ASSD. Experts' answers were analysed using the Potentially All Pairwise Rankings of all possible Alternatives method to determine the weights of the key variables to formulate the MCDA-based classification criteria. Clinical vignettes scored by the experts as consensus cases or controls and real-world data collected in participating centres were used to test the performance of candidate classification criteria using receiver operating characteristic curves and diagnostic accuracy metrics.

Results: Positivity for antisynthetase antibodies had the highest weight for ASSD classification. The highest-ranked clinical manifestation was ILD, followed by myositis, mechanic's hands, joint involvement, inflammatory rashes, Raynaud phenomenon, fever, and pulmonary hypertension. The candidate classification criteria achieved high areas under the curve when applied to the consensus cases and controls and real-world patient data. Sensitivities, specificities, and positive and negative predictive values were >80%.

Conclusions: The MCDA-driven candidate classification criteria were consistent with published ASSD literature and yielded high accuracy and validity.

WHAT IS ALREADY KNOWN ON THIS TOPIC

- Multicriteria decision analysis (MCDA) has been successfully used in developing classification criteria for rheumatoid arthritis, systemic sclerosis, and systemic lupus erythematosus.

WHAT THIS STUDY ADDS

- Positivity for antisynthetase antibodies had the highest weight for antisynthetase syndrome (ASSD) classification.
- Interstitial lung disease, myositis, mechanic's hands, joint involvement, and inflammatory rashes were highly weighted by the experts.
- MCDA was successfully used to develop candidate classification criteria for ASSD with high accuracy and validity.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- The MCDA-driven candidate classification criteria will be slightly adjusted to increase their face validity and clinical applicability without compromising the performance characteristics.

INTRODUCTION

Antisynthetase syndrome (ASSD) is characterised by the presence of autoantibodies that target the aminoacyl tRNA synthetase (ARS). To date, 10 distinct anti-ARS autoantibodies have been identified (Supplementary Table S1) [1–5]. The most common is anti-histidyl-tRNA synthetase (anti-Jo1). Anti-ARS autoantibodies are identified in >20% of idiopathic inflammatory myopathy patients [6,7]. However, considering that 20 ARS exist, it is speculated that other anti-ARS autoantibodies directed against the remaining 10 ARS could still be identified [8,9]. Despite being considered myositis-specific autoantibodies, anti-ARS autoantibodies are found in individuals without myositis and in patients with myositis overlapping with other systemic autoimmune rheumatic diseases (SARDs) [10]. The

prevalence of anti-ARS autoantibodies in nonmyositis settings is not defined.

Clinically, ASSD is generally characterised by the occurrence of the classic clinical 'triad' of arthritis, myositis, and interstitial lung disease (ILD) and by accompanying features such as mechanic's hands (MH), Raynaud phenomenon (RP), and fever. Triad features may present simultaneously or, more typically, months to years apart [11–14]. ILD is the most common feature of ASSD (70%–90% of cases) [15] and a major cause of morbidity and mortality [16–19].

Recent pathophysiological and therapeutic advances [20–23] could enable robust ASSD clinical trials, but widely accepted classification criteria are still lacking [24–26]. Despite the recognition of ASSD as a distinct clinical entity within the myositis-spectrum diseases [27] and previous initiatives to define it [28–30], no unified effort to develop data- and expert consensus-driven classification criteria for ASSD has been performed. Previously proposed criteria for ASSD lacked validation and broad consensus, and none have been widely used or recognised in the field [10,11,28–30].

The classification criteria for ASSD (CLASS) project is an international study aimed at developing classification criteria for ASSD using both data-driven and expert consensus-driven methods. The expert consensus-driven candidate criteria were based on multicriteria decision analysis (MCDA), a technique used in the development of classification criteria for other SARDs [31,32]. MCDA is based on the interpretation of the human decision-making process as an intuitive weighting of trade-offs between alternatives, which can be affected by various factors, including personal experiences [33]. These factors produce significant variability even among experts. MCDA potentially includes several rounds of surveys and discussion, with the ultimate goal of defining the weights of the variables in the decision-making process, representing their relative importance. The results summarise the experts' judgements and guide subsequent discussions aiming to simplify and refine the mathematical models representing their decision-making process. This

way, a significant element of the decision variability can be eliminated, and the truly important variables can be identified.

Our main objective was to develop and analyse the performance of MCDA-based candidate classification criteria for ASSD.

METHODS

The CLASS project

Nineteen international experts on myositis and ILD were selected as CLASS project Steering Committee (SC) members. The SC comprised 10 rheumatologists, 4 pulmonologists, 2 dermatologists, 2 neurologists, and 1 internist. The Executive Committee (EC) comprised co-Principal Investigators (PIs) from the University of Pittsburgh (RA) and University of Pavia (LC), lead CLASS project Rheumatology fellows from Pavia (GZ) and Stanford University (SFK), and a lead statistician (DR) from the Italian Society of Rheumatology. The CLASS project Working Group included 14 myositis investigators (GZ, ED, IBV, SFK, AY, AL, DRG, DL, FB, GS, SSB, YY, LC, and RA) from 12 centres across 7 countries from 4 continents. Patients and the public were not directly involved in the writing of this manuscript but were involved in other steps of the CLASS project.

Ranking survey and the development of the ASSD key domains and variables list

A systematic literature review (SLR) was conducted to identify studies and existing classification criteria for ASSD to generate a broad set of variables commonly used to define ASSD [34], yielding the SLR variable list. This list was then reviewed and simplified by the SC and participating centres' representatives, resulting in the original variable list (Fig 1).

The EC drafted 20 fictional clinical vignettes using the original variable list to represent the full spectrum of cases from clearly ASSD to definitely not ASSD. Most vignettes did not include anti-ARS information to avoid bias towards the syndrome's serological features and to emphasise its clinical characteristics. Many of the vignettes were deliberately ambiguous to stimulate debate among the experts and facilitate the identification of key diagnostic variables. The SC experts were asked to rank the 20 fictional clinical vignettes from the least to most likely to be ASSD. Each expert completed the survey individually, and the results were discussed collectively in an online meeting with the objectives of (i) highlighting differences in experts' opinions and (ii) reaching a consensus on which variables should be considered for the ASSD key variable list. At the end of the meeting, the experts reranked the vignettes, and a final consensus ranking was developed from the least to the most likely to be ASSD. The survey results were discussed for variable item reduction, leading to the ASSD key variable list. Key variables referring to similar clinical manifestations were grouped into domains. These domains and variables were then used as the base for the preferences surveys.

Multicriteria decision analysis

The MCDA software 1000minds, which implements the Potentially All Pairwise Rankings of all possible Alternatives (PAPRIKA) method, was utilised to create a series of preferences surveys based on the ASSD key domain and variable list.

Preferences survey

The 1000minds preferences survey is a type of conjoint analysis survey [33] or discrete choice experiment [35]. In the surveys, the experts were presented with series of pairwise comparisons of hypothetical cases with only 2 different variables from the same 2 domains. Assuming all other aspects were the same, experts were asked to select the case with a higher likelihood of ASSD (Supplementary Fig S1). Alternatively, they could mark the pairs of combinations as equally indicative of ASSD or skip the comparison. Based on the experts' answers, the PAPRIKA method identifies and eliminates all other combinations that can be automatically pairwise ranked by applying the logical property of transitivity, thereby significantly reducing the number of pairwise comparisons required.

After the ranking of the combinations of variables, the relative importance of the variables was codified by the PAPRIKA method as weights. These weights were used to develop a scoring system such that evaluating all ASSD key variables yields a score in the range of 0 to 100, with a high score reflecting a higher likelihood of ASSD classification. These results were used to formulate the candidate classification criteria.

Consensus case and control clinical vignettes for internal validation

Two hundred additional clinical vignettes were created using the ASSD key domains and variables list to represent the entire spectrum of cases from clearly ASSD to definitely not ASSD. The SC reviewed all the clinical vignettes and categorised them as unlikely, possible, probable, or definite ASSD. For internal validation, unlikely and possible ASSD were grouped as 'controls' and probable or definite ASSD as 'cases.' The consensus threshold was defined as two-thirds or more interrater agreement. Clinical vignettes that did not reach the consensus threshold for 'case' or 'control' were excluded. This exercise, called 'categorisation survey,' aimed at creating a *pseudo gold standard* for the internal validation of the candidate classification criteria.

Data collection, data management, and cohort selection

The CLASS project participating centres reported data from patients with ASSD (cases) or ASSD mimickers (controls), including all the key domains and variables for each patient, on REDCap, a web-based platform hosted at the University Hospital of Ferrara. Data was collected from August 2020 to April 2021.

All imputed data underwent quality control, and the diagnoses of both cases and controls were verified by the CLASS project Working Group. Each record was assessed by at least 2 reviewers, including at least 1 of the 2 PIs. Queries were sent to the participating centres, and the centres were allowed to revise or enter new data. Equivocal records were reviewed by the 2 PIs, and the final decision to include the patients as a case or control was based on their consensus. Inconsistent patient records were excluded from the analysis.

A derivation cohort and a validation cohort comprised of revised cases and controls were randomly selected using the sample function provided in the base R package.

Statistical analysis

The concordance of the 20 fictional clinical vignettes ranking was calculated using Kendall coefficient of concordance

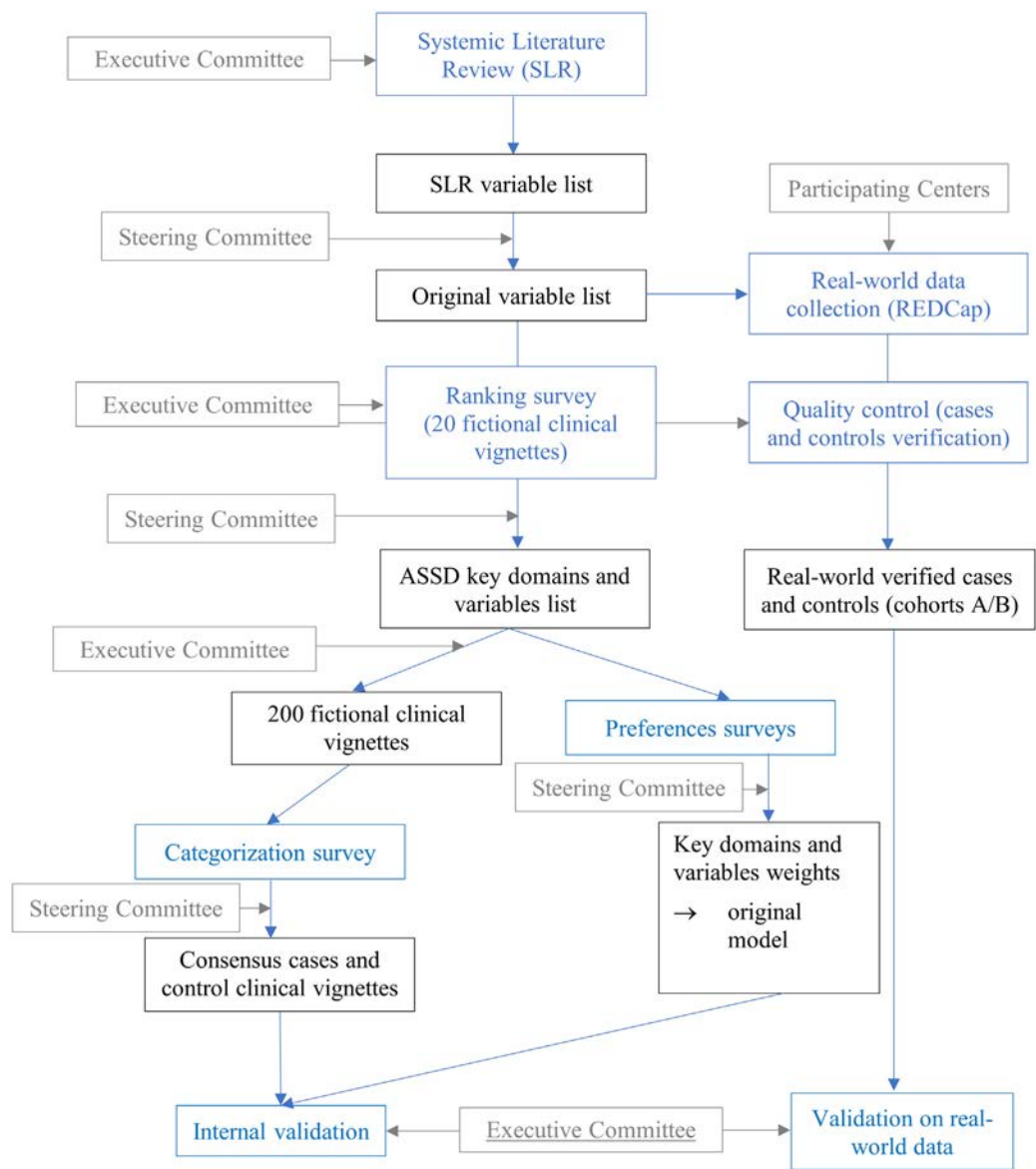


Figure 1. Stepwise description of the study procedures. The roles of the Executive Committee, the Steering Committee, and the Participating Centres are coloured grey, and the main methodologies used are coloured blue. ASD, antisynthetase syndrome; SLR, systematic literature review.

(Kendall W) and the interrater agreement using Fleiss κ , whereas the concordance in categorisation on the 200 clinical vignettes was calculated using Fleiss κ .

The internal validity of the ASD candidate classification criteria was assessed by plotting receiver operating characteristic (ROC) curves and calculating their areas under the curve (AUCs) using the *pseudo gold standard* (consensus cases and controls) as the internal validation cohort.

The validity of the ASD candidate classification criteria was also assessed by plotting ROC curves and calculating their AUCs using the derivation and validation cohorts (real-world verified cases and controls). Additionally, the optimal cutoff for each model was defined as the one simultaneously having >80% sensitivity and specificity and the highest Youden index in the derivation cohort. Each model's diagnostic accuracy metrics (sensitivity, specificity, negative predictive value [NPV], and positive predictive value [PPV]) for the derivation and validation cohorts were calculated and reported.

RESULTS

Ranking survey and the development of the ASD key domains and variables list

All 19 SC experts ranked the 20 clinical vignettes before and after the consensus meeting. Kendall W of the first round of the ranking survey was 0.387 (Fig 2A). The interrater agreement was $\kappa = 0.12$ (95% CI, 0.06-0.17), indicating slight agreement. In the reranking exercise after SC discussion of the original ranking, Kendall W was 0.808 (Fig 2B), and the interrater agreement improved to $\kappa = 0.4$ (95% CI, 0.26-0.52), indicating fair agreement.

The ranking survey prompted several rounds of discussion within the SC, leading to item reduction and, ultimately, to the development of the ASD key domains and variables list (Table 1). A final consensus on 12 domains and 33 variables was reached. The ASD key domains were joint involvement, muscle involvement, ILD, MH/hiker's feet, RP, inflammatory rashes,



Figure 2. Scatter plot of the ranking survey conducted before (A) and after (B) the consensus meeting. The black dashed lines represent the ranking means for each clinical vignette. Each coloured form represents one Steering Committee expert.

pulmonary hypertension, unexplained fever, anti-Jo1 autoantibodies, non–anti-Jo1 anti-ARS, antinuclear antibodies by indirect immunofluorescence assay (IIFA), and anti-Ro52/Sjögren syndrome-related antigen A (SSA) autoantibodies. Each domain included different levels defined by the 33 key variables (eg, ‘not present,’ ‘inflammatory rashes not specific for dermatomyositis [DM],’ and ‘DM-specific rashes’ for the inflammatory rashes domain). A glossary was developed to ensure clarity on the definitions of domains and variables (Supplementary Table S2).

Multicriteria decision analysis

Preferences survey

The overall positivity for anti-ARS autoantibodies had the highest weight for ASSD classification (Fig 3). The weight was numerically higher for non–anti-Jo1 anti-ARS tested by immunoprecipitation (IP) than for anti-Jo1 tested by any method or for non–anti-Jo1 anti-ARS tested by methods other than IP. Identifying a cytoplasmic pattern in antinuclear antibody testing

by IIFA was also considered important by the experts for defining ASSD.

Given the suboptimal performance of methods other than IP in detecting anti-ARS autoantibodies [36], especially for non–anti-Jo1 anti-ARS, the experts had considered the positivity for anti-Jo1 and non–anti-Jo1 anti-ARS as distinct domains in the ASSD candidate classification criteria original model (Table 1A, B). However, because anti-ARS autoantibodies are generally considered mutually exclusive, a modified model of the ASSD classification criteria was also created by merging the anti-Jo1 and non–anti-Jo1 anti-ARS domains (Table 1A, C). Therefore, the modified model had 11 domains despite having the same 33 variables. The scores of the modified model were automatically recalculated by redistributing the total score, keeping the relative weight between domains the same. The weights and rankings for each key variable and domain did not substantially differ between the original and modified models (Table 2 and Supplementary Tables S3 and S4).

In both models, the highest-ranked clinical manifestation was ILD with a predominant nonspecific interstitial pneumonia (NSIP) and/or organising pneumonia (OP) pattern, followed by

Table 1
List of domains of the candidate classification criteria and their levels (variables)

Domains	Levels
A) Domains included in the original model and the modified model	
Joint involvement	Not present
	Inflammatory arthralgia (without joint swelling)
	Oligoarthritis involving less than 4 joints of the hands and/or feet (Wrist, Ankle, MCPs, PIPs, MTPs)
	Polyarthritis involving 4 or more joints of the hands and/or feet (Wrist, Ankle, MCPs, PIPs, MTPs)
Muscle involvement	Not present
	Myalgia alone (without subclinical, clinical, or biopsy evidence of myositis)
	Sub-clinical evidence of myositis (elevated muscle enzyme, EMG, or Muscle MRI consistent with myopathy) without muscle weakness or biopsy evidence of myositis.
	Clinical myositis without muscle biopsy evidence of myositis (not done or not available or biopsy findings not consistent with myositis).
	Muscle biopsy evidence of myositis with clinical and/or subclinical myositis
Interstitial lung disease	Not present
	ILD with pattern unknown or unclassifiable ILD/Other ILD patterns
	ILD with predominant UIP (usual interstitial pneumonia) pattern on chest HRCT or pathology on lung biopsy
	ILD with predominant NSIP (non-specific interstitial pneumonia) and/or OP (organizing pneumonia) pattern on chest HRCT or pathology on lung biopsy
Mechanic's hands/Hiker's feet	Not present
	Present
Raynaud's phenomenon	Not present
	Present
Inflammatory rashes	Not present
	Inflammatory rashes not specific for DM
	DM specific rashes (Gottron's papules or sign or heliotrope, V sign, Shawl, holster, sleeve sign)
Pulmonary Hypertension	Not present/not evaluated
	Present (based on right heart catheterization (preferred) or Echocardiogram)
Unexplained fever	Not present
	Present
Antinuclear antibodies (ANA, by indirect immunofluorescence)	Negative
	Positive in any pattern except cytoplasmic pattern
	Positive with cytoplasmic pattern with or without other patterns
Anti-Ro52 or Anti-SSA antibody	Negative
	Unknown/pending
	Positive
B) Domains included only in the Original Model	
Anti-Jo1 antibodies	Negative by any method
	Not tested/unknown
	Positive by any method
Other (non-anti-Jo1) anti-ARS	Negative by Immunoprecipitation (IP, the reference standard testing)
	Negative by the commercial panel (i.e. any testing method other than IP)
	Not tested/unknown/pending
	Positive by any testing methods other than IP
	Positive by IP

(continued)

the presence of myositis assessed through muscle biopsy and MH. However, ILD with predominant usual interstitial pneumonia (UIP) pattern and clinical or even subclinical evidence of myositis were also highly weighted. The occurrence of symmetrical polyarthritis or inflammatory rashes were moderately valued, whereas RP, unexplained fever, and pulmonary hypertension obtained lower scores.

Consensus case and control clinical vignettes for internal validation

From the 200 clinical vignettes, 67 (33.5%) were classified as definite ASSD, 26 (13.0%) as probable ASSD, 33 (16.5%) as possible ASSD, and 28 (14.0%) as unlikely ASSD by the SC members, with 46 vignettes not reaching the consensus for a unique

Table 1 (Continued)

C) Domains included only in the Modified Model	
Anti-ARS autoantibodies	Anti-ARS autoantibodies negative by IP
	Anti-Jo1 and non-anti-Jo1 negative by commercial assays
	Anti-Jo1 negative (any method) and non-anti-Jo1 unknown
	Anti-Jo1 and non-anti-Jo1 unknown/not tested
	Non-anti-Jo1 positive by commercial assays
	Anti-Jo1 positive (by any method) or non-anti-Jo1 positive by IP
Abbreviations: ANA – antinuclear antibodies; anti-ARS – anti-synthetase antibodies; DM – dermatomyositis; EMG – electromyogram; HRCT – high-resolution computed tomography; ILD – interstitial lung disease; IP – immunoprecipitation; MCP – metacarpophalangeal joints; MRI – magnetic resonance imaging; MTP – metatarsophalangeal joints; PIPs – proximal interphalangeal joints. Clinical domains are coloured blue and serological domains are coloured green.	

definition (Supplementary Fig S2A). Regarding the classification as ‘cases’ or ‘controls,’ which was the *pseudo gold standard* for internal validation, 104 (52.0%) clinical vignettes were cases, and 73 (36.5%) were controls (Supplementary Fig S2B) with a $\kappa = 0.46$ (95% CI, 0.34–0.58). For 23 clinical vignettes (11.5%), consensus among experts was not achieved (interrater agreement <65%), and they were removed from the analysis. The interrater agreement improved to $\kappa = 0.53$ (95% CI, 0.41–0.66) after the exclusion of the nonconsensual vignettes.

Internal validation of the candidate classification criteria

The original model had an AUC of 0.935, and the modified model had an AUC of 0.931 compared to the *pseudo gold standard*. The ROC curves of the 2 models mostly overlapped (Supplementary Fig S3).

Data collection, data management, and cohort selection

The local investigators submitted 2035 cases and 2140 controls. The diagnosis was verified in 1952 and 2097 records, respectively. The derivation cohort included 948 cases and 1077 controls, and the validation cohort included 497 cases and 514 controls.

Validation of the candidate classification criteria on real-world data

The AUC of the original model in the derivation cohort was 0.922 (Fig 4). The optimal cutoff of the original model was 29.1. This cutoff corresponded to a sensitivity of 88.4%, specificity of 81.8%, PPV of 81.1%, NPV of 88.9%, and Youden index of 0.702 (Fig 5).

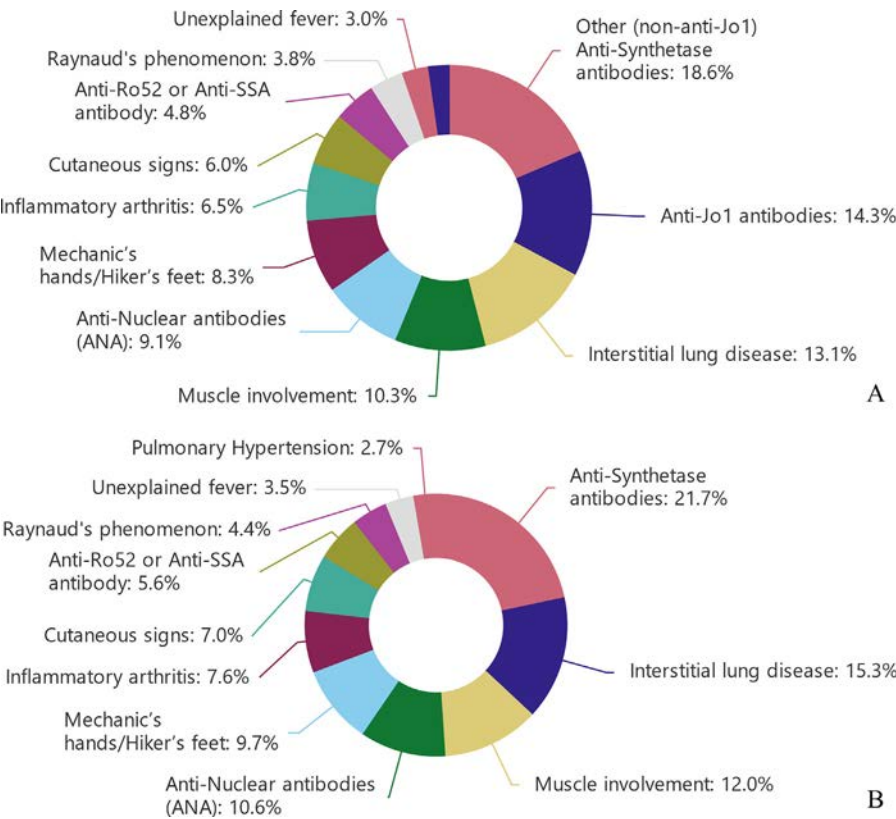


Figure 3. Weights attributed to the different domains in the original model (A) and modified model (B) of the multicriteria decision analysis-based candidate classification criteria. ANA, anti-nuclear antibody; SSA, Sjögren syndrome-related antigen A.

Table 2
Original and modified models' weights (maximum score 100) and ranks

Domains and variables	Original Model		Modified Model	
	Weight	Rank	Weight	Rank
Clinical domains				
Triad manifestations				
Interstitial lung disease		3 rd		2 nd
ILD with predominant NSIP and/or OP	13.1		15.3	
ILD with predominant UIP	7.0		8.2	
Unclassifiable/not specific ILD	4.3		5.0	
Muscle involvement		4 th		3 rd
Myositis assessed through biopsy	10.3		12.0	
Clinical myositis	7.9		9.2	
Sub-clinical evidence of myositis	6.3		7.3	
Myalgia alone	2.4		2.8	
Joint involvement		7 th		6 th
Polyarthritis	6.5		7.6	
Inflammatory arthralgia	4.4		5.1	
Oligoarthritis	3.9		4.7	
Non-triad manifestations				
Mechanic's hands/Hiker's feet	8.3	6 th	9.7	5 th
Inflammatory rashes		8 th		7 th
DM specific rashes	6.0		7.0	
Inflammatory rashes not specific for DM	2.9		3.4	
Raynaud's phenomenon	3.8	10 th	4.4	9 th
Unexplained fever	3.0	11 th	3.5	10 th
Pulmonary Hypertension	2.3	12 th	2.7	11 th
Serological domains				
Antinuclear antibodies		5 th		4 th
Positive with cytoplasmic pattern	9.1		10.6	
Positive (NO cytoplasmic pattern)	2.1		2.4	
Anti-ARS autoantibodies				
Anti-ARS autoantibodies (anti-Jo1 and non-anti-Jo1)				1 st
Anti-Jo1 positive (by any method) or non-anti-Jo1 positive by IP			21.7	
Non-anti-Jo1 positive by commercial assays			15.7	
Anti-Jo1 and non-anti-Jo1 unknown/not tested			7.0	
Anti-Jo1 negative (any method) and non-anti-Jo1 unknown			4.8	
Anti-Jo1 and non-anti-Jo1 negative by commercial assays			2.3	
Anti-ARS autoantibodies negative by IP			0.0	
Anti-Jo1 antibodies		2 nd		
Positive by any method	14.3			
Not tested/unknown	1.9			
Negative by any method	0.0			
Other (non-anti-Jo1) anti-ARS		1 st		
Positive by IP	18.6			
Positive by any testing (NO IP)	13.5			
Not tested/unknown/pending	4.1			
Negative by a commercial panel	2.0			
Negative by IP	0.0			
Anti-Ro52 or Anti-SSA antibody		9 th		8 th
Positive by any assay	4.8		5.6	
Unknown/pending/indeterminate by any assay	1.3		1.5	
Negative by any assay	0.0		0.0	

Abbreviations: anti-ARS – anti-synthetase antibodies; DM – dermatomyositis; ILD – interstitial lung disease; IP – immunoprecipitation; MDW – mean decimal weight (maximum score 1.000); NA – not applicable; NSIP – non-specific interstitial pneumonia; OP – organising pneumonia; UIP – usual interstitial pneumonia; % – percentage weights (maximum score 100%). Clinical domains are coloured **blue** and serological domains are coloured **green**.

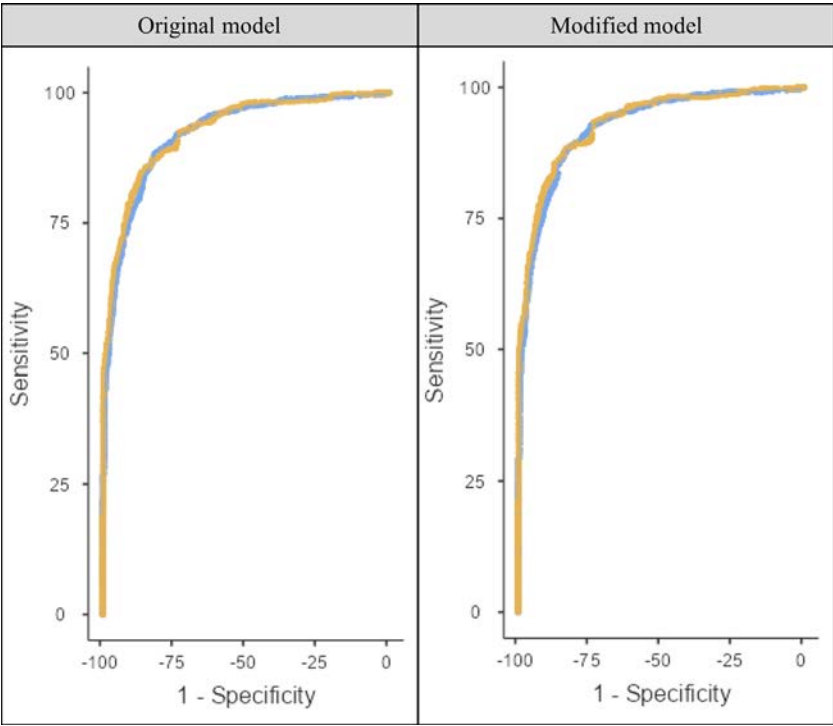


Figure 4. Receiver operating characteristic curves for the multicriteria decision analysis-driven candidate classification criteria's original model (left panel) and modified model (right panel) in the derivation cohort (blue lines) and validation cohort (yellow lines).

In the validation cohort, the 29.1 cutoff of the original model had a sensitivity of 87.7%, specificity of 80.4%, PPV of 81.2%, NPV of 87.1%, and Youden index of 0.681. The AUC of the original model in the validation cohort was 0.926.

The AUC of the modified model in the derivation cohort was 0.926. The optimal cutoff of the modified model was 35.5. This cutoff corresponded to a sensitivity of 87.6%, specificity of 84.0%, PPV of 82.9%, NPV of 88.5%, and Youden index of 0.716 (Fig 6).

In the validation cohort, the 35.5 cutoff of the modified model had a sensitivity of 86.1%, specificity of 86.0%, PPV of 85.6%, NPV of 86.5%, and Youden index of 0.721. The AUC of the modified model in the validation cohort was 0.932.

DISCUSSION

ASSD is a rare and complex syndrome characterised by a wide range of symptoms and signs that are commonly observed in other SARDs or ILDs. Therefore, defining ASSD based solely on clinical features would lead to frequent misclassification. Anti-ARS autoantibodies may be useful in this context, but the varying performance of commercially available assays, its low reliability in identifying non-anti-Jo1 anti-ARS autoantibodies (especially in low pretest clinical probability settings), and the undefined prevalence of anti-ARS autoantibodies in clinical settings other than ASSD challenge their interpretation [36–38]. Furthermore, the substantial heterogeneity of ASSD clinical

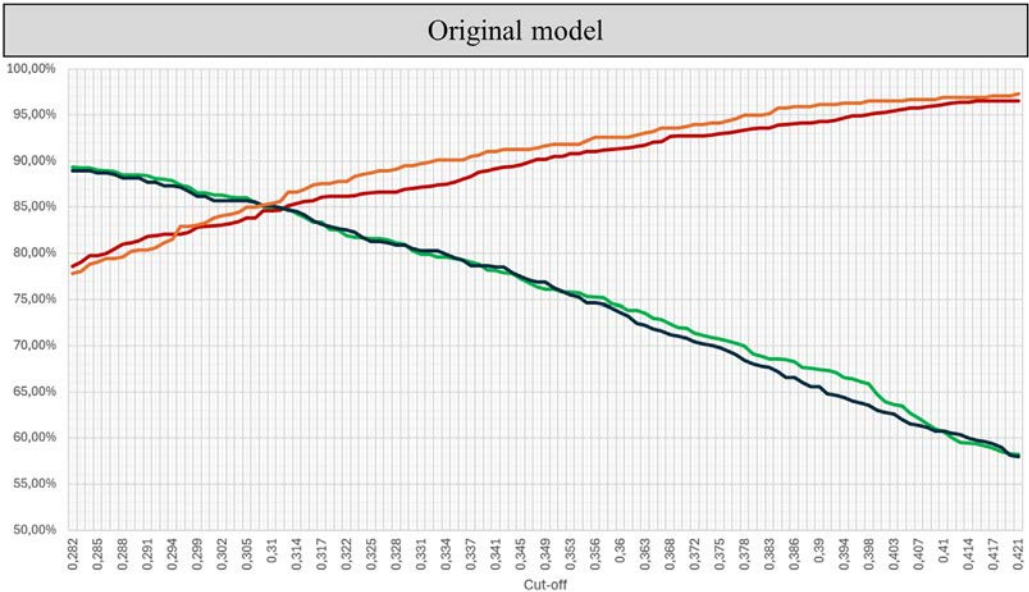


Figure 5. Threshold plot for the multicriteria decision analysis-driven candidate classification criteria's original model in the derivation cohort (green line—sensitivity; red line—specificity) and validation cohort (dark blue line—sensitivity; orange line—specificity).

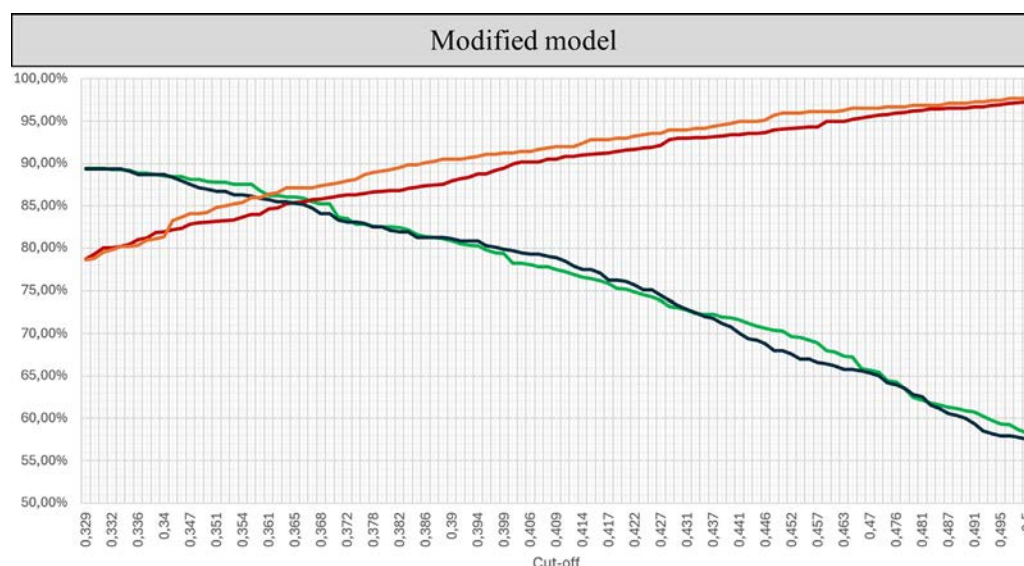


Figure 6. Threshold plot for the multicriteria decision analysis-driven candidate classification criteria's modified model in the derivation cohort (green line—sensitivity; red line—specificity) and validation cohort (dark blue line—sensitivity; orange line—specificity).

presentations leads to experts' different impressions of the syndrome. For these reasons, classification criteria based solely on antibodies or on expert opinion alone would likely lack clarity and mitigate their practical use within the medical community. The CLASS project encompasses both data-driven and expert consensus-driven methods, and this study focused on the development of candidate definitions for ASSD using MCDA followed by its validation with a *pseudo gold standard* and on real-world data.

The polymorphic nature of ASSD calls for a multispecialty international collaboration, which was achieved in the formation of the CLASS project SC. This careful and comprehensive approach to understanding ASSD produced a widely applicable classification tool that can be utilised irrespective of a physician's specialty or geographic location.

The first round of the ranking survey, in which the SC ranked the 20 fictional clinical vignettes from the least to the most likely to be ASSD, highlighted the heterogeneity of ASSD definitions among experts. On the other hand, the ranking survey consensus meeting led to fruitful discussions, culminating in a significantly higher interrater agreement in the reranking exercise and a consensus on the ASSD key domains and variables list that was used for the construction of the ASSD candidate classification criteria.

The list of key domains and variables was used to shape the preferences survey, which enabled the weighting of the variables according to the collective experts' judgements over a large number of clinical scenarios. The 2 models highlighted anti-ARS positivity as the most important factor that experts considered in classifying a patient as having ASSD. However, different assays yielded different weights. In both models, non-anti-Jo1 anti-ARS positivity tested by methods other than IP resulted in lower weights, reflecting the lower reliability of these assays compared to IP, the gold standard method for anti-ARS detection [38]. Nevertheless, even a positive non-anti-Jo1 anti-ARS tested using a method other than IP was valued higher than any clinical manifestation, highlighting the importance of these biomarkers and the poor diagnostic accuracy of isolated clinical manifestations. The magnitude of the anti-ARS weight in our models is consistent with their key importance in the 3 previously published ASSD criteria [28–30], in which anti-ARS

positivity is a mandatory criterion for ASSD diagnosis. More accessible worldwide than the anti-ARS assays, the antinuclear antibody IIFA was of great value in the identification of ASSD, as the presence of a cytoplasmic pattern was valued higher than clinical manifestations typically associated with ASSD, such as MH. Although less impactful, anti-Ro52/SSA autoantibodies were also judged as supportive of ASSD. These 2 serological criteria were not included in the previously published criteria but are consistent with the serological findings of ASSD patients published in the literature [22,39,40].

From the clinical perspective, the experts highly valued ILD, myositis, MH, joint involvement, and inflammatory rashes. In particular, ILD with a predominant NSIP and/or OP pattern was weighted almost twice as highly as a predominant UIP pattern for ASSD classification. Unclassifiable or unknown ILD patterns obtained lower weights than UIP but were still considered valuable. This is consistent with all previously published ASSD criteria, which considered ILD as a criterion, regardless of the ILD pattern [28–30]. This is also concordant with the remaining ASSD published literature, considering ILD is consistently reported as part of the syndrome but heterogeneously and usually poorly defined [34]. Muscle involvement was the second most important clinical domain. Although biopsy-proven myositis had the highest weight in the muscle domain, the experts also weighted clinical myositis and even subclinical evidence of myositis highly. The relatively high weight of clinical evidence of myositis likely reflects the clinical practice, in which the muscle biopsy is often deferred when ASSD is suspected and anti-ARS autoantibodies are detected [34]. While distinct muscle biopsy findings could potentially increase the specificity of the criteria, we did not include detailed biopsy parameters due to variability in techniques and interpretation across centres, a lack of consensus among experts regarding which muscle biopsy findings are ASSD-specific, because few centres reported specific stains or findings for ASSD, and to emphasise more readily available clinical and serological parameters. Despite not being pathognomonic of ASSD [41,42], MH are very commonly associated with ASSD in the literature. All 3 previously published ASSD criteria included MH as a criterion [28–30]. Therefore, its high attributed weight was not surprising. Joint involvement was highly valued as well, especially if there was joint pain and

swelling involving ≥ 4 joints of the hands and/or feet bilaterally. Of note, inflammatory arthralgia (without joint swelling) was also considered part of the ASSD spectrum of manifestations by the experts. Arthralgia is common in ASSD patients, has been widely reported as a manifestation of the disease [34], and has also been included in 1 of the 3 previously published ASSD criteria [30]. Inflammatory rashes, including the so-called ‘DM-specific’ rashes, were also considered suggestive of ASSD by the experts. This is backed by previously published data reporting a prevalence of ‘DM-type skin involvement’ in approximately 30% of ASSD patients [41,43]. Pulmonary hypertension was the lowest-scored key variable. This may be due to the higher prevalence of pulmonary hypertension in some ASSD mimickers and because pulmonary hypertension highly correlates with ILD in ASSD patients [44].

Because anti-ARS autoantibodies are generally considered mutually exclusive, the classification cutoffs—29.1 in the original model and 35.5 in the modified model—cannot be achieved with anti-ARS positivity alone. However, the maximum score obtained through serological scores alone (positive anti-ARS, antinuclear cytoplasmic pattern on IIFA, and positive anti-Ro52/SSA) is 32.5 in the original model and 37.9 in the modified model. Therefore, these classification cutoffs can be achieved in the absence of any clinical manifestations. This is an important face validity issue that will be addressed by changes to the candidate classification criteria recommended by the CLASS project SC. Raising these cutoffs could be an option, but sensitivity would be compromised. The addition of entry or mandatory criteria is an alternative option to address this limitation of the MCDA models without compromising their performance. On the other hand, even if all serological biomarkers are negative, patients can still be classified as ASSD as long as they display a combination of ≥ 3 clinical domains, including ILD with predominant NSIP and/or OP pattern, myositis assessed through muscle biopsy, and either MH or polyarthritis. The ability to reach the cutoffs regardless of antibody status is an advantage of these models for clinical practice and research, considering some anti-ARS autoantibodies have still not been identified. However, in other settings, such as clinical trials, it may also be a concern that a patient could be classified as having definite ASSD in the absence of an anti-ARS. This is another face validity issue that the SC will address. Possible solutions include defining different degrees of certainty of ASSD diagnosis using different cutoffs or additional criteria. Most importantly, and more reflective of a real-life clinical-serological assessment, a patient can also be classified as ASSD if appropriate combinations of serological biomarkers and clinical manifestations score higher than the cutoffs for classification.

The original and the modified models performed very similarly in the internal validation exercise, reaching similarly high AUCs. This showed that both models represented the experts’ opinions and had high internal validity.

The AUCs were also high and similar in the real-world data validation exercise, proving that these models can also distinguish real-world ASSD patients from mimickers. Importantly, both models had high sensitivity, specificity, PPV, and NPV when applied to the validation cohort, proving that the cutoffs originating from the derivation cohort could discriminate cases from controls in an independent cohort.

The MCDA has previously been used to develop classification criteria for rheumatic diseases, such as rheumatoid arthritis, systemic sclerosis, or systemic lupus erythematosus [31,32]. Strengths of this work include the comprehensive SLR performed to capture all possible ASSD definitions as the solid basis for the

exercises that followed, the multidisciplinary and internationally diverse SC, the 100% response rate from the SC in every exercise of the project, the use of a validated methodology for MCDA using a reliable instrument such as the 1000minds software, the expert consensus attainment, the validation on real-world data, and the relative weighting of each key domain and variable, yielding MCDA-based candidate criteria for ASSD classification.

