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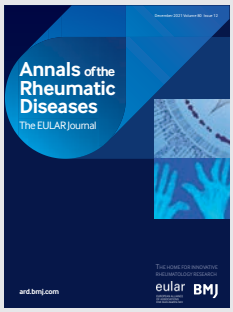

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Towards clinical significance of the *MUC5B* promoter variant and risk of rheumatoid arthritis-associated interstitial lung disease

Jeffrey A Sparks [1,2]

While rheumatoid arthritis-associated interstitial lung disease (RA-ILD) has been known to be a serious extra-articular rheumatoid arthritis (RA) manifestation for decades, RA-ILD and other pulmonary sequelae of RA have been of intense interest in recent years. Patients with RA have excess respiratory mortality of RA compared with the general population.[1 2] This respiratory burden of RA seems to be specific for patients with seropositive RA,[1] and RA-ILD is likely a key contributor to the respiratory burden of RA. Median survival after clinical RA-ILD detection is poor, ranging from 3 years to 8 years in previous studies.[3–5] Unlike nearly all other outcomes in RA, prevalence of RA-ILD does not seem to be decreasing over calendar time.[5] This may be explained by several factors that include increased longevity of patients, improved articular disease activity unmasking symptoms of dyspnoea on exertion, increased awareness by clinicians of RA-ILD, greater ease in obtaining advanced chest imaging, or perhaps by medications used to treat RA-ILD. Thus, RA-ILD is a serious public health condition for patients with RA. Establishing the risk and identifying risk factors for RA-ILD are therefore of utmost importance. In *Annals of the Rheumatic Diseases*, Palomäki and colleagues investigate the lifetime risk of RA-ILD related to the *MUC5B* promoter variant.[6]

RA-ILD is a heterogeneous condition that is notoriously difficult to diagnose. Usual interstitial pneumonia (UIP) is the most common RA-ILD, considered to be fibrotic and progressive. A recent meta-analysis found that the UIP subtype had worse prognosis compared with other RA-ILD subtypes.[7] Idiopathic pulmonary fibrosis (IPF) shares many of the same clinical and imaging features of UIP in RA-ILD. A common promoter variant of *MUC5B* (G>T at the rs35705950 single-nucleotide polymorphism) was identified as an important genetic risk factor for IPF.[8] Subsequently, the *MUC5B* promoter variant was associated with overall RA-ILD risk, specifically the UIP subtype, compared with patients with RA without ILD as well as general population controls.[9] However, in that previous study, there was no association of the *MUC5B* promoter variant with RA risk or serostatus, and there was no gene-smoking interaction for RA-ILD risk.[9]

People with the *MUC5B* promoter variant produce higher quantities of mucin 5B in lung parenchyma and airways.[10] While the exact mechanisms linking the *MUC5B* promoter variant with IPF and UIP risk are still being elucidated, the relative overabundance of the mucin 5B protein may lead to local recruitment of immune cells that eventually leads to long-term damage and fibrosis that presents clinically as fibrotic lung disease. The parallels between IPF and UIP have led some to speculate that these may be the same entity, the latter in a patient that just happens to also have RA. However, the prevalence of UIP is generally reported to be higher than would be expected by chance alone, considering the independent prevalence of IPF and RA. Previous studies have also identified RA-specific characteristics, such as RA-related autoantibodies and articular disease activity, as risk factors for RA-ILD.[11–14] Thus, there may be a synergistic relationship between the *MUC5B* promoter variant and RA for RA-ILD risk. Other established RA-ILD risk factors include older age at RA onset, male sex, cigarette smoking and longer RA duration, among others.[13]