Potential limitations of this study include the fact that the 19 experts of the SC may not fully represent the large, diverse myositis and ILD community across the world. Nonetheless, the CLASS project SC included experts from 16 centres across 10 countries from 4 continents, and the CLASS project Working Group included 14 investigators from 12 centres across 7 countries from 4 continents. This highly engaged and diverse working group provided the necessary feedback, including the feasibility of proposed candidates for classification criteria. Future iterations of these criteria should consider expanding the SC and working group to include more experts from underrepresented regions to further enhance the criteria’s relevance and applicability. Second, the study did not address the lack of standardisation and high variability in the testing of anti-ARS autoantibodies, which may hinder the performance of the criteria. This issue has been addressed separately [37]. Third, both models include low-impact variables and attribute nonrounded weights for several variables, limiting the clinical application and ease of use of the models in routine clinical practice. However, these models will be further simplified, and the variable scores rounded to increase their clinical applicability, feasibility, and acceptability without compromising the performance characteristics. Cutoffs may be adjusted slightly and entry, exclusion, or mandatory criteria for classification could be added to address face validity issues of the models without compromising the criteria’s performance. The final ASSD classification criteria will be further validated in a third independent cohort comprised of the remaining 25% of verified cases and controls that have not been used this far.

Competing interests

The authors report the following potentially relevant financial activities: GS has received speaking fees from Boehringer Ingelheim. FB has received consulting fees from Boehringer Ingelheim, Sanofi, Bristol Myers Squibb, and Savara Pharma; speaking fees from Boehringer Ingelheim and Sanofi; and support for attending meetings from Boehringer Ingelheim, AstraZeneca, Atyr, and Savara Pharma. TC has received consulting fees from 4D, Avalyn Therapeutics, Boehringer Ingelheim, Bridge Biotherapeutics, Bristol Myers Squibb, Cincera, DevPro, Endeavour BioMedicine, Pharmaxis, Pliant, and Roche; and speaking fees from Boehringer Ingelheim, Bristol Myers Squibb, and Roche. TD has received grants from Bayer, Genentech, and Sanofi. DFF has received consulting fees from Annexon, Argenx, Biogen, Bristol Myers Squibb, IgM, Kyverna, Nuvig, Octapharma, Paragon, Pfizer, and Priovant. MH has received grants from AstraZeneca, Boehringer Ingelheim, Bristol Myers Squibb, and Pfizer; and speaking fees from AstraZeneca and Boehringer Ingelheim. MK has received research grants from Boehringer Ingelheim, MBL, and Ono Pharmaceuticals; speaking fees from Asahi Kasei Pharma, Boehringer Ingelheim, Chugai, Eisai, Janssen, Kissei, Mochida, Nippon Shinyaku, and Ono Pharmaceuticals; and consulting fees from AstraZeneca, Boehringer Ingelheim, Chugai, GSK, Kissei, and Mochida. IL has received consulting fees from Argenx, Bristol Myers Squibb, Chugai, Corbus Pharmaceutical, EMD Serono Research & Development Institute, Galapagos, and Pfizer; speaking fees from Boehringer

Ingelheim; and has stock or stock options from Novartis and Roche. JS has received grants from Kezar; consulting fees from CSL Behring, DSMB, Grifols, Janssen, and Takeda; speaking fees from CSL Behring, Takeda, and UCB; and support for attending meetings from CSL Behring. VW has received grants from Amgen, AstraZeneca, Biogen, Bristol Myers Squibb, Caribou, Corbus Pharmaceuticals, CSL Behring, Gilead, Horizon, Pfizer, Priovant, Regeneron, Rome Pharmaceuticals, Ventus, and Viela; and consulting fees from AbbVie, Alpine Immune Sciences, Amgen, Anaptysbio, Argenx, AstraZeneca, Biogen, Bristol Myers Squibb, Cabaletta Bio, Calyx, Crialis, CSL Behring, Cugene, EMD Sorona, Evommune, Gilead, GSK, Horizon, Immunovant, Innovaderm, Janssen, Kwoya Kirin, Lilly, Merck, Nektar, Nuvig Pharmaceuticals, Pfizer, Regeneron, Rome Pharmaceuticals, Sanofi, Takeda, UCB, Ventus, Viela Bio, and Xencor. CS has received grants from AbbVie, Galapagos, and Lilly; and consulting fees from AbbVie. GS has received speaking fees from Boehringer Ingelheim. SC has received speaking fees from Eli Lilly, GSK, Novartis, and UCB; support for attending meetings from Eli Lilly, Janssen, and Pfizer; and has received equipment, materials, drugs, medical writing, gifts or other services from Grunenthal and UCB. MLA has received speaking fees from Boehringer Ingelheim and Tecnofarma; and support for attending meetings from Boehringer Ingelheim, Tuteur, and Raffo. MG has received consulting fees from AstraZeneca and UCB; and speaking fees from AbbVie, AstraZeneca, Eli Lilly, and GSK; support for attending meetings from AstraZeneca. RN has received speaking fees from Astellas Pharma, Boehringer Ingelheim, Daiichi Sankyo, Medical & Biological Laboratories, and Mitsubishi Tanabe Pharma Corporation. EA has received speaking fees from AbbVie, Johnson & Johnson, Lilly, and UCB. HC has received grants from Pfizer; and consulting fees from AstraZeneca, Horizon Therapeutics, PTC Therapeutics, and Pfizer; speaking fees from GSK, and UCB. EE has received consulting fees from Bristol Myers Squibb, Galapagos, and Novartis; and support for attending meetings from AbbVie, Novartis, and UCB. APS has received speaking fees from Nordic Pharma; and has stock or stock options from Pfizer. EC has received consulting fees from GSK; and speaking fees from Eli Lilly and GSK. LNN has received support for attending meetings from AbbVie, Nordic Pharma, and Novartis. MP has received speaking fees from Boehringer Ingelheim. LC has received consulting fees from Boehringer Ingelheim; and speaking fees from Alexion, Boehringer Ingelheim, and GSK. RA has received grants from Boehringer Ingelheim, Bristol Myers Squibb, EMD Serono, Janssen, Mallinckrodt, Pfizer, and Q32; and consulting fees from Actigraph, Alexion, ANI Pharmaceuticals, Argenx, AstraZeneca, Boehringer Ingelheim, Bristol Myers Squibb, Cabaletta Bio, Capella Bioscience, Corbus, CSL Behring, EMD Serono, Galapagos, Horizon Therapeutics, I-Cell, Janssen, Kezar, Kyverna, Merck, Novartis, Nuvig Therapeutics, Octapharma, Pfizer, Regeneron, Roivant, Sanofi, Teva, Artsome, Capstanx, and Manta. The remaining authors have no conflicts of interest to disclose.

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Contributors

Conceptualisation, GZ, SFK, RA, LC; methodology, GZ, SFK, ED, PH, DR, CAS, RA, LC; software, GZ, ED, PH, DR; formal analysis, GZ, PH; investigation, all the authors; resources, CAS; data curation, GZ, DR; writing—original draft preparation, ED, GZ, YBV; writing—review and editing, all the authors; visualisation, ED; supervision, RA, LC; project administration, RA, LC; funding acquisition, GZ, SFK, RA, LC; guarantor, RA. All authors have read and agreed to the final version of the manuscript.

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Patient consent for publication

Written informed consent was obtained from all patients at each centre.

Ethics approval

The CLASS project was approved by the Ethical Committee of the IRCCS Policlinico S. Matteo Foundation of Pavia, Pavia, Italy (P-201190088730; Prot. 20190094533) and the local IRBs in each participating centre. The study was conducted according to the Declaration of Helsinki.

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Data availability statement

The data underlying the findings reported herein are available on a reasonable request from the corresponding author.

Patient and public involvement

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

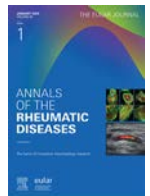
Supplementary materials

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Myositis

The Prospective Registry of Myositis (PROMIS): I. Next-generation sequencing identifies *HLA-DQA1* as a novel genetic risk of anti-MDA5 antibody-positive dermatomyositis

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ARTICLE INFO

ABSTRACT

Objectives: Antimelanoma differentiation-associated gene 5-positive dermatomyositis (anti-MDA5⁺ DM) is a rare subtype of myositis with a poor prognosis and insufficient genetic understanding. Our study aimed to identify risk variants associated with anti-MDA5⁺ DM and to assess their correlation with clinical outcomes.

Methods: We enrolled 231 anti-MDA5⁺ DM patients, 127 anti-MDA5⁻ DM patients, 1468 patients with non-DM rheumatic disease, and 1027 healthy controls, with an additional 51 anti-MDA5⁺ DM patients for validation. Whole-exome sequencing was used to identify human leukocyte antigen (HLA) alleles, amino acids, and single nucleotide polymorphisms (SNPs) in major histocompatibility complex (MHC) and non-MHC regions. Association studies were performed to identify genetic variants associated with anti-MDA5⁺ DM by comparing different sets of

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control groups. A clinical association study was conducted to further explore the influence of genetic factors on clinical manifestations.

Results: Two novel genetic risks, HLA-DQA1*06:01 and HLA-DQB1*06:09, were significantly associated with anti-MDA5⁺ DM in both the discovery and validation cohorts. HLA-DQA1*06:01 was particularly prevalent in anti-MDA5⁺ DM (20.4%) compared with anti-MDA5⁻ DM (6.69%) and non-DM rheumatic disease (5.93%). Besides, the strongest associated amino acid was HLA-DQ α 1:69 ($P = 3.27 \times 10^{-11}$), and 1 SNP in the ARHGAP22 gene (rs76208937) showed suggestive significance ($P = 7.99 \times 10^{-6}$). In addition, HLA-DQA1*06:01 was linked to acute rapidly progressive interstitial lung disease ($P < .001$), elevated levels of lactate dehydrogenase ($P = .026$), and death ($P = .049$) in patients with anti-MDA5⁺ DM.

Conclusions: HLA-DQA1*06:01 is a new genetic and prognostic factor for anti-MDA5⁺ DM, potentially serving as a biomarker for early diagnosis and intervention.

WHAT IS ALREADY KNOWN ON THIS TOPIC

- Antimelanoma differentiation-associated gene 5-positive dermatomyositis (anti-MDA5⁺ DM) is a rare, severe myositis subtype with skin ulceration, myopathy, and rapidly progressive interstitial lung disease (RPILD) prevalent in Asia.
- Some HLA-DRB1 alleles and a variant of the WDFY4 gene are associated with anti-MDA5⁺ antibodies, but comprehensive genetic analyses across the entire genome is lacking.
- Clinical risk factors like antibody titer and lactate dehydrogenase levels are known, but they are insufficient and lack genetic risk factors for early prediction.

What this study adds

- We identified HLA-DQA1*06:01 as a new genetic risk factor for anti-MDA5⁺ DM.
- HLA-DQA1*06:01 is associated with worse outcomes like acute RPILD and death.
- This study provides comprehensive insights into the genetic architecture of HLA alleles, amino acids, and single nucleotide polymorphism analysis in a relatively large anti-MDA5⁺ DM patient cohort.

How this study might affect research, practice or policy

- This study may stimulate deeper genetic research and biomarker discovery, offering tools for early diagnosis and personalised treatment, and may influence resource allocation while raising awareness of genetic factors in autoimmune diseases.

INTRODUCTION

Antimelanoma differentiation-associated gene 5 (MDA5) antibody-positive (anti-MDA5⁺) dermatomyositis (DM; anti-MDA5⁺ DM), a subtype of idiopathic inflammatory myopathy (IIM), is an inflammatory and autoimmune disorder characterised by skin ulceration, myopathy, and rapidly progressive interstitial lung disease (RPILD) [1,2]. RPILD is one of the most severe complications and leading cause of high mortality in patients with anti-MDA5⁺ DM [3]. How to predict and identify this critical type is a clinical challenge that needs to be addressed. However, because of the multidimensional clinical phenotypes of anti-MDA5⁺ DM patients, it is difficult to predict or assess disease severity at an early stage. In addition, more than 75% of patients die within 6 months to 1 year [4,5]. Several risk factors, such as anti-MDA5 antibody titer, lactate dehydrogenase (LDH) level, serum ferritin level, and cluster analysis, have been implicated in the development of RPILD [6]. However, the genetic signatures of anti-MDA5⁺ DM have not been well studied, yet an understanding of these signatures may help to optimise the model and improve early detection.

The research on anti-MDA5⁺ DM is predominantly found in Asian populations, especially in China and Japan [7]. This focus is likely reflective of the fact that Asian patients with anti-MDA5⁺ DM and RPILD tend to face more severe prognoses [6]. To date, there are no large-scale populational genetic studies on anti-MDA5⁺ DM. Prior research has primarily utilised targeted sequencing platforms, concentrating on specific genes of interest [8]. Human leukocyte antigen (HLA)-DRB1 alleles such as 01:01, 04:05, 12:02, 04:01, 12:01, and rs7919656 on the WDFY4 gene were reported to be associated with the production of anti-MDA5⁺ antibodies [9–13]. However, these studies lack comprehensive whole-genome association analyses, which are necessary for a more detailed understanding of the genetic factors involved in anti-MDA5⁺ DM.

Therefore, we assembled the largest anti-MDA5⁺ DM cohort to date and, for the first time, explored the relationships between HLA alleles, single nucleotide polymorphisms (SNPs), and amino acids (AAs) with the occurrence of disease on a genome-wide scale by comparing with anti-MDA5⁻ DM patients, non-DM rheumatic patients, and healthy controls. Additionally, we conducted a clinical association study to delve deeper into the influence of genetic factors on the clinical manifestations of the disease.

METHODS

Study population

This study is based on the Chinese Rheumatology Data Center–Prospective Registry Of Myositis (PROMIS) biobank in Peking Union Medical College Hospital (PUMCH) [14] and the Second Affiliated Hospital of Nanchang University. Patients were recruited consecutively from our clinical centre from November 2017 to October 2024. Patients >18 years of age with a diagnosis based on the Bohan–Peter criteria [15], the 2017 European League Against Rheumatism (EULAR)/American College of Rheumatology (ACR) criteria for DM [16], or the 2002 Sontheimer criteria for clinical amyopathic DM [17] were recruited. The detection of anti-MDA5 antibodies was performed by using a specific line immunoassay (Euroimmun) or a specific dot immunoassay (D-Tek). In total, 231 anti-MDA5⁺ DM patients were collected in the discovery cohort and 51 in the validation cohort.

For the control group, whole-exome sequencing (WES) data from 1027 healthy individuals were collected from the Shunyi Study of China [18]. For negative controls, 127 anti-MDA5⁻ DM patients (antisynthetase syndrome [ASS] patients) and 1468 patients with non-DM rheumatic diseases, including systemic lupus erythematosus (SLE), takayasu arteritis (TAK), primary

Sjögren's syndrome (pSS), and antiphospholipid syndrome (APS), were included from January 2008 to September 2023 in PUMCH. We included cases that fulfilled at least 1 of the following classification criteria: the 2012 Systemic Lupus International Collaborating Clinics classification criteria [19] or the 2019 ACR/EULAR classification criteria for SLE [20]; the 1990 ACR classification criteria for TAK [21]; the 2002 American-European Consensus Group classification criteria [22] or the 2016 ACR/EULAR classification criteria for pSS; and the 2006 Sydney revised classification criteria for APS [23].

WES

Patient blood samples were subjected to WES, including anti-MDA5⁺ DM patients in the discovery cohort, anti-MDA5⁻ DM, and non-DM rheumatic patients. The exome sequences were efficiently enriched from 0.4 µg genomic DNA extracted from peripheral blood using the Agilent liquid capture system (Agilent SureSelect Human All Exon V6) according to the manufacturer's protocol. Captured libraries were enriched in a polymerase chain reaction (PCR), and index tags were added to prepare for sequencing. Products were purified using the AMPure XP system (Beckman Coulter) and quantified using the Agilent High Sensitivity DNA Assay on the Agilent Bioanalyzer 2100 system. DNA libraries were sequenced on Illumina Nova-Seq for paired-end 150 bp reads. In the validation cohort, 38 samples underwent WES, while the other 13 samples were subjected to PCR sequence-based typing (SBT) for HLA-DQA1*06:01 genotyping.

Reads mapping and SNP calling

Valid sequencing data were mapped to the reference genome (GRCh38/hg38) by the Burrows-Wheeler Aligner software [24] (v0.7.17) to get the original mapping reads in BAM format. Samtools [25] (1.16.1) was utilised to sort bam files. Genome Analysis Toolkit [26] (GATK v4.3.0.0) was used for duplicate marking and identifying SNPs and small insertions/deletions. Next, we performed quality control (QC) on variants and individuals using vcftools [27] (0.1.16) and PLINK [28] (v1.90b). Briefly, variants with multialleles were first split and only variants located in the 100 bp flanking range of captured regions were retained. Besides, the variant had to pass the following criteria: (1) genotype quality ≥ 10 , (2) $10 \leq \text{read depth} \leq 3500$, and (3) variant calling rate among all participants ≥ 0.9 . We also excluded individuals from the association analysis who (1) were sex-discordant/ambiguous; (2) had an excessive heterozygosity rate (>3 SDs from the mean); (3) outlier samples in principal component analysis (Supplementary Fig S1); (4) low sample call rate (≤ 0.9); and (5) relatedness among individuals was calculated using the identity by descent analysis. For each pair of individuals with PI_HAT (Proportion IBD) > 0.2 , we excluded the one with a lower call rate. These remaining samples were applied to HLA allele genotyping.

HLA allele genotyping and AA identification

HLA genotyping was performed using the HISAT-genotype software, which is accessible through a public GitHub repository (1.3.2 release; <https://daehwankimlab.github.io/hisat-genotype/>). Individual HLA genotyping was conducted using 2 programmes: 'hisatgenotype' and 'hisatgenotype_toolkit.' A total of 26 HLA genes in the major histocompatibility complex (MHC) region (A, B, C, DMA, DMB, DOA, DOB, DPA1, DPB1, DPB2,

DQA1, DQB1, DRA, DRB1, E, F, G, H, HFE, K, L, MICA, MICB, TAP1, TAP2, and V) were typed with default parameters using WES data. To assess the accuracy of the HLA alleles inferred from the sequencing data, classical HLA genes were validated by PCR-SBT sequencing in a subset of 50 samples. A direct comparison of the 2 methods gave a concordance of 97.5% at 4-digit resolution (Supplementary Table S1). The AA sequence was determined according to the ImMunoGeneTics (IMGT)/HLA database (v.3.55.0) based on HLA alleles.

Genetic association analyses

Association analyses for SNPs in non-MHC regions, as well as HLA gene alleles and AAs in the MHC region, were carried out by PLINK (v1.90b). An additive logistic regression adjusting for sex, age, and the first 8 principal components (PCs) was applied to evaluate the association at a biallelic locus for variants with a minor allele frequency $>1\%$. For a multiallelic HLA allele locus, we collapsed the locus into a series of biallelic loci and then tested the association of each allele separately. For a multiallelic AA locus, we conducted an omnibus test to locate the risk loci before collapsing the significant locus into biallelic loci to assess the contribution of each specific AA residue. PLINK was used to perform linkage disequilibrium analysis and conditional analysis with the most significant allele (DQA1*06:01). The Bonferroni corrected *P* value significance threshold, adjusted for multiple comparisons, was 3.86×10^{-07} ($.05/[196 \text{ alleles} + 128,654 \text{ SNPs} + 617 \text{ AAs}]$). The genomic inflation factor (λ) was calculated with original *P* values obtained from the association test of SNPs to assess the stratification of the population.

Three-dimensional protein structure modelling

Protein Data Bank entries for 3pl6 [29] were used to produce the 3-dimensional models of the HLA-DQ molecule with PyMOL Molecular Graphics System version 2.6 (Schrödinger, LLC.).

Clinical association study

Demographic and clinical data were retrieved from the PROMIS registry, including age, sex, lifestyle factors (smoking history, environmental exposure), diagnosis, clinical manifestations, disease activity assessments, laboratory tests, imaging data, and treatment information. All enrolled patients were followed up until the date of the last visit. In patients whose date of death was during the follow-up period, their follow-up duration was calculated from the time of disease onset to the date of death. A radiologist, a pulmonologist, and a rheumatologist independently evaluated the high-resolution chest computed tomography (HRCT) of each patient to determine the presence and types of ILD [30], and discussions were held until a consensus was reached in cases of discrepancies. The ILD types can be distinguished as organising pneumonia (OP), nonspecific interstitial pneumonia (NSIP), usual interstitial pneumonia, or a combination of different patterns. RPILD was defined as the rapid deterioration of dyspnoea secondary to ILD within 1 month of the diagnosis of ILD [31]. ILD was divided into non-RPILD and RPILD based on the course of the disease and imaging manifestations. RPILD was further categorised into acute or subacute groups based on the following specific criteria:

1. Acute RPILD: patients were classified as acute if their imaging findings resembled those of acute interstitial pneumonia (AIP) or if they required mechanical ventilation due to

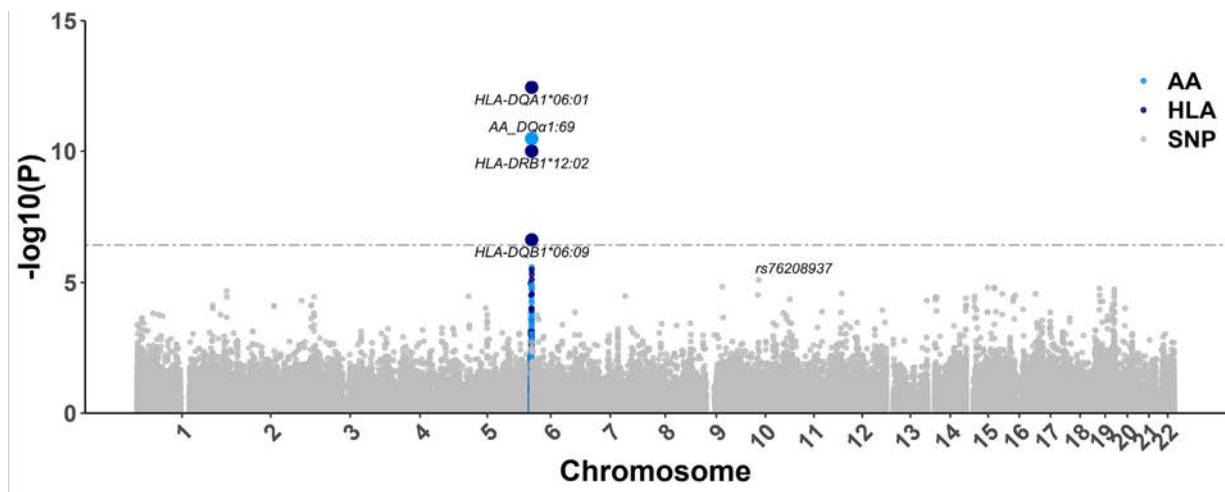


Figure 1. Genetic association analysis for antimelanoma differentiation-associated gene 5-positive dermatomyositis. The $-\log_{10}$ of P values are plotted against their chromosomal position. The dotted line represents the level of statistical significance ($P = 3.86 \times 10^{-7}$). Coloured signals represent alleles (dark blue), amino acids (AAs, light blue), and single nucleotide polymorphisms (SNPs; grey) associated with antimelanoma differentiation-associated gene 5-positive dermatomyositis. HLA, human leukocyte antigen. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

respiratory failure. Key HRCT features included bilateral areas of ground-glass attenuation, airspace consolidation with basal and subpleural predominance, traction bronchiectasis, and architectural distortion [32,33] (Supplementary Fig S2A, as an example).

2. Subacute RPILD: patients who did not meet the AIP criteria above and presented with none or mild hypoxemia that did not require mechanical ventilation were classified as subacute. Imaging findings included patterns resembling OP [34] or NSIP [35] (Supplementary Fig S2B, as an example).

Clinical characteristics were compared between patients with and without risk alleles using the Mann-Whitney U test or t-test for continuous values and Fisher's exact test for categorical variables. Clinical outcomes, namely all-cause mortality, were used in this study. A Kaplan-Meier Plot was used to calculate the cumulative probabilities of survival. Statistical analyses were computed using R statistical software (R Foundation for Statistical Computing). P values $< .05$ were considered statistically significant.

RESULTS

A total of 231 anti-MDA5⁺ DM patients and 1027 geographically and ethnically matched healthy controls of Chinese descent were recruited ($\lambda = 1.022$; Supplementary Fig S1, Supplementary Table S2) and underwent WES, with an average sequencing depth of 178; and more than 98.84% of the targeted bases had a read depth of more than 10 (Supplementary Table S3). After QC, sequencing data of 225 patients and 990 controls were included for further analyses (see Materials and Methods, Supplementary Table S4). Finally, a total of 129,467 variants were identified, including 196 four-digit HLA alleles, 617 AA positions from MHC regions, and 128,654 common SNPs from non-MHC regions.

*HLA-DQA1*06:01 and DQB1*06:09 were identified as risk alleles for anti-MDA5⁺ DM*

In most current genetic association studies of HLA, the unit of variation is not usually an SNP but rather a whole HLA gene

version or haplotype. To investigate the contribution of HLA alleles to anti-MDA5⁺ DM in our cohort, we conducted an association analysis across all HLA genes using a logistic regression model adjusting for age, sex, and PCs. Notably, *HLA-DQA1*06:01* exhibited the most significant association with the occurrence of anti-MDA5⁺ DM ($P = 3.54 \times 10^{-13}$; odds ratio [OR] = 3.52). Additionally, *DRB1*12:02* ($P = 9.86 \times 10^{-11}$; OR = 3.39) and *DQB1*06:09* ($P = 2.44 \times 10^{-7}$; OR = 11.68) also reached genome-wide significance compared with healthy controls (Fig 1 and Supplementary Table S5). Among them, *DRB1*12:02* has been previously reported to be linked to DM [10]. However, conditional association analyses with the most significant allele, *HLA-DQA1*06:01*, revealed that the signal for *DRB1*12:02* disappeared ($P = .67$), suggesting a close genetic relationship between these 2 alleles. This hypothesis was further supported by linkage disequilibrium analysis, exhibiting a strong correlation ($R^2 = 0.83$). Despite *DQB1*06:09* maintaining significance after conditioning ($P = 7.11 \times 10^{-10}$), it exhibited a relatively low allele frequency within the population (4.0%), indicating that only a minority of individuals are affected.

Given the rarity of the disease, we validated our findings using a separate cohort of 51 patients with anti-MDA5⁺ DM. Notably, 22 individuals carried *HLA-DQA1*06:01*; 1 was homozygous, and 21 were heterozygous, resulting in an allele frequency of 22.5%. A comparison with the healthy controls confirmed the significant association for *HLA-DQA1*06:01* ($P = 1.11 \times 10^{-6}$; OR = 4.16). When we combined the discovery and validation cohorts, the statistical significance of *DQA1*06:01* was further improved ($P = 2.45 \times 10^{-16}$; Table 1).

Table 1
HLA-DQA1*06:01 allele frequencies in the discovery, validation, and combination cohorts of antimelanoma differentiation-associated gene 5-positive dermatomyositis

Sample group	N	AF (%)	P value	OR	95% CI
Discovery cohort	225	20.4	3.54×10^{-13}	3.52	2.51-4.95
Validation cohort	51	22.5	1.11×10^{-6}	4.16	2.34-7.83
Combination	276	20.8	2.45×10^{-16}	3.77	2.74-5.17

AF, allele frequency; OR, odds ratio.

Table 2
Comparisons of allele frequencies of *HLA-DQA1*06:01* among various participant cohorts

Disease	N	AF (%)	Control	OR	P value	Control	OR	P value
ASS	127	6.69	MDA5 ⁺	0.27	1.19×10^{-7}	HC	1.02	.93
SLE	689	3.99	MDA5 ⁺	0.16	$<2.2 \times 10^{-16}$	HC	0.63	.0059
TAK	200	5.25	MDA5 ⁺	0.21	1.28×10^{-12}	HC	0.84	.56
pSS	280	8.92	MDA5 ⁺	0.37	1.93×10^{-8}	HC	1.49	.03
APS	299	8.03	MDA5 ⁺	0.33	4.00×10^{-10}	HC	1.33	.11

AF, allele frequency; APS, antiphospholipid syndrome; ASS, antisynthetase syndrome; HC, healthy controls; MDA5⁺, antineutrophil cytoplasmic antibody 5-positive dermatomyositis; N, sample number; OR, odds ratio; pSS, primary Sjögren's syndrome; SLE, systemic lupus erythematosus; TAK, Takayasu arteritis.

To further investigate whether *HLA-DQA1*06:01* was specifically associated with anti-MDA5⁺ DM or not, we genotyped *HLA-DQA1*06:01* in 127 anti-MDA5⁺ DM patients and 1468 patients with non-DM rheumatic disease. The allele frequency ranged from 3.99% to 8.92%, comparable to the healthy control group but significantly different from that observed in the anti-MDA5⁺ DM cohort, indicating the high probability specificity of *HLA-DQA1*06:01* for anti-MDA5⁺ DM (Table 2, see Materials and Methods). To ensure genotyping accuracy, we randomly selected 20 anti-MDA5⁺ DM patients carrying the *DQA1*06:01* allele and subsequently verified their genotypes through PCR-SBT, achieving a 100% accuracy rate (Supplementary Table S6).

HLA-DQα1:69 is significantly associated with anti-MDA5⁺ DM

The AA position with the strongest association with anti-MDA5⁺ DM belonged to HLA-DQα1, with a peak at HLA-DQα1 AA 69, when comparing anti-MDA5⁺ DM patients with healthy controls ($P_{\text{omnibus}} = 3.27 \times 10^{-11}$; Fig 1). HLA-DQα1:69 is in the α1 domain of the HLA-DQ molecule, close to residues forming pockets (Fig 2). Subsequent analysis collapsing the locus into biallelic loci indicated that the highest contribution to this association was made by HLA-DQα1:69T ($P = 3.12 \times 10^{-12}$; OR = 3.20; Supplementary Tables S7 and S8), a substitution encoded by *HLA-DQA1*06*, which coincides with the association results of the HLA allele.

Association study for SNPs in non-MHC regions

To investigate the relationship between exonic SNPs in non-MHC regions and anti-MDA5⁺ DM, we conducted an association analysis for common SNPs. However, after adjusting for age, sex, and PCs, no variant reached genome-wide significance. Notably, we identified 1 SNP on chromosome 10 (rs76208937, $P = 7.99 \times 10^{-6}$; OR = 2.64) that exhibited suggestive

significance for association with anti-MDA5⁺ DM. It is located within the exon region of the Rho GTPase activating protein 22 (ARHGAP22) gene and results in a synonymous code changing. The variant (rs76208937) presents a relatively low allele frequency in the population, with 2.20% in the gnomAD dataset and 2.04% in our in-house healthy control cohort. However, among anti-MDA5⁺ DM patients, the allele frequency was nearly 5.23% (Fig 1). Subsequently, we further investigated the genetic profile of this SNP across various disease groups. The results revealed that the allele frequency among anti-MDA5⁺ DM patients was also notably higher compared with the anti-MDA5⁺ patient group ($P = .0067$) and the non-DM patient cohort ($P = .0077$; Supplementary Table S9). This finding suggests a potential role for rs76208937 in the pathogenesis of anti-MDA5⁺ DM, although further studies are needed to confirm its significance.

*HLA-DQA1*06:01 was associated with worse clinical outcomes in anti-MDA5⁺ DM*

We stratified 276 patients with anti-MDA5⁺ DM into two groups: those carrying the *HLA-DQA1*06:01* allele and those without the allele. As detailed in Table 3 and Supplementary Table S10, patients harbouring this allele exhibited a higher prevalence of hypoxemia compared with the others (17.3% vs 7.6%, $P = .018$). Although there was no significant difference in the frequency of RPILD between the 2 groups, patients with the *DQA1*06:01* allele had a significantly increased incidence of acute RPILD (60.6% vs 27.2%, $P < .001$). Pulmonary imaging revealed that OP was less common in patients with the allele (31.7% vs 51.7%, $P = .001$). Besides, they also exhibited elevated levels of LDH ([219, 355] U/L vs [202, 304] U/L, $P = .026$) and C-reactive protein (2.6 [1.0, 10.1] mg/L vs 0.9 [0.4, 2.4] mg/L, $P = .007$). Kaplan-Meier survival analysis revealed that patients with *DQA1*06:01* had a lower cumulative

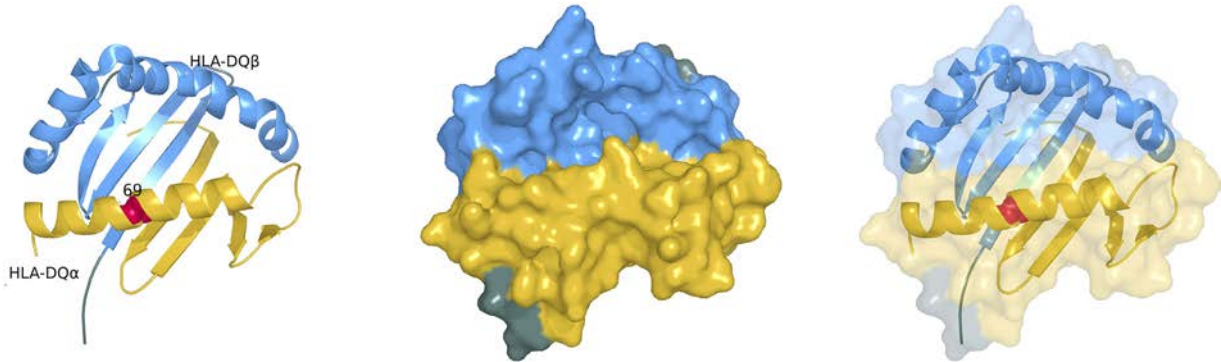


Figure 2. The 3-dimensional model visualises the peptide binding groove and associated amino acid residues of the HLA-DQ heterodimer. Position 69 amino acid residue is highlighted in the ribbon model. DQα1:69 is located in the α-helix domain, close to antigen-binding pockets.

Table 3
Demographic and clinical features of antimeelanoma differentiation-associated gene 5-positive dermatomyositis patients carrying and not carrying HLA-DQA1*06:01

Variables	N = 276 n (%) ^a	With DQA1*06:01 (N = 104) n (%) ^a	Without DQA1*06:01 (N = 172) n (%) ^a	P value
Female	197 (71.4)	75 (72.1)	122 (70.9)	.891
Age (y) of onset, M (Q1, Q3)	46 (36, 53)	47 (37, 53)	45 (35, 55)	.755
Smoked	52 (18.8)	16 (15.4)	36 (20.9)	.271
Myalgia	18 (6.52)	3 (2.9)	15 (8.7)	.077
Muscle weakness	115 (41.7)	41 (39.4)	74 (43.0)	.615
Dysphagia	29 (10.5)	7 (6.7)	22 (12.8)	.155
Hoarseness	47 (17.0)	15 (14.4)	32 (18.6)	.412
Arthritis	135 (48.9)	55 (52.9)	80 (46.5)	.322
Rash	234 (84.8)	87 (83.7)	147 (85.5)	.731
Vasculitis	87 (31.5)	31 (29.8)	56 (32.6)	.689
Mechanic hand	21 (7.6)	6 (5.8)	15 (8.7)	.484
ILD				
Number	258 (93.5)	98 (94.2)	160 (93.0)	.805
Age (y) of onset, M (Q1, Q3)	46 (39, 54)	47 (39, 53)	46 (37, 55)	.664
RPILD	207 (75.0)	71 (68.3)	136 (79.1)	.062
subacute	127 (61.4)	28 (39.4)	99 (72.8)	<.001
acute	80 (38.6)	43 (60.6)	37 (27.2)	—
HRCT				
UIP	33 (12.0)	14 (13.5)	19 (11.0)	.570
NSIP	149 (54.0)	52 (50.0)	97 (56.4)	.321
OP	122 (44.2)	33 (31.7)	89 (51.7)	.001
Pneumomediastinum	6 (2.2)	4 (3.8)	2 (1.2)	.203
Hypoxemia	31 (11.2)	18 (17.3)	13 (7.6)	.018
Laboratory test				
LY no. (10 ⁹ /L), ^b M (Q1, Q3)	1.2 (0.8, 1.8)	1.0 (0.7, 1.6)	1.3 (0.8, 1.9)	.103
CK (U/L), M (Q1, Q3)	47 (31, 85)	46 (32, 70)	48 (30, 85)	.628
LDH (U/L), M (Q1, Q3)	244 (205, 330)	277 (219, 355)	237 (202, 304)	.026
ESR (mm/1h), M (Q1, Q3)	13 (6, 28)	15 (8, 30)	12 (6, 26)	.412
Ferritin (ng/mL), M (Q1, Q3)	196 (60, 519)	216 (58, 625)	185 (60, 437)	.820
CRP (mg/L), M (Q1, Q3)	1.5 (0.6, 3.9)	2.6 (1.0, 10.1)	0.9 (0.4, 2.4)	.007
Anti-Ro 52 antibody	132 (47.8)	49 (47.1)	83 (48.3)	.901
Treatment				
GCs	271 (98.2)	103 (99.0)	168 (97.7)	.653
GCs + CNIs + CYC (triple immunosuppressive therapy)	142 (51.4)	52 (50.0)	90 (52.3)	.711
GCs + CNIs/CYC (dual immunosuppressive therapy)	108 (39.1)	39 (37.5)	69 (40.1)	.704
bDMARDs/tsDMARDs	78 (28.3)	30 (28.8)	48 (27.9)	.891
IVIG	51 (18.5)	19 (18.3)	32 (18.6)	1.000

bDMARDs, biologic disease-modifying antirheumatic drugs; CK, creatine kinase; CNI, calcineurin inhibitors; CRP, C-reactive protein; CYC, cyclophosphamide; ESR, erythrocyte sedimentation rate; GC, glucocorticoid; HRCT, high-resolution computed tomography; ILD, interstitial lung diseases; IVIG, intravenous immunoglobulin; LDH, lactate dehydrogenase; LY, lymphocyte; NSIP, nonspecific interstitial pneumonia; OP, organising pneumonia; RPILD, rapidly progressive interstitial lung disease; tsDMARDs, targeted synthetic disease-modifying antirheumatic drugs; UIP, usual interstitial pneumonia.

^a Unless otherwise specified.
^b One patient with the mutation was lost to follow-up.

survival rate compared with those without the allele ($P = .049$; Fig 3).

DISCUSSION

In our study, we assembled the largest anti-MDA5⁺ DM cohort published to date and performed genome-wide analyses encompassing HLA alleles, AAs, and SNPs using next-generation sequencing data. We identified 2 novel genetic risk factors, namely *HLA-DQA1*06:01* and *HLA-DQB1*06:09*, that exhibited significant associations within the Chinese anti-MDA5⁺ DM cohort. Notably, *HLA-DQA1*06:01* demonstrated priority genetic correlation with anti-MDA5⁺ DM compared with anti-MDA5⁻ DM and other rheumatic diseases. Moreover, this HLA allele was also linked to worse clinical outcomes in anti-MDA5⁺ DM patients. In addition, further analysis revealed significant associations between the HLA-DQα1 position 69 and rs76208937 within the ARHGAP22 gene and the occurrence of anti-MDA5⁺ DM.

*HLA-DQA1*06:01* is an allele type that exhibits relatively low frequency, with its prevalence varying significantly across different populations. Reported allele frequencies for *HLA-DQA1*06:01* range from 0.65% to 8.60% in Japan [36], Brazil [37], Russia [38], and the USA [39]. In China, a large population-based study has revealed a frequency of 7.86% for this allele [40], while our internal control group of 1027 healthy individuals exhibited a frequency of 6.1%. The allele frequency for *HLA-DQA1*06:01* in anti-MDA5⁺ DM was 20.8%, which was significantly higher than those in anti-MDA5⁻ DM patients, non-DM rheumatic disease cohorts, and healthy controls (Table 2). *HLA-DQA1*06:01* was reported as a risk factor for several immune-mediated conditions, including the progression of chronic hepatitis C to liver cirrhosis [41] and vitiligo [42], and this is the first time it is linked to DM. HLA-DQα is encoded by *HLA-DQA1* and forms the α chain of MHC class II molecule HLA-DQ, which has a peptide binding groove formed by the β-sheet floor and 2 α-helices, as well as pockets into which anchor residues of the antigen can fit. HLA-DQ plays a role in thymic selection, eliminating self-reactive T-cell clones, which

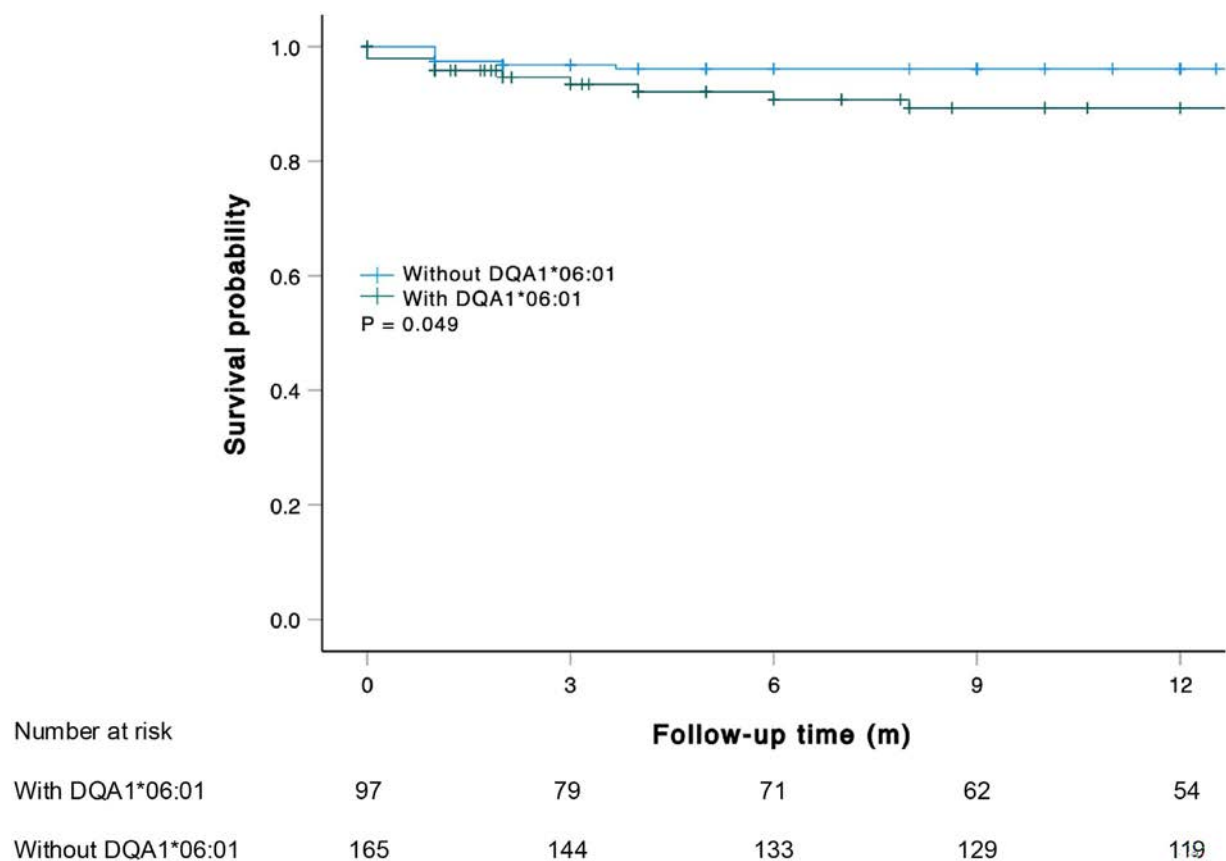


Figure 3. Comparisons of survival probability between antimelanoma differentiation-associated gene 5-positive dermatomyositis patients with and without *HLA-DQA1*06:01*.

offers a plausible explanation for its association with autoimmune diseases [43]. To investigate the possible mechanism of *HLA-DQα1* contributing to disease susceptibility, we performed an AA association study. Notably, the AA residue that conferred the strongest and independent association was *HLA-DQα1:69*, which is in the $\alpha 1$ domain of the *HLA-DQα1* molecule, close to the residues forming pockets P6 and P9. The substitution of threonine for alanine at this position, encoded by *HLA-DQA1*04* and *HLA-DQA1*06*, could be associated with peptide binding affinity and selectivity, resulting in susceptibility and worse prognosis of anti-MDA5⁺ DM.

In addition to *HLA-DQA1*06:01*, the second significant allele was *HLA-DRB1*12:02*, which has been previously reported in other genetic studies of DM. However, after conditional analyses, we found that the positive signal of *HLA-DRB1*12:02* disappeared. *DRB1* encodes the DR β protein, which is the β chain of the *HLA-DR* molecule. Together with the α chain encoded by *DRA1*, it forms the *HLA-DR* molecule. The association signal of *HLA-DRB1*12:02* disappeared after conditioning, possibly due to the proximity of *DQA1* and *DRB1* in genetic distance. These results demonstrated that *HLA-DQA1*06:01* was novel and the most important risk allele in our anti-MDA5⁺ DM cohort. *DQB1*06:09* also presented a significant correlation with anti-MDA5⁺ DM in both the discovery and validation cohorts but with extremely low allele frequency (anti-MDA5⁺ DM: 4.0%, healthy control: 0.4%) and a relatively high OR of 11.68. In addition to this, we also investigated the prevalence of *DQB1*06:09* in other disease cohorts. Although the allele frequencies of *DQB1*06:09* in the SLE, TAK, and pSS cohorts were comparable to that of healthy controls and significantly lower than that in the anti-MDA5⁺ DM cohort, the carrying rate was

relatively high in the ASS and APS cohorts (more than 2%), presenting a significant difference compared with the healthy control group (P value < .001; [Supplementary Table S11](#)). Thus, while *DQB1*06:09* could serve as an indicator for anti-MDA5⁺ DM, we do not regard it as a genotype that is specifically linked to this condition at present. Subsequent clinical correlation analysis did not show a significant correlation with phenotypes, perhaps due to a relatively small number of individuals carrying this allele. Thus, a larger sample cohort is needed to further validate this allele and its potential biological functions.

Considering the complexity of the HLA region, the use of HLA alleles in association tests has higher statistical power than SNP-based approaches and allows for a better understanding of the role of the HLA region. Therefore, we performed HLA allele associations for the HLA gene and focused on SNPs on non-HLA genes. Our exome-wide association analysis of common variants showed that rs76208937 reached a suggestive significance level compared with healthy controls. Furthermore, we found that the allele frequency of rs76208937 in the anti-MDA5⁺ cohort was also significantly higher when compared with both ASS and the overall non-DM rheumatic patients (P < .05). However, when looking at specific disease groups, the significance of the differences varied. We observed that when compared with the TAK and APS groups, the P values did not reach significance (P > .05; [Supplementary Table S9](#)), which may be related to the sample size or the effect size of the SNP itself. Further research with larger cohorts is needed to confirm these findings. rs76208937 is a synonymous variant located within *ARHGAP22* and was defined as ‘Benign,’ according to ClinVar. The *ARHGAP22* gene encodes a Rho family GTPase protein and is involved in regulating the signalling pathways of endothelial

cell capillary formation during angiogenesis and cell movement. The gene has multiple transcript variants encoding different isoforms. Research has substantiated that the protein ARHGAP22 plays a pivotal role in modulating the inflammatory response associated with vascular pathologies [44]. There is a correlation between *ARHGAP22* and diabetic retinopathy, suggesting that vascular damage may be linked to the expression or function of *ARHGAP22* [45]. In our study, we observed concomitant skin vasculitis in 87 patients with anti-MDA5⁺ DM, which could potentially implicate *ARHGAP22* in the pathogenesis of vascular inflammation within the context of this disease.

RPILD has a high incidence rate in anti-MDA5⁺ patients [46]. Consistent with previous studies, our study revealed a significant incidence of RPILD in both groups carrying or not carrying *HLA-DQA1**06:01 (67.1% and 78.3%, respectively). Moreover, the acute form of RPILD stands out as a primary contributor to the unfavourable prognosis in anti-MDA5⁺ DM patients [47]. Predicting the onset of acute RPILD in this patient population is of paramount importance. Our subgroup analysis provided intriguing insights: among those with RPILD, a higher proportion of patients with *HLA-DQA1**06:01 exhibited increased inflammation and a more acute ILD (40 [60.6%] vs 37 [27.2%]), indicating that the patients in the *HLA-DQA1**06:01 group were more prone to severe lesions. Serum cytokines are significantly increased in the setting of anti-MDA5⁺ DM and correlate with pulmonary severity and poor prognosis. Uncontrolled hyperinflammation and persistent immune activation lead to severe lung injury, which might resemble the ‘cytokine storm’ [48]. Excessive activation of the type I interferon pathway may also be related to cytokine storms [49]. However, there is currently no research on the *HLA-DQA1**06:01 and type I interferon pathways. The correlation between the 2 in the pathogenesis of RPILD still needs further research. Patients with *HLA-DQA1**06:01 were associated with high mortality, as evidenced by distinct phenotypic profiles. Anti-MDA5 antibodies are an independent risk factor for mortality in IIM [50]. It was discovered that most deaths occurred in the early stages of the disease, with 89% of deaths occurring within the first year after diagnosis [5]. In our study, the mortality rate of patients carrying *HLA-DQA1**06:01 was significantly higher than that of patients without the allele.

This study also has limitations. First, although it is the largest genetic study of anti-MDA5⁺ DM conducted using deep sequencing methods, due to the rare nature of the disease, this study did not undergo large-scale validation. We validated the results of *HLA-DQA1**06:01 in another group of 51 patients who had an even higher allele frequency than the discovery cohort, which may suggest the value of this variant. Further validation studies on the value of *HLA-DQA1**06:01 as a predictive and prognostic factor need to be performed. Second, although our findings reveal that *HLA-DQA1**06:01 is highly associated with disease occurrence and prognosis, the underlying mechanism of *HLA-DQA1**06:01 in increasing anti-MDA5⁺ DM susceptibility is not well understood. Further study is also needed. Third, we conducted an initial comparative analysis of survival rates among patients with and without *HLA-DQA1**06:01. However, due to the scarcity of death events, the statistical power was hampered when accounting for confounding factors. Therefore, a future study is imperative to assess the long-term prognostic significance of this genetic variant.

Competing interests

None declared.

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Contributors

XY, QW, and ML had the idea for and designed the study; they had full access to all data in the study and take responsibility for the integrity of the data analysis. XY, CY, CW, and X Zhang wrote the first full draft of the report. LS, HH, and QW independently evaluated the high-resolution chest computed tomography. QW, XY, CY, CW, XZ, ZP, YG, ML, and XZ contributed to the critical revision of the report. All authors contributed to data acquisition, data analysis, or data interpretation, and they all reviewed and approved the final version. QW and ML are responsible for the overall content as guarantors.

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Patient consent for publication

Consent obtained directly from patient(s).

Ethics approval

This study was approved by the Peking Union Medical College Hospital Institutional Review Board (ethical number JS-2038), and we obtained written informed consent from each patient.

Provenance and peer review

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Data availability statement

Individual participant data are available from the corresponding author on reasonable request.

Supplementary materials

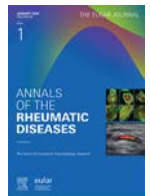
Supplementary material associated with this article can be found in the online version at [doi:10.1016/j.arid.2025.02.017](https://doi.org/10.1016/j.arid.2025.02.017).

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Systemic sclerosis

Spatial multiomics decipher fibroblast–macrophage dynamics in systemic sclerosis

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ABSTRACT

Objectives: Stromal-immune crosstalk shapes the pathogenic microenvironment of systemic sclerosis (SSc), but the spatial regulatory networks underlying fibrogenesis remain poorly defined. We aimed to explore tissue organisation and cell coordination in SSc skin, providing spatiotemporal insights into disease mechanisms and bridging the gap between omics discovery and precision medicine.

Methods: We performed spatial transcriptomics on skin biopsies from 10 patients with diffuse cutaneous SSc and 4 healthy controls using the 10× Visium platform. These findings were confirmed using higher-resolution Stereo-seq transcriptomics, spatial proteomics, and single-cell RNA sequencing data from patients with SSc, SSc mouse models, and wound-healing reindeer models. *In vivo* and *in vitro* studies were conducted to validate the key regulatory pathways.

Results: Fourteen skin biopsies were analysed, revealing significant expansion of fibrotic niches enriched with fibroblasts and macrophages in SSc, correlating with clinical severity. We revealed disease-specific cell states of fibroblasts and macrophages and evaluated their spatial

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dependency and cell–cell communication. Stratification based on signature genes enabled the identification of patients with SSc with progressive disease and treatment-nonresponsive phenotype. ACKR3 (a CXCL12 decoy receptor) was selectively expressed in myofibroblast progenitors, which diminished during differentiation towards mature myofibroblast, potentially serving to regulate CXCL12/CXCR4-mediated proinflammatory macrophage recruitment. Inhibition of CXCR4 attenuated skin and lung fibrosis in experimental fibrosis mouse models.

Conclusions: Our spatially resolved atlas uncovered dynamic fibroblast–macrophage interplay as a hallmark of fibrotic niche expansion. These findings offer spatiotemporal insights into disease mechanisms and pave the way for advanced mechanistic and therapeutic studies, bridging the gap between omics discovery and precision medicine.

WHAT IS ALREADY KNOWN ON THIS TOPIC

- Translating omics discoveries into clinically actionable strategies, such as biomarker-driven patient stratification or mechanism-based target identification, remains a critical unmet challenge in systemic sclerosis (SSc).
- While single-cell omics have revolutionised our understanding of cellular heterogeneity in SSc, integrating spatial context is essential to unravel tissue architecture and cell–cell coordination driving fibrogenesis.
- Although stromal-immune crosstalk, particularly fibroblast–macrophage interactions, has been implicated in tissue fibrosis, the niche-specific spatiotemporal dynamics of these interactions in SSc are not fully resolved.

WHAT THIS STUDY ADDS

- Integrated spatial omics elucidated the fibrotic microenvironment in SSc skin, showing spatial organisation, transcriptional profiles, dynamic interactions, and their links with clinical outcomes.
- Cross-tissue and cross-species analyses identified a core differentiation axis from myofibroblast progenitors to terminally differentiated myofibroblasts, highlighting shared mechanisms in fibrotic remodelling.
- In-depth cell communication analysis uncovered fine-tuned regulatory events involving fibroblasts and macrophages, which may potentially drive the progression of SSc.
- A myofibroblast progenitor-specific chemokine regulatory mechanism is diminished with the disease progression, implicating fibroblast–macrophage crosstalk in sustaining fibrosis and nominating targetable pathways for intervention.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- Our study deciphers the microenvironment driving SSc progression, identifies potential therapeutic targets, and highlights the potential of omics-based stratification to predict treatment response, bridging the gap between omics discovery and precision medicine in clinical practice.

INTRODUCTION

Systemic sclerosis (SSc), an orphan autoimmune disease, is characterised by the progressive fibrosis affecting skin and multiple organs, holding the highest mortality among all rheumatic diseases [1]. The development of effective therapeutic strategies for SSc is impeded by a lack of comprehensive understanding of its pathogenesis and the limited translatability of current pre-clinical models. Importantly, studying SSc as a prototypical systemic fibrotic disease could yield new insights into the pathogenesis of fibrotic diseases and aid in the discovery of novel therapeutic approaches.

SSc is characterised by a complex interplay of features that include vasculopathy, inflammation, and fibrosis. Within this triad, fibroblasts have been recognised as pivotal effector cells in the production of extracellular matrix, largely contributing to developing fibrotic tissue remodelling in SSc. Fibroblasts sense and receive signalling from the neighbouring cells, which are conveyed and converged to the shared and distinct profibrotic pathways [2]. The coordination of fibroblasts with macrophages can shape their cellular response, therefore orchestrating the initiation or resolution of fibrosis across tissues [3–9]. Beyond the conventional macrophage polarisation paradigm, new populations of macrophages have been linked with tissue repair and fibrotic remodelling [3–7,10]. For instance, phenotypically distinct macrophages (LYVE1^{lo}MHCII^{hi} and LYVE1^{hi}MHCII^{lo}) manifested specific niche-dependent functional roles in pulmonary and cardiac fibrosis [10]. However, it remains largely unexplored whether and how the spatial neighbourhood in the tissue architecture affects the crosstalk between fibroblast and macrophage subsets, potentially driving fibrotic remodelling in SSc.

Recent studies using single-cell RNA sequencing (scRNA-seq) have provided an unprecedented opportunity to study the molecular and cellular mechanisms of SSc at single-cell resolution [11–15]. Pioneering studies have characterised fibroblast types and states in SSc [11–13,15]. While these studies have provided valuable insights, they are significantly constrained by the lack of spatial resolution due to the reliance on disaggregated tissue analysis. We recently demonstrated cellular niches centred around endothelial cells (ECs) or fibroblasts in SSc using a panel of up to 40 markers through multiplex spatial proteomics [16,17]. Thus, a spatially resolved characterisation of genome-wide transcriptomics of SSc is needed.

In this work, we profiled human skin from patients with SSc and healthy controls using spatial transcriptomics based on 10× Visium formalin-fixed paraffin-embedded platform and spatial-enhanced resolution omics sequencing (Stereo-seq) [18]. To increase resolution, we integrated 2 publicly available scRNA-seq data sets, creating a human skin transcriptome atlas and mapping cell type information onto spatial regions. Validation included higher-resolution spatial profiling (eg, imaging mass cytometry [IMC]) and time-course experimental skin fibrosis. These methods characterised cellular heterogeneity and pathologic interactions between fibroblast and macrophage subsets, identifying potential therapeutic targets through disruption of disease-associated cellular niches. This work connects multi-omics findings to clinically relevant strategies for SSc.

METHODS

A detailed description of all materials and methods are provided in [Supplementary Methods](#). All human studies were approved by the ethical committee of the Medical Faculty of

Fudan University. All patients and controls signed an informed consent form approved by the local institutional review board. Statistical measures, including the number of samples, descriptive statistics (median and IQR), and significance, are reported in the figure and figure legends. *P* values of <.05 were considered statistically significant.

RESULTS

Profiling of SSc and healthy skin with spatially resolved transcriptomics

We collected 14 human skin biopsies from 10 patients with diffuse cutaneous SSc with a median disease duration of 8 months since the first non-Raynaud phenomenon (RP), as well as from 4 sex-matched and age-matched normal controls (Fig 1A; Supplementary Table S1). For each biopsy, we generated spatially resolved transcriptomics using the 10× Visium platform and retained 13,204 high quality spots, with a median detection of 2265 genes per spot after removing low quality data (Supplementary Fig S1A-D; Supplementary Table S2). To enhance spatial resolution, we also performed Stereo-seq on 4 of these skin samples, including 3 from patients with SSc and 1 from a healthy donor. After controlling data quality, we retained 40,566 spots across these samples. We additionally analysed 2 scRNA-seq data sets generated from skin tissues of patients with SSc (*n* = 104) and healthy donors (*n* = 64) [11,12] (Fig 1B,C; Supplementary Fig S2). We deconvoluted the cell type compositions of each spot in 10× Visium data using the scRNA-seq as reference. The estimated cell type abundances from spatial transcriptomics were generally colocalised with their respective marker genes and locations (Fig 1D). For instance, keratinocytes were found localised within the epidermis and surrounding the hair follicle. We also inferred the pathway activity for each spot and observed a strong association between marker expression and some well-known targetable pathways, such as Janus kinase signal transducer and activator of transcription protein (JAK-STAT) [19–22], transforming growth factor (TGF)- β [2], and interleukin (IL)-6 [23–25] using PROGENy pipeline (Fig 1D). We next transferred the cell type annotation from scRNA-seq to Stereo-seq through data integration and observed a similar cellular distribution as 10× Visium data (Fig 1E,F). Collectively, our analyses of spatial transcriptomics and scRNA-seq data enables us to map cell type information to spatial for skin samples.

Characterisation of tissue organisation in SSc and healthy skin

To characterise the cellular colocalisation events in human skin, we clustered the spots based on estimated cell type proportions and identified 12 cell type niches (Fig 2A; Supplementary Fig S3A). Visualisation of the cell type abundances and marker genes of these niches revealed that they were strongly associated with the corresponding cell types (Fig 2B,C; Supplementary Fig S3B,C). Furthermore, we observed a significant increase in the proportions of 3 niches (0, 1, and 4) in the SSc skin compared with those in healthy skin (Fig 2D,E; Supplementary Fig S3D,E). Notably, niche 0 was infiltrated with a high prevalence of fibroblasts, macrophages, conventional dendritic cells (cDCs), and mast cells, indicating a potential immune microenvironment within a fibrogenic niche. Niche 1 was characterised by an enrichment of ECs, pericytes, macrophages, cDCs, and mast cells, suggesting a perivascular niche associated with immune cell infiltration in SSc skin. Niche 4 was distinguished by a significant presence of plasma cells, melanocytes, and neural cells.

Decoupler pathway activity analyses for each niche demonstrated the highest activity of the TGF- β signalling pathway in fibroblast-dominant niche 0 (Fig 2F). Niche 9, composed of various immune cells, showed an increased trend in proportion and exhibited the strongest activity of JAK-STAT signalling. KEGG pathway enrichment analysis based on marker genes also revealed high activity in the TGF- β signalling in niche 0 (Supplementary Fig S3F). These findings supported the essential roles of TGF- β and JAK-STAT signalling in the pathogenesis of SSc as expected [2], suggesting a convergence of multiple signalling activities within the structural organisation in SSc fibrosis.

Notably, we observed colocalisation of fibroblasts and macrophages in our 10× Visium and Stereo-seq data (Fig 2G,H). To validate these results, we performed IMC and identified the cellular neighbourhood (CN) community as described previously [17]. We observed similar niches comprising stromal cells and macrophages in CN8, while we found CN5 containing vascular ECs and immune cells, such as macrophages (Supplementary Fig S4A-C).

We then correlated the niche proportions detected in 10× Visium data with clinical data across the SSc samples and observed a positive Spearman correlation between the proportion of niche 0 and the current total modified Rodnan skin score (mRSS) (r = 0.733; P = .023), C-reactive protein levels (r = 0.750; P = .066), and platelet counts (r = 0.697; P = .031), which are interpreted as clinical indicators for disease severity or activity of SSc (Fig 2I). Additionally, we observed a significant negative correlation between the proportion of niche 1 and the non-RP disease duration (r = -0.756; P = .014). No correlations were observed between niche proportion and clinical outcomes, such as forced vital capacity (FVC)% predicted or diffusing capacity of the lungs for carbon monoxide% predicted (Supplementary Fig S3G). In summary, our spatial multiomics revealed a distinct structural organisation associated with disease severity and activity, suggesting a potential coordinated regulation of immune-stromal interactions that may drive SSc progression.

Characterisation of fibroblast subclusters in healthy and SSc skin

We next clustered the scRNA-seq data for fibroblasts and identified 10 distinct subclusters. Consistent with source studies [11,12], we found the corresponding fibroblast populations, including *PCOLCE2*⁺/*LGR5*⁺ (Fib1), *MYOC*⁺/*C3*⁺/*APOD*⁺ (Fib3), *CCL19*⁺/*C7*⁺/*APOE*⁺ (Fib4), *SFPR2*⁺/*WIF1*⁺/*APCDD1*⁺ (Fib6), *COCH*⁺/*SPARCL1*⁺ (Fib7), *COMP*⁺/*SFPR2*⁺/*PRSS23*⁺ (Fib9), and *IGFBP2*⁺/*ANGPTL7*⁺ (Fib10) (Fig 3A,B; Supplementary Fig S5A). Additionally, Fib5 was characterised by the expression of *FOS* and *JUNB*, which were components of activator protein 1 known as the important transcription factors (TFs) in SSc fibrosis [26,27]. Fib1 demonstrated the upregulation of *SCARA5*, which has been recently identified as a marker for myofibroblast progenitor in human myocardial and kidney fibrosis [28–30]. Fib2 and Fib9 were characterised by the expression of periostin (*POSTN*), encoding an extracellular matrix protein that can amplify TGF- β signalling [31,32]. In addition, Fib2 also exhibited a high expression of myofibroblast hallmark *ACTA2* (Fig 3B). Moreover, we observed a significant reduction of myofibroblast progenitor Fib1 and an increase of myofibroblast Fib2 and Fib9 in SSc skin, compared with those in healthy skin (Fig 3C).

Interestingly, Fib2 was characterised by high TF activity of the Hedgehog transcription factor *GLI2* (Supplementary Fig S5B). This convergent driver can mediate Hedgehog pathways

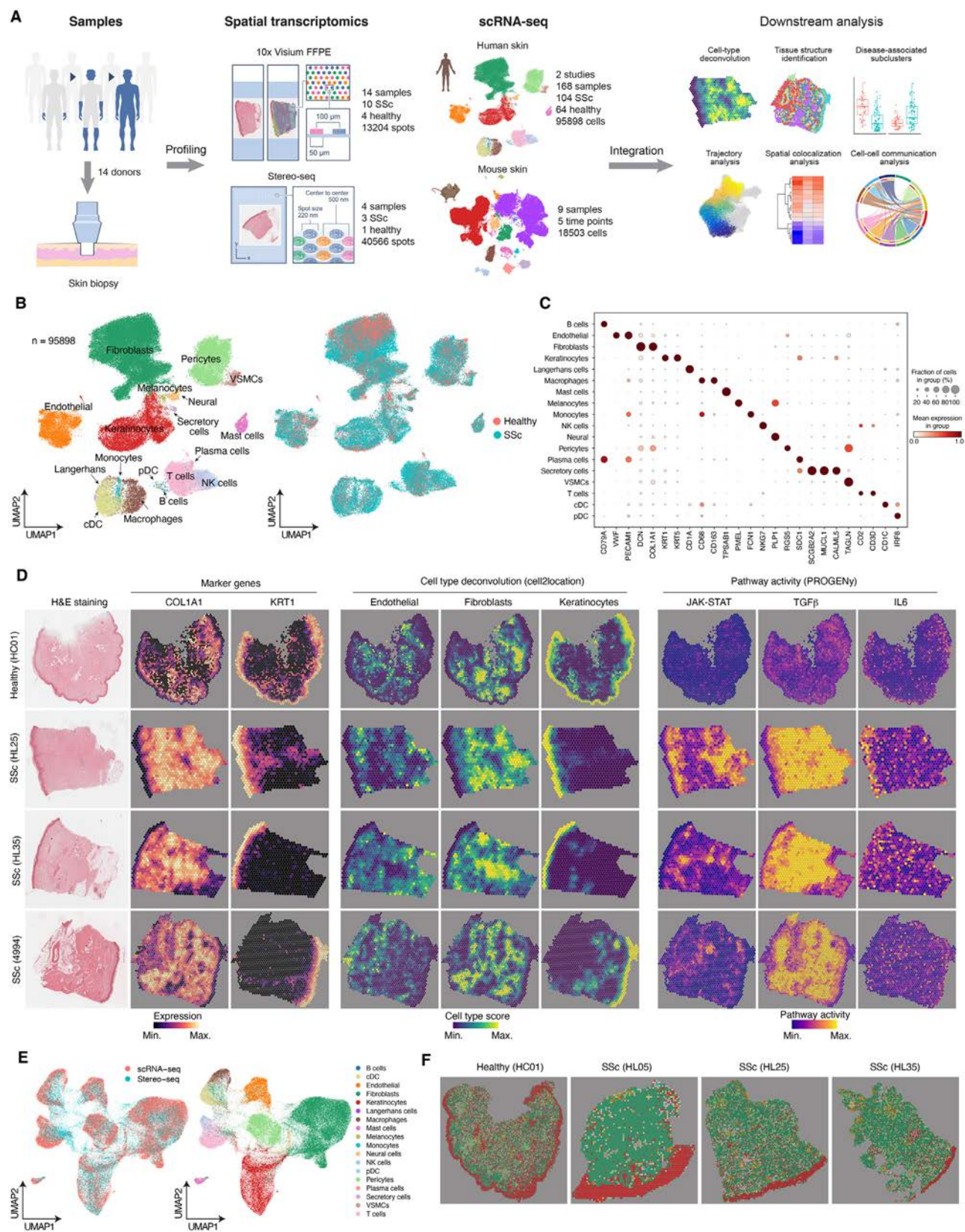


Figure 1. Spatial profiling of human skin. (A) Overview of experimental design and analysis workflow. (B) Uniform manifold approximation and projection (UMAP) showing integrated single-cell RNA sequencing (scRNA-seq) data sets as coloured by cell types (left) and conditions (right). Systemic sclerosis (SSc) skin, n = 104; healthy skin, n = 64. (C) Dotplot showing gene expression for each cell type. The colours refer to the mean expression of the genes and the dot sizes the proportion of cells expressing the marker genes. (D) Hematoxylin and eosin staining and the corresponding visualisation maps of based on x10 Visium (SSc skin, n = 10; healthy skin, n = 4). Left: *COL1A1* and *KRT1* gene expressions in spatial transcriptomics data. Middle: representative cell types estimated by cell2location. Right: visualisation of pathway activity estimated by their target genes in spatial space. (E) Integration of scRNA-seq and Stereo-seq data coloured by protocols (left) and cell types (right). (F) Visualisation of cell types in spatial space based on Stereo-seq data. SSc skin, n = 3; healthy skin, n = 1. The experiments were performed on adjacent tissue sections to those used for x10 Visium.

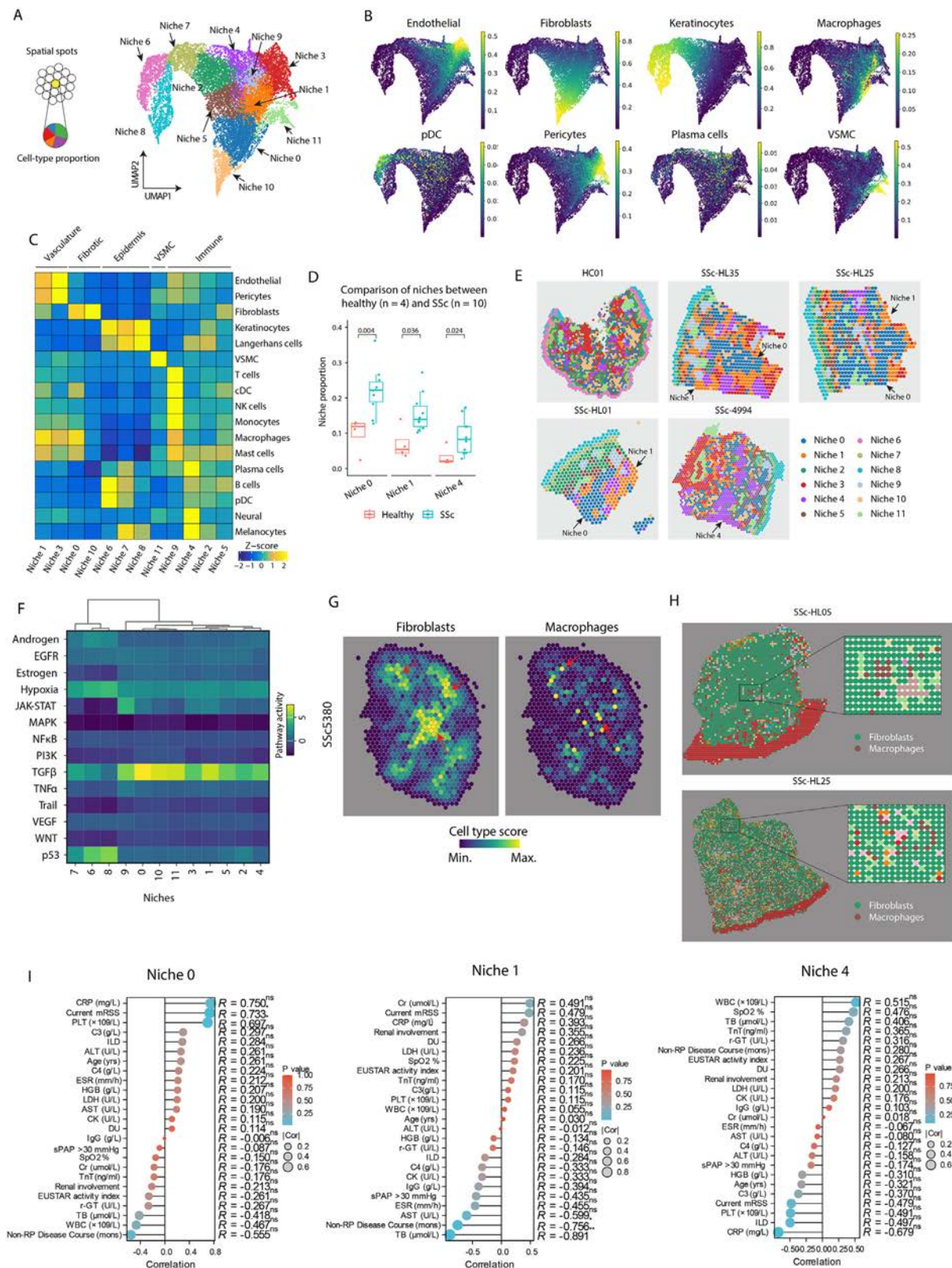


Figure 2. Characterisation of cell type niches in systemic sclerosis (SSc) and healthy skin. (A) Uniform manifold approximation and projection (UMAP) showing the integrated spatial data as coloured by cell type niches. (B) Visualisation of cell type proportion in UMAP space. (C) Heatmap showing the enrichment of cell types in niches. Each column represents a niche and each row a cell type. Colours refer to the z-score—transformed proportions of each cell type across different niches. (D) Boxplot comparing the proportion of each niche between SSc and healthy skin samples. Each dot represents a sample. P values are calculated using the Wilcoxon rank-sum test (unpaired; 2 sides). (E) Visualisation of cell type niches in spatial space for the healthy and SSc samples using $\times 10$ Visium data. (F) Heatmap showing pathway activity for each niche. (G) Visualisation of fibroblasts and macrophages in spatial space using $\times 10$ Visium data. (H) Visualisation of cell types in spatial space using Stereo-seq data. (I) Correlations between the proportions of the indicated niches with clinic statistics across all subjects with SSc. Statistical significance was determined by the Spearman test for a 2-variable correlation. The correlation coefficient r is indicated in figure (* $P < .05$). ALT, alanine aminotransferase; AST, aspartate aminotransferase; C4, complement component 4; CK, creatine kinase; Cr, creatinine; CRP, C-reactive protein; C3, complement component 3; DU, digital ulcer; ESR, erythrocyte sedimentation rate; EUSTAR, European Scleroderma Trials and Research Group; HGB, haemoglobin; IgG, immunoglobulin G; ILD, interstitial lung disease; LDH, lactate dehydrogenase; mRSS, modified Rodnan skin score; PLT, platelet count; r-GT, gamma-glutamyl transferase; RP, Raynaud phenomenon; sPAP, systolic pulmonary arterial pressure; SpO₂, oxygen saturation; TB, total bilirubin; TnT, troponin T; WBC, white blood cell.

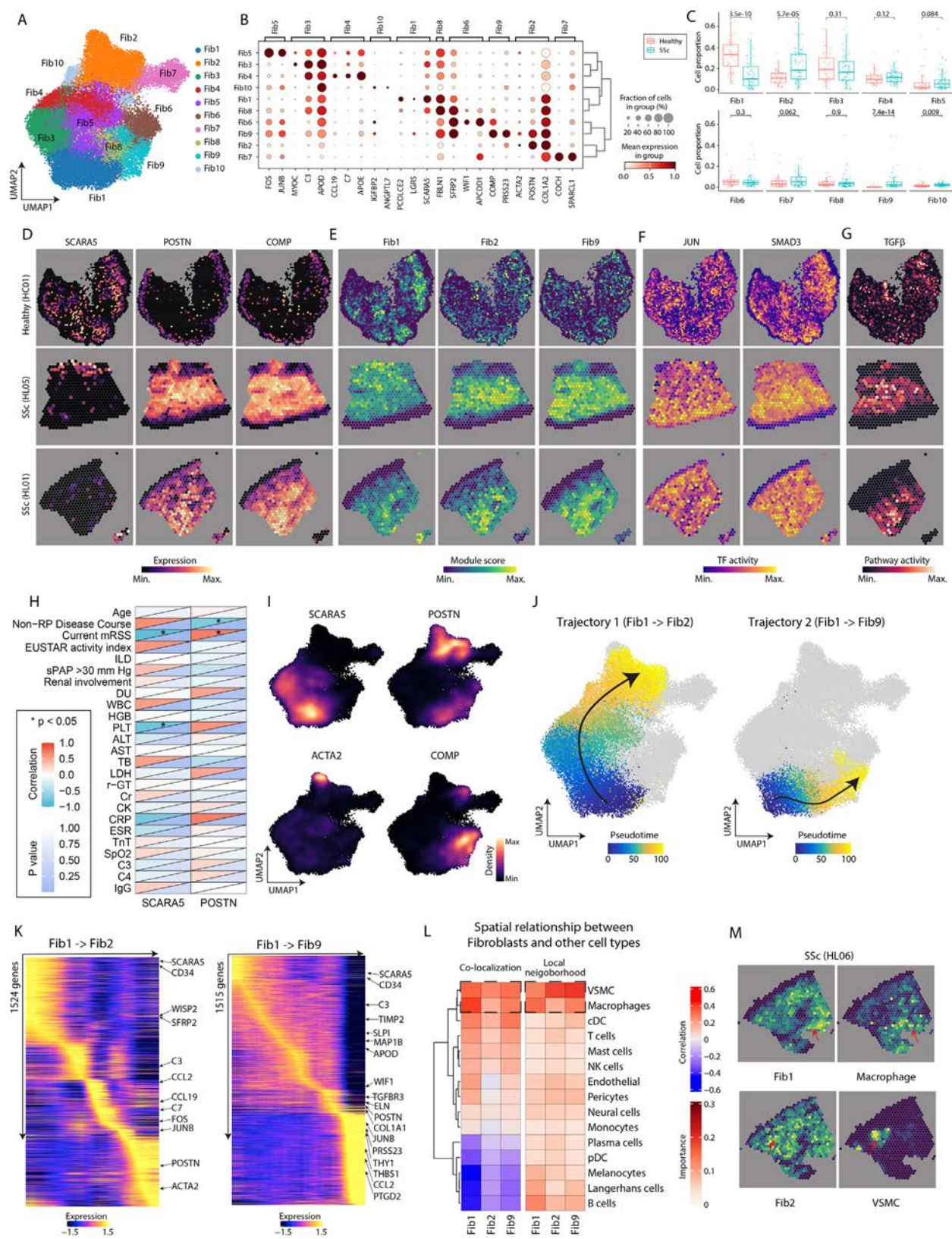


Figure 3. Characterisation of fibroblast subpopulations. (A) Uniform manifold approximation and projection (UMAP) showing subpopulations of fibroblasts based on single-cell RNA sequencing (scRNA-seq) data. (B) Dot plot showing the marker genes of each subset. (C) Bar graph comparing the proportion of each fibroblast subset between healthy and systemic sclerosis (SSc) samples. Each dot represents a sample. P values are calculated using the Wilcoxon rank-sum test (unpaired; 2 sides). (D) Visualisation of *SCARAS*, *POSTN*, and *COMP* expression in spatial space. (E) Visualisation of module score of Fib1, Fib2, and Fib9 in spatial space. (F) Visualisation of transcription factor (TF) activity of JUN and SMAD3 in spatial space. (G) Visualisation of transforming growth factor (TGF)- β pathway activity in spatial space. (H) Heatmap showing correlations between expressions of *SCARAS* and *POSTN* and different clinical outcomes of SSc. The colours in the upper left square represent Spearman r value, while those in the lower right square represent P values ($*P < .05$). (I) Expression of *SCARAS*, *POSTN*, *ACTA2*, and *COMP* overlaying with UMAP. (J) UMAP showing the differentiation trajectory from Fib1 to Fib2 (left) and Fib1 to Fib9 (right). Colours refer to pseudotime points. (K) Heatmap showing the gene expression dynamics during the trajectory from Fib1 to Fib2 (left) and Fib1 to Fib9 (right). (L) Colocalisation between major cell types and fibroblast subclusters (Fib1, Fib2, and Fib9) as measured within the same spot (left) and neighbouring spots (right). (M) Visualisation of the abundance of macrophages and

in activating fibroblast and promoting fibrosis in SSc [27,33]. Fib9 was marked by *SMAD3*, *SMAD4*, and *JUN*, all of which are TGF- β downstream pathways to promote fibrosis. We also analysed a bulk RNA-seq dataset from the prospective registry of early systemic sclerosis (PRESS) cohort [34] and observed the significant increase of *POSTN* and decrease of *SCARA5* in SSc skin compared with healthy skin (Supplementary Fig S5C). An inverse correlation was also observed in the gene expressions between *SCARA5* and *POSTN*. Overall, these findings further supported the potential involvement of *SCARA5*⁺ and *POSTN*⁺ fibroblasts in the pathogenesis of SSc.

We next analysed our spatial transcriptomics data and found that *SCARA5* was significantly downregulated in SSc skin, whereas *POSTN* was upregulated in SSc skin (Supplementary Fig S5D). Additionally, *SCARA5* exhibited distinct spatial enrichment patterns that were mutually exclusive to regions enriched for *POSTN* and *COMP* across multiple samples (Fig 3D; Supplementary Fig S5E). Estimation of module scores using marker genes for each subset revealed that Fib2 and Fib9 were spatially colocalised (Fig 3E; Supplementary Fig S5F). Moreover, we observed increased activities of JUN, SMAD3, and the TGF- β pathway, which were closely associated with the spatial localisation of Fib2 and Fib9 (Fig 3F,G; Supplementary Fig S5G,H). These findings aligned with their known profibrotic roles, further confirming the spatial mapping of these subclusters. Furthermore, we found that *SCARA5* expression negatively correlated with the mRSS, whereas *POSTN* expression positively correlated with mRSS (Fig 3H). Additionally, *SCARA5* showed an inverse correlation with platelet counts, while *POSTN* negatively correlated with non-RP disease duration. These results collectively suggested divergent effects of *SCARA5*⁺ and *POSTN*⁺ fibroblasts in SSc.

Differentiation of *SCARA5*⁺ to *POSTN*⁺ fibroblasts in SSc skin

Several studies have identified *SCARA5*⁺ fibroblasts as myofibroblast progenitors in fibrosis [28–30]. To determine whether this is true for SSc skin fibrosis, we integrated scRNA-seq data from mouse skin challenged by bleomycin (BLM) at various time points (days 0, 3, 7, 14, and 28) [35], identifying 14 major cell types and 9 fibroblast subclusters (Supplementary Fig S6A,B). Interestingly, we found a progressive decrease in *Scara5*⁺ fibroblasts following BLM treatment, while *Postn*⁺ fibroblasts substantially increased on days 14 and 28 (Supplementary Fig S6C,D). These temporal changes suggested a potential differentiation process from *Scara5*⁺ to *Postn*⁺ fibroblasts. Treatment of TGF- β or IL-1 β induced the downregulation of *SCARA5* and upregulation of *POSTN* and *ACTA2* in primary cultured human dermal fibroblasts (Supplementary Fig S6E).

Based on these observations, we next inferred 2 pseudotime trajectories from Fib1 to Fib2 (trajectory 1) and Fib1 to Fib9 (trajectory 2) (Fig 3I,J). We next estimated the expression variation of each gene along the pseudotime and identified 1524 and 1515 genes associated with trajectories 1 and 2, respectively. For trajectory 1, we found many genes were downregulated during the differentiation, including precursor marker *CD34* [36] and *SFRP2* [11] (Fig 3K; Supplementary Fig S6F). Moreover, we

identified a set of genes, including *C3*, *C7*, *CCL2*, and *CCL19*, which were particularly enriched at the intermediate stage of the trajectory, suggesting the involvement of chemokine and complement signalling during the differentiation process. Genes associated with the late stage of this trajectory included *FOS* and *JUNB*. For trajectory 2, we observed that *TIMP2*, *TGFBR3*, and *APOD* were additionally associated with the intermediate stage (Fig 3K; Supplementary Fig S6G). These results revealed shared and distinct gene regulatory networks that are involved in the divergent differentiation of fibroblasts.

Interestingly, Sinha et al [7] conducted comparative single-cell multiomics in reindeer following full-thickness injury and found that the velvet skin regenerates, while the back skin heals by forming a scar [7]. Similarly, in reindeer skin, *SCARA5* gradually decreased but *ACTA2* increased after wound in their back, but not in velvet (Supplementary Fig S7). This pattern is reminiscent of fibrotic tissue remodelling in human, which indicates the fibroblast dynamic changes could be conserved across species and tissues.

Spatial colocalisation of fibroblast subclusters and other cell types

To investigate spatial dependencies between fibroblast subsets and other major cell types, we conducted spatial colocalisation analyses in 2 distinct proximity areas: (1) co-occurrence within 1 single spot, determined by Spearman correlation between the module score of each fibroblast subcluster and the proportion of each major cell type, and (2) extended neighbourhood within an annular region encompassing up to 3 surrounding spots for each target spot analysed with the MISTy pipeline [37]. We focused specifically on Fib1, Fib2, and Fib9, as they have been demonstrated to exhibit significant altered in SSc (Fig 3C). We observed a consistent spatial dependency in both models across tissues, characterised by an increased co-occurrence between myofibroblast progenitor *SCARA5*⁺ Fib1 and macrophages, whereas *POSTN*⁺/*ACTA2*⁺ myofibroblasts (Fib2 and Fib9) were found in proximity to vascular smooth muscle cells (Fig 3L,M). These findings highlighted the potential critical role of macrophage in establishing the spatial microenvironment surrounding fibroblast from the early stage.