Palomäki and colleagues used FinnGen to study the relationship of the *MUC5B* promoter variant with the lifetime risk of RA-ILD.[6] FinnGen was assembled from other prospective studies and linked to nationwide registers in Finland to link genetic and clinical data with up to 50 years of follow-up. They analysed 293 972 individuals to determine presence and dates of RA and ILD, identified using medication reimbursement codes and diagnoses from hospital inpatient and outpatient registries.[6] They then stratified by presence or absence of the *MUC5B* promoter variant and also performed separate analyses among men and women. In the entire population, about 20% had at least one copy of the *MUC5B* promoter variant.[6] Overall, the estimated risk of ILD was 1.5% by 80 years of age (considered as a surrogate for lifetime risk). Within RA, the lifetime risk of RA-ILD was higher, at 6.1%.[6] Presence of the *MUC5B* promoter variant was strongly associated with ILD risk within RA compared with absence of the variant (HR 2.27, 95% CI 1.75 to 2.95).[6] The lifetime risk of ILD in RA for those with the *MUC5B* promoter variant was 16.8% (compared with 4.4% in the general population) and seemed to specifically emerge after age of 65 years.[6] Consistent with other studies, men with RA had a higher lifetime risk of ILD than women with RA. However, the lifetime risk for RA-ILD among those with the *MUC5B* promoter variant was quite high for both men (20.9%) and women (14.5%).[6] Considering that the *MUC5B* promoter variant is relatively common, this means that many patients with RA may be harbouring this genetic variation that could dramatically alter their lifetime risk for RA-ILD.

Beyond risk for RA-ILD, there was also some evidence that the *MUC5B* promoter variant could also impact overall RA risk.[6] Those with the variant had slightly increased risk for RA in FinnGen, which was also replicated in the UK Biobank. The relationship persisted when eliminating patients from the analysis who developed ILD prior to RA. A possible mechanism for this observation could be that the *MUC5B* promoter variant could impact RA-related autoantibody production, perhaps by leading to pulmonary mucosal inflammation and immune tolerance loss prior to articular RA onset. However, large genetic studies have not identified a relationship of *MUC5B* with overall or seropositive RA risk,[6] so these findings should be considered preliminary.

Some limitations need to be considered. Most notably, cigarette smoking was not measured and so could not be considered in the analysis. Smoking likely mediates the relationships between *MUC5B*, RA, and RA-ILD. It would be

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of clinical and public health importance to determine how smoking may modify the RA-ILD risks observed in this study, particularly among patients with the *MUC5B* promoter variant. Likewise, RA-related autoantibody levels, disease activity, medications and severity likely impact RA-ILD risk[13] and were unable to be incorporated in analyses. The past and current RA disease states would need to be considered for clinical application of the *MUC5B* promoter variant. The authors did not consider patients with two copies of the promoter variant who are likely at even higher risk of RA-ILD. As already noted, RA-ILD is a heterogeneous phenotype and the *MUC5B* promoter variant is only expected to strongly impact UIP risk within RA. Identifying RA-ILD in administrative datasets and institutional biobanks may be inaccurate[15] and could have at least slightly overestimated its incidence in this study.[6] The results emphasise how much more common RA-ILD is compared with the general population, again suggesting that RA-ILD is a distinct entity and not IPF in a patient who happens to have RA. However, without data on the UIP subtype, this is not yet definitive.

While lifetime risk of ILD is an important clinical metric to consider, these findings suggest relatively little impact of the variant until patients have reached the age of 60 years. Even then, this would be predictive of RA-ILD within 20 years, a relatively long window to monitor patients for signs and symptoms. If this genetic variant was measured many years before this 'risk window of RA-ILD', it may invoke either decades of anxiety or a false sense of reassurance if RA-ILD does not immediately develop when checked. As also noted in other studies, RA-ILD risk seems to be most pronounced in older patients.[16 17] More research is needed to understand how *MUC5B* and the ageing process may impact RA-ILD risk.[18]

In conclusion, the *MUC5B* promoter variant has clearly emerged as the single most important genetic risk factor for RA-ILD. This is evidenced by one in every six patients with RA with this variant developing RA-ILD by age of 80 years in this study. As such, any model of RA-ILD pathogenesis, particularly UIP, needs to incorporate the variant into its framework, analogous to the *HLA-DRB1* shared epitope in RA pathogenesis and *HLA-B27* in spondyloarthritis pathogenesis. While these findings have clear research importance for the field of RA-ILD, clinical application is not yet determined. This would require a clear determination on who to order the test, the diagnostic and prognostic implications, and actions to mitigate risk. This study suggests an age window and provides prognostic implications on which testing the *MUC5B* promoter variant may be considered as appropriate. Further studies are needed to determine actions such as smoking cessation or pharmacological therapies, such as anti-inflammatories or antifibrotics, that could possibly alter the natural history of RA-ILD.