Correlations of the cell type niche and cellular phenotypes with clinical outcomes of SSc

We next associated the cell type niches with the clinical outcomes and treatment responsiveness in patients with SSc. All patients with SSc included in our study were under the standard treatment with mycophenolate mofetil (MMF) for consecutive 52 weeks. The proportion of niche 0, mainly comprising fibroblasts and macrophages, was significantly higher in the patients with SSc who were nonresponsive to a 52-week standard treatment of MMF, compared with those who were responsive (median proportion in responder, 18.28%, *n* = 5; median proportion in nonresponder, 25.00%, *n* = 5; *P* = .0079) (Fig 4A). The responsiveness was defined according to the criteria of the revised ACR-CRISS (American College of Rheumatology Composite Response Index in Systemic Sclerosis), which is the

vascular smooth muscle cells (VSMCs) and the state scores of Fib1 and Fib2 in a representative SSc skin sample. ALT, alanine aminotransferase; AST, aspartate aminotransferase; C4, complement component 4; CK, creatine kinase; Cr, creatinine; CRP, C-reactive protein; C3, complement component 3; DU, digital ulcer; ESR, erythrocyte sedimentation rate; EUSTAR, European Scleroderma Trials and Research Group; HGB, haemoglobin; IgG, immunoglobulin G; ILD, interstitial lung disease; LDH, lactate dehydrogenase; mRSS, modified Rodnan skin score; PLT, platelet count; γ -GT, gamma-glutamyl transferase; RP, Raynaud phenomenon; sPAP, systolic pulmonary arterial pressure; SpO₂, oxygen saturation; TB, total bilirubin; TnT, troponin T; WBC, white blood cell.

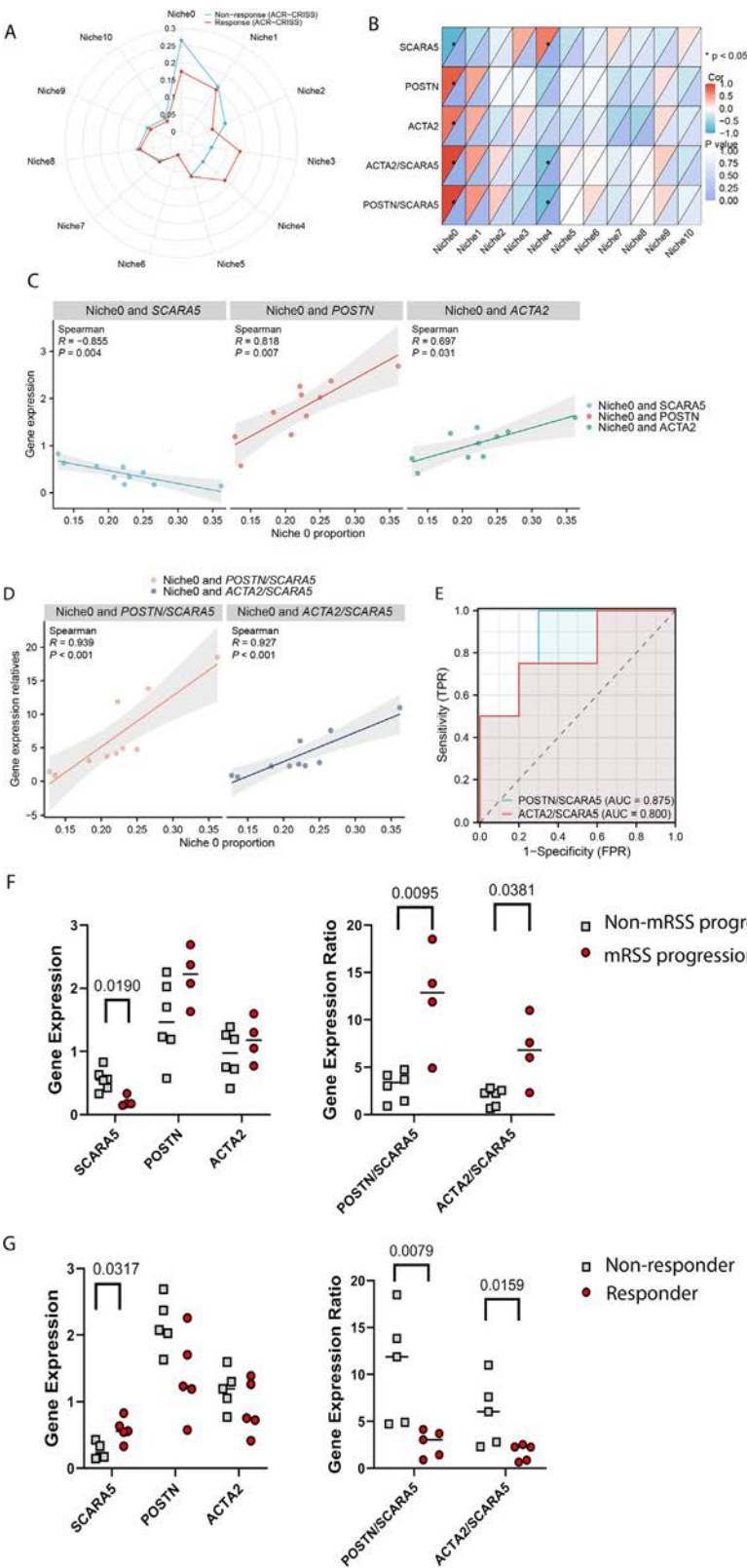


Figure 4. Correlations of the cellular phenotype with clinical outcomes. (A) A radar plot displaying the proportions of the identified niches in patients with systemic sclerosis (SSc), distinguishing between those who were responsive to the standard treatment of mycophenolate mofetil (MMF) and those who were not. (B) Heatmap showing correlations between the proportion of each niche and the pseudobulk gene expression, along with the ratios of *POSTN/SCARA5* and *ACTA2/SCARA5*. The colours in the upper left square represent Spearman r value, while those in the lower right square represent P values ($*P < .05$). (C, D) Correlations between the proportion of niche 0, which was predominantly composed of fibroblasts and macrophages, and the gene expressions of *SCARA5*, *POSTN*, and *ACTA2* (C), along with their ratios (D). Correlation data were analysed using nonparametric Spearman tests. Each data point represents 1 SSc sample, and the CI is indicated by grey shadows. (E) Receiver operating characteristic (ROC) curve showing the discrimination of patients with SSc from healthy controls based on the gene expression ratios of *POSTN/SCARA5* and *ACTA2/SCARA5*, respectively. The area under the curve (AUC) values are indicated in graphs. (F) The gene expressions of *SCARA5*, *POSTN*, and *ACTA2*, along with their ratios, comparing the patients with SSc who experienced skin fibrosis progression with those who were not. (G) The gene expression of *SCARA5*, *POSTN*, and *ACTA2*, along with their ratios, comparing the patients with SSc who were responsive to the standard treatment of MMF with those who were not, evaluated at a 52-week follow-up. Two-sided Wilcoxon rank-sum tests were conducted to compare the differences between the 2 groups.

improvement in ≥ 3 of 5 core clinical measurements with minimal clinically important difference of 25% for mRSS, 5% for FVC% predicted, 20% for health assessment questionnaire-disability index, and 20% for patient and clinician global assessments [38].

Consistently, the proportions of niche 0, enriched with fibroblasts and characterised by high activity of TGF- β signalling, correlated negatively with *SCARA5* and positively with *POSTN* and *ACTA2*, and more pronounced with the gene expression

ratios of *POSTN/SCARA5* and *ACTA2/SCARA5* (Fig 4B-D). Furthermore, the ratios of *POSTN/SCARA5* and *ACTA2/SCARA5* manifested the ability to discriminate the patients with SSc from healthy controls with the area under the curve of 0.875 and 0.800 ($P = .0339$ and $.0897$, respectively) (Fig 4E). Those baseline ratios were observed to be significantly increased in the patients who would experience skin fibrosis progression (Fig 4F), determined as over 25% increment in mRSS assessed at 52-week follow-up. Notably, the higher baseline ratios of

POSTN/SCARA5 and *ACTA2/SCARA5* were closely linked to the nonresponsiveness for the standard treatment with MMF for 52 weeks (Fig 4G). These data highlighted the potentials of aforementioned gene expression ratio in identification of the standard treatment–refractory patients with SSc, who may require more advanced treatment regimens rather than standard MMF.

To further validate our findings, we analysed the basal gene expression using bulk RNA-seq data from 6 patients with SSc who received various immunosuppressant and antifibrotic treatment (Supplementary Table S3). We observed an upwards trend in *POSTN/SCARA5* in patients with SSc with skin fibrosis progression and nonresponsiveness to treatment (Supplementary Fig S5I). After normalising and pooling RNA-seq data from 16 patients with SSc with different treatment backgrounds, we confirmed these trends, with higher *POSTN/SCARA5* levels in patients showing mRSS progression or treatment refractoriness (Supplementary Fig S5J). Similar results were seen in the genetics of inflammatory skin disease and systemic outcomes study (GENISOS) cohort (Supplementary Fig S5K; Supplementary Table S4). These data may provide insights into the future precision stratification strategy for patients with SSc.

Characterisation of macrophage heterogeneity

We next clustered the scRNA-seq data of macrophages and identified 3 distinct subsets (Fig 5A; Supplementary Fig S8A). We observed 1 cluster highly expressing proinflammatory cytokine *IL-1 β* , as well as *CXCL8*, *CCL3*, *CXCL2*, and *PTGS2*, thus termed as proinflammatory macrophages (Fig 5B). This subset also highly expressed *NR4A1*, *NR4A2*, and *NR4A3*, which have been identified as immediate early genes in response to various stimuli and have been linked to the pathogenesis of fibrotic tissue remodelling [39,40]. We also identified a subcluster characterised by phagocytic markers, including *MARCO*, *LYVE1*, *CD36*, *FCGRT*, and *C1QA*, therefore defined as phagocytic macrophages. The scavenging receptor CD36, known to mediate uptake in macrophage, has been implicated in the pathogenesis of tissue fibrosis [41,42]. *MARCO* was found to be upregulated in tissue-resident macrophages and circulating monocytes upon activation, and *MARCO*⁺ myeloid cells have been recognised as therapeutic targets for organ fibrosis in SSc [43,44]. *LYVE1*-expressing macrophage has been demonstrated to form coordinated multicellular nest structures that are distributed proximal to vasculature [45]. Another subset of macrophage that may be involved in the antigen presentation process is characterised by their overexpression of *HLA-B* and *HLA-C*, hereafter referred to as antigen-presenting macrophages. Indeed, the antigen-presenting macrophage and phagocytic macrophage defined in this study largely resembled previously identified *LYVE1*^{lo}*MHCII*^{hi} and *LYVE1*^{hi}*MHCII*^{lo} macrophages, which are conserved across tissues [10]. Notably, the *LYVE1*^{hi}*MHCII*^{lo} macrophages have been demonstrated to play a critical role in maintaining homeostasis, as depletion of *LYVE1*^{hi}*MHCII*^{lo} macrophages exacerbated experimental lung and heart fibrosis, along with exacerbated vessel permeability, immune infiltration, and collagen deposition [10].

The comparison of cell proportion between healthy and SSc samples showed that proinflammatory macrophages were significantly enriched in SSc, whereas the abundance of phagocytic macrophages was significantly decreased in SSc samples (Fig 5C). We also estimated TF activity for each cluster and observed a high activity of REL, RELA, and RELB in proinflammatory macrophages (Supplementary Fig S8B). These TFs are key members of the NF- κ B pathway that regulates immune and

inflammatory responses [46], further supporting the functions of proinflammatory macrophages.

Crosstalk between fibroblast and macrophage subclusters

Our analysis of spatial transcriptomic data revealed a strong spatial association between macrophages and fibroblasts, underscoring their potential functional interplay within the tissue microenvironment (Figs 2C and 3L). To further investigate the cellular crosstalk between specific macrophage and fibroblast subtypes, we performed a detailed cell–cell communication analysis using scRNA-seq data based on ligand–receptor (L–R) networks, identifying 393 significant interactions (Fig 5D). Among macrophage subsets, proinflammatory macrophages exhibited the highest number of interactions with fibroblast subsets ($n = 179$ interactions), suggesting that they are central players in fibroblast-mediated signalling (Fig 5E). Particularly, Fib9 emerged as a key fibroblast subtype with the strongest connectivity to macrophages ($n = 83$ interactions), outpacing other fibroblast subsets in its interaction capacity.

Visualisation of the specific L–R interactions revealed that macrophages receive signals from fibroblasts through multiple signalling pathways, including THBS, MIF, CXCL, LAMININ, and COLLAGEN (Supplementary Fig S8C). Among them, receptor CXCR4 was observed to interact with fibroblast-derived ligands such as MIF and CXCL12 (Fig 5F). This suggested a mechanism by which fibroblasts recruit and retain proinflammatory macrophages, thereby sustaining local inflammation and contributing to the progression of fibrosis. On the contrary, fibroblasts also acted as signal receivers, engaging the receptor ACKR3 to interact with MIF and CXCL12 (Fig 5G; Supplementary Fig S8D). Notably, *MIF* and *CXCL12* were expressed by nearly all subtypes of fibroblasts and macrophages (Fig 5H,I), underscoring their widespread involvement in cellular crosstalk.

However, the expression of CXCR4 was specific to proinflammatory macrophages, while ACKR3 was uniquely expressed in the Fib1 myofibroblast progenitor subcluster (Fig 5H). A significant decrease in ACKR3 expression in SSc fibroblasts has been observed (Supplementary Fig S8E,F). To confirm at the protein levels, we applied tyramide signal amplification (TSA) high-plex immunofluorescence onto adjacent tissue sections. Our TSA staining confirmed the overlapping expression of ACKR3 in SCARA5⁺ fibroblast (Fig 5J). Again, we observed IL-1 β ⁺ macrophages, which were also positive for CXCR4, closely distributed adjacent to fibroblasts (Fig 5K). These results revealed a highly organised and reciprocal signalling network between fibroblasts and macrophages, underscored by distinct L–R interactions. Of particular interest, the chemokine CXCL12 can bind to its classic receptor CXCR4, a G protein–coupled receptor, facilitating the recruitment of CXCR4⁺ cells, such as proinflammatory macrophages in our case. Alternatively, CXCL12 can interact with its atypical chemokine receptor ACKR3 independent of G protein–coupled receptor mediated signalling. ACKR3 is capable of actively internalising its chemokine ligands CXCL12, through scavenging. This function helps to modulate the availability and gradient of CXCL12 in the extracellular environment [47]. The selective expression of ACKR3 in myofibroblast progenitors may indicate an endogenous compensation at an early stage. A dysfunction in this mechanism could lead to the subsequent CXCR4 mediated recruitment of macrophages, during the transition from myofibroblast progenitor to mature myofibroblast over the course of SSc progression. Pharmacologic inhibition of CXCR4 attenuated tissue fibrosis in bleomycin-induced

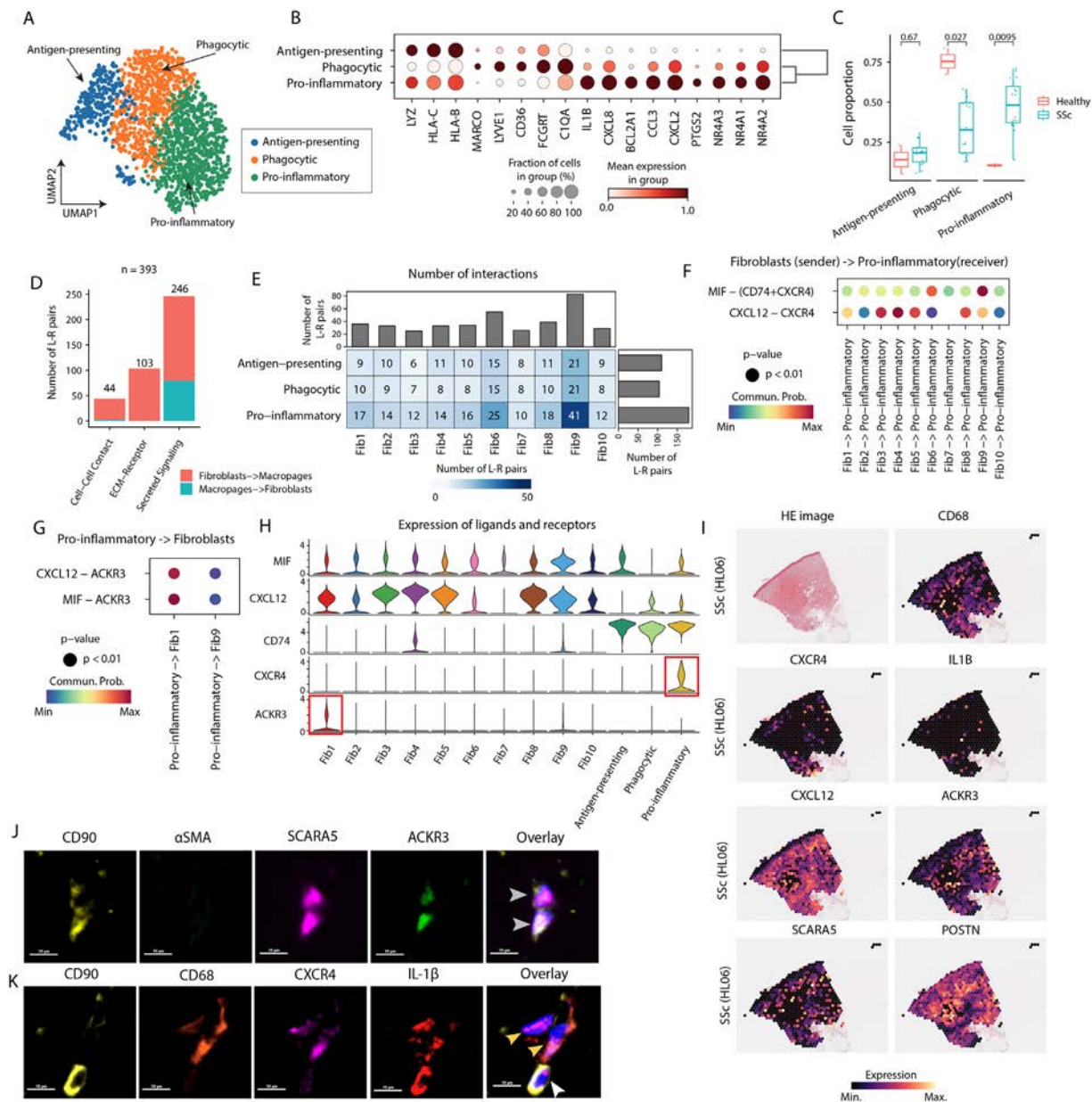


Figure 5. Characterisation of macrophage subclusters. (A) Uniform manifold approximation and projection (UMAP) showing subclusters of macrophages based on single-cell RNA sequencing (scRNA-seq) data. (B) Dotplot showing the marker genes for each subcluster. (C) Boxplot comparing the proportion of each subcluster between healthy and systemic sclerosis (SSc) samples. Each dot represents 1 sample. *P* values are calculated using the Wilcoxon rank-sum test (unpaired; 2 sides). (D,E) Number of significant ligand–receptor (L–R) pairs between the fibroblasts and macrophages. (F) Heatmap showing significant L–R pairs between fibroblast subsets and proinflammatory macrophages, with fibroblast serving as signalling senders and macrophage as receivers. The colours refer to the communication probability, and the sizes represent *P* values. (G) Heatmap showing significant L–R pairs between proinflammatory macrophages and fibroblast subsets (Fib1 and Fib9), as macrophage serving as signalling senders and fibroblast as receivers. (H) Violin plot showing the expression of ligands and receptors in scRNA-seq. (I) A representative hematoxylin and eosin (HE) staining and the expression of ligands and receptors in spatial space. (J) Representative images of tyramide signal amplification (TSA) staining on the expression of ACKR3 in SCAR5⁺ fibroblast, which are labelled with CD90 (indicated by grey arrows). (K) Representative images of TSA staining on the co-occurrence of fibroblasts (marked by CD90 and indicated by a white arrow) with interleukin (IL)-1β⁺ macrophages, which are also positive for CXCR4 (indicated by yellow arrows). The staining in (J) and (K) were performed on adjacent tissue sections to those used for ×10 Visium. Horizontal scale bars represent 10 μm.

experimental models, as evidenced by decreased dermal thickness in the skin, reduced Ashcroft scores in the lungs, and lower myofibroblast counts in both tissues (Fig 6A–D; Supplementary Fig S9A,B).

A data-driven model of SSc

Collectively, we proposed a data-driven and experimentally validated disease model of SSc that elucidated the mechanisms under investigation in this study (Fig 7). An imbalance in fibroblast subsets in SSc was characterised by a decrease in

SCAR5⁺ myofibroblast progenitors and an increase in POSTN⁺ and ACTA2⁺ terminally differentiated myofibroblasts. The decreased phagocytic macrophages (LYVE1^{hi}MHCII^{lo}) may contribute to the impairment of vessel integrity. The infiltration of IL-1β⁺ proinflammatory macrophages was observed actively interact with fibroblast subsets. These proinflammatory macrophages were overexpressed with CXCR4, which may mediate their recruitment by interacting with CXCL12. Additionally, the expression of ACKR3 on fibroblasts, particularly in progenitors under homeostatic conditions, may regulate the local levels CXCL12 by internalising the ligand,

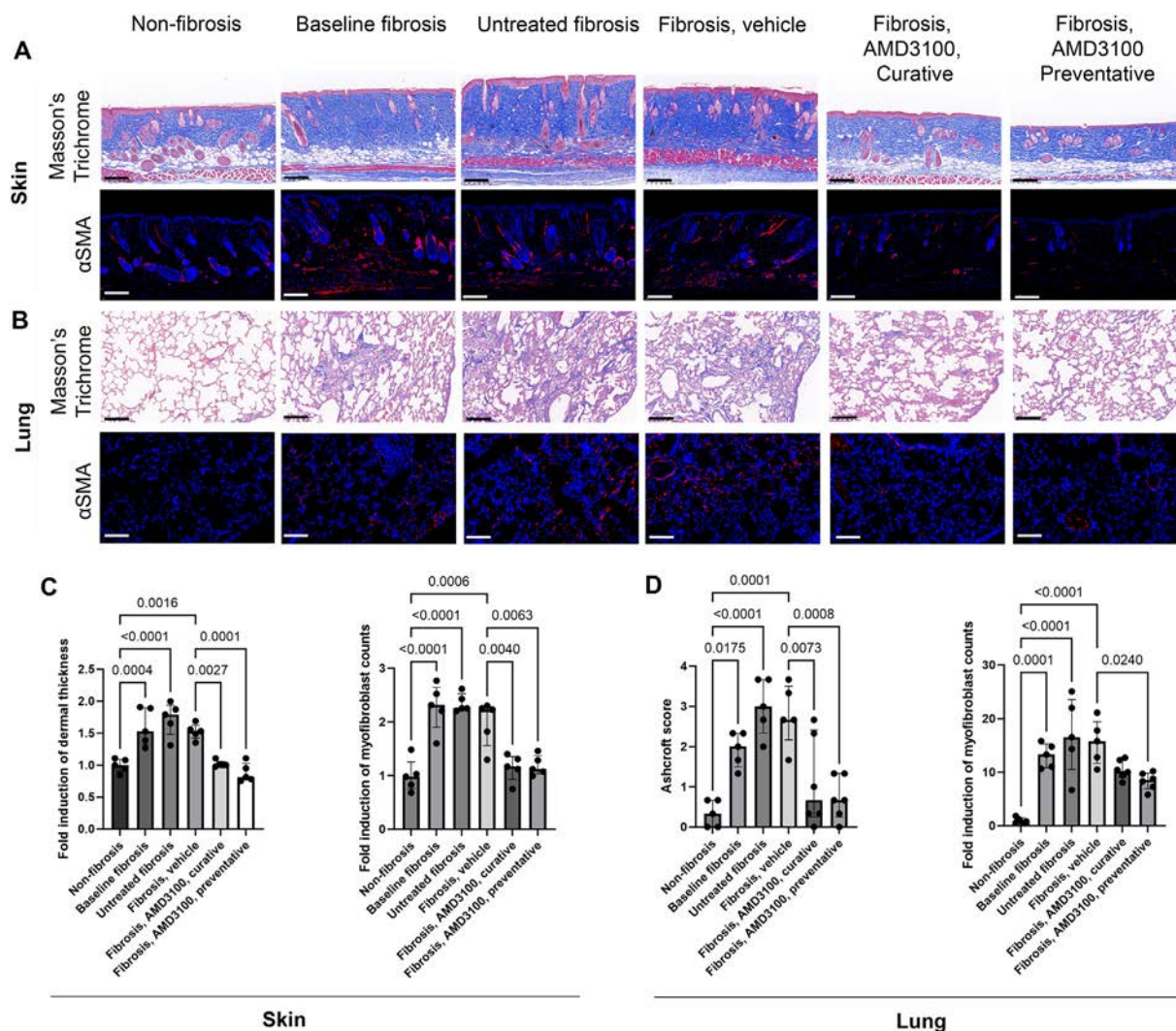


Figure 6. Pharmacologic inhibition of CXCR4 ameliorates experimental fibrosis models. The effect of CXCR4 inhibitor AMD3100 on experimental skin fibrosis (A,C) and lung fibrosis (B,D) induced by bleomycin is shown. (A) Representative Masson trichrome staining and immunofluorescent staining for the myofibroblast marker α SMA in skin sections are shown at $\times 100$ magnification (scale bars = 200 μ m). (B) Representative Masson trichrome staining and immunofluorescent staining for α -smooth muscle actin (SMA) in lung sections are shown at $\times 200$ magnification (scale bars = 100 μ m). (C) Quantification of dermal thickness and α SMA-positive myofibroblast in skin, normalised to control values. (D) Assessment of Ashcroft scores and quantification of α SMA-positive myofibroblast in the lungs, normalised to control values. Data in (C) and (D) are presented as median $\pm$ IQR from $n = 5$ -6 independent biological samples per group. P values determined by One-way analysis of variance (ANOVA) with Tukey multiple comparisons test as post hoc analysis.

thereby limiting excessive leukocyte recruitment and preventing unwanted tissue inflammation. However, this regulatory mechanism might diminish in effectiveness as myofibroblast progenitor differentiate to mature myofibroblast, ultimately resulting in uncontrolled CXCL12/CXCR4-mediated inflammatory cell attraction. This regulatory network fosters a shift from tissue homeostasis to a fibrogenic state, driving the progression of SSc. Therefore, the integration of spatial omics with scRNA-seq provides insight into the immune-stromal crosstalk involved in the pathogenesis of SSc.

DISCUSSION

In this study, we provided a comprehensive spatially resolved map of the skin ecosystem in SSc by integrating spatial transcriptomics with scRNA-seq data. Our results revealed a disease-specific microenvironment enriched with fibroblasts and macrophages exhibiting high TGF- β signalling pathway activity and strong correlation with clinical outcomes in SSc. Moreover, we uncovered a proportional imbalance of fibroblast subsets in

SSc, with a significant reduction in *SCARA5*⁺ fibroblasts and an upregulation in *POSTN*⁺/*ACTA2*⁺ fibroblasts compared with healthy controls. Of note, *SCARA5*⁺ fibroblasts have been recently reported as myofibroblast progenitors in heart and kidney [28,29,48]. In addition, a cross-tissue human fibroblast atlas also demonstrated the substantial overlaps among *SCARA5*⁺ fibroblasts and progenitor-like fibroblasts, as well as between *POSTN*⁺/*ACTA2*⁺ fibroblasts and myofibroblasts [49]. These findings suggest that the differentiation from *SCARA5*⁺ myofibroblast progenitors to *POSTN*⁺/*ACTA2*⁺ myofibroblasts may represent a conserved fibrotic mechanism across different tissues, organs, and diseases. Targeting the shared mechanism has emerged as a strategy for basket trial design especially for rare diseases that share the same triggering or perpetuating signalling pathways [50].

Of note, we characterised the intricate interplay of fibroblasts and macrophages within niches that potentially drive the transition from a homeostatic to a fibrogenic state. Further investigation into cell–cell communications offered potential for identifying targetable disease-driving molecules for therapeutic

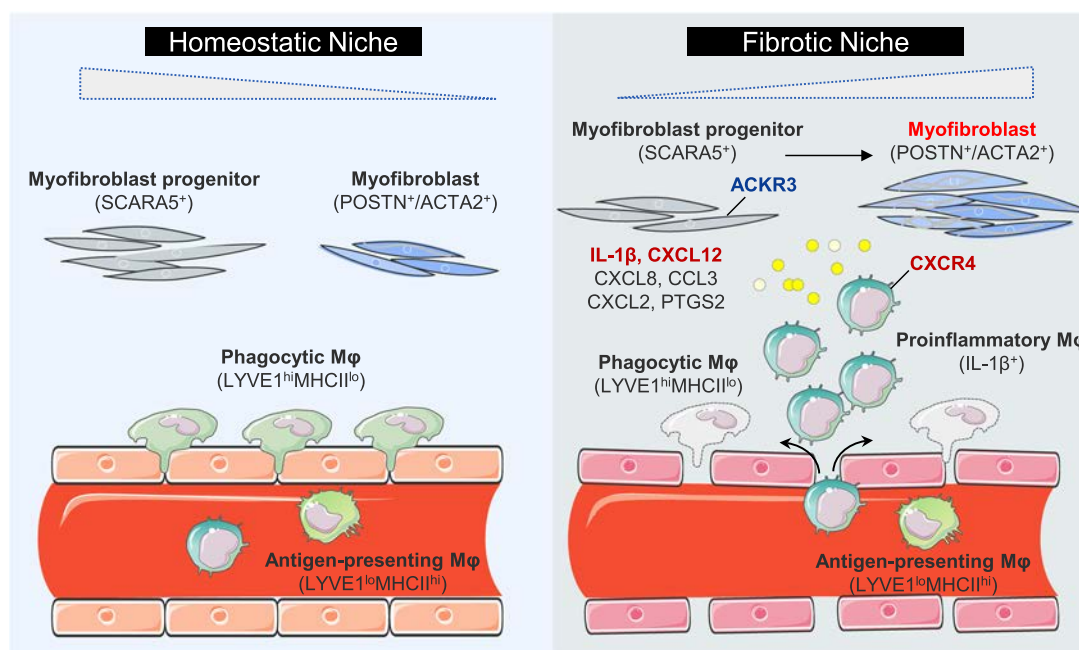


Figure 7. A data-driven model of systemic sclerosis (SSc). Schematic summary illustrates the proposed cellular interplay in SSc skin, which leads to a shift from tissue homeostasis to a fibrogenic environment. The illustration was created by using images from Servier Medical Art (<http://smart.servier.com/>), licensed under CC BY 3.0 (<https://creativecommons.org/licenses/by/3.0/>).

intervention. We revealed an enhanced interaction between $IL-1\beta^+$ proinflammatory macrophages and fibroblast subsets in SSc. Infiltrating macrophages activate fibroblasts by secreting $IL-1\beta$, while systemic $IL-1\beta$ inhibition or targeted $IL-1\beta$ deletion in macrophages prevents fibroblast activation [3,4,51]. The role of $IL-1$ family of cytokines, including $IL-1$, $IL-18$, $IL-33$, and $IL-36$, has been implicated in the pathogenesis of SSc [3,4,51,52]. For instance, the elevated $IL-36$, which has been reported to mediate cellular crosstalk in SSc, represents a promising area for further research [52].

Furthermore, we found this crosstalk was predicted to be mediated by the interaction of $CXCL12$ with $CXCR4$ in macrophages and $ACKR3$ in myofibroblast progenitors. Moreover, we also found the deregulation of $LYVE1^{hi}MHCII^{lo}$ macrophages in SSc, which are known to play a protective role by maintaining vascular integrity and preventing immune cell infiltration in fibrotic conditions [10]. These sequential events such as tissue entry and immune infiltration occurring in SSc may shift the hemostatic condition towards a profibrotic environment. Importantly, the inability to constrain $CXCL12$ overexpression in the milieu through internalisation by myofibroblast progenitors via $ACKR3$ leads to $CXCR4$ -mediated proinflammatory macrophage recruitment, which is reflective of the fine-tuned regulation by local fibroblasts.

Translating omics discoveries into clinically actionable strategies remains a critical unmet challenge in SSc. Our research contributes novel insights to clinical practice in biomarker-driven patient stratification and mechanism-based target identification:

Firstly, we found the gene ratio of $POSTN/SCARA5$ emerges as a predictive indicator for the clinical progression of skin fibrosis and treatment refractoriness in SSc, which actually aligned and reinforced the predictive values of basal molecular pattern. The study by Hinchcliff et al [53] showed that 571 genes (false discovery rate < 10%) had changed between pre-MMF and post-MMF treatment biopsies for patients demonstrating mRSS improvement [53]. The study by Assassi et al [54] showed the 12-month treatment of MMF yield changes in peripheral blood gene expression profiling, highlighting the predictive

significance of baseline peripheral blood cell transcript scores for response to MMF treatment in SSc–interstitial lung disease [54]. We additionally validated and extended these data in the patients with SSc with different treatment background, highlighting the potential of using gene expression profiling in personalised stratification strategies.

Secondly, we identified that the interplay between fibroblasts and macrophages may drive the transition from homeostasis to a profibrotic environment. Targeting the key regulators within this network may abrogate the profibrotic transition and ameliorate disease. We validated our data-driven model by inhibiting $CXCR4$ in 2 preclinical murine fibrosis models. Our study demonstrated a promising approach to translating omics findings into therapeutic target identification.

Beyond the molecular signature, restoring the disorganised distribution in spatial space might represent a new dimension in pathogenesis exploration and therapy development [55]. Likewise, we found a loss of the polarised distribution of fibroblast subsets in SSc skin shown in Figure 3D. In healthy skin, the $SCARA5^+$ Fib1 are predominantly located in the papillary dermis in comparison with the reticular dermis, while $POSTN^+$ Fib2 are scarcely observed in the dermis and mainly in the upper layer. In SSc, both $SCARA5$ and $POSTN$ shift to a reticular expression pattern. Therefore, this phenomenon indicates the need in the investigation into the mobility and migration of fibroblasts during tissue remodelling as the future effort. Genome-wide spatial omics exhibited the advantage in characterising this physiologic skin organisation.

While spatial transcriptomics provides a unique advantage in analysing the structured tissue niches, our study was limited by the relatively low resolution of the $10\times$ Visium platform ($55\text{-}\mu\text{m}$ per spot). To mitigate this limitation, we leveraged Stereo-seq, an emerging spatial transcriptomics technology with an unprecedented resolution of $0.5\text{ }\mu\text{m}$ per spot. To achieve a comprehensive description of the skin landscape in SSc, we also recognised that 3-dimensional profiling of tissue space and subcellular localisation of protein signals will significantly expand our understanding in SSc pathogenesis.

We also acknowledge the limitations of bleomycin-induced fibrosis models applied in our study. We selected this model since we primarily focused on the immune-stromal crosstalk in SSc, as Sargent et al [56] showed that cutaneous gene expression patterns in bleomycin-induced skin fibrosis overlapped with the inflammatory intrinsic gene subset of human patients with SSc [56]. While they mimic early inflammatory and fibrotic phases of SSc, they are limited to the injection site and primarily driven by inflammation, which is not entirely consistent with human SSc. Subcutaneous injection of bleomycin will not affect lung remarkably; therefore, we also included a bleomycin-induced lung fibrosis mouse model to compensate for this limitation. Moreover, the potential immunostimulatory effects of AMD3100 through bone marrow stem cell mobilisation, which indeed complicate translation from murine models to human disease. However, this effect is transient, reversible, and tolerable in human [57]. Inhibition of CXCR4 using AMD3100 exhibited antifibrotic effect in multiple preclinical fibrosis models [58–60]. Further optimising the route and dosage of AMD3100 administration—such as through localised delivery—could minimise unintended stem cell mobilisation while maximising therapeutic efficacy.

In summary, this study reveals a fibroblast–macrophage connectivity network underlying SSc pathogenesis by integrating spatial multiomics with scRNA-seq data. Within this network, fibroblasts and macrophages with distinct phenotypes contribute to tissue fibrotic remodelling in a coordinated manner. This approach enables the identification of key regulatory interactions and potential therapeutic targets for fibrosis intervention.

Competing interests

Although no disclosures directly relate to the present study, JHWD has consultancy relationships with Active Biotech, Anamar, ARXX, AstraZeneca, Bayer Pharma, Boehringer Ingelheim, Callidatas, Celgene, Galapagos, GSK, Inventiva, Janssen, Kyverna, Novartis, Pfizer, Quell Therapeutics, and UCB; has received research funding from Anamar, ARXX, BMS, Bayer Pharma, Boehringer Ingelheim, Cantargia, Celgene, CSL Behring, Exo Therapeutics, Galapagos, GSK, Incyte, Inventiva, Kiniksa, Kyverna, Lassen Therapeutics, Mestag, Sanofi-Aventis, RedX, UCB, and ZenasBio; and is the CEO of 4D Science and scientific lead of FibroCure. LP has financial interests in Edilytics Inc, and SeQure Dx Inc. All other authors declare they have no competing interests.

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Contributors

ML and ZL were responsible for conceptualisation of the study. ZL, ML, ARR, WX, LH, WY, Y-NL, A-EM, CB and XS were responsible for methodology. ZL, ML, and ARR performed investigations and visualised the study. ML, WY, LH, and JW were responsible for resources. ML, JHWD, LP, and HZ were responsible for funding acquisition. ML administered the project. ML, ZL, JHWD, and RH supervised the study. ML and ZL wrote the

original draft. All authors reviewed, edited, read, and approved the final manuscript.

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Patient consent for publication

All patients and controls signed an informed consent form approved by the local institutional review board.

Ethics approval

All human studies were approved by the ethical committees of Huashan Hospital of Fudan University.

Provenance and peer review

Not commissioned; externally peer reviewed.

Data availability statement

The spatial transcriptomics data presented in this study are available at the Zenodo data archive (<https://zenodo.org/records/14577696>). The scRNA-seq data for human skin were downloaded from GEO with the accession number GSE195452 (Gur2022) and GSE138669 (Tabib2021). A cross-tissue human fibroblast atlas was analysed with the interface website <http://pan-fib.cancer-pku.cn> [49]. The scRNA-seq data of full-thickness injuries in skin from reindeer was analysed with the interface website w.biernaskielab.ca/reindeer_atlas [7]. The scRNA-seq data for mouse skin were downloaded from the Zenodo data archive (<https://zenodo.org/records/7455989>). The codes used in this study are available on Github: https://github.com/lzj1769/Spatial_SSc_project

Supplementary materials

Supplementary material associated with this article can be found in the online version at [doi:10.1016/j.jard.2025.04.025](https://doi.org/10.1016/j.jard.2025.04.025).

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IgG4-related disease

Thalidomide can effectively prevent relapse in IgG4-related disease outweighing its side effects: a multicentre, randomised, double-blinded, placebo-controlled study

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ARTICLE INFO

ABSTRACT

Objectives: To investigate the effects of thalidomide in preventing disease relapse with ‘zero’ glucocorticoids (GCs) usage in IgG4-related disease (IgG4-RD).

Methods: This was a multicentre, randomised, double-blinded, placebo-controlled study, in which eligible patients in disease active status were randomised into 2 groups (group 1: GCs + Thalidomide; group 2: GCs + Placebo) at a 1:1 ratio. The primary outcome of this trial was the disease relapse rate at month 12, whereas the secondary outcomes were the disease remission rate at month 12 and the incidence of adverse events (AEs).

Results: A total of 60 patients were randomised, and 57 patients (GCs + Thalidomide: 29; GCs + Placebo: 28) finished the study per protocol. The relapse rates of the GCs + Thalidomide and GCs + Placebo groups at month 12 were 13.8% and 67.8%, respectively. A 100% response rate was observed in both treatment group, while the remission rates of the GCs + Thalidomide and GCs + Placebo groups were 75.8% and 32.1%, respectively. In total, 49 AEs were recorded in 35 participants, in which 4 were graded as moderate, and 45 were graded as mild. The risk–benefit analysis based on the evaluation of rates of disease relapse and moderate AEs within the 12-month follow-up showed a NNT (number needed to treat) of 2 and a NNH (number needed to harm) of 8 for thalidomide treatment.

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Conclusions: Thalidomide can effectively prevent relapse in IgG4-RD outweighing its side effects, which indicating that thalidomide can be a potential safe therapeutic option for disease relapse prevention in parallel with steroid sparing in IgG4-RD.

WHAT IS ALREADY KNOWN ON THIS TOPIC

- IgG4-related disease (IgG4-RD) is a rheumatic disease entity with autoimmune features predisposing to disease relapse during the remission induction and maintenance therapy, in which the long-term usage of glucocorticoids can bring various treatment-related side effects.
- Thalidomide is an immunomodulatory imide drugs applied in the treatment of multiple myeloma, which is a haematologic malignancy characterised by monoclonal expansion of abnormal plasma cells, and shares some lymphoplasmacytic proliferative features with IgG4-RD.

WHAT THIS STUDY ADDS

- Thalidomide can be an effective and safe regimen in preventing the disease relapse of IgG4-RD under 'zero' steroid usage conditions.
- This study indicated the potential pathogenic roles of the proteins downstream of CRL4 (CUL4-RBX1-DBP1)–CRBN (cereblon) complex (target of thalidomide) in IgG4-RD.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- Thalidomide can be a potential effective 'steroid-sparing' agent for preventing disease relapse of IgG4-RD.

INTRODUCTION

IgG4-related disease (IgG4-RD) is a recently defined rheumatic disease entity with features of autoimmune conditions, of which the most cardinal histopathological features are dense lymphoplasmacytic infiltrate and fibrosis in involved tissue [1]. Typically, remission of IgG4-RD can be induced with glucocorticoids (GCs). However, disease relapses are frequently observed after GC withdrawal (from 34% to 58%) [2]. Although, several studies have revealed that continuous administration of low-dose GCs can help ameliorate the disease relapse, long-term usage of GCs, even at low-dose, can bring adverse effects including diabetes, dyslipidaemia, osteoporosis, hypertension, and infection [3,4]. Therefore, identification of optimal agents balancing both the efficacy and tolerability, which can help steroid sparing, and even realisation of 'zero steroid usage' during maintenance therapy, is necessary.

Thalidomide, a well-known teratogen during the pregnancy, has been re-evaluated as clinically effective drug for the treatment of multiple myeloma (MM), a haematologic malignancy characterised with monoclonal expansion of abnormal plasma cells derived from postgerminal centre B cells, due to its immunomodulatory effects primarily resulting from selective degradation of zinc-finger B-cell transcription factors IKZF1 and IKZF3, of which the dysregulated expression or function were also tightly correlated with autoimmunity [5–9]. Considering the potential similar lymphoplasmacytic proliferative features between MM and IgG4-RD, and the potential therapeutic effects of thalidomide targeting the autoimmune responses, we initialised this multicentre, randomised, double-blinded, placebo-controlled study, aiming to elucidate the therapeutic effects of disease remission maintenance and assess the safety and benefit-risk profile of thalidomide under 'zero steroid usage' condition after routine remission induction in IgG4-RD patients.

METHODS

Study design and participants

This is a multicentre, randomised, double-blinded, placebo-controlled study performed in the departments of rheumatology and immunology of 6 medical centres in China between June 2020 and December 2023.

Participants meeting the following criteria were eligible for this study: (1) aged 18 years or older; (2) diagnosed with IgG4-RD based on the comprehensive diagnostic criteria for IgG4-RD described in 2011 by the Centers for Disease Control (CDC) or the 2019 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) classification criteria for IgG4-related disease (ACR/EULAR criteria) [10,11]; (3) had not received the treatment of drugs with immunosuppressive/modulatory effects (including glucocorticoids, hydroxychloroquine/chloroquine, leflunomide, thalidomide, azathioprine, cyclophosphamide, methotrexate, rituximab, Janus kinase inhibitors, tumour necrosis factor inhibitor/receptor-Fc fusion protein, nonsteroidal anti-inflammatory drug), within 6 months before final enrolment; (4) should agree to adopt a reliable method of contraception before enrolment and during the trial, unless the patient is non-reproductive. The exclusion criteria included patients who were (1) diagnosed with diseases mimicking IgG4-RD; (2) currently pregnant, in breastfeeding, or intending to become pregnant during the trial or within 6 months after trial; (3) suffering from severe life-threatening diseases (eg, severe renal/hepatic insufficiency, acute coronary syndrome, and etc.) based on the evaluation of physicians; (4) suffering from active infection (eg, infection of mycobacterium tuberculosis, hepatitis B virus, hepatitis C virus, and human immunodeficiency virus); (5) had history of malignancy within 5 years before enrolment; (6) and allergic to thalidomide. All the participants should sign the written informed consent approved at the sites' institutional review boards before the study, which was registered at www.chictr.org.cn (registration number ChiCTR2000028918).

Randomisation and treatment regimens

Eligible participants were randomly assigned into group 1 (GCs + Thalidomide) or group 2 (GCs + Placebo) with a 1:1 ratio using a computer-generated randomisation list prepared by the third party, of which the block size was 4, and the stratification factor was the IgG4-RD responder index (RI) [12] (Supplementary Fig S1). All patients, investigators, and study site personnel were masked to group assignments.

Participants in both groups received the prednisone treatment for disease remission induction with an initial dose of 0.6 mg/kg/day, which was then tapered in a speed of 5 mg/day every 2 weeks, and should be discontinued within 3 months. Patients in group 1 (GCs + Thalidomide) and patients in group 2 (GCs + placebo) received thalidomide and placebo (identical to the thalidomide tablets in appearance) orally once daily in the whole study, respectively. It should be mentioned that patients received thalidomide or placebo with an initial dose of 25 mg/day, which can be increased to no more than 75 mg/day within 1 week based on patients' tolerability to them.

Outcome measure

The primary outcome of this study was rate of relapse at month 12 of 2 treatment groups, which was defined as the elevation of IgG4-RD RI ≥ 2 in any one of the single domains compared with the last follow-up. The secondary outcomes were the disease remission rate at month 12 (disease response was defined as an improvement of IgG4-RD RI ≥ 2 in any single domain at any visit without disease relapse, remission was defined as achievement of IgG4-RD RI ≤ 3 without any prednisone usage and disease relapse at month 12). For patients whose IgG4-RD RI was equal to 3 at baseline, remission should only be defined if there was a disease response), and the incidence of adverse events (AEs) recorded based on the Common Terminology Criteria for Adverse Events (v5.0).

The IgG4-RD RI (without the inclusion of serum IgG4 level) [12,13] and Physician's Global Assessment (PGA) scores (ranging from 0 to 10) were assessed by an experienced rheumatologic expert from the corresponding centre in each follow-up visit and reviewed independently by the same 2 experts of Tongji hospital.

Statistical analysis

Sample size was calculated with PASS software (version 11, NCSS, LLC, Kaysville, UT, USA) based on the following assumptions: the relapse risk of GCs + Placebo and GCs + Thalidomide was 50% and 18%, respectively. The statistical power was set to 90%, and the two-sided type I error rate was set to 5%. Considering the potential drop out in the trial (assumed to be 25%), the final sample size of each group was set as 51. However, the steering and safety committee elected to stop randomisation on November 30, 2022. This decision was driven by slowed recruitment due to the COVID-19 pandemic, and the resultant

impending expiration of trial drugs. No interim analyses or reassessments of sample size were undertaken.

The quantitative and categorical data were presented as median (IQR), and number (%), respectively. Mann–Whitney U tests were applied for the comparisons of continuous data between 2 groups, whereas the χ^2 tests or Fisher's exact tests were applied for the analyses of categorical data. Log-rank tests were applied to compare the survival curves, whereas the linear mixed model was applied to analyse the data with repeated measures (eg, IgG4-RD RI and PGA). For the benefit-risk analysis, NNT (number needed to treat) and NNH (number needed to harm) were calculated respectively based on the difference of ratio of moderate AEs and disease relapse between 2 treatment groups, of which the 95% CI were estimated with the 'Wilson' method using binomDiffCI function embedded in R package Mkinfer (v1.2). A *P* value $< .05$ (2-sided) was regarded as statistic significant in all comparisons, except in the analyses with linear mixed model, of which the α was adjusted to 0.0083 (0.05/6) due to multiple comparisons. All the statistical analyses and figures were performed/plotted with R software 4.3.1.

RESULTS

Baseline features of the participants

A total of 60 allocated treatment received eligible patients were randomised to 2 treatment groups. In total, 53 of them fulfilled the ACR/EULAR criteria for IgG4-RD, and 7 of them fulfilled only the CDC criteria. The median score for the ACR/EULAR criteria for all 60 patients was 28.5, and 57 of them (GCs + Thalidomide group, *n* = 29; GCs + Placebo group, *n* = 28) finished the study based on the protocol. Based on the protocol, all the participants discontinued the prednisone usage within 3 months and were administered thalidomide or placebo throughout the entire study (Fig 1A,

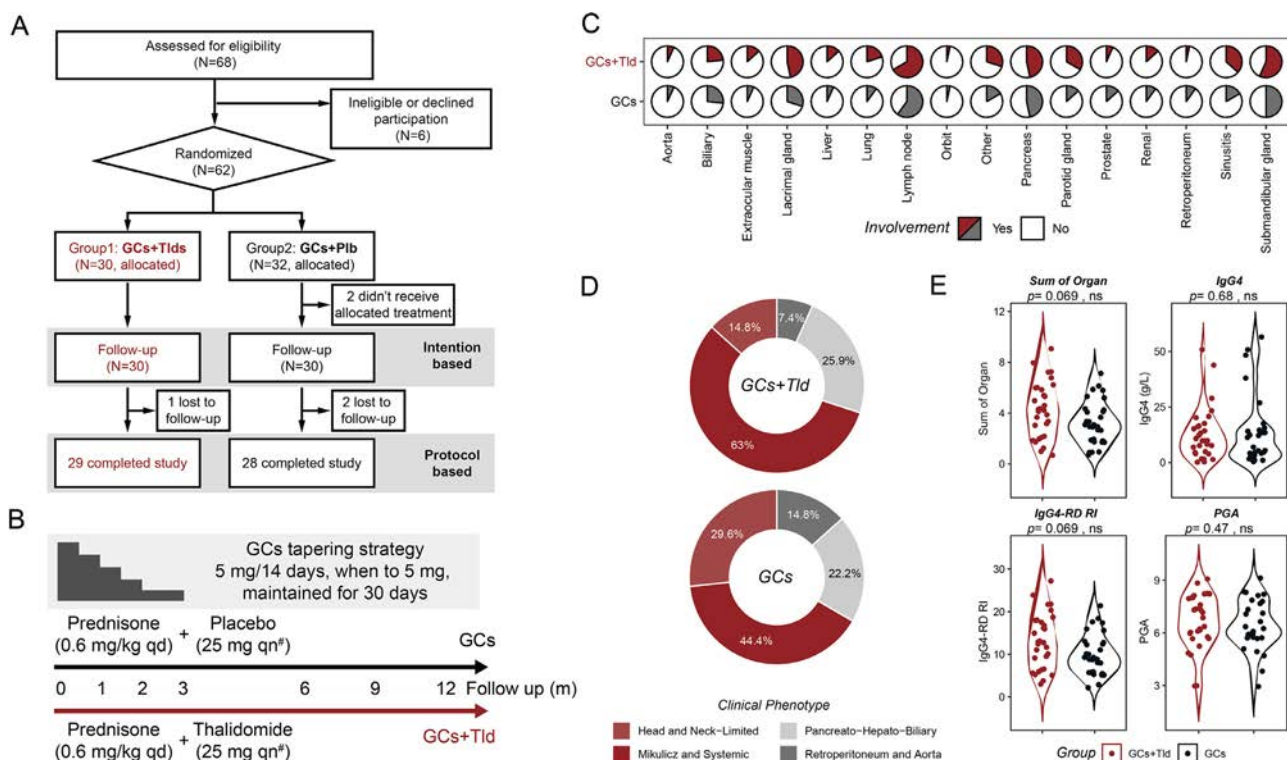


Figure 1. The trial profile and baseline data of enrolled participants. (A) The trial profiles. (B) The therapeutic plan of the 2 different treatment groups. (C) The organ involvement profiles of patients. (D) The clinical phenotypes of patients. (E) Parameters reflecting the disease severity of patients between 2 different treatment groups. Group glucocorticoids (GCs)/GCs + Plb, GCs + Placebo group; group GCs + Tld, GCs + Thalidomide; #, as initial dosage, can be added to no more than 75 mg (3 tablets) qn within 1 week since the beginning of trial; qn, quaque nocte.

B). The most involved organ of patients in this study was lymph node, followed by the submandibular gland, pancreas, lacrimal glands, and biliary tracts (Fig 1C). There was no significant statistical difference of the ratio of involved organs in patients between 2 treatment groups (Supplementary Table). The majority of the participants in this study were classified as the ‘Mikulicz and Systemic’ phenotype based on their patterns of organ involvements [14] (Fig 1D). No statistically significant differences of the involved organs’ sums, serum IgG4 levels (g/L), IgG4-RD RIs, and PGAs at baseline were observed between the participants from 2 treatment groups, of which the median values for all the participants were 3.50 [2.00, 5.00], 9.88

[4.14, 15.72], 10.50 [6.00, 15.00], and 6.00 [6.00, 8.00], respectively (Fig 1E, Supplementary Table).

Outcomes

The IgG4-RD RI and PGA of individual participants during the follow-up were shown in Figure 2A. Significant statistical differences between the Kaplan–Meier curves recording the nonrelapse probability of 2 different treatment groups were observed in both intention-based and protocol-based analyses (Fig 2B). In participants who finished the whole study based on the protocol, 13.8% (4 out of 29) and 67.8% (19 out of 28) of

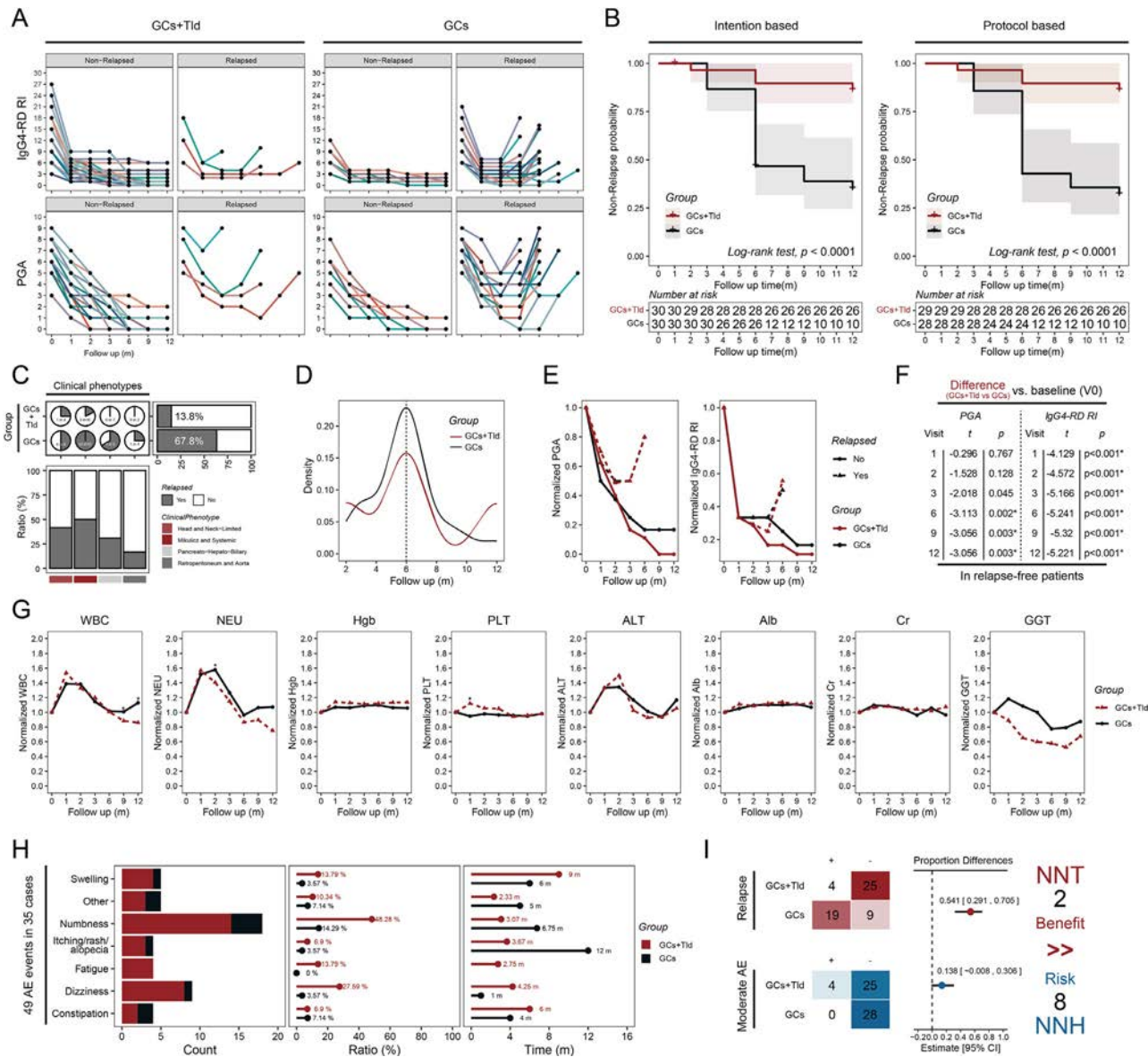


Figure 2. The therapeutic effects and risk–benefit analysis of thalidomide administration in in IgG4-related disease (IgG4-RD). (A) The IgG4-RD responder index (RI) and Physician’s Global Assessment (PGA) of individual participants during the 12-month follow-up. (B) The Kaplan–Meier curves and the corresponding log-rank tests between 2 different treatment groups in intention-based and protocol-based analysis. (C) The relapse profiles in 2 different treatment groups (up), and different clinical phenotypes (down). (D) Distribution of relapse time. (E) Tendency of IgG4-RD RI and PGA in relapsed and non-relapsed patients of different treatment groups. Points represent the median value normalised to the baseline data of that follow-up of the corresponding indices; data after month 6 of the relapsed patients were not shown due to the observation that relapse happened primarily around the month 6 follow-up. (F) Linear mixed effect model analysis in relapse-free patients focusing on the comparisons of IgG4-RD RI and PGA (original scores, not normalised data) difference of 2 different groups between different follow-up points and baseline. Due to multiple comparisons, the α was adjusted to 0.0083 (0.05/6). (G) Tendency of routinely detected laboratory indices during the follow-up. *, $P < .0083$. (H) The adverse event (AE) profiles of the participants reported in this trial. Left: the absolute counts; Middle: prevalence in participants of the corresponding groups; Right: average time of the first report. (I) Risk–benefit analysis based on the difference (middle) of ratio (left) of relapse and moderate AEs in different groups, on which based, NNT (number needed to treat) and NNH (number needed to harm) were further calculated (right).

the patients in the GCs + Thalidomide group (mean dosage for maintenance during the study: 50 mg quaque nocte) and the GCs + Placebo group, respectively, experienced disease relapse within 12 months. In the GCs + Thalidomide group, a significant decrease of serum IgG4 level (g/L, from 9.89 [4.54, 16.50] to 1.99 [1.40, 3.89], $P < .001$), a potential decrease of IgE level (IU/mL, from 181.50 [101.80, 593.40] to 90.29 [43.40, 232.45], $P = .065$), and a recovery of C3 level (g/L, from 0.88 [0.77, 1.05] to 1.04 [0.95, 1.15], $P = .019$) can be observed at the end of the study, when compared with baseline levels, whereas no statistical difference of these indices was identified in the GCs + Placebo group (Supplementary Fig S2). Patients with different clinical phenotypes in this study showed potential different predisposition of disease relapse: patients with 'Mikulicz and Systemic' clinical phenotype were more predisposed to disease relapse, which was particularly significant in the GCs + Placebo group, where the disease of all the patients with that clinical phenotype experienced disease relapse. A high relapse rate was also demonstrated in patients with 'Pancreato-hepato-Biliary' phenotype in the GCs + Placebo group (66.7%, 4 out of 6). However, no relapse was observed in 'Pancreato-Hepato-Biliary' involving patients treated with GCs + Thalidomide (Fig 2C). The distributional analysis of the relapse time showed that most disease relapse happened around 6 months after the beginning of therapeutic intervention, there was no significant difference in the median relapse time between 2 groups (Fig 2D). The univariate logistic analysis to the potential risk factors of disease relapse based on the laboratory parameters at baseline indicated that, in patients treated with GCs + Placebo, numbers of involved organs and disease severity indices (IgG4-RD RI and PGA) were positively associated with disease relapse, whereas the association between percentage of CD19⁺ B cells in the total lymphocytes and disease relapse was negative. In the GCs + Thalidomide group, the serum IgG4 level at baseline was positively associated with disease relapse (Supplementary Fig S3).

A 100% response rate was observed in both treatment groups, whereas the remission rates of the GCs + Thalidomide and GCs + Placebo groups were 75.8% (22 out of 29) and 32.1% (9 in 28), respectively. The tendency of IgG4-RD RI and PGA changes grouped by therapeutic regimens and disease relapse within 12 months were recorded in Figure 2E. To investigate the therapeutic effects of thalidomide administration, trend analyses based on the linear mixed model were also made in relapse-free patients, which revealed that thalidomide can help significantly reduce the IgG4-RD RI score since the first month follow-up, and the PGA score since the sixth month follow-up (Fig 2F).

AEs and risk–benefit analysis

In general, at the majority of time points during follow-up, no significant difference in the counts of white blood cells, neutrophils, and platelets as well as the levels of haemoglobin, alanine aminotransferase, serum albumin, serum creatinine, and γ -glutamyl transferase were observed between the GCs + Placebo and GCs + Thalidomide groups, indicating the safety of thalidomide usage within a 12-month period in IgG4-RD patients (Fig 2G).

A total of 49 AEs had been recorded in 35 participants in this trial. Of these, 45 were graded as mild (grade 1), whereas 4 were graded as moderate (grade 2). In total, 77.5% of the total AEs occurred in participants treated with thalidomide, in which numbness of limbs was the most frequently observed AE in 48.3% of the participants (14 in 29, average time of first report: 3.07 months), followed by dizziness (27.59%, 8 in 29, average

time of first report: 4.25 months), swelling (13.79, 4 in 29, average time of first report: 9 months), and fatigue (13.79, 4 in 29, average time of first report: 2.75 months) (Fig 2H). The risk–benefit analysis was realised based on the evaluation of rates of disease relapse and moderate AEs within the 12-month follow-up in 2 different groups. The proportion differences in disease relapse and moderate AEs between the GCs + Thalidomide and GCs + Placebo groups were 0.541 (95% CI: [0.291, 0.705]) and 0.138 (95% CI: [−0.008, 0.306]), respectively, indicating that the benefit of disease relapse prevention of thalidomide intervention in IgG4-RD patients outweighed the potential harm (moderate AEs) it may bring (NNH vs NNT: 8 vs 2) (Fig 2I).

DISCUSSION

Studies in early years found that thalidomide can exert anti-inflammatory, immunomodulatory, and antiangiogenic effects [15]. Recent studies have identified, that thalidomide exerts its immunomodulatory effects primarily by binding to a ubiquitously expressed protein of the E3 ubiquitin ligase cullin-4A RING ligase complex (CUL4-RBX1-DDB1-CRBN, CRL4-CRBN), leading to the ubiquitination and the following degradation of a set of downstream protein targets [6,16]. In MM cells, lenalidomide (one of the modern analogues of thalidomide) can cause selective degradation of zinc-finger B-cell transcription factors IKZF1 (also called Ikaros) and IKZF3 (also called Aiolos) and lead to decreased levels of IRF4 and c-Myc and cell death [6,7]. IKZF1 can play important roles in regulating the functional status of T and B lymphocytes. Hoshino et al [17] revealed that gain-of-function IKZF1 variants in human can cause inflammation, autoimmunity, allergic symptoms, impaired T cell production of interleukin-2, enhanced helper T cell (Th) skewing to Th2, low numbers of regulatory T cells (Treg), eosinophilia, and abnormal PC proliferation, which is very similar to the immunological features of IgG4-RD [1,18]. Recently, a gain-of-function variant of Ikaros (Arg183His) has also been observed to promote the transcription of Src kinase FYN to participate in the potential pathogenesis of a familial IgG4-RD via enhancing FYN activity with the help of the UBR4 (Cys4179Ter) variant and the following Th2 polarisation [19]. Dysregulation of IKZF3 may also lead to the abnormality of adaptive immune response. Signal transducer and activator of transcription 3 can physically interact with IKZF3 to form a transcription factor complex capable of inducing the expression of BCL6, the lineage-defining transcription factor for follicular helper T cells, which can promote the differentiation (into plasma cells or memory cells), proliferation, and survival of germinal centre B cells that consistently exert pathogenic effects in various autoimmune conditions, including IgG4-RD [20–23].

Our study demonstrated that thalidomide can help improve conditions in IgG4-RD patients since the first month of treatment and effectively prevents disease relapse during a 12-month follow-up. From the perspective of the therapeutic effects, therefore, this study indicated that thalidomide can be a potential therapeutic option for IgG4-RD. However, on the other hand, thalidomide is well-known for its teratogenicity tragedy in the 1950s and 1960s, which led to its subsequent withdrawal from the market for nearly 50 years until it was re-evaluated as a drug for MM [6]. In addition to teratogenicity, thalidomide monotherapy may cause several side effects [15]. In this study, several thalidomide-related AEs were reported during the follow-up. However, only 4 moderate AEs were recorded, which was the most severe grade of AEs in this study. Compared with the potential benefit in decreasing the relapse rate with 'zero'

steroid usage, the possible risk of moderate AEs was not significant. Taking into account that the majority of IgG4-RD patients were middle-aged and elderly, who may exhibit reduced intention of fertility. Therefore, disease remission induction and maintenance therapy with thalidomide in IgG4-RD is feasible.

It is worth mentioning that not all IgG4-RD patients responded good to thalidomide therapy in this study, which seemed to relates with the clinical phenotypes of patients. There was a relapse rate of 13.8% in the GCs+ Thalidomide group in this study, in which most of the patients (75%, 3 out of 4) were categorised as the ‘Mikulicz & Systemic’ clinical phenotype. The potential relationship between the ‘Mikulicz & Systemic’ phenotype and disease relapse can also be observed in the study by Peng et al [24], which revealed the predisposition of disease relapse in patients with submandibular gland involvement, which is a typical organ involved in Mikulicz disease. The precise reasons underlying the connections between these 2 conditions is still unknown; however, clues at the levels of both ‘quantitative’ (number of organ involvement and disease activity) and ‘qualitative’ (potential different pathogenic immune mechanisms) may provide some indications. First, patients with salivary gland involvement were predisposed to develop multi-organ disease in IgG4-RD (tightly correlates with higher IgG4-RD RI) and have higher eosinophil counts and serum IgG4 and IgE levels, which were also identified as predictors of disease relapse in IgG4-RD patients following rituximab treatment [25,26]. Second, patients with the ‘Mikulicz & Systemic’ phenotype may have different immune cell profiles in the peripheral blood, indicating the potential existence of different immune phenotypes in IgG4-RD patients [27]. Immune phenotypes may also help predict the potential response to certain therapeutic regimen in IgG4-RD, which has been observed in a previously published study by us [28]. However, phenotypic identification at the molecular level, as has been already attempted in systemic lupus erythematosus and Sjögren’s syndrome, is still lacking in IgG4-RD [29,30].

In summary, as one of the few clinical trials with multicentre, randomised, double-blinded, placebo-controlled design focusing on one specific therapeutic regimen in the field of IgG4-RD, this study has observed the potential efficacy of thalidomide on helping remission induction and preventing disease relapse in parallel with steroid sparing, for which the potential benefit outweighed the risk, indicating that thalidomide is a potentially effective drug for the treatment of IgG4-RD. In addition, this study also provided supporting data for further mechanistic research focusing on the pathogenic roles of the proteins downstream of CRL4-CRBN and clinical trials with more potent modern derivatives of thalidomide in IgG4-RD. The limitations of this study were the relatively small sample size and lack of the identification of strategy predicting the thalidomide’s therapeutic response. Therefore, further large-scale studies and basic studies based on systematic and computational biology should be performed to promote progress in the development of precise medicines for IgG4-RD.

Competing interests

All authors declare they have no competing interests.

Contributors

YC participated in the eligibility assessment, disease activity evaluation, and manuscript drafting, and is the first author of this study. CY, PY, WZ, LD, WW, and RW participated in the

eligibility assessment. SD, LC, XW, JZ, and CL participated in disease activity evaluation. YC and CY participated the study design and reviewed the disease activity evaluation. WS and HU provided advice for this study. SC performed the analysis, interpreted the data, drafted the manuscript, and participated the study design. LD designed, provided important advice for this study, provided project administration, and interpreted the data. LD and SC are the corresponding authors of this study, and LD is the guarantor of this manuscript.

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Patient consent for publication

Consent obtained directly from patients.

Ethics approval

This study was approved by the Institutional Review Board and Medical Ethics Committee of Tongji Hospital, Tongji Medical College of Huazhong University of Science and Technology (2019S1175). Written informed consent was acquired before medication administration, sample/data collection, and subsequent analyses. The trial registration number is ChiCTR2000028918.

Provenance and peer review

Externally peer reviewed.

Data availability statement

Data are available upon reasonable 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.jard.2025.01.033](https://doi.org/10.1016/j.jard.2025.01.033).

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Crystal arthropathies

Integrative analysis reveals the multilateral inflammatory mechanisms of CD14 monocytes in gout

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ABSTRACT

Objectives: Gout, prevalent inflammatory arthritis caused by urate crystal deposition, involves immune cell activation, yet the precise role of CD14 monocytes in initiating the inflammatory response is poorly understood. This study aimed to characterise the molecular and cellular landscape of CD14 monocytes in gout using single-cell transcriptomic analysis.

Methods: Single-cell RNA sequencing was performed on peripheral blood mononuclear cells from 8 gout patients and 6 age- and sex-matched healthy controls. The findings were validated using publicly available datasets. Differential gene expression and pathway enrichment analyses were conducted to identify gout's key molecular regulators and cellular subclusters.

Results: At the molecular level, we identified hypoxia-related pathways, including *HIF1A*, as key regulators of interleukin-1 β production in CD14 monocytes in gout. We also observed significant downregulation of *CLEC12A* across all CD14 monocyte subclusters. At the cellular level, an S100A^{high} CD14 monocyte subcluster, characterized by high expression of S100A8/A9/A12 and linked to inflammatory and metabolic pathways, was found to drive NLRP3 and CLEC7A inflammasome activation, as well as prostaglandin secretion. *In vitro* stimulation with monosodium urate crystals revealed that the differentially expressed genes were enriched in S100A^{high} monocytes, highlighting the synergistic role of these pathways in driving gout inflammation. Additionally, gout genome-wide association study-prioritised genes underscored the role of fatty acid metabolism in inflammation, promoting prostaglandin secretion from S100A^{high} monocytes.

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Conclusions: These findings provide new insights into the role of CD14 monocytes in gout pathogenesis, particularly the contribution of hypoxia and fatty acid metabolism pathways, and suggest potential therapeutic targets for precision medicine in gout treatment.

WHAT IS ALREADY KNOWN ON THIS TOPIC

- Gout is an autoinflammatory disease driven by the deposition of monosodium urate crystals, which trigger an innate immune response.
- Interleukin (IL)-1 β production is central to the pathogenesis of gout, with both CD14 monocytes and neutrophils playing key roles in this inflammatory cascade.

WHAT THIS STUDY ADDS

- Using single-cell RNA sequencing, this study identifies a novel S100A^{high} CD14 monocyte subset, marked by high S100A8/A9/A12 expression, enriched in pathways regulating neutrophil-mediated immunity and NLRP3 inflammasome activation, driving the inflammatory response in gout.
- HIF1A is identified as a key transcriptional regulator linking hypoxia to enhanced IL-1 β production in CD14 monocytes, contributing to the inflammatory cascade.
- Fatty acid metabolism is significantly involved in the inflammatory response of the S100A^{high} monocyte subset, promoting prostaglandin secretion, which is known to amplify inflammation and enhance immune cell recruitment.
- Downregulation of *CLEC12A* across CD14 monocyte subsets highlights a loss of negative regulation, leading to sustained immune activation in gout.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- The findings suggest that targeting the S100A^{high} monocyte subset and key pathways, such as hypoxia signalling and fatty acid metabolism, could modulate the innate immune response and IL-1 β -driven inflammation in gout. This presents a novel therapeutic approach for more effective gout management.

INTRODUCTION

Gout is a common and debilitating form of inflammatory arthritis with an increasing prevalence of up to 4% in most developed countries [1]. The disease is characterised by recurrent, self-remitting episodes of intense pain and inflammation, predominantly affecting the joints, known as gout flares. The main risk factor for gout development is hyperuricemia, defined as elevated blood urate levels [2]. When urate exceeds a critical threshold, typically reported around 6.8 mg/dL, urate precipitates into monosodium urate (MSU) crystals, which deposit in joints, initiating an acute innate immune response [3]. Although necessary, hyperuricemia alone is insufficient for gout development, as most individuals with hyperuricemia never develop gout [4]. This discrepancy can be attributed to individual genetic and molecular factors affecting the immune response to gout [5].

The inflammatory response to MSU crystals is primarily mediated by the activation of the NLRP3 inflammasome in monocytes and macrophages [6]. Initiation of the NLRP3-dependent inflammatory cascade requires a 2-step process. In the first step, a priming signal generated through TLR2 and TLR4 engagement results in nuclear factor (NF)- κ B activation and transcriptional upregulation of prointerleukin (IL)-1 β and inflammasome components. In the second step, the activation of the NLRP3 inflammasome is provided by MSU crystals [5,7].

This results in inflammasome assembly and processing of pro-IL-1 β into the biologically active form, IL-1 β , triggering an inflammatory cascade. While the role of MSU crystals in the activation step is well-established, the priming signal is believed to be influenced by individual factors, such as the presence of fatty acids. This is supported by the observation that gout flares are often triggered after large meals [8].

Despite its high prevalence and its association with a wide range of cardiometabolic comorbidities and an increased mortality rate, gout remains suboptimally treated [9,10]. Current clinical management of gout involves 2 approaches: first, the control of serum urate concentration with urate-lowering therapies; second, the management of inflammation using colchicine, non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, or anti-IL-1 β biologics aimed at treating acute gout flares [2,5,9,11]. Given the potential side effects associated with long-term usage of these therapies, including infection and haematologic disorders, it is essential to investigate the molecular features of the inflammation cascade within IL-1 β -producing cells. This will allow the identification of new molecular therapeutic targets for safer and more effective clinical management of gout and its associated comorbidities [12].

At a significance level equivalent to that of the molecular components of this disease, understanding the specific role of immune cells in driving inflammation in gout remains at the forefront of ongoing research. To gain a deeper understanding of the involvement of peripheral blood immune cells, it is necessary to magnify the intricate molecular profile within these cells, revealing cell type-specific gene expression profiles associated with inflammation. To date, only a few studies have utilised this technology for investigating gout, focusing on Asian male donors [13,14]. It is necessary to encompass diverse ethnicities, a larger sample size, and both sexes in gout studies. Thus, this study aimed to compare the molecular profiles of CD14 monocytes in gout patients and healthy controls to investigate how transcription factors (TFs) and inflammatory pathways regulate IL-1 β expression in these cells. We examined the impact of gout and MSU crystals on CD14 monocytes and their subclusters, aiming to understand genetic predispositions and pathways identified through genome-wide association studies (GWAS), scrutinising the transcription profile and enriched pathways to elucidate subcellular changes in CD14 monocytes.

Our findings, consistently examined in the validation group data, led to the identification of molecular candidates and CD14 monocyte subclusters that may serve as future therapeutic targets in modulating gout.

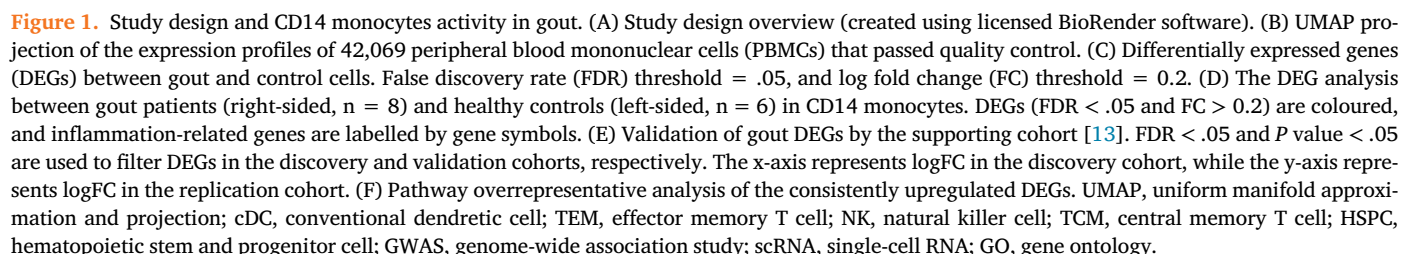
METHODS

The method section is provided as online [Supplementary Material](#).

RESULTS

The transcriptome of CD14 monocytes shows the largest difference between gout and control groups with high enrichment of genes and pathways involved in gout inflammation

We investigated transcriptomic differences in the discovery group, comprising 8 gout and 6 healthy donors with similar age



To annotate these clusters, top cluster-specific differentially expressed genes (DEGs) were matched with known cell type-specific marker genes (Supplementary Fig S1B). Cell population percentages did not show significant enrichment between gout and control groups (Supplementary Fig S1C). Examining the DEGs per cell type between gout and control groups, we

observed the largest transcriptomic alterations in CD14+ monocytes (Fig 1C), underscoring the importance of this cell type in gout. Examining the DEGs in CD14+ monocytes, the expression of inflammation-related genes such as *IL1β*, *NLRP3*, and *PTGS2* showed upregulation in gout. In contrast, genes such as *CLEC12A* and *IRF1* showed downregulation (Fig 1D). We validated these and other findings using previously published data as a validation group. Among 229 DEGs (false discovery rate < .05, absolute log fold change [FC] > 0.2), we validated 116 DEGs. (Fig 1E). Pathway enrichment of the validated upregulated DEGs (circled by the dashed box) showed significant enrichment of pathways related to nitrogen metabolism, NF-κB activation, and neutrophil degranulation (Fig 1F). The demographic and clinical characteristics of the donors in the discovery group are provided in Supplementary Table S1, while the lists of DEGs per cell type from both the discovery and validation groups can be found in Supplementary Table S2.

High regulatory potential of HIF1A on IL1β expression from CD14 monocytes in gout patients

Within CD14 monocytes, we identified *IL1β* and its receptor accessory protein (*IL1RAP*) as the most upregulated IL genes in gout (Fig 2A). To pinpoint which TFs are predominantly involved in *IL1β* expression regulation in CD14 monocytes from gout patients, we conducted TF regulatory potential analysis starting

from TFs known to regulate *IL1β* expression from curated datasets (see Methods). *HIF1A* emerged as a top-ranked TF potentially regulating *IL1β* expression in CD14 monocytes from gout patients (Fig 2B, Supplementary Fig S2B). This finding aligns with the significantly positive correlation observed between the expression of *IL1β* and *HIF1A* in CD14+ monocytes in gout patients across both discovery and validation groups. In contrast, marginal or no significant correlation was found in the healthy control group, suggesting that this regulation is specific to gout (Fig 2C, Supplementary Fig S2C). Using TF target-weight scoring analysis (see Methods), we further verified the increased expression of *HIF1A* targets in gout CD14 monocytes. The mean expression of its reported curated targets was significantly different between gout and control groups and higher in CD14 monocytes from gout patients (Fig 2D, Supplementary Fig S3A,B).

S100A high CD14 monocytes exhibit elevated inflammatory markers in gout

To elucidate the contribution of CD14 monocyte subsets in the inflammatory cascade of gout, we applied unsupervised clustering to 8132 high-quality CD14 monocyte-derived transcriptomes from all donors, resulting in 5 distinct subclusters (Fig 3A). We found 1 subcluster significantly depleted in people with gout (Dirichlet test adjusted *P* value = .023), a subculture characterised by significant elevation of *CCL3* and *CCL4*

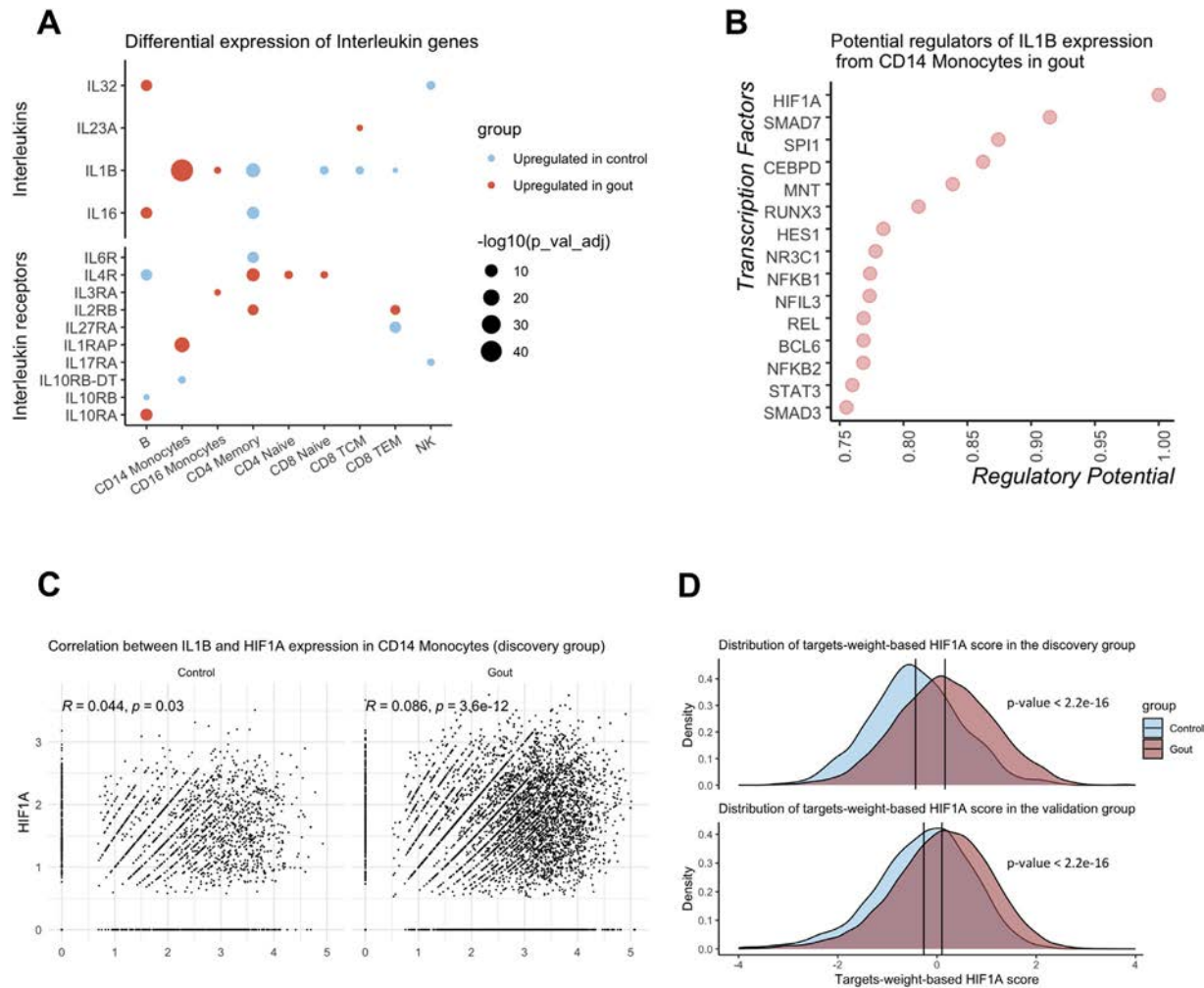


Figure 2. Interleukin (IL)-1B regulation in CD14 monocytes from gout patients. (A) IL-related genes targeted differential expression analysis in all cell types. (B) Regulatory potential scoring of transcription factors on IL-1B expression in gout. (C) Correlation between HIF1A and IL-1B expression in CD14 monocytes in the discovery group. (D) Density plot of the HIF1A target weight-based score in gout and healthy cells. (Kolmogorov-Smirnov test *P* value < 2.2e-16).