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Nothing about us without us: involving patient collaborators for machine learning applications in rheumatology

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ABSTRACT

Novel machine learning methods open the door to advances in rheumatology through application to complex, high-dimensional data, otherwise difficult to analyse. Results from such efforts could provide better classification of disease, decision support for therapy selection, and automated interpretation of clinical images. Nevertheless, such data-driven approaches could potentially model noise, or miss true clinical phenomena. One proposed solution to ensure clinically meaningful machine learning models is to involve primary stakeholders in their development and interpretation. Including patient and health care professionals' input and priorities, in combination with statistical fit measures, allows for any resulting models to be well fit, meaningful, and fit for practice in the wider rheumatological community. Here we describe outputs from workshops that involved healthcare professionals, and young people from the Your Rheum Young Person's Advisory Group, in the development of complex machine learning models. These were developed to better describe trajectory of early juvenile idiopathic arthritis disease, as part of the CLUSTER consortium. We further provide key instructions for reproducibility of this process.Involving people living with, and managing, a disease investigated using machine learning techniques, is feasible, impactful and empowering for all those involved.

UNSUPERVISED MACHINE LEARNING IN HEALTHCARE

Recent years have seen a rapid growth in artificial intelligence applications, such as machine learning, to healthcare[1] allowing for the prediction of outcomes and identification of patterns within increasingly complex datasets. Therefore, applications such as automated interpretation of X-ray or MRI, decision support for therapy selection and data-driven classification of heterogeneous conditions may become common practice.[2]

In rheumatology, these approaches could help better define and map outcomes in patients with complex diseases such as juvenile idiopathic arthritis (JIA) or systemic lupus erythematosus. While supervised machine learning applications are trained to classify or make predictions for patients, unsupervised machine learning methods allow for data-driven pattern detection, or clustering, of people without using predefined clinical criteria.[1 2] These clusters may represent those with unique disease features at a single clinic visit or with distinct disease trajectories over time. Although each person with a disease is unique and the entirety of their disease impact should be considered when providing treatment, guidelines for treatment are developed for mass application and rely on population-based criteria. Identifying similar experiences within groups of people can allow for tailoring of therapies and forecasting of disease course in a more pragmatic paradigm that can be applied to treatment guidelines. In addition, people within groups with similar disease manifestations or experiences may have separate clinical and biological mechanisms that underpin their data-driven clusters, for example, following specific antirheumatic therapies.[3 4] Data-driven clustering methods are, therefore, a potential gateway to stratified medicine across rheumatology.

Clusters identified through unsupervised machine learning methods may prove to be more clinically relevant than those defined by preset clinical criteria; participants are grouped using factors that may be crucial in terms of outcome but not evident to clinicians; however, their flexibility means that modelling of noise within a dataset, which does not represent true variation between patients or disease courses in clinical practice, is a possibility.[2] Furthermore, a lack of a ground truth means that researchers are faced with several potential 'optimal' models to choose from, and validation of any resulting groupings is not straightforward. Current machine learning paradigms suggest that final model selection be driven through optimising parsimony without compromising model fit, for example, selecting the model at the 'elbow' of a Bayesian Information Criterion (BIC) curve.[5 6] However, the selection of a more parsimonious model might miss a true clinical phenomenon, while a more complex, better-fitting model may inadvertently overfit the data, modelling data quirk rather than clinically meaningful differences. Both of these scenarios result in the potential for suboptimal patient care where subgroups with unique disease features are missed or subgroups that do not exist clinically are incorrectly treated differently due to these analytical constraints for current model selection.

WHY INVOLVE PEOPLE WITH THE DISEASE AND MEDICAL PRACTITIONERS IN MACHINE LEARNING RESEARCH?

One proposed solution to improve selection of clinically meaningful models is to include primary stakeholders in the construction of machine

learning applications. People living with a given disease have unique viewpoints, allowing for research to be directed and refined based on first-hand experiences. Excluding these people from the research process means that both their agency and their priorities are likely overlooked.[7] Healthcare professionals play a crucial role in the management of disease in these primary stakeholders; their priorities often differ from people directly affected by disease[8] but should also be reflected in machine learning models built for healthcare.

Key instruction 1

Involve people who live with and those who treat diseases in research about their condition of interest.

An example: finding clusters of children and young people (CYP) with JIA

JIA is the most common inflammatory arthritis of childhood. It is a heterogenous condition and approach to, as well as response to, treatment is not universal, with a significant proportion of children known to have persistent disease, chronic symptoms and associated comorbidity[9 10] despite treatment. Through the CLUSTER consortium (www.clusterconsortium.org.uk), as part of our efforts to improve personalised treatment in JIA, we aimed to identify clusters of CYP who experience distinct patterns of arthritis-related outcomes.[11] These outcomes were assessed following diagnosis based on clinical data captured within the Childhood Arthritis Prospective Study, a UK multicentre inception cohort of JIA. Using group-based trajectory models, a form of unsupervised machine learning, we clustered approximately 1200 CYP, based on their recorded number of affected joints, a physician global assessment of their disease activity and a patient global assessment of their well-being over time.[12]

The initial results from the clustering analysis revealed a shortlist of models that all fit criteria for good model adequacy, fit and discrimination between identified clusters.[6] These models were brought forward for discussion with key stakeholders through structured workshops.

Key instruction 2

Present and discuss with involvement groups only well-fitting models, to ensure final results both well describe the data captured and are clinically meaningful.

Patient and healthcare professional involvement in model selection

Potential models were discussed in separate focus groups with CYP and healthcare professionals. The CYP group included members of the young person's advisory group Your Rheum,[13 14] consisting of CYP aged 11–24 years with musculoskeletal conditions across the UK. Seven of these members (aged 14–22 years) were involved in the current study. The healthcare professional group consisted of 12 multidisciplinary rheumatology specialists (paediatric rheumatology, physiotherapy, occupational health, nursing, research practitioner, trainees) within Royal Manchester Children's Hospital at Manchester Foundation NHS Trust. These groups were consulted with the specific aims of identifying the most clinically relevant models that represent real-world experiences of CYP and healthcare professionals, but avoiding modelling of noise, from our shortlist of well-fitting models. Both groups undertook four activities supervised by the researcher, to initially ground them in interpretation of trajectory plots through drawing their own experiences, to aid in outcome selection through a group discussion and then to select and discuss models most relevant to their experiences (figure 1).

Key instruction 3

Include an educational and interactive activity before discussing complex research, to ground the involvement groups in the methods, bring key experiences to the forefront of their minds and facilitate initial discussion.

Choosing outcomes for machine learning studies

Core outcomes in JIA represent patient-reported and physician-assessed variables which may hold different levels of importance to people with the disease and healthcare professionals, respectively. Machine learning research should consider consulting involvement groups to select and include outcomes relevant to both parties.

The models presented to the focus groups clustered CYP with JIA based on changes across multiple core outcomes, which can be combined into a composite outcome, producing a single score to represent overall disease impact.[15] Both groups suggested that their experiences or treatment decisions would hinge on specific outcomes within those included in the models, thus bringing into question the utility of a composite score for research assessing clusters of disease. For young people, clusters identified by modelling the outcomes separately rather than using the composite score were deemed more meaningful (figure 2). For this group, separating changes in well-being from physician-assessed measures was key to understanding how they would experience their disease over time. Young people were particularly concerned that the research should demonstrate separate trajectories for physician global scores and patient well-being scores, since these measure different aspects of disease impact and many young people had experienced physician-assessed disease activity and self-perceived well-being not aligning with one another.

For healthcare professionals, modelling the outcomes separately rather than as a composite score was also important (figure 2); modelling all outcomes as a composite score would not delineate changes in joint count from changes in more subjective measures on which they would be less likely to base antirheumatic drug or physiotherapeutic decisions.

Based on discussions with both groups, multivariate modelling was therefore preferred to allow the identification of clusters with unique characteristics across outcomes prioritised by different key stakeholder groups who may have different goals of treatment.

Key instruction 4

Involve key stakeholders in selecting outcomes of research to best fit their priorities. Note that different groups of stakeholders may have different priorities, and efforts should be made to facilitate each of these.