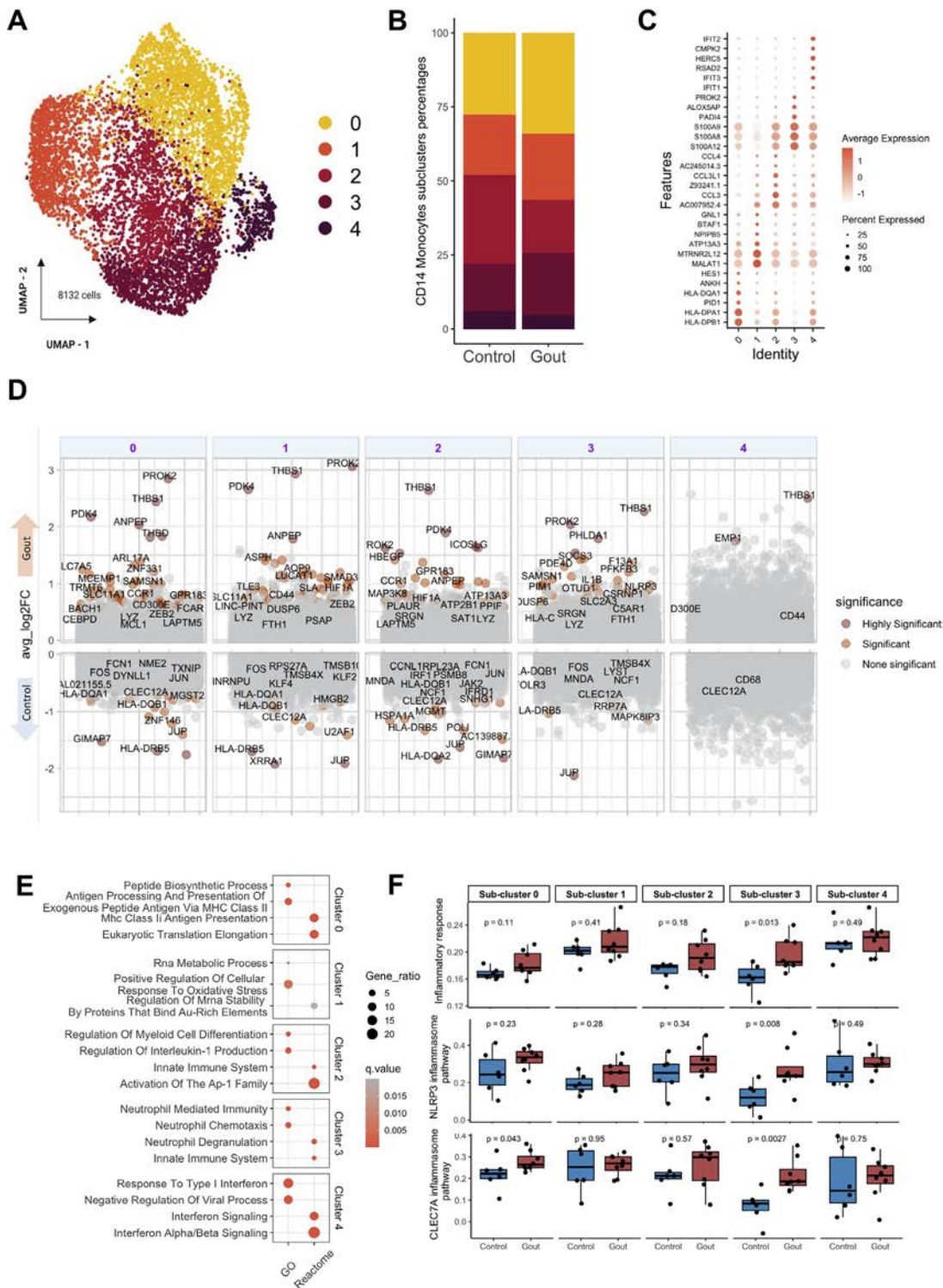


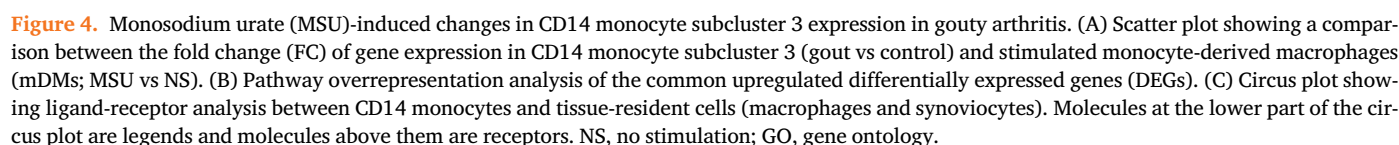
Figure 3. Profiling CD14 monocyte subclusters and examining the activity of S100A^{high} monocytes. (A) UMAP projection of CD14 monocyte subclusters. (B) Proportions of monocyte subclusters in gout and control cells. (C) Dotplot of the markers used in subclusters annotation. (D) The differential gene expression (DEG) analysis between gout patients (upside, n = 8) and healthy control (downside, n = 6) in CD14 monocyte subclusters. DEGs (false discovery rate < .05 and fold change [FC] > 0.2) are labelled by gene symbols. (E) Pathway overrepresentation analysis of the cluster-specific top 50 DEGs. (F) Boxplot showing the module score of distinct pathway sets in cluster 3 of CD14 monocytes in gout and control samples. P values were calculated using the Wilcoxon test. UMAP, uniform manifold approximation and projection; GO, gene ontology.

expression (Fig 3B,C, Supplementary Fig S4A). Examining gout vs control DEGs within each subcluster revealed consistent upregulation of *THBS1* expression and downregulation of *CLEC12A* expression in all subclusters (Fig 3D). On the other hand, subcluster 3 showed significant upregulation of different inflammatory genes such as *IL1 β* , *NLRP3*, and *SOC33*. Subcluster 3 was characterized by high expression of S100A family markers (Fig 3C), thus termed ‘S100A^{high} monocytes,’ in subsequent

analyses. To understand the functional activity of these subclusters, we performed a functional enrichment analysis of the top 50 significant subcluster-specific DEGs (Fig 3E). Particularly, S100A^{high} monocyte (subcluster 3)-specific DEGs were highly enriched in neutrophil-mediated immunity, chemotaxis, and degranulation, but the other subclusters were not highly enriched in these functions. Next, to track *IL1 β* expression from these cells and the inflammatory CD14 monocyte compartments,

emphasise the pivotal role of S100A^{high} monocytes in the regulation of inflammation in gout, modulating diverse inflammatory pathways.

Given the causal role of MSU crystals in gout, we assessed the overlap in DEGs identified in our study and DEGs identified by Cobo et al [17] in MSU crystal-treated monocyte-derived macrophages (mDMs). We identified 32 common DEGs upregulated in both S100A^{high} monocytes (this study) and in cells stimulated with MSU. Interestingly, HIF1A and PTGS2 were significantly upregulated in both S100A^{high} monocytes and mDMs, highlighting a shared effect of gout and MSU on these cells (Fig 4A). Functional enrichment analysis of these 32 DEGs showed their involvement in angiogenesis, cell death regulation, and vitamin B3 metabolism (Fig 4B). Next, to illustrate the molecular interactions between different cell populations within the synovial fluid in gout, we included synovial fluid scRNA data from the



validation group. These data were clustered into different cell types and annotated based on cluster-specific markers (Supplementary Fig S6A,B). We identified several ligand-receptor pairs between monocytes and other cell types, including inflammatory ligands and adhesive or regulatory glycoproteins (Fig 4C). Alongside *IL1 β* , the *CCL3* chemokine and its receptors were consistently expressed by monocytes and other cell types, including nonimmune cells. Although we did not observe the upregulation of *CCL3* in gout monocytes, it was reported as an effect of MSU crystal stimulation on mDMs [17]. The diverse array of inflammatory molecular interactions underscores the intricate nature of synovial tissue inflammation in gout, involving multiple cell types.

GWAS-prioritised gene expression reveals the significance of fatty acid metabolism in gout pathogenesis

To examine the impact of genes genetically associated with gout on the inflammatory phenotype in gout patients, we analysed 100 genes identified in the most recent and comprehensive gout GWAS [18]. These 100 genes were not only associated with gout but also prioritised as potentially involved in the inflammatory process of gout based on 7 criteria defined by the authors

(see Methods) and fulfil at least 4 criteria out of 7. We module-scored the cells based on their expression of these 100 GWAS-prioritised genes, and we found their highest average expression to be in CD14 monocytes and higher in gout CD14 monocytes (Fig 5A). Furthermore, 11 of the GWAS-prioritised genes were also found to be DEGs between gout and controls within the CD14 monocyte subsets (Fig 5B). Functional enrichment of these 100 GWAS-prioritised genes revealed their involvement in multiple fatty acid metabolism-related pathways (Fig 5C). The fatty acid metabolism module score was significantly different between gout and controls in the S100A^{high} monocytes subcluster (*P* value = .013, Wilcoxon test) but not for the other subclusters. We tested the prostaglandin production score as a potential end product of fatty acid metabolism, which also showed a significant difference between gout and control in the S100A^{high} monocytes subcluster (*P* value = .0027, Wilcoxon test) (Fig 5D). This finding was validated within CD14 monocyte subclusters from the validation group, where the highest significant difference in the prostaglandin production score between gout and control groups was also found in the S100A^{high} monocyte subcluster (Fig 5D, Supplementary Fig S7A, B). The fatty acid metabolism score was significantly correlated with NF- κ B signaling, CLEC7 and NLRP3 inflammasomes, and prostaglandin

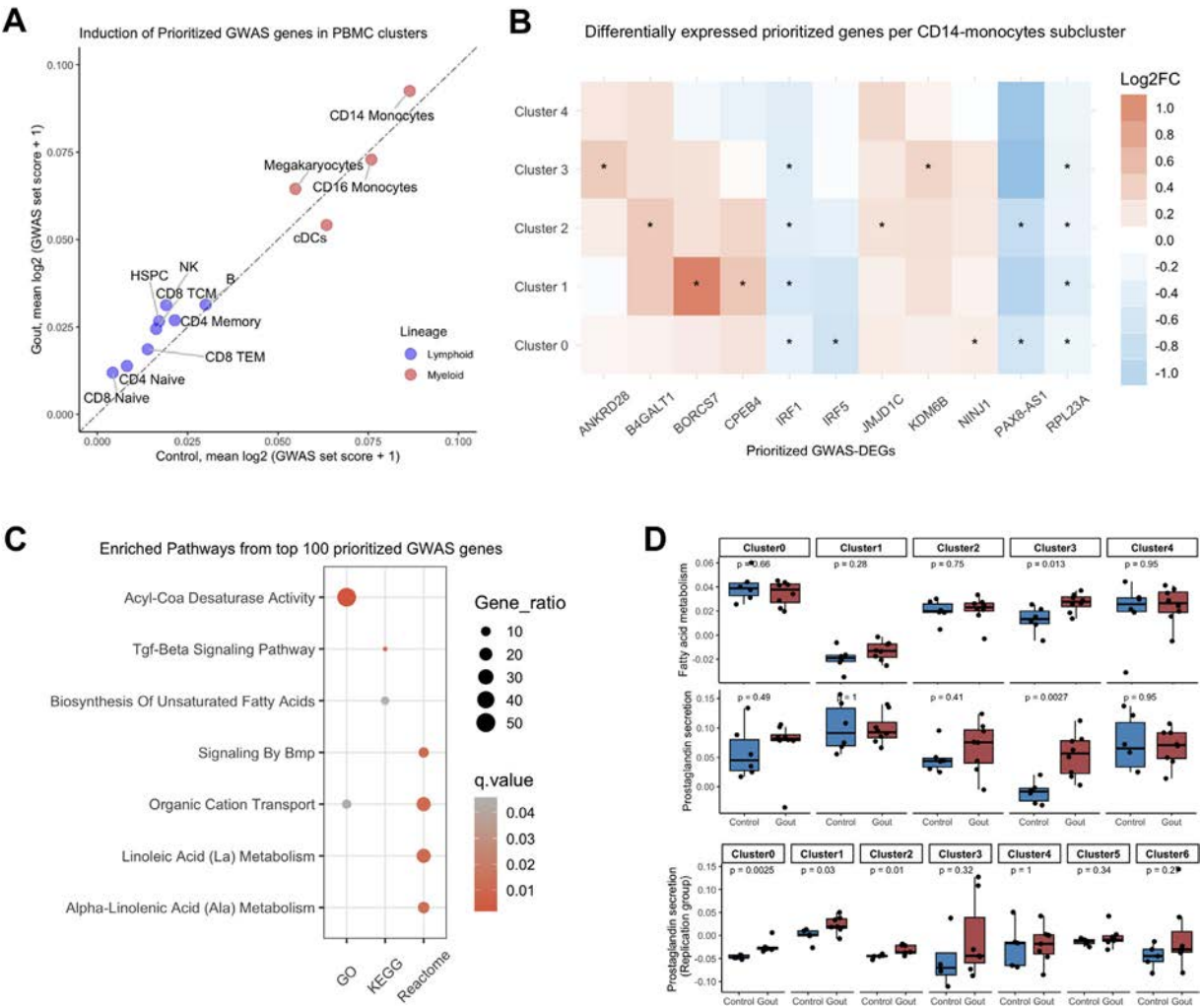


Figure 5. Genome-wide association study (GWAS)-prioritised genes in differentially expressed genes (DEGs; prioritisation score ≥ 4). (A) Scatter plot showing the top 100 GWAS-prioritised gene module scores in gout (y-axis) and control (x-axis) samples in each cell type (dots) coloured by lineage. (B) Differentially expressed GWAS genes in CD14 monocyte subclusters. (C) Pathway overrepresentation analysis of the top 100 GWAS-prioritised genes. (D) Boxplot showing the module score of distinct pathway sets in cluster 3 of CD14 monocytes in gout and control samples. *P* values were calculated using the Wilcoxon test. FC, fold change; PBMC, peripheral blood mononuclear cell; GO, gene ontology; KEGG, Kyoto encyclopedia of genes and genomes.

synthesis scores within S100A^{high} monocytes (Supplementary Fig S7C). DEGs from other cell types showed different intersections with the GWAS-prioritised genes from both discovery and validation groups (Supplementary Fig S8A, B). By comparing the gout effect and different *in vitro* stimulation effects on the GWAS-prioritised gene expression, we found a strong and significant correlation between gout and the *in vitro* MSU crystal stimulation effect but not lipopolysaccharide (LPS) stimulation (Supplementary Fig S8C). In summary, the GWAS-prioritised genes underscore the significant role of fatty acid metabolism pathways in gout pathogenesis, particularly within S100A^{high} monocytes, revealing key genetic contributions to the inflammatory processes underlying this disease.

DISCUSSION

This is the first integrative approach study examining the immune transcriptome profile of gout at single-cell resolution. The study identified CD14 monocytes as the primary cell type exhibiting significant differences in gene expression between gout patients and controls. A notable upregulation of inflammation-related genes and pathways, particularly influenced by HIF1A and MSU crystal, was observed. Our findings further highlight the pivotal role of S100A^{high} CD14 monocytes and fatty acid metabolism in gout inflammation.

We combined our findings with prior knowledge regarding gout inflammation to advance our understanding of the disease and highlight potential therapeutic targets. In this study, we recruited 8 gout and 6 healthy donors for PBMC collection as a discovery group and utilised recently published sequencing data as a validation group [13]. Starting from cell type percentages, we did not observe a statistically significant difference between gout and control populations, unlike the reported observations on the increased naïve lymphoid and CD14 monocyte population in gout [13,14]. Remarkably, the gout upregulated DEG count was consistently higher across all cell types except CD16 monocytes, reflecting broad hyperactivation of the immune system in gout.

Given the high count of DEGs between gout and nongout and established involvement in the pathogenesis of gout, this study centred on elucidating the activity of CD14 monocytes in gout. Studies on CD14 knockout mice have demonstrated impaired inflammatory responses to MSU crystals, with reduced neutrophil recruitment and IL-1 β production, underscoring the pivotal role of CD14 in gout-related inflammation [19]. In our analysis, the differential expression (DE) testing in the CD14 monocyte transcriptome revealed downregulation of *CLEC12A* expression in gout, a finding that was also observed in all CD14 monocyte subclusters. *CLEC12A* depletion is directly linked to the induction of gout inflammation. *CLEC12A* has been shown to act as an innate immune receptor for MSU crystals, limiting MSU crystal-induced immune activation and inflammation both *in vitro* and in mouse models [20]. Interestingly, we document here the downregulation of the *IRF1* TF in both cohorts, which is a key TF in type I interferon signalling [21]. After validation, the upregulated DEGs exhibiting the highest FC values were selected for functional enrichment testing. By this, we aimed to select the DEGs most likely relevant to the disease. These DEGs exhibited their involvement in the inflammatory pathway, including neutrophil degranulation and NF- κ B signalling, nitrogen compound metabolism, and the biosynthesis of chondroitin sulfate.

As expected, IL1 β expression was also significantly upregulated and validated in the gout CD14 monocytes. This IL is the pivotal inflammatory cytokine in gout and is currently targeted

for treatment [2]. DE testing of selected IL genes showed that the upregulation of IL1 β expression was the strongest, a phenomenon uniquely observed in CD14 monocytes (Fig 2A). We then examined the regulatory potential of several TFs on IL1 β expression from gout CD14 monocytes to improve the understanding of the molecular cascades ending with IL1 β expression. *HIF1A* demonstrated a regulatory influence on IL1 β expression in gout CD14 monocytes, a discovery consistent with observations made in the validation group. Furthermore, the expression level of the known targets of *HIF1A* was significantly higher in gout CD14 monocytes [22]. Despite the published finding that directly links *HIF1A* activity to increased IL1 β secretion and monocyte polarisation into M1 macrophages [23], other studies suggest that *HIF1A* activation can directly trigger NF- κ B-mediated inflammation—which ultimately leads to IL1 β gene regulation [24]. This is consistent with the above-mentioned enrichment of the validated DEGs in NF- κ B TF activity, as well as the high potential regulation score of *NFKB1* and *NFKB2* TFs (Figs 1F, 2B). Previous studies have suggested that innate immune memory mechanisms may be involved in the proinflammatory reprogramming of myeloid cells of patients with gout [7,25]. Innate immune memory is dependent on HIF1A activation via the AKT-mTOR pathway [26]. Our group has previously demonstrated that *ex vivo* monocytes from patients with gout exhibit increased mTOR signalling [27]. This activation of the AKT-mTOR pathway was replicated *in vitro* in response to MSU crystal stimulation and elevated urate concentrations, and it was shown to mediate IL-1 β production [27,28]. The present findings reinforce the role of the AKT-mTOR-HIF1A axis in the proinflammatory adaptation observed in gout, suggesting a rewiring of inflammatory pathways similar to those described in innate immune memory [26].

In pursuit of pinpointing the specific cellular compartment implicated in gout-related inflammatory pathways, our investigation extended to the characterisation of CD14 monocyte subclusters. Through the functional enrichment results of these subcluster DEGs, we identified a subset notably enriched in pathways pertinent to neutrophil activity (S100A^{high} monocytes). The S100A protein family—especially S100A8 and S100A9—are well-characterised inflammatory proteins that contribute highly to metabolic and degenerative diseases [29]. In the arthritis context, they facilitate unsaturated fatty acid transport and arachidonic acid conversion to leukotrienes, strongly stimulated by IL-1 β in chondrocytes, resulting in proinflammatory cytokine generation and priming gouty inflammation by binding to TLR4 and activating the NF- κ B axis [30–34]. Notably, S100A^{high} monocytes were aligned with previously validated pathways linked to neutrophil activity. While our analysis did not directly include neutrophils, residual neutrophil precursors from PBMC isolation may contribute to the observed inflammatory signature, emphasising the broader role of S100A^{high} monocytes in driving neutrophil activity and gout pathogenesis [35]. The gout upregulated DEGs in this subcluster include several genes reported in gout inflammation, such as *NLRP3*, *IL1 β* , *SOCS3*, and *PTGS2* [1,14,36]. Consequently, we detected a significant increase in NLRP3/CLEC7A inflammasome and canonical NF- κ B pathway module scores in gout S100A^{high} monocytes.

To quantify the overlap and assess the mutual influence between the gout effect identified in the discovery group and the MSU crystal effect on monocytes, we intersected the DEGs from the inflammatory CD14 monocyte subcluster (S100A^{high} monocytes) with *in vitro* MSU crystal stimulation on mDMs [17]. Remarkably, 86% of the intersected DEGs were commonly

upregulated, likely reflecting the inflammatory gene expression profile characteristic of gout patients with hyperuricemia. These shared DEGs were enriched in niacin metabolism, cell death regulation, and hypoxia signs (angiogenesis and HIF1A). The repeated evidence of *HIF1A* upregulation from the TF analysis, hypoxia module score, and MSU crystal effect support its role mentioned above. Interestingly, the nicotinamide metabolism pathway was highly enriched, which could be due to compensation for the depletion of Nicotinamide adenine dinucleotide (NAD⁺) molecules as a consequence of the MSU crystal effect [37]. Meanwhile, nicotinamide is also known for its ability to induce hyperuricemia, which would sustain the MSU crystal effect [38]. Besides the identified gout-induced inflammatory molecules in our study, other MSU crystal-induced molecules, such as CCL3 and tumour necrosis factor, as well as their receptors, are actively expressed by synovial fluid cells [17,39].

Lastly, we examined the convergence of the gout-inflammatory GWAS-prioritised genes and our single-cell transcriptomes. The genetically prioritised gene module scores were found to be pre-eminent in myeloid cells, especially gout CD14 monocytes. Those genes were mainly enriched in fatty acid metabolism-related pathways, bone morphogenetic protein signalling, and transforming growth factor (TGF)- β signalling. Starting from these findings, the fatty acid metabolism module scores were accessed within CD14 monocyte subclusters. Again, S100A^{high} monocytes showed a significant increase in this score and the expected resulting prostaglandin secretion score. Fatty acid metabolism was consistently correlated with the previously identified inflammatory pathways, including CLEC7/NLRP3 inflammasome, NF- κ B signalling, and prostaglandin secretion in the S100A^{high} monocytes (Supplementary Fig S8C). This subcluster expresses the *ALOX5AP* gene (a subcluster-specific marker; Fig 5C), which is an essential gene in arachidonic acid-induced inflammation [40]. We illustrated the association between the prominent fatty acid metabolism pathway and various identified CD14⁺ monocyte-associated inflammatory mechanisms within the S100A^{high} monocyte subcluster. Fatty acid metabolism is a metabolic pathway linked with innate immune memory formation [41,42], supporting a potential role for fatty acid metabolism in the immunometabolic circuitry associated with long-term proinflammatory reprogramming in gout. Another pathway that we identified as significantly over-represented among the GWAS-prioritised genes is TGF- β (Fig 5C). We have previously shown that TGF- β is upregulated in both gout and hyperuricemia [43] and that the proinflammatory myeloid phenotype induced by urate acts through TGF- β signalling [44]. Additionally, TGF- β was found to engage in cell-cell interactions with dendritic cells in the validation study [13], emphasising the complex interplay between genetic factors, cellular metabolism, and inflammatory pathways in gout.

Limitations of this study include the inability to analyse neutrophils despite their well-established role in gout-related inflammation, which restricts a comprehensive understanding of the cellular mechanisms involved in the disease. Additionally, further *in vivo* validation of the identified pathways and regulatory mechanisms is necessary to fully elucidate their contributions to gout pathogenesis. The study's sample size was limited and did not accurately reflect the broader gout patient population, where male prevalence is at least 3-fold higher than females [45]. Furthermore, the exclusion of 2 female samples during mandatory quality control procedures disrupted sex matching among control donors, even though the gout donor group maintained equal sex distribution. Lastly, our efforts to replicate findings in the validation group were sometimes

unsuccessful due to differences in group composition and challenges associated with matching subclusters across groups, likely due to the fact that the validation group consisted of acute gout patients, whereas our study focused primarily on intercritical patients. Longitudinal studies capturing transcriptional changes across both flare and remission phases are warranted to address these gaps.

CONCLUSION

Our findings underscore the significance of *CLEC12A*, *HIF1A*, and the S100A^{high} monocyte subset as pathophysiological pathways and potential therapeutic targets in gout management. Downregulation of *CLEC12A* expression highlights its crucial role in limiting the inflammatory response, while *HIF1A* emerges as a central regulator of *IL1 β* expression, linking hypoxia signalling to gout inflammation. Furthermore, the S100A^{high} monocyte subset stands out for its involvement in neutrophil-mediated immunity and its association with NLRP3 inflammasome activation and prostaglandin secretion. Integration with genetic data identifies fatty acid metabolism as a potential immunometabolic process of interest for inflammation in gout. In conclusion, our work advances the current understanding of gout inflammation, offering insights into immune cell dynamics and highlighting novel avenues for targeted therapeutic intervention in this prevalent inflammatory disorder.

Competing interests

LABJ is a member of the scientific advisory board of Olatec Therapeutics. MGN and LABJ are scientific founders of TTXD and Lemba. All other authors declare no competing financial interests in relation to the work described.

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Contributors

Study concept and design: LABJ, YL, TOC, C-JX, and AA. Acquisition of data: AA, GC, and TOC. Cohort recruitment and peripheral blood mononuclear cell isolation: GC, TOC, and CP. Single-cell experiment: AA and LZ. Analysis and interpretation of data: AA, GC, LABJ, YL, TOC, C-JX, JB-B, MZ, and WL. Drafting of the manuscript: AA and GC. Critical revision of the manuscript for important intellectual content: all authors. Statistical analysis: AA, C-JX, and YL. Obtained funding: YL and LABJ. Administrative, technical, or material support: all authors. Study supervision: TOC, C-JX, YL, and LABJ. Guarantor: YL and LABJ (shared correspondence).

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Patient consent for publication

Informed consent was obtained from all participants.

Ethics approval

Ethical approval was obtained from the University of Medicine and Pharmacy of Cluj-Napoca, Romania (approval number: 422/08.10.2019).

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Not commissioned; externally peer reviewed.

Data availability statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Patient and public involvement

Not applicable.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ard.2025.01.046.

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Osteoarthritis

Lipidomics unravels lipid changes in osteoarthritis articular cartilage

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ABSTRACT

Objectives: Osteoarthritis (OA) is linked to disrupted lipid metabolism. We aimed to profile the lipid composition of human articular cartilage, investigate OA-associated lipidome changes, and explore biological effects.

Methods: Lipidomic profiling and computational analyses were performed on human articular chondrocytes (hACs) from non-OA (n = 13) and OA (n = 14) hips. Lipid changes were confirmed in the destabilisation of the medial meniscus (DMM) mouse model. The effect of specific lipids was evaluated by *in vitro* supplementation and gene silencing.

Results: We identified 573 lipid species covering 11 lipid classes in hACs. OA and non-OA hACs showed distinct lipid profiles. Most ceramides and dihydroceramides were increased, while cholesterol esters, diacylglycerols, triacylglycerols, sphingomyelins, hexosylceramides, and lactosylceramides were predominantly decreased in OA chondrocytes. Most upregulated lipids in OA contained C18:1, C20:4, or C22:4 side chains. Many downregulated lipids contained C18:2 or odd-chain C17:0. Lipid profiling of articular cartilage from the DMM mouse model paralleled changes in OA hACs, including odd-chain C17:0 reduction. Further analysis showed that deficiency in enzyme 2-hydroxyacyl-CoA lyase 1 (HACL1), responsible for odd-chain fatty acid synthesis, leads to accumulation of 2-hydroxy C18:0, precursor of C17:0, which results in a shift in hACs from an anabolic to a catabolic state.

Conclusions: Our study maps the hAC lipid composition and highlights changes in lipid profiles associated with OA. Dysregulation of certain lipids, especially odd-chain fatty acids, linked to a deficiency in the enzyme HACL1, leads to pathological changes. This understanding opens potential avenues for therapies aimed at targeting lipid imbalances to slow down or treat OA.

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WHAT IS ALREADY KNOWN ON THIS TOPIC

- Osteoarthritis (OA) is a widespread, yet incurable, joint disease characterised by cartilage degradation.
- Several risk factors for OA, such as ageing, obesity, and metabolic syndrome, are strongly associated with disrupted lipid metabolism. Prior proteomic analyses and gene expression studies in cartilage of OA patients have revealed dysregulations in lipid-related pathways. However, the lipidome of articular cartilage has not been systematically characterised, and how the lipid profile changes in OA remains poorly defined.

WHAT THIS STUDY ADDS

- We mapped the lipid profile of human articular cartilage and identified OA-associated lipid changes. We found that specific lipid alterations in classes, species, and side chain composition are associated with human OA.
- We validated our findings in a mouse model of OA. Our data indicate that lipidome changes in articular cartilage from osteoarthritic mice largely mirror the changes observed in human osteoarthritic cartilage.
- Our detailed fatty acid analysis highlighted a marked reduction in odd-chain fatty acid C17:0 associated with OA. We identified that deficiency in the enzyme 2-hydroxyacyl-CoA lyase 1 (HACL1), responsible for odd-chain fatty acid synthesis, leads to accumulation of 2-hydroxy C18:0, precursor of C17:0, causing detrimental effects on chondrocytes.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- Our study provides a detailed resource on the lipidome of articular cartilage and its changes in OA.
- HACL1-mediated dysregulation of odd-chain fatty acids is linked to pathological changes in human articular chondrocytes, suggesting opportunities for therapeutic targeting.

INTRODUCTION

Osteoarthritis (OA), the most common chronic joint disease, affects over 500 million people globally, emerging as a leading cause of disability and source of persistent pain, thereby imposing a substantial socioeconomic burden [1]. Current treatment strategies for OA are very limited, offering only relief of symptoms and, in severe cases, requiring joint replacement surgery. Disease-modifying or curative approaches are entirely lacking, underscoring an urgent medical need. OA is primarily characterised by progressive degradation of the articular cartilage, often accompanied by increased joint-associated bone remodelling and varying degrees of inflammation [2]. The articular cartilage, crucial for normal joint mobility, consists of articular chondrocytes embedded within a self-produced extracellular matrix (ECM) mainly composed of type 2 collagen fibres and proteoglycan aggrecan [3]. In OA, molecular alterations in chondrocytes lead to the synthesis of an ECM of inferior biomechanical quality and increased production of tissue-destructive enzymes, together exacerbating tissue damage and contributing to functional impairment [3].

OA is a complex multifactorial disease. Several important risk factors for OA such as ageing, obesity, and metabolic syndrome are strongly associated with dysregulations in lipid metabolism [4]. In line with this, proteomic analyses [5,6] and gene expression studies [7,8] in cartilage of OA patients have revealed dysregulations in lipid-related pathways [5–8]. Despite these accumulating observations that suggest an important role for lipids in OA, the composition of the chondrocyte's lipidome

remains poorly defined, and the specific lipid changes within OA cartilage are largely unknown.

Lipids represent a diverse array of water-insoluble molecules such as fatty acids, triacylglycerols (TAG), phospholipids, and cholesterol, playing pivotal roles as constituents of cellular membranes, energy sources, and signalling molecules [9]. Despite these important biological functions, lipid research has not attained the same level of scientific attention as studies focusing on proteins, RNA, and DNA. This discrepancy partly arises from the extensive variety of lipid species and the high structural complexity of lipids, and their lack of genetic encoding, necessitating the use of direct chemical analysis methods for scientific investigations. However, recent advances in technology have facilitated lipidomics, enabling more comprehensive investigations into the structure and function of lipids within cells and tissues [10,11]. Such technologies offer unprecedented opportunities to understand the role of lipids in the pathogenesis of OA.

Here, using targeted shotgun lipidomics, we mapped the lipid landscape of human articular cartilage and its disease-associated changes in osteoarthritic patients. Importantly, we validated these findings in a standardised surgical mouse model of the disease. Our data reveal significant changes in odd-chain fatty acids within the chondrocyte lipidome in both humans and mice with OA, suggesting a novel molecular mechanism driving disease progression. These findings highlight potential avenues for therapeutic intervention by targeting lipid dysregulation, with potential to modify the disease course and to improve patient outcomes.

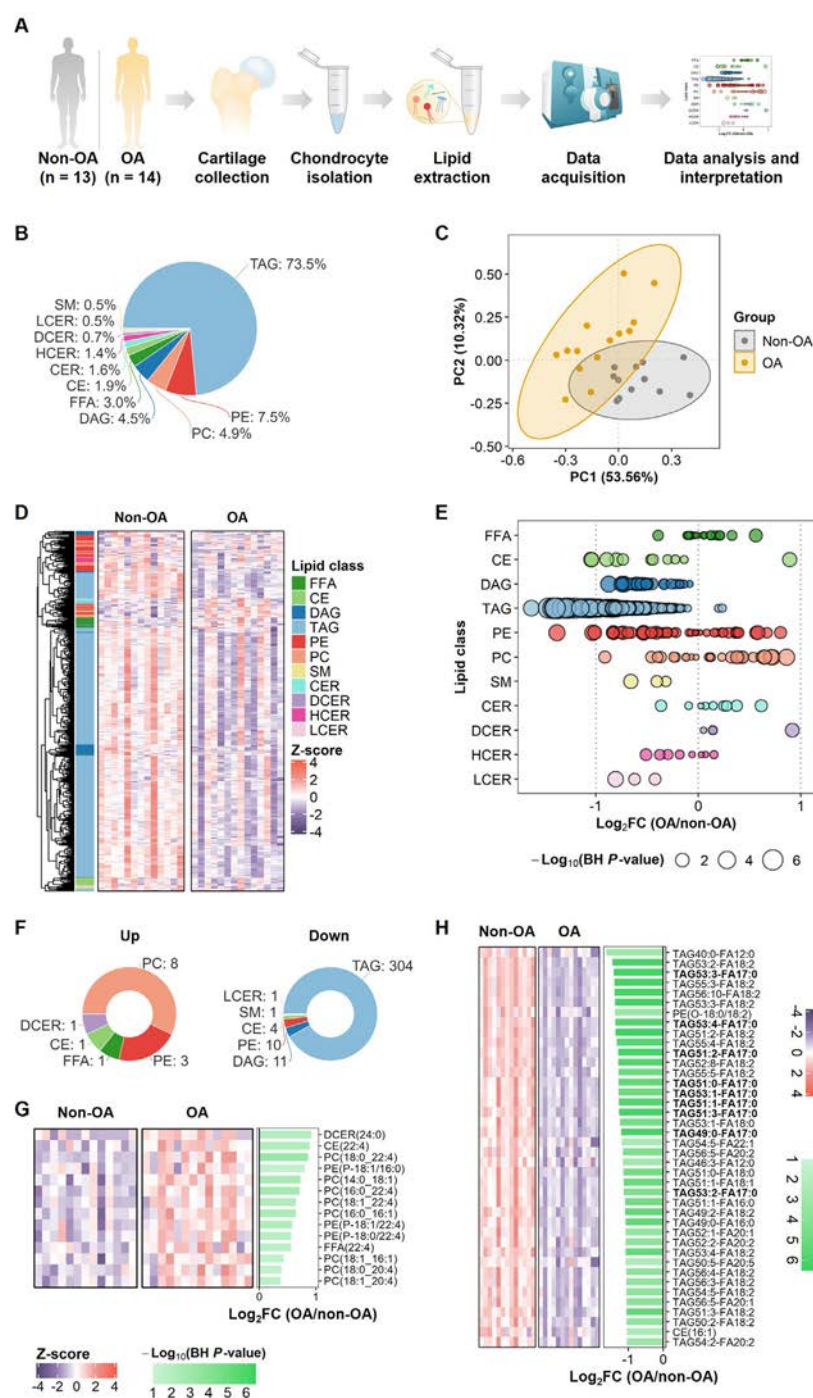
METHODS

Extended materials and methods are provided in the supplementary materials.

RESULTS

Lipidomic profiling of articular chondrocytes reveals alterations in lipid classes and species associated with human OA

To map the lipid profile of human articular cartilage and identify OA-associated lipid changes, we performed a comprehensive lipidomics analysis of human articular chondrocytes isolated from OA patients (n = 14) and non-osteoarthritic controls (n = 13) (Fig 1A; Supplementary Table S1). This analysis covered 13 lipid classes: free fatty acids (FFA), sterol lipids (cholesteryl esters [CE]), glycerolipids (including diacylglycerols [DAG] and TAG), glycerophospholipids (including phosphatidylethanolamines [PE], phosphatidylcholines [PC], lysophosphatidylethanolamines [LPE], and lysophosphatidylcholines [LPC]), and sphingolipids (including sphingomyelins [SM], ceramides [CER], dihydroceramides [DCER], hexosylceramides [HCER], and lactosylceramides [LCER]). Representative structures of these lipid classes [12] are presented in Supplementary Figure S1. The result of data quality control indicated that most lipids had no missing values (Supplementary Fig S2A) and technical reproducibility was examined prior to full data analysis (Supplementary Fig S2B). Lipid species from the LPE and LPC classes were excluded from further analyses because quality control standards were not met for these groups. After data processing and normalisation, we identified 573 lipid species from 11 lipid classes across the articular chondrocytes of different individuals in both groups (Supplementary Table S2). Most identified lipid species belonged to the TAG class (73.5% of total identified



lipids), followed by PE (7.5%), PC (4.9%), DAG (4.5%), FFA (3.0%), CE (1.9%), CER (1.6%), HCER (1.4%), DCER (0.7%), LCER (0.5%), and SM (0.5%) classes (Fig 1B).

Next, we aimed to determine differences in lipid classes and species between osteoarthritic and non-osteoarthritic human articular chondrocytes. Principal component analysis (PCA) separated the disease and control samples (Fig 1C). The heatmap depicting the relative abundance of lipid species revealed lipidomic signatures that distinguish between the 2 groups (Fig 1D). Hierarchical clustering analysis of lipid species showed that TAG, DAG, glycerophospholipid (PC and PE), CE, and FFA species clustered together respectively (Fig 1D). Differential analysis of individual lipid species showed that lipids within the CE, DAG, TAG, SM, HCER, and LCER classes were predominantly decreased, with TAG showing the largest effect (Fig 1E). In contrast most FFA, CER, and DCER species were increased in

osteoarthritic vs non-osteoarthritic chondrocytes (Fig 1E). A total of 345 lipid species were significantly changed (false discovery rate [FDR] < 0.05), with 14 lipids increased and 331 lipids decreased in the osteoarthritic samples (Fig 1F; Supplementary Table S3). In detail, upregulated lipids consisted of 8 PC, 3 PE, 1 FFA, 1 CE, and 1 DCER species, while downregulated lipids included 304 TAG, 11 DAG, 10 PE, 4 CE, 1 SM, and 1 LCER species (Fig 1F). Most upregulated lipids contained, as side chains, C18:1 (eg, oleic acid), C20:4 (eg, arachidonic acid), or C22:4 (eg, adrenic acid) (FDR < 0.05; Fig 1G). The top-40 downregulated lipid species in osteoarthritic chondrocytes highlighted markedly low levels of lipids (mostly TAG) that contained C18:2 (eg, linoleic acid) or C17:0 (eg, margaric acid), an odd-chain fatty acid (FDR < 0.05; Fig 1H). The presence of TAG with odd-chain fatty acids was further confirmed through high resolution mass spectrometry-based lipid analysis. We

specifically examined TAG with odd-chain fatty acids and ether-linked TAG with highly similar molecular masses. For example, TAG 16:0_17:0_16:1 and TAG-O 18:0_16:0_16:1 would present with $[M+NH_4]^+$ molecular ions at 836.7709 and 836.7904 Da, respectively (Supplementary Fig S3A,B). We could confirm these structures by tandem mass spectrometry analysis and further validate our finding of decreased TAG with the odd-chain fatty acid, not ether-linked TAG, in OA samples (Supplementary Fig S3C,D). This observation was striking because odd-chain fatty acids have low abundance and are generally considered to be insignificant or nonfunctional in humans [13]. However, an inverse association between circulating odd-chain fatty acids and disease risk has been reported, for instance, in coronary heart disease and type 2 diabetes [14,15], suggesting that these rare lipids may play a role in disease pathogenesis. Overall, the enrichment of the indicated fatty acid chains in differentially regulated lipid classes and species in diseased chondrocytes shows that specific lipid alterations are associated with human OA.

Lipidomics profiling of articular cartilage from mice with surgically induced OA validates lipid insights in human osteoarthritic cartilage

The lipid profile of patients can be influenced by genetic factors [16,17] and by lifestyle and environmental cues, such as diet [18] and physical exercise [19]. We therefore sought to verify our findings obtained in human cartilage by using a standardised animal model of OA in which genetic, environmental, and dietary factors are strictly controlled. To this end, we used the surgical destabilisation of the medial meniscus (DMM) model to induce OA in mice, characterised by gradually progressing disease development driven by altered joint biomechanics [20]. We first analysed the lipidome in articular cartilage from DMM-operated mice and sham controls 12 weeks after surgery ($n = 5$ animals per group) (Fig 2A). Data quality control revealed that most lipids had no missing values (Supplementary Fig S2C), and technical reproducibility of lipid screening was examined before subsequent data analysis (Supplementary Fig S2D). In mouse articular cartilage, we identified 762 lipid species covering 13 lipid classes, and most identified lipids were TAG (Fig 2B; Supplementary Table S4), similar to our observations in human cartilage (Fig 1B). Sham control and DMM mouse samples were not completely separated by PCA, but most variation was explained by PC1 (Fig 2C). The heatmap of relative abundance of lipid species evidenced a distinct lipidomic signature between the groups and, mirroring the signature of human OA cartilage, most lipids were decreased in the articular cartilage of osteoarthritic mice (Fig 2D). Hierarchical clustering analysis of lipid species showed the clustering of TAG and glycerophospholipids (PC and PE) (Fig 2D). Differential analysis of individual lipid species indicated that HCER and LCER were increased in articular cartilage of osteoarthritic mice compared with sham controls, while most FFA, CE, DAG, TAG, PE, PC, LPE, LPC, SM, and CER species were reduced (Fig 2E). Upregulated lipids included CE(20:2), LCER(24:0), and TAG36:0-FA12:0 ($FDR < 0.05$; Fig 2F,G; Supplementary Table S5). Downregulated lipids included 465 TAG and 13 DAG as the main decreased lipid classes ($FDR < 0.05$; Fig 2F; Supplementary Table S5). Most of top-40 decreased lipid species in articular cartilage from mice with OA contained fatty acid chains including C14:0 (eg, myristic acid), C16:0 (eg, palmitic acid), odd-chain C17:0 (eg, margaric acid), C20:0 (eg, arachidic acid), or C20:1 (eg, gadoleic acid) ($FDR < 0.05$; Fig 2H). Altogether, these data indicate that lipidome changes in articular cartilage from osteoarthritic mice largely mirror the

changes observed in human osteoarthritic cartilage, in particular the reduction in overall TAG levels and the decrease in TAG species that contain odd-chain fatty acid C17:0.

We further analysed the time course changes in lipid species and classes 2, 4, and 12 weeks after the induction of OA. No lipid species were significantly altered at 2 and 4 weeks after surgery in contrast to the changes detected at 12 weeks (Supplementary Fig S4A). At 4 weeks, the total levels of most lipid classes showed a transient increase in osteoarthritic mice compared with the sham control, with LPC and SM showing the most pronounced changes (Supplementary Fig S4B). After 12 weeks, most lipid classes were decreased, with TAG displaying the most substantial reduction (Supplementary Fig S4B).

Specific shifts in fatty acid side chain composition in articular cartilage are associated with human OA

We then performed a deeper analysis of the fatty acid side chain composition of the different lipids in human articular chondrocytes and the changes therein that are associated with OA. Fatty acids are aliphatic chains with different numbers of carbons and double bonds, contributing to the diversity in lipids by varying chain length and degree of unsaturation, respectively. We analysed fatty acid side chains in 3 dimensions: (1) individual species (Fig 3A-C), (2) length (Fig 3D-F), and (3) degree of unsaturation (Fig 3G-I). For each dimension, we analysed the data at 3 different levels: (1) the number of measured fatty acid chains within the different lipid classes, (2) the fold change of specific fatty acid chains in osteoarthritic chondrocytes compared with non-osteoarthritic chondrocytes, and (3) the fold change of specific fatty acid chains that are statistically significantly different between osteoarthritic chondrocytes and non-osteoarthritic chondrocytes presented by lipid class. Twenty-six different fatty acid chains were detected in human articular chondrocytes (Fig 3A). C16:0, C18:0, C18:1, and C18:2 showed the highest counts in our analysis (Fig 3A). In osteoarthritic chondrocytes, fatty acid chains C12:0, C18:0, C18:2, and odd-chain C17:0, and to a lesser extent C15:0, were among those predominantly reduced compared with non-osteoarthritic chondrocytes (Fig 3B). The analysis of the significantly dysregulated fatty acid chains in osteoarthritic chondrocytes provided more details about their type and distribution among lipid classes (Fig 3C). Most significantly decreased fatty acid side chains in osteoarthritic chondrocytes were found in the TAG class of lipids (Fig 3C). Only C14:0, C16:0, C16:1, C18:0, C18:1, C20:4, C22:4, and C24:0 were significantly increased in specific species, mainly of the PC and PE classes (Fig 3C).

We next interrogated differences in fatty acid chain length between osteoarthritic and control human articular chondrocytes. Long-chain fatty acids (LCFA) were the most abundant fatty acids found in articular chondrocytes, with fewer very-long-chain fatty acids (VLCFA) and medium-chain fatty acids (MCFA) showing the lowest number (Fig 3D). Specifically, the most abundant fatty acids were 18- and 16-carbon LCFA (Supplementary Fig S5A). Moreover, most MCFA, LCFA, and VLCFA were downregulated in OA (Fig 3E), especially 17- and 18-carbon LCFA (Supplementary Fig S5B). Additionally, among significantly regulated fatty acids, most downregulated MCFA, LCFA, or VLCFA were TAG; all the increased LCFA were species of the PE or PC class, while increased VLCFA were PC, PE, DCER, CE, or FFA (Fig 3F; Supplementary Fig S5C).

Next, we analysed differences in fatty acid chain unsaturation between osteoarthritic and control human articular chondrocytes. Higher counts of saturated fatty acids (SFA) and

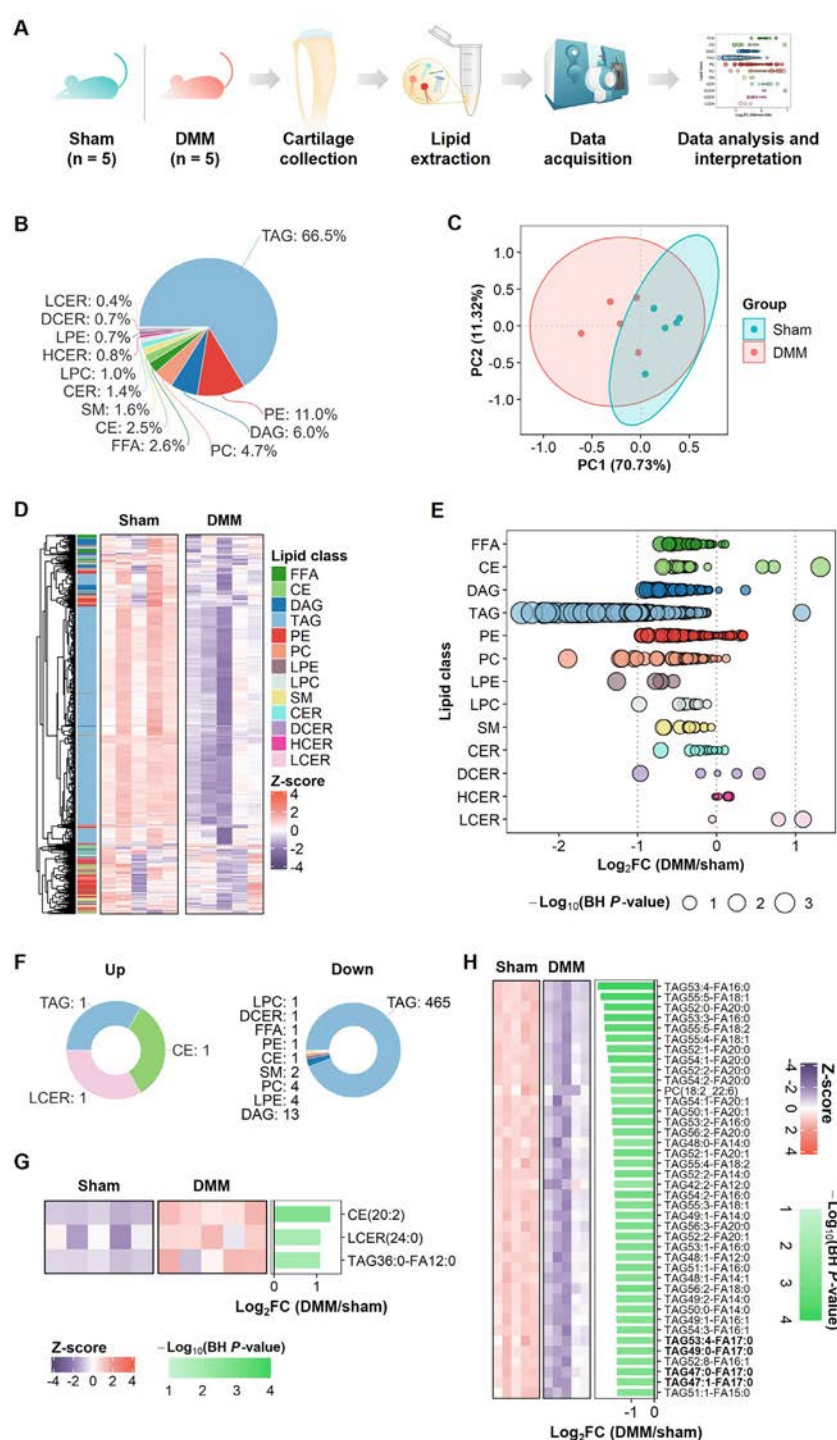


Figure 2. Changes in lipid classes and species associated with mouse osteoarthritis. (A) Overview of the lipidomics analysis of mouse articular cartilage. (B) Pie chart indicating the proportions of identified lipid species within each lipid class in articular cartilage. (C) PCA showing clustering of sham and DMM mice. Each dot represents 1 mouse. (D) Heatmap displaying z-scored abundance of lipid species in articular cartilage from sham and DMM mice. Hierarchical clustering of lipid species was performed. (E) Log₂ fold changes of lipid species summarised by lipid classes in articular cartilage of DMM vs sham mice. Each bubble indicates a lipid species. The bubble size represents the statistical significance level. (F) Donut plot indicating the number of significantly up- and downregulated lipid species within each lipid class (FDR < 0.05). (G,H) Heatmap displaying z-scored abundance of significantly (G) increased and (H) top-40 decreased lipid species in articular cartilage of DMM compared with sham mice (FDR < 0.05). The bar plot shows the log₂ fold changes of lipid species in articular cartilage of DMM vs sham mice. The bar colour represents the significance level. BH, Benjamini-Hochberg; CE, cholesteryl esters; DAG, diacylglycerols; DCER, dihydroceramides; DMM, destabilisation of the medial meniscus; FC, fold change; FDR, false discovery rate; FFA, free fatty acids; LCER, lactosylceramides; LCFA, long-chain fatty acids; MCFA, medium-chain fatty acids; MUFA, monounsaturated fatty acids; OA, osteoarthritis; PC, phosphatidylcholines; PCA, principal component analysis; PE, phosphatidylethanolamines; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; SM, sphingomyelins; TAG, triacylglycerols; VLCFA, very-long-chain fatty acids.

polyunsaturated fatty acids (PUFA) were detected compared to monounsaturated fatty acids (MUFA) in these cells (Fig 3G; Supplementary Fig S5D). Most SFA, MUFA, and PUFA were decreased in osteoarthritic chondrocytes (Fig 3H), particularly those with 0-2 double bonds (Supplementary Fig S5E). One hundred forty-six of 249 SFA, 85 of 161 MUFA, and 146 of 260 PUFA were significantly changed in osteoarthritic human articular chondrocytes, mainly in species from the TAG, DAG, PE, and PC classes (Fig 3I). The analysis of significantly regulated fatty acids grouped by unsaturation degree exhibited similar changes among SFA, MUFA, and PUFA, as reduced fatty acids were mainly species from the TAG class and increased lipids were mainly PC or PE (Fig 3I; Supplementary Fig S5F). In summary, the observed shifts in the fatty acid side chains of lipids from osteoarthritic human articular chondrocytes, at the 3

dimensions including individual species, chain length, and unsaturation, document the fatty acid spectrum in articular chondrocytes and demonstrate that specific changes in the lipidome are associated with human OA.

Fatty acid side chain analysis of articular cartilage from mice with surgically induced OA validates identifies shifts in human osteoarthritic cartilage

We then examined whether identified lipid side chain shifts in osteoarthritic human articular chondrocytes are also observed in the standardised DMM mouse model of the disease. We first profiled the fatty acid composition at 12 weeks after DMM induction. Twenty-seven different fatty acid chains were detected in mouse articular cartilage (Fig 4A). C16:0, C18:0,

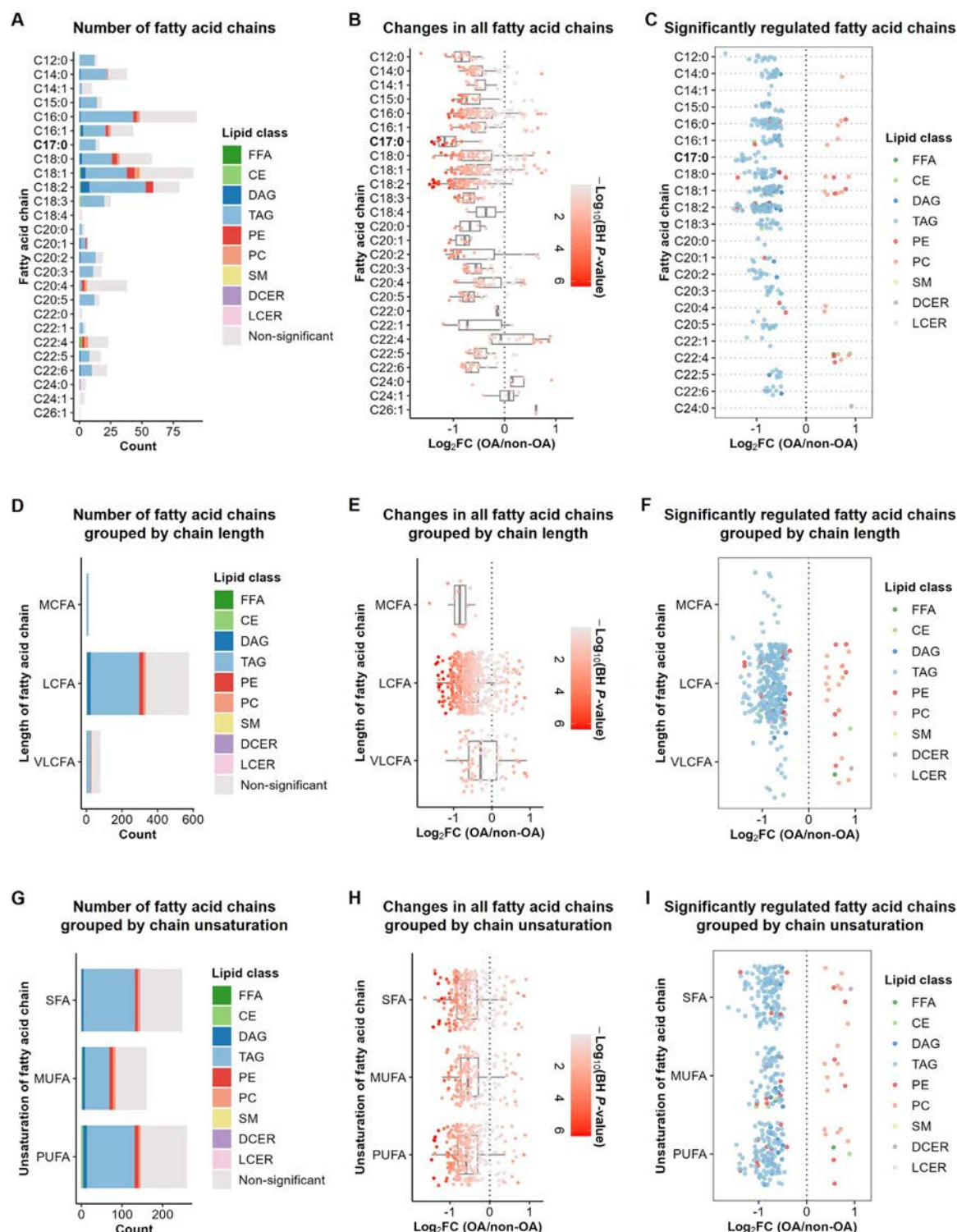


Figure 3. Changes in fatty acid chain composition associated with human osteoarthritis. (A) Number of fatty acid chains identified in hACs by lipid class (bar colour). (B) Log₂ fold changes of individual fatty acid chains in OA vs non-OA hACs. Each dot represents 1 fatty acid chain. The dot colour indicates the statistical significance level. (C) Log₂ fold changes of significantly altered fatty acid chains in OA vs non-OA hACs (FDR < 0.05) by lipid class (dot colour). (D) Number of fatty acid chains categorised by chain length in hACs per lipid class (bar colour). (E) Log₂ fold changes of fatty acid chains categorised by chain length in OA vs non-OA hACs. Each dot represents 1 fatty acid chain. The dot colour indicates the significance level. (F) Log₂ fold changes of significantly altered fatty acid chains categorised by chain length in OA vs non-OA hACs (FDR < 0.05) per lipid class (dot colour). (G) Number of fatty acid chains categorised by chain unsaturation in hACs by lipid class (bar colour). (H) Log₂ fold changes of fatty acid chains categorised by chain unsaturation in OA vs non-OA hACs. Each dot represents 1 fatty acid chain. The dot colour indicates the significance level. (I) Log₂ fold changes of significantly altered fatty acid chains categorised by chain unsaturation in OA vs non-OA hACs (FDR < 0.05) by lipid class (dot colour). BH, Benjamini-Hochberg; CE, cholesteryl esters; DAG, diacylglycerols; DCER, dihydroceramides; DMM, destabilisation of the medial meniscus; FC, fold change; FDR, false discovery rate; FFA, free fatty acids; hAC, human articular chondrocyte; LCER, lactosylceramides; LCFA, long-chain fatty acids; MCFA, medium-chain fatty acids; MUFA, monounsaturated fatty acids; OA, osteoarthritis; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; SM, sphingomyelins; TAG, triacylglycerols; VLCFA, very-long-chain fatty acids.

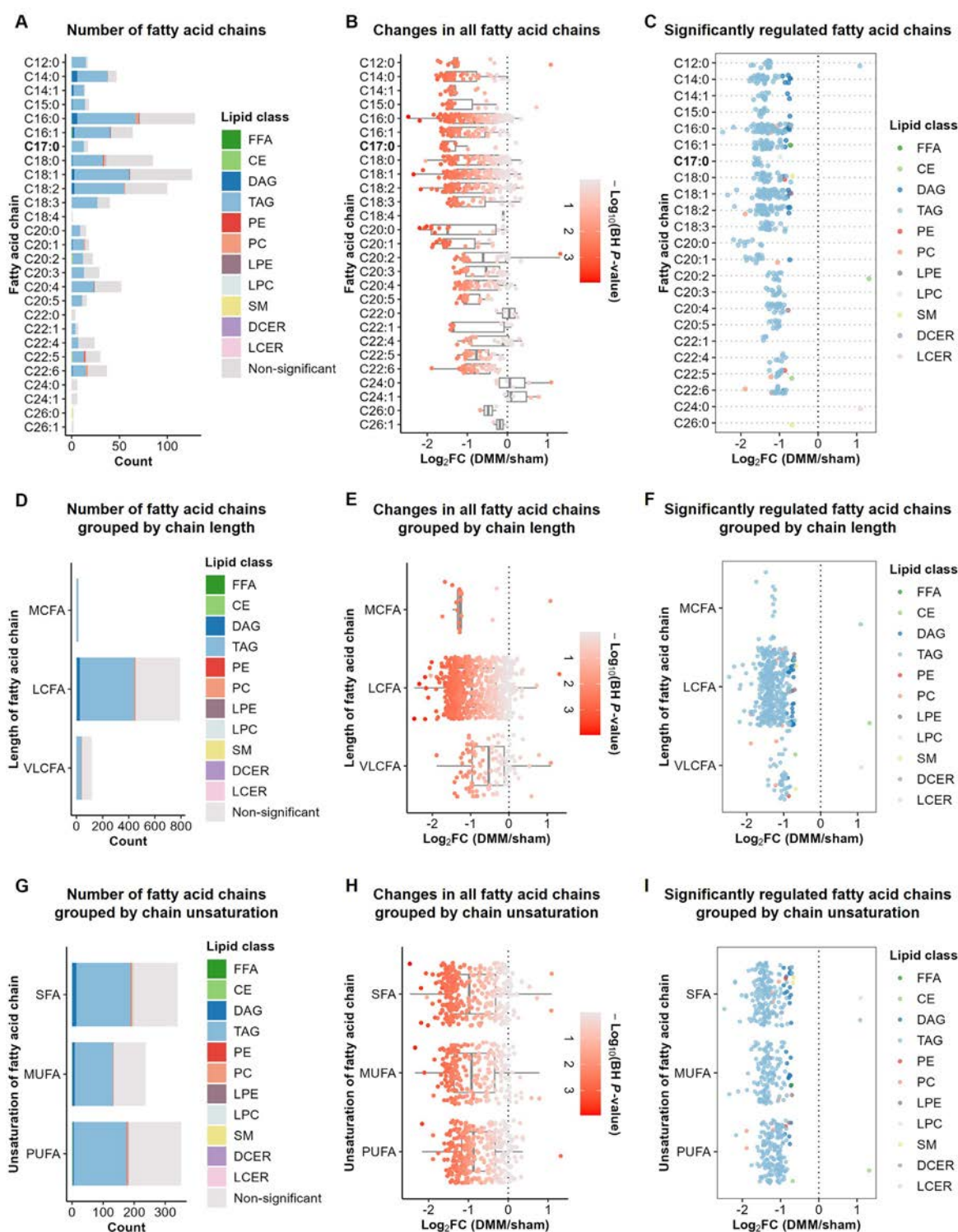


Figure 4. Changes in fatty acid chain composition associated with mouse osteoarthritis. (A) Number of fatty acid chains identified in mouse articular cartilage by lipid class (bar colour). (B) Log₂ fold changes of individual fatty acid chains in articular cartilage of DMM vs sham mice. Each dot represents 1 fatty acid chain. The dot colour indicates the statistical significance level. (C) Log₂ fold changes of significantly altered fatty acid chains in articular cartilage of DMM vs sham mice (FDR < 0.05) by lipid class (dot colour). (D) Number of fatty acid chains categorised by chain length in mouse articular cartilage by lipid class (bar colour). (E) Log₂ fold changes of fatty acid chains categorised by chain length in articular cartilage of DMM vs sham mice. Each dot represents 1 fatty acid chain. The dot colour indicates the significance level. (F) Log₂ fold changes of significantly altered fatty acid chains categorised by chain length in articular cartilage of DMM vs sham mice (FDR < 0.05) per lipid class (dot colour). (G) Number of fatty acid chains categorised by chain unsaturation in mouse articular cartilage by lipid class (bar colour). (H) Log₂ fold changes of fatty acid chains categorised by chain unsaturation in articular cartilage of DMM vs sham mice. Each dot represents 1 fatty acid chain. The dot colour indicates the significance level. (I) Log₂ fold changes of significantly altered fatty acid chains categorised by chain unsaturation in articular cartilage of DMM vs sham mice (FDR < 0.05) by lipid class (dot colour). BH, Benjamini-Hochberg; CE, cholesteryl esters; DAG, diacylglycerols; DCER, dihydroceramides; DMM, destabilisation of the medial meniscus; FC, fold change; FDR, false discovery rate; FFA, free fatty acids; LCER, lactosylceramides; LCFA, long-chain fatty acids; LPC, lysophosphatidylcholines; LPE, lysophosphatidylethanolamines; MCFA, medium-chain fatty acids; MUFA, monounsaturated fatty acids; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; SM, sphingomyelins; TAG, triacylglycerols; VLCFA, very-long-chain fatty acids.

C18:1, and C18:2 again showed the highest counts in our analysis (Fig 4A). Among the decreased fatty acids in osteoarthritic mouse cartilage, a striking reduction in odd-chain C17:0 and to a lesser extent C15:0 was again observed (Fig 4B). The analysis of the significantly dysregulated lipids in osteoarthritic mouse cartilage provided more details about their type and distribution among lipid classes (Fig 4C). Most significantly decreased fatty acids side chains in the articular cartilage of mice with OA were again found in TAG species (Fig 4C), thereby also confirming a striking reduction in the concentrations of TAG-associated odd-chain fatty acids (Fig 4C). Therefore, the analysis in osteoarthritic mouse articular cartilage parallels and validates key observations on fatty acid side chain shifts in osteoarthritic human chondrocytes.

We also interrogated differences in fatty acid chain length between osteoarthritic and sham-operated mouse articular cartilage. As for human chondrocytes, LCFA were the most abundant fatty acids found in mouse articular cartilage, with fewer VLCFA and MCFA showing the lowest number (Fig 4D; Supplementary Fig S6A). Most MCFA, LCFA, and VLCFA were downregulated in mouse osteoarthritic cartilage (Fig 4E; Supplementary Fig S6B). Among the significantly regulated fatty acids, most downregulated MCFA, LCFA, or VLCFA were TAG and to a lesser extent DAG (Fig 4F; Supplementary Fig S6C).

Next, we analysed differences in fatty acid chain unsaturation between osteoarthritic and control mouse articular cartilage. As in human samples, higher counts of SFA and PUFA were detected compared to MUFA (Fig 4G; Supplementary Fig S6D). Most SFA, MUFA, and PUFA were decreased in osteoarthritic cartilage (Fig 4H; Supplementary Fig S6E). The analysis of significantly regulated fatty acids grouped by unsaturation degree exhibited similar changes among SFA, MUFA, and PUFA because the reduced fatty acids were mainly species from the TAG class and to a lesser extent from the DAG class (Fig 4I; Supplementary Fig S6F). Taken together, all these data from a controlled animal model validate our findings obtained in human chondrocytes.

We also performed a time course analysis of the fatty acid chains after OA induction. At 4 weeks, the total levels of C22:4 and C24:0 were significantly elevated in the articular cartilage of DMM-operated mice compared with sham controls (Supplementary Fig S4C). At 12 weeks, most fatty acids, including C12:0, C14:0, C14:1, C15:0, C16:0, C16:1, C17:0, C18:0, C18:1, C18:2, C18:3, C20:0, C20:1, C22:1, C22:5, C22:6, and C26:0, were markedly reduced (Supplementary Fig S4C).

Depletion in 2-hydroxyacyl-CoA lyase 1-mediated odd-chain fatty acid C17:0 synthesis triggers a shift in the molecular signature of human articular chondrocytes towards an OA-like profile

The intriguing reduction in odd-chain fatty acids, especially in C17:0, in osteoarthritic chondrocytes from human and mouse cartilage triggered us to investigate their functional role in the disease. In mammals, odd-chain fatty acids originate from food sources such as dairy products but are also produced endogenously. The C15:0 level appears to be largely influenced by exogenous dietary sources, whereas C17:0 appears to be substantially generated de novo [21]. The observed reduction in odd-chain fatty acids in cartilage of the diet-standardised OA mouse model (Fig 2H; Fig 4A–C) excluded the diet factor as causative of this dysregulation and thus suggested a deficient endogenous synthesis of odd-chain fatty acids in osteoarthritic chondrocytes. Odd-chain fatty acids can be endogenously synthesised by 1-carbon removal of corresponding 2-hydroxy fatty

acids via 2-hydroxyacyl-CoA lyase 1 (HACL1)-catalysed alpha-oxidation in the peroxisome or via HACL2-catalysed alpha-oxidation in the endoplasmic reticulum [22,23]. In particular, the synthesis of C17:0, also known as margaric acid, involves 1-carbon removal from 2-hydroxy stearic acid (2-OH C18:0), in which HACL1 and HACL2 act as key enzymes [22,23] (Fig 5A).

To evaluate the effects of reducing the synthesis of odd-chain fatty acid C17:0 in cartilage, we knocked down HACL1 or HACL2 in human articular chondrocytes from non-osteoarthritic individuals using small interfering RNA (siRNA) (Supplementary Fig S7). Silencing of HACL1 (siHACL1) in chondrocytes led to a decrease in C17:0, whereas silencing of HACL2 (siHACL2) had no consistent effect (Fig 5B). The amount of C15:0 was not affected by siHACL1 or siHACL2 (Fig 5B). These data indicate that HACL1 contributes to the synthesis of odd-chain C17:0 in human articular chondrocytes. Then, to investigate the role of C17:0 in OA, we triggered its depletion by knocking down HACL1 (and HACL2 as negative control) and examined effects on articular chondrocyte markers. Silencing of HACL1 and subsequent reduction in odd-chain C17:0 resulted in a downregulation of chondrocyte anabolic markers type 2 collagen (COL2A1) and aggrecan (ACAN), essential constituents of the healthy articular cartilage ECM (Fig 5C). Conversely, silencing of HACL1 led to an up-regulation in disease-associated markers including alpha-1 type 1 collagen (COL1A1) and tissue-destructive matrix metalloproteinases 1 and 3 (MMP1 and MMP3) (Fig 5C). Silencing of HACL2 had no effect on the expression of these chondrocyte markers (Fig 5C). These data indicate that a reduction in the synthesis of odd-chain C17:0 by HACL1 leads to a shift in the molecular signature of healthy articular chondrocytes towards an OA-like profile.

We then interrogated whether supplementation with exogenous C17:0 in human articular chondrocytes with deficient C17:0 synthesis could restore a healthy molecular profile. We silenced HACL1 in chondrocytes from non-osteoarthritic patients and treated these cells with increasing concentrations of C17:0, but this supplementation did not lead to beneficial effects in healthy or disease chondrocyte markers (Fig 5D; Supplementary Figs S8A, S9A). In line with this observation, supplementation with C17:0 in articular chondrocytes from osteoarthritic patients, which have reduced C17:0 levels, did not ameliorate their disease-associated phenotype (Fig 5E; Supplementary Fig S9B). Together, these data indicate that odd-chain C17:0 itself may not exert beneficial effects in articular chondrocytes, at least under our experimental conditions. This suggests that a reduction of C17:0 is likely not causative of joint damage in OA but is indicative of deficient synthesis by HACL1.

HACL1 expression is downregulated in human and mouse osteoarthritic articular cartilage

We then investigated potential differences in the expression of HACL1 in OA and control articular cartilage. First, looking at transcriptome datasets from literature, we found that HACL1 was significantly downregulated in human OA articular cartilage compared with control cartilage (Table 1) [24–26]. We then specifically investigated available single-cell transcriptome datasets of human osteoarthritic and control cartilage (GSE220244 [27] and GSE104782 [28]) and found a decreased number of chondrocytes with detectable HACL1 in OA samples compared to the non-OA controls and in late stages compared to early stages of OA (Fig 5F). We additionally found that protein amounts of HACL1 in articular cartilage from mice with DMM-induced OA were substantially decreased compared with

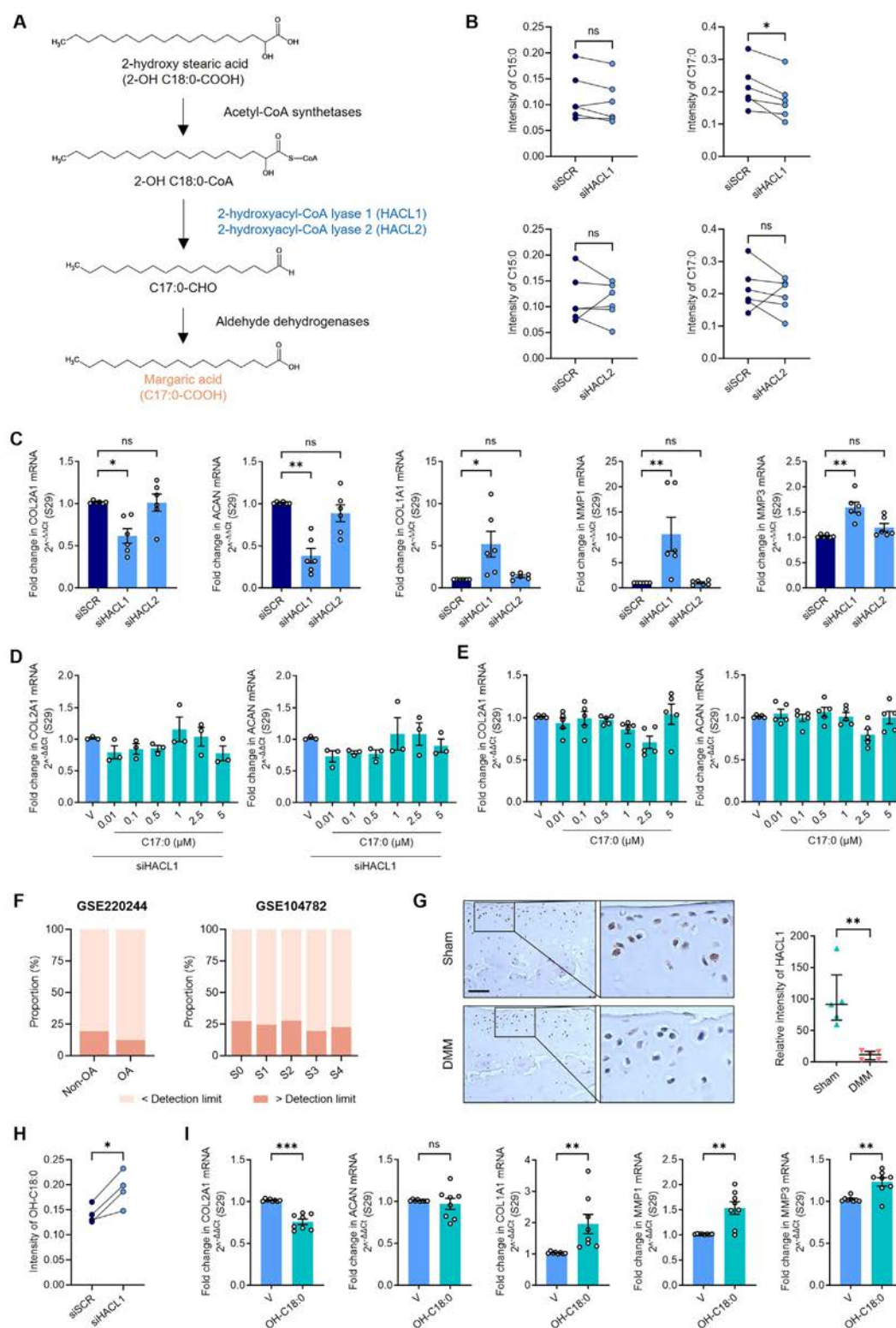


Figure 5. Reduction of odd-chain fatty acid lipid synthesis by HACL1 shifts the molecular profile of articular chondrocytes towards osteoarthritis. (A) Scheme of endogenous biosynthesis of odd-chain fatty acids via alpha-oxidation. (B) Measurement of C17:0 and C15:0 levels in primary hACs of non-OA patients after treatment with siRNA-mediated silencing of *HACL1* (siHACL1), *HACL2* (siHACL2), or scrambled control (siSCR) ($n = 6$, $*P < .05$ by paired two-tailed Student *t*-test). (C) Real-time PCR analysis of *COL2A1*, *ACAN*, *COL1A1*, *MMP1*, and *MMP3* in primary hACs of non-OA patients after treatment with siHACL1, siHACL2, or siSCR ($n = 6$, $*P < .05$, $**P < .01$ by RM 1-way ANOVA with Dunnett's test for multiple comparisons to siSCR). (D) Real-time PCR analysis of *COL2A1* and *ACAN* in primary hACs of non-OA patients treated with C17:0 upon siHACL1 ($n = 3$, analysed by linear mixed model). (E) Real-time PCR analysis of *COL2A1* and *ACAN* in primary hACs of OA patients treated with C17:0 ($n = 5$, analysed by linear mixed model). (F) Proportions of primary hACs with detectable HACL1 from public single-cell transcriptome datasets GSE220244 and GSE104782. (G) Immunohistochemical detection of HACL1 in the articular cartilage of OA mouse model triggered by DMM surgery compared with sham-operated mice. Images are representative of 5 mice per group. Scale bar: 50 μm ($n = 5$, $**P < .05$ by 2-tailed Mann–Whitney test, data are shown as individual points and mean with IQR). (H) Measurement of 2-OH C18:0 level in primary hACs of non-OA patients upon siHACL1 ($n = 4$, $*P < .05$ by paired 2-tailed Student *t*-test). (I) Real-time PCR analysis of *COL2A1*, *ACAN*, *COL1A1*, *MMP1*, and *MMP3* in primary hACs of non-OA patients treated with OH-C18:0 at 40 μM ($n = 8$, $**P < .01$, $***P < .001$ by paired 2-tailed Student *t*-test). (B–E, H–I) Data are shown as individual points with mean $\pm$ SEM where indicated. ACAN, aggrecan; ANOVA, analysis of variance; CoA, coenzyme A; COL1A1, alpha-1 type 1 collagen; COL2A1, type 2 collagen; DMM, destabilisation of the medial meniscus; hAC, human articular chondrocyte; HACL, 2-hydroxyacyl-CoA lyase; MMP, matrix metalloproteinase; ns, not significant; OA, osteoarthritis; PCR, polymerase chain reaction; RM, repeated measures; SCR, scrambled.

Table 1
HACL1 expression in publications

Publication (OA vs non-OA patients)	HACL1 expression
Karlsson et al [24]. Osteoarthritis Cartilage. 2010	Fold change = −2.908 (<i>P</i> = 3.606E-16)
Fisch et al [25]. Osteoarthritis Cartilage. 2018	Log ₂ fold change = −0.277 (<i>P</i> = .066)
Soul et al [26]. Ann Rheum Dis. 2018	Log ₂ fold change = −0.408 (<i>P</i> = .002, in 1 subgroup)

HACL1, 2-hydroxyacyl-CoA lyase 1; OA, osteoarthritis.

cartilage from control mice (Fig 5G). Hence, these data indicate that HACL1 expression is downregulated in osteoarthritic articular cartilage. We also investigated whether HACL1 expression is affected by some key signalling pathways involved in cartilage homeostasis, including IL-1 β [29], Wnt [30], and hypoxia [31]. Wnt activation reduced HACL1 expression, hypoxic conditions led to a slight increase, and IL-1 β stimulation did not affect HACL1 expression (Supplementary Fig S10).

Treatment with 2-hydroxy C18:0, precursor of C17:0, shifts the molecular signature of human articular chondrocytes towards OA

Based on our observations, we hypothesised that deficient HACL1 activity in osteoarthritic cartilage would lead to a subsequent accumulation of 2-hydroxy stearic acid (2-OH C18:0), precursor of C17:0 (Fig 5A), with potential detrimental effects. As expected, we found that 2-OH C18:0 is increased in articular chondrocytes from non-osteoarthritic patients upon silencing of HACL1 (Fig 5H). Then, we investigated the potential effects of 2-OH C18:0 on cartilage health. Treatment of human articular chondrocytes from non-osteoarthritic patients with 2-OH C18:0 led to a decrease in anabolic marker COL2A1 and an increase in catabolic markers COL1A1, MMP1, and MMP3 (Fig 5I; Supplementary Fig S8B), mirroring detrimental changes in chondrocyte markers that were triggered by silencing of HACL1 (Fig 5C). Altogether, our data show that deficient HACL1-mediated synthesis of odd-chain fatty acid C17:0 in osteoarthritic cartilage results in the accumulation of its precursor 2-OH C18:0, which leads to detrimental changes in the articular chondrocytes.

DISCUSSION

Our study unravels the lipidome of human articular cartilage and its shifts in OA. Analysis of patient samples compared to non-OA controls identified notable changes in different lipid classes and a clear separation of the control vs diseased profiles. These findings were validated in a standardised mouse model of OA, with largely analogous lipid alterations observed in articular cartilage from the diseased animals. Detailed fatty acid analysis highlighted a marked reduction in odd-chain fatty acid C17:0 associated with OA. Subsequent functional studies indicated that reduced HACL1-mediated C17:0 synthesis in chondrocytes led to molecular shifts resembling OA pathology. Our mechanistic investigations showed that, instead of the reduction of C17:0, the accumulation of its precursor, 2-hydroxy C18:0, due to HACL1 deficiency, triggers pathological changes in articular chondrocytes. Together, our results provide novel insights into lipid changes in OA and unravel a lipid-related mechanism underlying disease pathogenesis.

The complexity of OA and the existing challenges to find effective strategies for the treatment of this disease underscore the enormous need for comprehensive investigations into its pathogenesis. Although the transcriptome and proteome of articular cartilage have been systematically studied, lipids have

received little attention, despite their critical roles in cellular function. Hence, our research fills an important knowledge gap by documenting the broad lipidomic profile of articular chondrocytes and its shifts in OA. Early morphologic studies by histochemical approaches and quantitative studies by chromatography suggested some lipid classes, such as neutral lipids and phospholipids, accumulate in human articular cartilage with age [32–34]. However, lipids can also accumulate without any sign of cartilage degeneration [32]. How this relates to OA remains unclear. Later quantitative studies looking at the level of total fatty acid composition reported that the predominant fatty acids in human articular cartilage are palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), and linoleic acid (C18:2) [35,36], in agreement with the observations in our detailed and comprehensive lipidome study. Limited functional studies suggested that lipids might exert diverse effects in OA development. For instance, inhibition of glycosphingolipid synthesis was reported to exacerbate structural damage in a mouse model of OA [37], while blocking cholesterol biosynthesis in mice was shown to protect against the disease [8]. In contrast to the limited existing insights into lipid profiles in articular cartilage health and OA, our global lipidomic analysis and functional experiments provide not only a comprehensive overview of lipids but also a more detailed view on the disease-associated and disease-contributing lipid changes.

Our lipid profiling of osteoarthritic articular cartilage prompted a functional investigation into the impact of the dysregulation of one specific lipid identified by our comprehensive approach. We found that deficient HACL1-mediated synthesis of odd-chain C17:0 in osteoarthritic cartilage results in the accumulation of its precursor, which leads to the loss of chondrocytic molecular identity. The specific deficiency of HACL1-mediated lipid synthesis we identify here may represent an attractive opportunity for the development of novel therapeutic strategies for OA. To date, the factors that regulate HACL1 expression and activity in articular cartilage remain unknown and are an important area for further research. Efforts to modulate HACL1 expression or activity could prevent or reduce the accumulation of 2-OH C18:0, which leads to detrimental effects in articular chondrocytes. This latter finding aligns with a previous report in which 2-OH C18:0 was found to be elevated in synovial fluid after intra-articular ankle injury [38]. There is evidence that 2-OH fatty acids can be further metabolised to 2-keto derivatives by peroxisomal 2-OH acid oxidases, leading to the production of hydrogen peroxide [39,40]. In addition, although in our study we demonstrate detrimental effects of 2-OH C18:0 on articular chondrocytes, whether 2-OH C18:0 is the only HACL1-related molecule responsible for the detrimental effects of HACL1 deficiency in articular cartilage remains to be determined.

As a key component of the lipidomic signature associated with OA pathology, C17:0 levels or other OA-associated lipid changes may serve as a potential biomarker reflecting disease severity or progression. However, to establish its utility as a diagnostic biomarker, further investigation is warranted, including the analysis of such markers in synovial fluid, blood samples,

or urine from OA patients, as direct access to the articular cartilage for biomarker testing is not within regular clinical reach. Furthermore, lipidomic changes in cartilage may also reflect systemic metabolic alterations that can be detected in peripheral fluids, offering a link between local joint pathology and broader physiological mechanisms.

The rapidly changing therapeutic landscape and new targets in the treatment of obesity and diabetes, combined with our data presented here, raise the question whether interventions targeting systemic metabolic pathways could influence lipid metabolism in cartilage and thus help preserve chondrocyte phenotype and cartilage integrity. Semaglutide, a glucagon-like peptide-1 receptor agonist, not only reduces body weight but also alleviates pain in OA patients, suggesting a clear link between metabolic regulation, weight loss, and joint health. An effect on systemic lipid metabolism, which might translate to favourable changes in the lipidome of cartilage, could be part of this. However, while semaglutide may hold promise, the evidence surrounding other metabolic interventions, such as statins, has been less conclusive. Preclinical models and human studies have not consistently shown that statin use leads to significant improvements in cartilage integrity or chondrocyte function, highlighting the complexity of translating metabolic effects to cartilage-specific outcomes. Thus, while treatments targeting systemic metabolic dysfunction may offer therapeutic potential, further research is needed to establish their effectiveness in modulating the lipid landscape of cartilage and preserving joint health.

Some study limitations should be acknowledged. Sample size and characteristics of the end-stage OA samples may limit generalizability of the findings. Clinical data about postsurgical OA subtypes are missing, and these samples cannot provide information over the evolution of the articular cartilage lipidome over time. The non-OA samples came mostly from patients with fractures, and underlying osteoporosis or malignancy may impact the lipidome of the cartilage. Therefore, the validation of our findings in a controlled mouse model was essential. Mouse tissues were used for the validation experiment and compared to human cells. The clear overlap between lipid shifts in human and mouse samples in OA vs non-OA controls showed good robustness and validation of our data. Additionally, the pipeline for the analysis of the lipidome data includes essential steps such as batch corrections, data imputation, and a set of criteria applied. This procedure could theoretically exclude certain lipids of interest or under- or overestimate differences between OA and non-OA samples. Despite batch correction steps, residual variation between batches may also impact the analysis. The cross-sectional design of the study in the human subjects from which biological material was obtained at the time of joint replacement surgery may limit the ability to establish true causal relationships between lipid alterations and OA development. However, our functional investigations support a causative role for HACL1-related lipid alterations in OA. Of note, our data cannot claim specificity of HACL1-mediated effects because the direction of changes seen with silencing of HACL2 was similar although the silencing efficacy was smaller. Nevertheless, further *in vitro* and *in vivo* experiments investigating the effects of modulating HACL1 expression on OA development and progression are needed to assess its therapeutic relevance.