Prioritising clinically meaningful models while minimising noise

Distinguishing features between competing, well-performing, models included the addition or removal of a cluster or differences in polynomial structure, or pattern of change, observed over time. For the young person's group, the most complex model (cubic polynomial) showed clusters with distinct, meaningful patterns of disease and well-being, even though this model would not have been objectively selected using the elbow approach. An additional cluster was depicted by this model (cluster 2, figure 2B) and the researchers were unsure of clinically meaningful difference to an existing cluster (cluster 1, figure 2B). The group suggested that this additional cluster represented a unique experience of disease over time. However, healthcare professionals noted that the profile of this new cluster would only change their treatment decisions compared with a

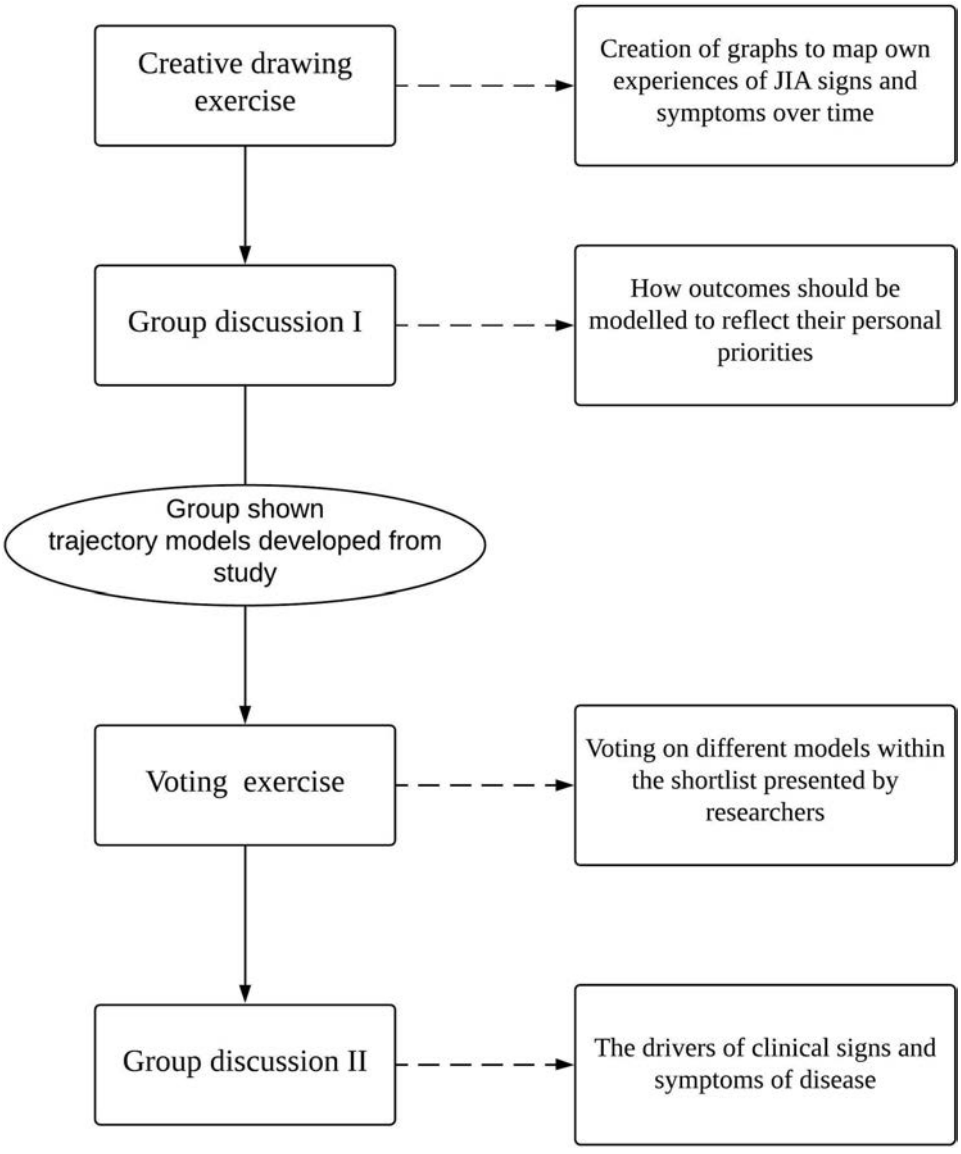

Figure 1 The process by which machine learning models were ratified through patient and healthcare professional involvement. JIA, juvenile idiopathic arthritis.

more parsimonious model if the magnitude of change was demonstrated in the active joint count outcome. Had a similar difference between clusters been observed in one of the other outcomes, it would not have influenced their treatment decisions, particularly for the paediatric rheumatologists considering antirheumatic drug therapy. This exemplifies the utility of presenting results to CYP and healthcare professionals, where better fitting, more complex models may not identify clinically distinct groups of patients in terms of the patient experience or management of disease in its current form. In these cases, more parsimonious models with less optimal fit may be more clinically useful and/or better describe the patient experience; however, in this instance, the most complex model was deemed to cluster young people based on meaningful differences in disease, rather than noise in the dataset, and was therefore selected.

Key instruction 5

Be prepared to balance clinical relevance and statistical fit. The objectively 'best-fitting' model may be noisy or overfit, and stakeholders can help identify when a more parsimonious model would be more clinically helpful.

Feasibility of involving primary stakeholders in machine learning research

Involving young people in interpreting research, particularly when asking them to recall their own disease journey, is a very personal experience and therefore requires a greater level of sensitivity than involvement for planning or disseminating research. Potential barriers to effective involvement with these young people were perceived to be the potentially sensitive nature of recall alongside the complexity of the models presented. Written and verbal feedback on the event from stakeholders was sought to evaluate the experiences of being involved, including difficulty, comfort and enjoyment of the exercises, and suggestions for future events (box 1).

Key instruction 6

Seek feedback on all involvement activities. This evaluation will improve future efforts for both stakeholders and the overall research.

Despite no previous education on machine learning, the creative drawing exercise successfully familiarised the young