While our study provides valuable insights into the dysregulation of odd-chain fatty acid C17:0 synthesis in OA chondrocytes and its impact on the disease, this represents just one facet of the complex changes in the lipidomic landscape associated with OA. Our comprehensive dataset highlights numerous

dysregulated lipids across various classes, emphasising the need to further investigate to understand their functional impact on modulating chondrocyte metabolism, cartilage integrity, and disease progression. Understanding how these lipid alterations influence cellular pathways offers a promising avenue for identifying novel therapeutic targets in OA. Future studies should aim to elucidate the functional roles of key dysregulated lipids and their interactions with metabolic and inflammatory processes within the cartilage, other joint tissues, and at the systemic level. By defining these mechanistic links, it may become possible to develop lipid-targeted interventions aimed at restoring homeostasis and slowing or reversing disease progression. Our dataset serves as a valuable resource for the wider OA research community. Moreover, integrating lipidomics with other -omics approaches, such as transcriptomics and proteomics, could provide a better understanding of the pathophysiology of OA and facilitate the discovery of biomarkers for disease diagnosis, prognosis, and treatment response. This comprehensive approach will be critical for translating mechanistic insights into clinically relevant strategies, ultimately driving the development of precision therapies that target lipid dysregulation and improve patient outcomes in OA.

Competing interests

Leuven Research and Development, the technology transfer office of KU Leuven, has received consultancy and speaker fees and research grants on behalf of RJL from AbbVie, Amgen (formerly Celgene), Biocon, Biosplice Therapeutics (formerly Samumed), Eli Lilly, Galapagos, Fresenius Kabi, Janssen, Novartis, Pfizer, and UCB. The other authors declare that they have no competing interests.

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Contributors

RJL, SM, and QZ planned the study and designed all experiments. QZ processed human samples. Mohan Ghorasaini (MG) performed lipidomics sample preparation and measurement. FMFC performed animal experiments. QZ performed data analysis and visualisation of lipidomics and single-cell transcriptome data. QZ, RA, and ADR performed *in vitro* experiments. Martin Giera (MG) was responsible for quality oversight of lipidomics experiments and made suggestions. RJL and QZ were responsible for statistical analyses. RJL, SM, and QZ wrote the manuscript. RJL is the manuscript guarantor.

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Patient consent for publication

Primary human articular chondrocytes were isolated from cartilage from patients with informed consent.

Ethics approval

Ethical approval for experiments with human tissue was granted by the University Hospitals Leuven Ethics Committee and Biobank Committee (Leuven, Belgium) (S56271). Experiments in mice were performed with approval from the Ethics Committee for Animal Research (P159-2016 and P013-2022; KU Leuven) (licence no. LA1210189).

Provenance and peer review

Not commissioned; externally peer reviewed.

Data availability

The datasets generated in this study are included within the article and supplementary files. Public data used in this work are accessible in the Gene Expression Omnibus database. Other data supporting the findings of this study are available from the corresponding authors upon request.

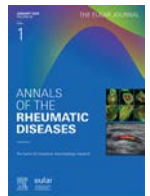
Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ard.2025.01.009.

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Letter

One-year remission of therapy-resistant SLE after a single course with the bispecific CD3: BCMA antibody teclistamab, but induction of a non-infectious Crohn's-like inflammatory bowel disease

B and plasma cell-targeting antibodies have opened new horizons in lupus therapy, but they carry the disadvantage of a limited distribution into the inflamed tissue [1,2]. Cytotoxic natural killer cells or targeted cytotoxic T cells promise superiority due to their active infiltration into the inflamed tissue. Bispecific CD3 and B cell maturation antigen (BCMA) antibodies combine initiation of efficient cell-mediated cytotoxicity and plasma cell tropism.

With this background, we hypothesised that treatment with the CD3:BCMA bispecific antibody teclistamab would be efficient in systemic lupus erythematosus (SLE) therapy. We treated a 42-year-old woman whose SLE had been diagnosed in 2005, based on arthralgias, skin lesions, mucosal ulcers, photosensitivity, leukocytopenia, thrombocytopenia, lupus nephritis World Health Organization type II, antinuclear antibody titre of 1:10,000, elevated double-stranded DNA antibody (tested using enzyme-linked immunosorbent assay and radioassay) and nucleosome antibody concentrations, and low serum concentrations of complements C3 and C4. She had been continuously treated with various conventional synthetic and targeted disease-modifying antirheumatic drugs without yielding a sufficient response. Rituximab (2 × 1 g) was infused 12 months (March 2023) and 5 months (October 2023) before teclistamab. Her skin symptoms did not change (Fig A), leukocyte and thrombocyte counts were consistently low, and serological signs of SLE activity did not improve (Fig B). Thrombocyte count fell to 52/nL in February 2024 despite 99% CD19⁺ B cell depletion in blood.

After discontinuation of all immunosuppressants (only hydroxychloroquine was continued and later stopped), teclistamab was injected in March 2024, with 60 µg/kg subcutaneously on day 1, 300 µg/kg on day 4, and 1500 µg/kg on days 8, 15, and 22. After each injection, the patient developed self-limiting fever up to 38.5°C for 1 to 2 days, reminiscent of cytokine-release syndrome grade 1.

Oral ulcers and skin lesions improved rapidly and have not recurred (Fig A; week 4, week 8, and 12 months). The

impressive cellular, clinical, and serological response over time is shown in Figure B-D. C4 serum concentrations normalised similar to C3 levels and stayed normal through the complete posttreatment period (data not shown). Antinucleosome antibodies fell below the detection level 10 weeks after start of therapy and remained undetectable until 12 months (data not shown). The patient felt considerably better; the visual analogue score fell from 50 of 100 before the start of treatment to 5 of 100 at 12 months. As a patient-reported outcome measure, we used the Short Form-36; all components improved, most impressively in social functioning (56% to 100%), energy viability (35% to 80%), and change in health (25% to 100%). For the first time in years, she spent her vacation at the beach, easily tolerating sun exposure, and she had persistent complete remission of her former skin lesions.

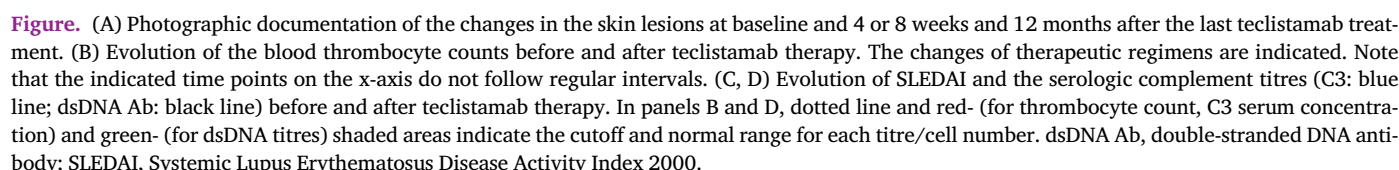
After rituximab, the number of peripheral B lymphocytes was already significantly diminished (2.2/µL) with a relatively high proportion of CD19⁺CD20⁻CD27⁺CD38⁺⁺ plasmablasts (1.3/µL). Twelve months after teclistamab treatment, all subpopulations are still significantly reduced except for naïve B cells with transitional phenotype, plasmablasts, and double-negative B cells (Table). As expected, total IgG serum concentration fell from a normal range before treatment to approximately 4 g/L after 2 months, requiring regular substitution. This included the typical vaccination antibodies such as measles, mumps, and rubella; titres fell below the limit of protection 3 to 8 weeks after start of therapy.

In the posttreatment period, she experienced a period of subpatellar bursitis and later, self-limiting scleritis. In September 2024, the patient-reported febrile diarrhoea, and we found flat ulcers along the colon and terminal ileum on endoscopy. The patient had never experienced any of these symptoms before. A broad microbiological workup did not reveal any infection: we checked specifically for cytomegalovirus and herpes simplex virus (both in serum and tissue using polymerase chain reaction), typical and atypical mycobacteria, cultured stool for atypical bacteria, vibrios, or parasites. Moreover, we performed 16S ribosomal RNA polymerase chain reaction of the gut tissue (all negative results). Histology revealed chronic granulomatous and erosive mucositis, no vasculitis, and no immune complex or complement deposition. After taking mesalazine (for only 4 months) and the gut-specific integrin antibody vedolizumab (in monotherapy since January 2025), the patient completely recovered. The Systemic Lupus Erythematosus Disease Activity Index 2000 remained 0 (Fig C).

Intriguingly, the patient's sister suffers from a severe case of Crohn's disease. Thus, given the endoscopic picture, the mucosal histology, the lack of any gut involvement in her previous SLE history, the efficacy of mesalazine/vedolizumab treatment, and the positive family history, we interpret colitis, bursitis, and

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Subpopulation (cells/mL)	Mar 1, 2024	Apr 8, 2024	May 6, 2024	Jun 3, 2024	Jul 4, 2024	Jul 29, 2024	Nov 18, 2024	Dec 18, 2024	Jan 30, 2025	Mar 13, 2025	Reference range (10%-90% quantile)
CD19 ⁺ B cells	2156	612	899	515	8055	50,093	18,466	56,465	102,450	28,010	190,167-367,515
CD20 ⁺ B cells	1954	294	571	379	369	435	524	1136	1049	3187	1529-9025
Class switch B cells	1348	0	19	16	61	64	553	932	1427	1667	18,070-63,219
MZ B cells	0	12	0	0	0	32	175	204	466	351	11,624-58,612
DN B cells	758	435	847	431	600	1095	291	3699	2418	2514	1861-9538
DN CD11c ⁺ B cells	270	106	372	264	261	113	58	379	291	234	xxx
Naïve B cells	51	165	32	68	7394	48,902	17,447	57,437	86,360	23,478	105,122-240,111
Naïve B cells with transitional phenotype	17	24	0	44	6764	47,742	13,107	44,476	26,651	8362	5398-25,662
Plasmablasts	1482	282	71	88	277	354	524	1282	816	2631	627-8266

In conclusion, we here show for the first time that lasting (12 months to date), complete remission of SLE is possible after only 1 course of teclistamab, confirming our hypothesis. Our data support and expand a recent report of Alexander et al. [5], who published data of a teclistamab response over 16 weeks in a single SLE patient.

CRedit authorship contribution statement

Hanns-Martin Lorenz: Supervision, Investigation, Formal analysis, Data curation, Conceptualization. **Wolfgang Merkt:** Investigation. **Juliane Brandt:** Investigation. **Thomas Giese:** Formal analysis, Data curation. **Tarik Exner:** Investigation, Data curation. **Norbert Blank:** Investigation, Data curation. **Margarida Souto-Carneiro:** Methodology, Data curation. **Annika Gauss:** Investigation. **Marc S. Raab:** Supervision, Investigation, Conceptualization.

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Letter

Effective use of anti-CD19 chimeric antigen receptor T cells in a case of treatment-resistant granulomatosis with polyangiitis

BACKGROUND

Granulomatosis with polyangiitis (GPA), the most common form of antineutrophil cytoplasmic antibody (ANCA)-associated small vessel vasculitis (AAV), can cause life-threatening organ damage, and its treatment, consisting of immunosuppressive therapy, is associated with serious side effects. Rituximab, an anti-CD20 monoclonal antibody, is considered the standard treatment for AAV; however, its effectiveness may be limited because autoantibody-producing plasmablasts and plasma cells are not targeted and tissue depletion is not profound. In rituximab-refractory cases, deep tissue depletion of B cells using anti-CD19 chimeric antigen receptor (CAR) T-cell therapy could provide an effective alternative [1]. CAR T cells have already been successfully used in patients with treatment-refractory autoimmune diseases, and 1 patient with AAV has recently been reported [2–4].

CASE REPORT

A 40-year-old male patient was diagnosed with GPA in April 2023, fulfilling 2022 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) classification criteria and having histologically proven small vessel vasculitis. Between April and December 2023, the patient received 7 rituximab infusions (cumulative dose, 5790 mg), 5 cyclophosphamide infusions (cumulative dose, 4200 mg), cyclosporin A, avacopan, and frequent prednisolone escalations (Fig. A). While C-reactive protein (CRP) and proteinase 3 (PR3)-ANCA levels showed partial normalization after 1 year of therapy, severe pulmonary inflammatory lesions persisted, requiring daily prednisolone (≥ 10 mg) to prevent relapses. Imaging studies revealed unresolved lung nodules and a cavitary lesion in the left lower lobe (Fig. B, top). Additionally, [18F]-fluorodeoxyglucose positron emission tomography/computed tomography ([18F]FDG PET/CT) demonstrated heightened glucose metabolism in the lungs, indicative of ongoing disease activity (Fig. C, top).

With limited therapeutic options remaining—despite normalized ANCA levels and near-complete B-cell depletion in peripheral blood—the patient was treated with a second-generation anti-CD19 CAR T-cell construct (KYV-101). This approach was based on the hypothesis that deep tissue B-cell depletion could resolve residual vasculitic lesions. The CAR T-cell construct featured a fully human CD19-binding domain, a CD8 α hinge and transmembrane domain, a CD28 costimulatory domain, and a CD3 ζ activation domain. Prior to CAR T-cell infusion, the patient received lymphodepleting therapy with cyclophosphamide (300 mg/m²) and fludarabine (30 mg/m²) on days –5, –4, and –3. A total of 10⁸ CAR T cells per kilogram of body weight were administered, and the treatment was well-tolerated, with no evidence of cytokine release syndrome (CRS) or immune effector cell-associated neurotoxicity syndrome (ICANS). Transient leukocytopenia persisted for 14 days after infusion, with no subsequent hematologic toxicity. CAR T-cell expansion in peripheral blood peaked on day 9 at 180 cells/ μ L.

Four months after treatment, [18F]FDG PET/CT imaging revealed near-complete resolution of inflammatory activity (Fig. C, bottom). Prednisolone was successfully tapered to 2 mg daily, without significant increases in CRP or PR3-ANCA levels. The patient, who had already achieved B-cell aplasia due to prior rituximab therapy, maintained this state for 300 days after CAR T-cell infusion (Fig. D, right), with detectable CAR T cells in peripheral blood (Fig. D, left). Clinically, the patient reported marked improvements in physical fitness and quality of life, as reflected in standardized assessment scores (Fig. E). This was accompanied by a significant improvement in his lung function parameters (Supplementary Fig S1B) with a total lung capacity (TLC) of 76.0%/Ref (percent of predicted value) and forced vital capacity (FEV) of 60.2%/Ref directly before CAR T-cell treatment to TLC of 85.6%/Ref and FEV of 72.6%/Ref at day 300 after CAR T-cell administration. Birmingham Vasculitis Activity Score (BVAS) decreased from 8/33 at baseline to 0/33 at day 300 after CAR T-cell therapy.

DISCUSSION

This case highlights the successful use of anti-CD19 CAR T-cell therapy in a patient with treatment-refractory GPA. Notably, the patient experienced no adverse events, such as CRS or ICANS, and achieved significant clinical improvement despite prior normalization of ANCA levels and peripheral B-cell depletion. The resolution of pulmonary inflammation suggests that residual pathogenic B cells—either in peripheral blood after rituximab treatment or within lymphoid tissues—may have been

LU, MP, and MK contributed equally and share the first authorship.

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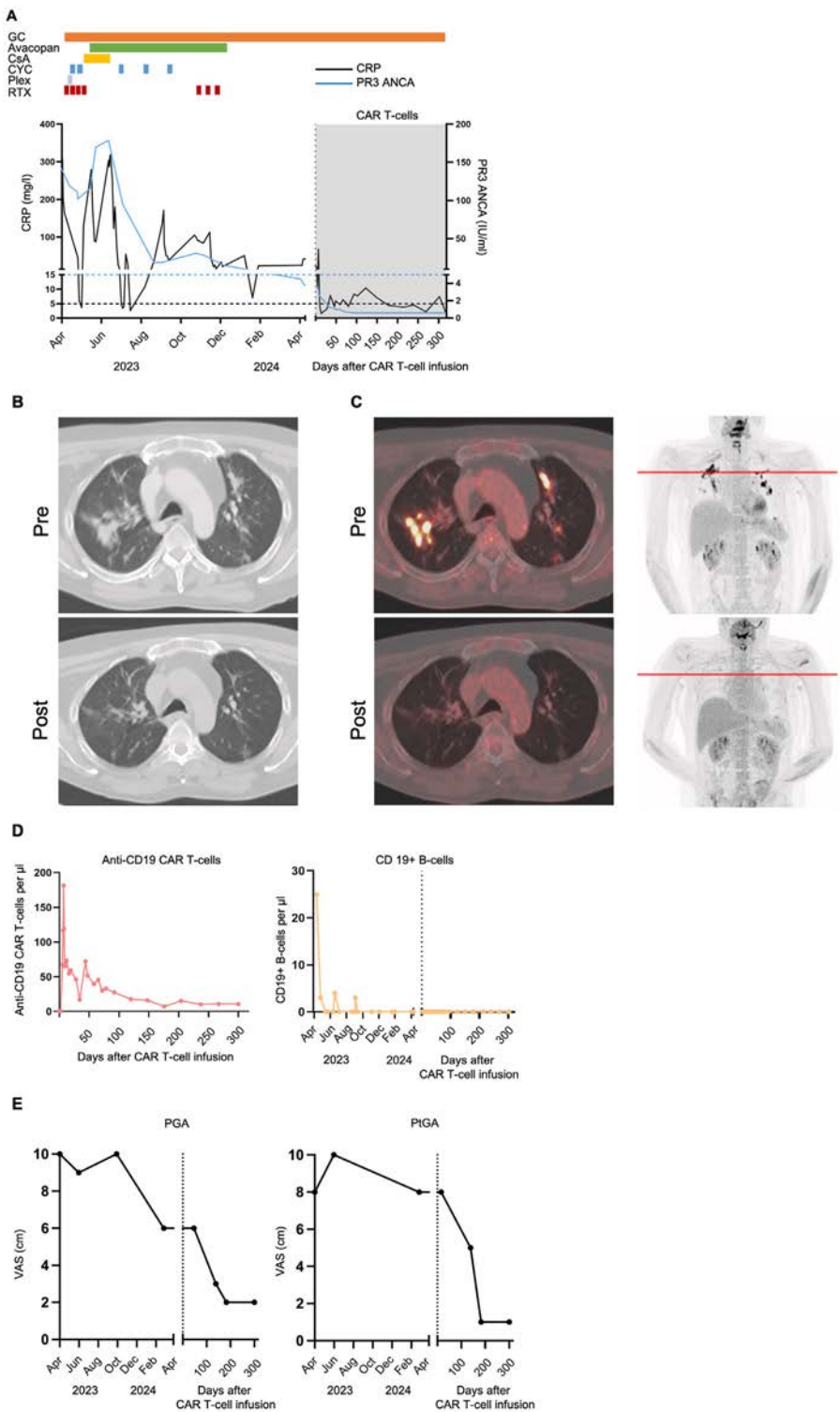


Figure. Effects of chimeric antigen receptor (CAR) T cells on the manifestations of granulomatosis with polyangiitis. (A) C-reactive protein (CRP) and proteinase 3–specific antineutrophil cytoplasmic antibody (PR3 ANCA) concentrations before and after treatment with CD19 CAR T cells and the timing of previous treatments. The horizontal dotted lines indicate the upper limit of the normal range (black: CRP = 5 mg/L; blue: PR3 ANCA = 5 UI/mL). (B) Chest computed tomography (CT) scans. Axial reconstructed CT scan of the lung showing pretherapeutic inflammatory round foci in all lobes and a cavern in the left lower lobe (top) and clear regression of the bipulmonary round foci and no cavernous formation 6 months after therapy (bottom). (C) Axial reconstructed 18F-fluorodeoxyglucose ([¹⁸F]FDG) positron emission tomography (PET)/CT scan with increased glucose metabolism in the bipulmonary nodules (top) and bipulmonary near-complete resolution of inflammatory activity in residual nodules (bottom). Right: [¹⁸F]FDG PET/CT; maximum intensity projection showing pretherapeutic increased glucose metabolism in multiple bipulmonary lesions especially in the upper lobes and nasopharynx (top), SUV-lbm max(lesions)/SUV-lbm mean(liver): 7.95; and near-total regression with residual pulmonary lesions 4 months after therapy (bottom), SUV-lbm mean(lesions)/SUV-lbm mean(liver): 2.16; red line indicates image position of axial CT (left) and PET/CT (right). (D) Concentration of circulating anti-CD19 CAR T cells (left) and CD19 B cells (right) over time. (E) Disease activity assessed using the physician global assessment (PGA) (left) and patient global assessment (PtGA) (right) measures. CsA, cyclosporine A; CYC, cyclophosphamide; GC, glucocorticoids; Plex, plasma exchange; RTX, rituximab; SUV, standard uptake volume; VAS, visual analogue scale.

effectively targeted by CAR T cells. At the time of this report (10 months after treatment), no B-cell re-emergence was observed, and CAR T cells remained detectable. This raises important questions regarding the durability of remission following B-cell reconstitution, warranting further investigation.

In conclusion, this case, together with a recently reported patient with AAV responding to CAR-T cell treatment [2], provides compelling evidence that anti-CD19 CAR T-cell therapy may serve as an effective alternative in treatment-resistant GPA. Further research is warranted to evaluate long-term safety, durability of remission, and the optimal therapeutic window for CAR T-cell intervention in AAV.

Competing interests

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CRediT authorship contribution statement

Luisa Uhlmann: Writing – original draft, Visualization, Data curation, Conceptualization. **Matthias Pierer:** Writing – review & editing, Supervision. **Marco Krasselt:** Writing – review & editing. **Georg Franke:** Supervision. **Robert Zeidler:** Visualization. **Osama Sabri:** Supervision. **Timm Denecke:** Visualization. **Ulrich Sack:** Supervision. **Ulrike Köhl:** Supervision. **Andreas Boldt:** Methodology. **Dominic Borie:** Supervision, Resources. **Ulf Wagner:** Supervision. **Uwe Platzbecker:** Supervision. **Vladan Vucinic:** Writing – review & editing, Supervision, Project administration.

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Patient consent for publication

We have obtained the patient's consent for publication.

Ethics approval

The patient's treatment was carried out as compassionate use due to a lack of alternative treatment options.

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Supplementary materials

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Letter

Relapse after anti-CD19 CAR T-cell therapy in a patient with severe rheumatoid arthritis and multiple sclerosis effectively treated by autologous stem cell transplantation

Recent studies have indicated that anti-CD19 chimeric antigen receptor (CAR) T-cell therapy has emerged as a promising therapeutic option for refractory autoimmune diseases (AIDs), with partially impressive long-lasting drug-free remissions observed in systemic lupus erythematosus or systemic sclerosis [1–3]. In multiple sclerosis (MS) and rheumatoid arthritis (RA), there have been encouraging case reports [4–6]. Autologous haematopoietic stem cell transplantation (HSCT) has been used for >20 years in patients with severe AID and has evolved into a standard treatment, particularly in patients with systemic sclerosis, but also in MS [7,8]. In RA, standard treatment improved that much over this time period that HSCT is not used anymore on a regular basis, as the results were not that convincing. The present case report details a patient treated with both CAR-T-cell therapy and HSCT for her respective diseases: RA and MS.

This 32-year-old (at time of HSCT) female patient was diagnosed with relapsing-remitting MS at the age of 20 years and has received sequential treatments with interferon beta-1a and fingolimod due to high disease activity. Following this, alemtuzumab (12 mg on 5 consecutive days) was administered in Spring 2016 and resulted in disease-free remission of the MS. However, in March 2017, the patient developed symmetric, erosive polyarthritis leading to the diagnosis of seronegative RA with a very aggressive and refractory course during the following years (Fig 1A). She was treated with methotrexate, rituximab, abatacept, anakinra, multiple Janus kinase inhibitors, tocilizumab, multiple tumour necrosis factor inhibitors, cyclophosphamide (6 cycles of 750 mg/m²), and secukinumab without lasting response. In the absence of effective therapeutic alternatives, treatment with academically manufactured CD19-targeting CAR T-cells was initiated in May 2023. The patient's RA had ongoing treatment with high doses of glucocorticoids (GC) for many years and did not require further specific MS therapy. Autologous T-cells were collected by leukapheresis and freshly transduced with a CD19-targeting second-generation CAR lentiviral vector containing a costimulatory

CD3-zeta signalling domain (Miltenyi Biotec), as previously reported [2]. Prior leukapheresis GC was reduced and switched to 30 mg hydrocortisone. CAR T-cells (1.1×10^6 /kg bodyweight) were infused on May 16, 2023, (day 0) after conditioning with fludarabine and cyclophosphamide (CYC). CAR-Ts expanded and B-cell depletion was observed in accordance with the expected parameters (Fig 1B,C). The patient showed no occurrence of ICANs (Immune effector cell-associated neurotoxicity syndrome) or MS activity despite the cessation of all immunosuppressive therapy. An early cytokine release syndrome grade II was treated with tocilizumab on days 1 + 2. The patient was discharged from the hospital on day 11, with hydrocortisone to prevent adrenal insufficiency, which was subsequently tapered until day 150 (Fig 1D). In the early visits after therapy, her C-reactive protein (CRP) levels and disease activity score (DAS28) improved and she achieved remission on day + 28; however, after 8 months, there was a severe recurrence of synovitis (Fig 1A,E). The B-cell count had normalised (i.e. >200 CD19 positive B-cell/ μ L) at that time point for several weeks (Fig 1C). Decision was taken to proceed with HSCT. Mobilisation was conducted with CYC and lenograstim. The conditioning regimen comprised CYC (4×50 mg/kg bodyweight) and rabbit anti-thymocyte globulin (Grafalon, NEO-VII, total dose 23.5 mg/kg), administered prior to reinfusion of CD34+ -selected stem cells in April 2024. No immunosuppressive therapy was given before or after HSCT. Following this treatment, a decline of DAS28 and CRP levels was observed and the patient expressed satisfaction with her state of health until today. As a side effect, she suffered from a severe zoster central nervous system infection despite prophylaxis. The B-cell compartment did not decline after HSCT, whereas a decline was observed in the T-cell compartment (Fig 1C,F).

To the best of our knowledge, this is the first report on a successful consecutive HSCT after non-lasting response to CAR T-cell therapy in a patient with 2 B-cell driven AID. The unusual character of the seronegative polyarthritis, which started after alemtuzumab, is of particular interest due to its atypical characteristics. Despite fulfilling the American College of Rheumatology and the European League Against Rheumatism criteria for RA, we believe that this polyrefractory condition might result from an alemtuzumab-induced secondary autoimmune arthritis. Although alemtuzumab has been observed to induce a range of secondary AIDs, arthritis has been reported on a relatively infrequent basis [9]. Consequently, we emphasise that anti-CD19 CAR T-cell therapy remains a promising option for classic (seropositive) RA. In this particular case, a profound immunoablation with HSCT proved to be more effective than the deep B-cell depletion. In patients with mixed and seronegative AIDs, HSCT is still a very potent treatment option and should be used where necessary.

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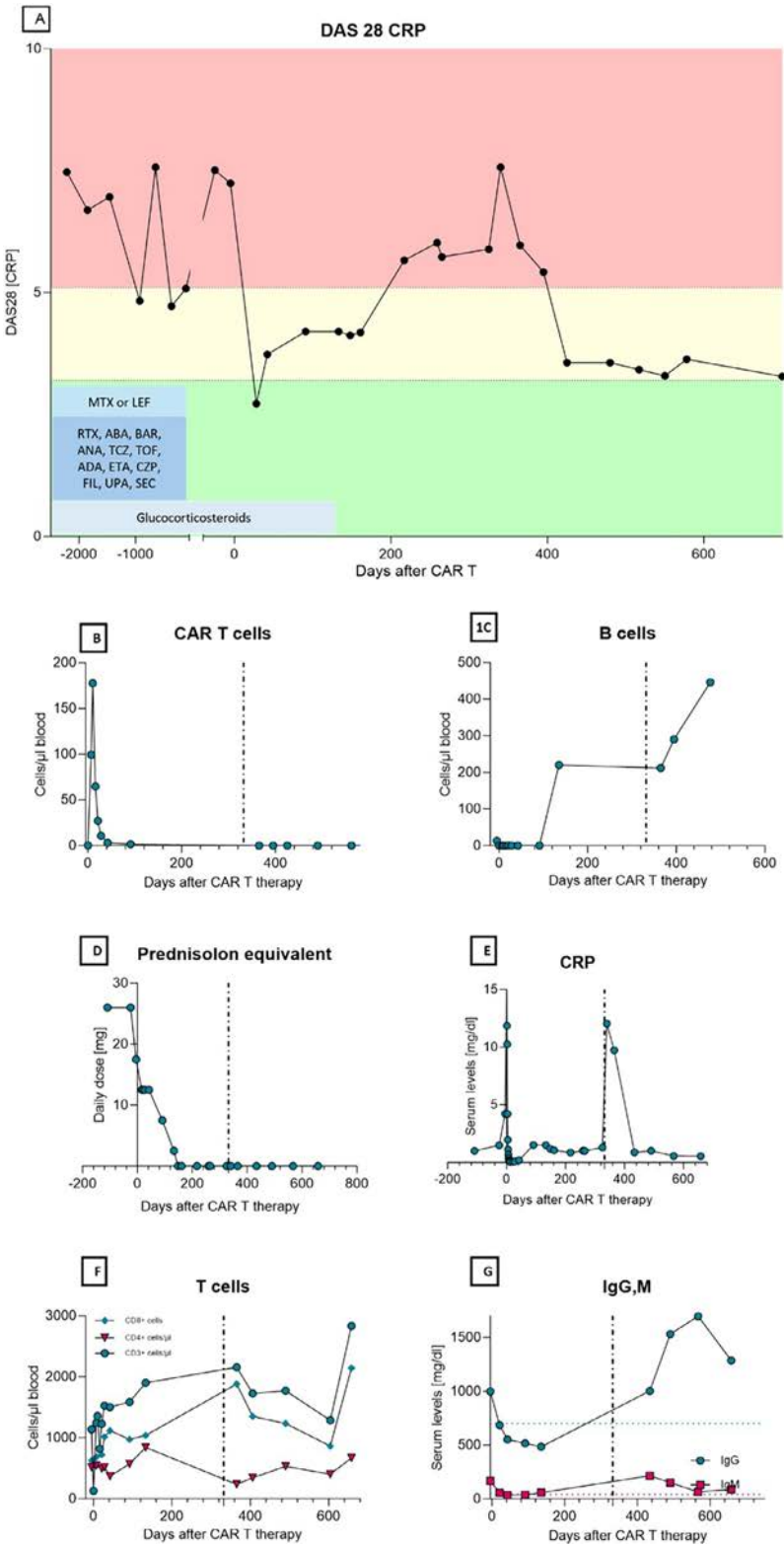


Figure 1. Results of the CAR T-cell therapy and the HSCT in terms of RA disease activity measured by DAS28 and concomitant medications given sequentially (A); CAR T-cell expansion (B), B-cell depletion (C), continued intake of prednisone dose (D), CRP levels (E), T-cells subpopulation (F), and immunoglobulin levels (G). The black line marks the time point of HSCT (day 332 after CAR T-cell treatment). ABA, abatacept; ADA, adalimumab; BAR, baricitinib; CRP, C-reactive protein; CZP, certolizumab pegol; DAS28, disease activity score; ETA, etanercept; FIL, filgotinib; LEF, leflunomide; MTX, methotrexate; RTX, rituximab; SEC, secukinumab; TCZ, tocilizumab; TOF, tofacitinib; UPA, upadacitinib.

Declaration of competing interest

The authors declare they have no competing interests.

Contributors

ACP, LH, JH, and CL participated in the design and implementation of the research; analysis of the results; writing of the manuscript. RS, MK, AGJ, VR, MS, and WB participated in the acquisition, analysis and interpretation of data; critical revision of the manuscript.

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Correspondence

Correspondence on 'Methotrexate continuation increases fracture risk in patients who sustained lower limb insufficiency fractures' by Hauser et al

We read with interest the study by Hauser et al [1], which investigated the relationship between continued methotrexate (MTX) use and fracture risk in patients with MTX osteopathy (MTXO). Previous studies suggested that MTX might increase the risk of osteoporosis and insufficiency fractures through impaired osteoblastic cell function [2,3]. Hauser et al [1] further reported an association between the continuation of MTX and an increased risk of further fracture. While their findings provide valuable insights, several points warrant further discussion before concluding that the continuation of MTX is associated with a high risk of further fracture, especially given the crucial role of MTX in rheumatology as an anchored therapy for rheumatoid arthritis (RA) and its common use in other rheumatic diseases.

First, the study employed Kaplan-Meier survival analysis to compare fracture-free survival between patients who continued and discontinued MTX and used Cox regression to examine differences. However, the Cox regression model did not adjust for potential confounders, such as glucocorticoid exposure, bone mineral density, RA disease activity (or inflammatory markers like erythrocyte sedimentation rate [ESR] and/or C-reactive protein [CRP]), and osteoporosis treatment. Without proper adjustment, the observed association between MTX continuation and increased fracture risk could be confounded by these factors. For outcomes such as pain improvement and weight-bearing capacity, using multivariable regression methods, such as logistic or linear regression, could determine whether the differences are due to MTX continuation or other factors like disease activity and prior fractures.

Second, the study does not report whether the continuation and discontinuation groups differed in RA disease activity, cumulative glucocorticoid exposure, or the use of other rheumatic medications and osteoporosis treatments. Patients who continued MTX may have more active RA, which is also a strong determinant of fracture risk [4]. Although the authors mentioned that the baseline disease activity score 28 was not available due to the retrospective study design, the adjustment for baseline inflammation, such as ESR and/or CRP, could still provide insights into differences in baseline disease activity between the MTX continuation and discontinuation groups.

Notably, the study reported that the fracture risk assessment tool (FRAX) -predicted fracture risk in the MTX continuation group was higher than in the discontinuation group; this suggests that the baseline fracture risk may not have been evenly distributed between the 2 groups. In the study, 90% of patients started osteoporosis medications after MTXO. It would be important to know whether the osteoporosis treatment was balanced between the MTX continuation and discontinuation groups.

Third, a significant proportion (two-thirds) of the study population was recruited from a tertiary metabolic bone clinic. This may introduce a potential selection bias, as patients seen in metabolic bone clinics may have underlying metabolic bone disorders such as chronic kidney disease–mineral and bone disorder and primary hyperparathyroidism, which independently affect fracture risk. However, the study does not report whether these conditions were present or controlled. Further clarification on baseline bone metabolism parameters and comorbid conditions would enhance the study's validity.

Whether to continue MTX is an important decision in rheumatology practice. Given the rarity of MTXO, it is extremely challenging to conduct prospective controlled studies to validate these findings. We acknowledge the inherent limitations in controlling confounders within retrospective cohort studies. Nonetheless, addressing the points mentioned above would strengthen the reliability of the conclusions.

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Competing interests

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Response

Response to: Correspondence on ‘Methotrexate continuation increases fracture risk in patients who sustained lower limb insufficiency fractures’ by Dai et al

We thank Dr Dai et al. [1] for their interest in our article and their thoughtful commentary on our study investigating the association between methotrexate (MTX) continuation and fracture risk in patients with MTX osteopathy (MTXO) [2].

We are grateful for their recognition of our study as a valuable contribution to the understanding of MTXO, and we note their caution regarding the interpretation of our findings.

Dr Dai et al. [1] raise valid concerns regarding the potential confounding effects of factors such as glucocorticoid (GC) use, bone mineral density (BMD), rheumatoid arthritis disease activity, and osteoporosis treatment, which were not accounted for in our Cox regression analysis. We agree that, in an ideal setting, these variables would be included in the analysis. However, as noted in our manuscript, the retrospective nature of the study, along with the limited sample size and incomplete data, precluded multivariable regression modelling. Nevertheless, we presented group comparisons in Table 3 [2] (patients who discontinued MTX versus those who continued), which offer insight into baseline characteristics and help contextualise potential differences.

With respect to fracture risk, we acknowledge that FRAX® (fracture risk assessment) scores differed numerically between groups; however, there was no statistically significant difference in FRAX® or BMD between the MTX continuation and discontinuation groups. Regarding GC exposure, Table 1 [2] indicates that none of the patients were on long-term, continuous systemic GC therapy, and fewer than 10% received systemic GCs in the 3 months preceding their first insufficiency fracture. These data suggest that GC exposure is unlikely to have been a major contributor to fracture risk in this cohort [3].

Importantly, 95% of patients who continued MTX sustained further fractures compared with 36% of those who discontinued the drug ($P < .0001$). As highlighted in the accompanying editorial by Rolvien [4], MTXO frequently presents with multiple bilateral fractures occurring in close succession, as documented

in prior case reports [5,6]. Prompt discontinuation of MTX may, therefore, be critical in mitigating the risk of subsequent fractures.

We also agree that a multivariable regression model would be ideal for identifying predictors of fracture healing and clinical outcomes. However, the rarity of MTXO, limited case numbers, and incomplete data restricted our ability to perform such an analysis. Notably, a recent study by von Brackel et al [7] demonstrated that MTX discontinuation was associated with improved mobility outcomes, lending further support to our findings.

In terms of disease activity, the average duration of MTX therapy exceeded 10 years, and two-thirds of patients were receiving MTX monotherapy—suggesting that MTX was effective in controlling inflammation in the majority of cases. If MTX discontinuation had led to disease flare and subsequent inflammation-driven bone loss, one would expect an increased fracture rate in the discontinuation group. However, the opposite was observed, making it unlikely that differences in inflammatory activity underlie the observed disparity in fracture incidence.

Regarding osteoporosis management, 90% of patients were initiated on antiosteoporotic therapy following their MTXO diagnosis. While we acknowledge that the type of osteoporosis treatment may influence outcomes, the short time interval within which most subsequent fractures occurred (as demonstrated in the Kaplan-Meier analysis, Fig 3 in our paper [2]) suggests that differential effects of treatment are unlikely to have significantly impacted short-term fracture risk.

Finally, we wish to clarify that the vast majority (21/22) of secondary care referrals were made by rheumatologists; only 1 referral originated from dermatology. All patients referred to the osteoporosis and metabolic bone clinic underwent screening for secondary causes of osteoporosis and were excluded if an alternative explanation was identified.

In conclusion, while we recognise the inherent limitations of retrospective studies and the challenges of adjusting for confounding variables in rare conditions such as MTXO, we believe our findings offer important clinical insights. The marked difference in fracture recurrence between patients who continued versus discontinued MTX underscores the need for careful consideration of MTX continuation in patients diagnosed with MTXO.

CONTRIBUTORS

Writing—original draft: BH; Writing—review and editing: BH, AM, JF, JG, EM, and SHR; BH was responsible for the overall

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content as the guarantor. All authors reviewed and approved the final manuscript.

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Patient consent for publication

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

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Not applicable.

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Data are available upon reasonable request.

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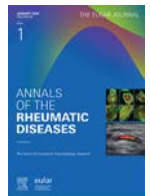
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Correspondence

Correspondence on
'2023 ACR/EULAR
antiphospholipid syndrome
classification criteria'

Following the revised Sapporo Antiphospholipid Syndrome (APS) criteria published in 2006 [1], American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) issued the latest classification criteria for APS in 2023 [2]. Compared with the revised Sapporo APS criteria in 2006, the specificity of the 2023 ACR/EULAR APS criteria has been significantly improved. However, when we carefully read the article '2023 ACR/EULAR antiphospholipid syndrome classification criteria' and carefully calculated the data in Table 5 in the article, we found that there were problems with the data regarding cohort 1 and cohort 2 used to verify the sensitivity and specificity of the revised Sapporo APS criteria. Our calculations are as follows:

Cohort 1

True Positive (TP) = $98 \times 100\% = 98$

True Negative (TN) = $180 \times 91\% = 164$

False Positive (FP) = $180 - 164 = 16$

The number of people who met the criteria = $TP + FP = 114$

However, the number in the Table 5 is 120.

Cohort 2

TP = $113 \times 99\% = 112$

TN = $162 \times 86\% = 139$

FP = $162 - 139 = 23$

Number of people who met the criteria = $TP + FP = 135$

However, the number shown in Table 5 is 143.

We firmly believe that this is not due to a calculation error. We hope that the editors and authors can carefully check the data in the form. We look forward to your reply.

Competing interests

None declared.

Contributors

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Table 5

Operating characteristics of the 2023 ACR/EULAR antiphospholipid syndrome (APS) classification criteria versus the revised Sapporo APS classification criteria compared against independent adjudicators' consensus in 2 distinct validation cohorts

	Validation cohort 1 (n = 278)		Validation cohort 2 (n = 275)	
	2023 ACR/EULAR APS criteria	Revised Sapporo APS criteria	2023 ACR/EULAR APS criteria	Revised Sapporo APS criteria
Criteria met, no. of subjects	83	120	97	143
Specificity (95% CI)	0.99 (0.98 to 1.00)	0.91 (0.86 to 0.95)	0.99 (0.97 to 1.00)	0.86 (0.81 to 0.92)
Sensitivity (95% CI)	0.83 (0.75 to 0.90)	1.00 (1.00 to 1.00)	0.84 (0.77 to 0.91)	0.99 (0.98 to 1.00)

ACR, American College of Rheumatology; APS, antiphospholipid syndrome; EULAR, European League Against Rheumatism.

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Data availability statement

All the data are included in the article or available from the authors for reasonable request.

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Response

Response to: Correspondence on '2023 ACR/EULAR Antiphospholipid Syndrome Classification Criteria' by Yeng et al

We appreciate the comments by Yeng et al [1] in response to the publication of the 2023 American College of Rheumatology/European Alliance of Associations for Rheumatology (ACR/EULAR) antiphospholipid syndrome (APS) classification criteria (CC) [2]. We want to assure them that our calculations of the specificity and sensitivity of the Revised Sapporo APS CC [3] are indeed correct. However, their reverse calculations revealed a typographical error in 2 columns (for 'criteria met, no of subjects') of Table 5, which we regret. As discussed in the original publication [2], the sensitivity and specificity of both 2023 ACR/EULAR and Revised Sapporo APS CC were calculated after excluding 6 and 9 cases from the first and second validation cohorts, respectively. The number of patients meeting the Revised Sapporo APS CC in Table 5 (columns 3 and 5) were erroneously reported as 120 and 143 for the first and second validation cohorts, respectively. These values represent the cohorts including those 6 patients in the first (5 met criteria) and 9 in the second (9 met criteria) validation cohorts. The correct values, which were used in our specificity and sensitivity calculations, should have been reported as 115 and 134 for the first and second validation cohorts, respectively.

Competing interests

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