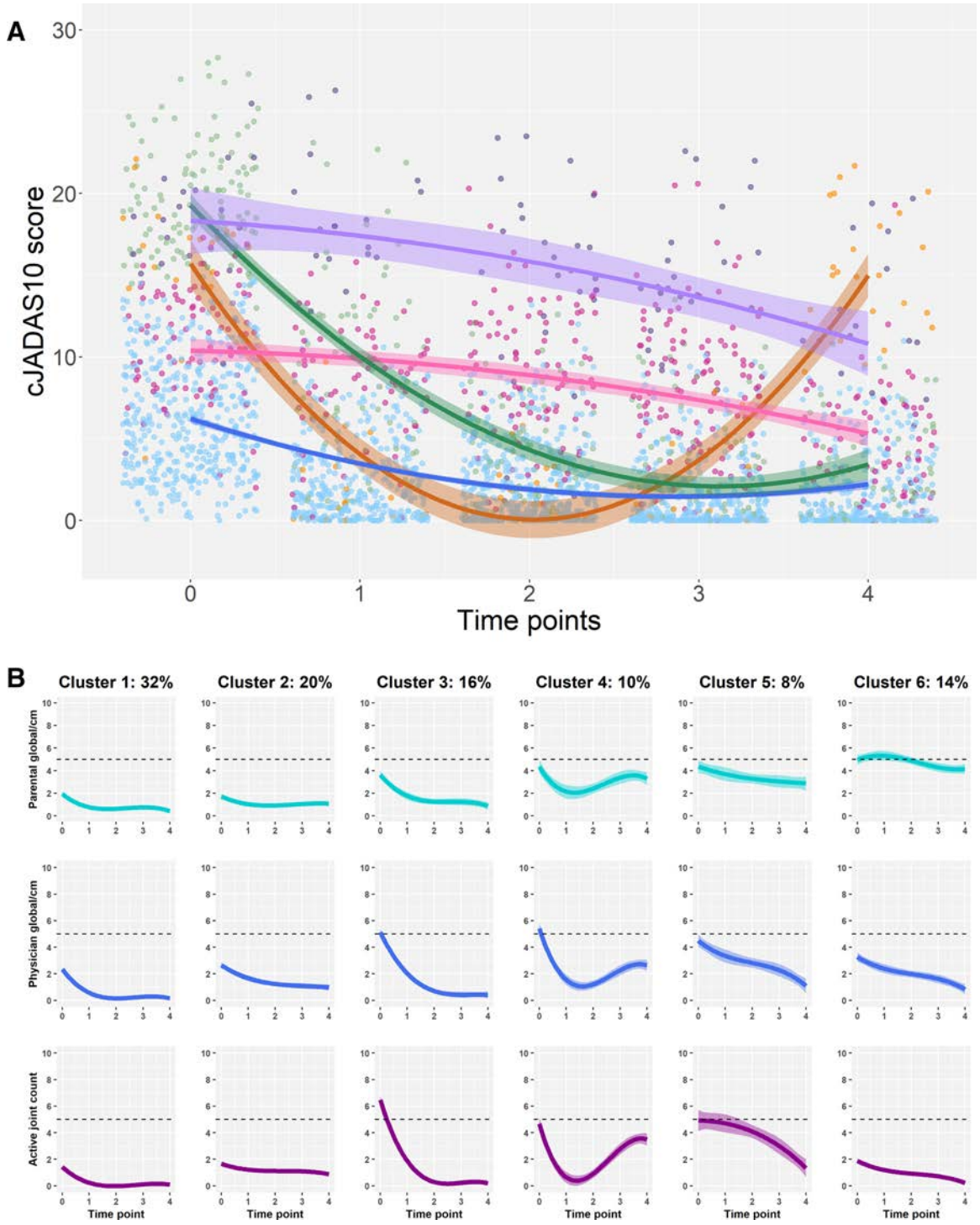

Figure 2 (A) Univariate and (B) Multivariate modelling approaches presented as alternatives to the focus groups, adapted from Shoop-Worrall *et al*.[12] Both groups considered multivariate modelling (B) more meaningful. Each trajectory represents an average outcome pattern for one cluster of CYP with JIA. For all outcomes, higher scores denote more severe outcomes. (A) Five clusters of CYP, each with a different average pattern of the cJADAS10 score over time. (B) Six overall clusters, each with unique shared patterns of parental global scores, physician global scores and active joint counts over time. CYP, children and young people; JIA, juvenile idiopathic arthritis.

people and healthcare professionals with trajectory graphs and the concept of multivariate modelling. Completing the tasks in a group setting appeared to facilitate understanding in addition to fostering reassurance among young people about shared experiences. Young people also felt that recalling past events could be distressing and to always clarify that participants could draw whatever they feel comfortable sharing, with suggestion that some distressing events may be cathartic to discuss in a sensitive and supportive environment (box 1). Young people over the age of 16 years provided written consent to taking part in the involvement group. Parents of young people under the age of 16 years signed consent forms and accompanied their children to the event but were not present for the duration of the meeting. Previous experience with this advisory group has suggested more open conversations and a greater sense of peer support and when young people are able to participate independently.

Box 1 Experiences completing the creative drawing and viewing printed model tasks.

(a) Difficulty:
- (The exercise was) difficult to begin with as there were things you had to decide, such as 'what is my timescale?' and 'what classes as good or bad?'
- Seeing other people do it made it easier.
- I really enjoyed the drawing.

(b) Recalling past events:
- Remembering important events was much easier to do as they are things that stick in your mind.
- I can remember the pattern of illness but can't remember what it felt like at age 2.
- It might have been easier to do if you were years away from diagnosis as you can look back…in the years closest to you, you almost remember too much.

(c) Potential for distress:
- (Looking at the graphs was) personally not distressing, more distressing was putting on the life events as they're reminders of upsetting times…it was refreshing to be fair.
- Some things may feel close to the bone.
- These are things you don't often get the chance to talk about, you hide them away.
- Even if it is upsetting, you are doing something useful with it. I find thinking about the future harder.

(d) Running the sessions:
- It helps having the right person run the session, somebody who wants to listen.
- I didn't feel at all forced into doing it. You choose what you want to put down, no-one is inside your head.
- Just make it clear that you don't have to put anything down you don't want to.

(e) Creative drawing as a means of understanding multitrajectory graphs presented:
- This helped engage with the graphs as you understand it.
- If you faced me with the graphs (without the creative drawing) I wouldn't have known where to begin.
- Drawing and the arts are really helpful…anything that puts it into perspective helps.
- Especially helpful if you are working with younger kids.
- I love a graph! It is not loads of things to read in complex language.
- I hate graphs but I found it quite enjoyable.

(f) Overall takeaways from being involved:
- Getting people talking about it helps you realise that you are not alone.
- The transparency of what (the research) will be used for and how it will help was good.
- It was refreshing to hear the purpose of the research, why we are doing it differently.
- (The research was) really, really worthwhile looking at.

Key instruction 7

Plan to facilitate sharing of experiences while minimising discomfort. Peer support is often helpful for both of these and so group sessions may be preferred to one-on-one sessions. Have a distress protocol in place for managing participants' potential distress.

CONCLUSIONS

Unsupervised machine learning approaches may be the key to unlocking stratified medicine across diseases, including in rheumatology. Involving people with first-hand experience of disease and healthcare professionals who treat it, in key methodological and interpretive decisions, including outcome selection, model selection and model interpretation, can significantly improve unsupervised machine learning based research. Involvement in this type of research is feasible even with young people through creative tasks. Once well-fitting models have been identified using mathematical measures, researchers should consider that their own second-hand or third-hand knowledge of a disease is insufficient to choose a final model. Leveraging experiences from these groups ensures that models produced are: (1) useful to key stakeholders, (2) do not exclude clinically meaningful outputs and (3) minimise identification of noise as a clinical finding. These insights can only be gained through discussions with those closest to the disease.

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Axial spondyloarthritis

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ABSTRACT

Axial spondyloarthritis (axSpA) encompasses both radiographic and non-radiographic axSpA. It is a chronic inflammatory disease with a predilection for involving the axial skeleton. The most common presenting symptoms are chronic back pain and spinal stiffness but peripheral and extra-musculoskeletal manifestations occur also frequently. The diagnosis of axSpA relies on the recognition of a clinical pattern of the disease, based on clinical, laboratory and imaging features. The Assessment in SpondyloArthritis international Society classification criteria for axSpA are valid and well implemented for research purposes. Sustained disease activity, measured by validated tools such as the Ankylosing Spondylitis Disease Activity Score, leads to irreversible structural damage and poor functioning and therefore should be abrogated. As part of the management algorithm, non-steroidal anti-inflammatory drugs remain as the first line of pharmacological treatment besides physiotherapy. As a second line, tumour necrosis factor inhibitor and interleukin-17 inhibitor are available but recently Janus kinase inhibitors have also shown efficacy in improving symptoms of the disease.

INTRODUCTION

Spondyloarthritis (SpA) is a chronic inflammatory disease that either involves predominantly the axial (ie, the sacroiliac joints (SIJ) and the spine; axial SpA (axSpA)) or the peripheral skeleton (ie, joints of the limbs; peripheral SpA (pSpA)).[1] The prototype of axSpA is radiographic axSpA (r-axSpA), also known as ankylosing spondylitis and historically described according to the modified New York classification criteria (mNY).[2] The hallmark feature of r-axSpA is commonly referred to as 'radiographic sacroiliitis'. The term, however, is misplaced because sacroiliitis implies inflammation, but only structural damage, rather than inflammation, is visible on radiographs.

By the time that structural abnormalities become apparent on pelvic radiographs, patients typically had already symptoms, such as pain, for several years. Efforts to reduce the diagnostic delay of axSpA led to the recognition of patients presenting with a clinical phenotype similar to r-axSpA except for the absence of definite damage visible on pelvic radiographs. Unlike radiographs, MRI allows direct visualisation of inflammation.[3] In the mid-1990s, MRI demonstrated that these patients have inflammation on the SIJ often predating radiographic damage for years.[4]

This evidence led the Assessment in SpondyloArthritis international Society (ASAS) to coin the term 'axial spondyloarthritis' to refer to the entire spectrum of the disease, covering both patients who have already developed definite radiographic damage in the SIJ (r-axSpA) and patients without such damage (non-radiographic axSpA (nr-axSpA)).[5 6] Patients with nr-axSpA represent early forms of axSpA, in a disease continuum, in which some, but not all, eventually progress to r-axSpA. For the purpose of diagnosis, in clinical practice, the distinction between r-axSpA and nr-axSpA is less relevant, and preference should be given to the term axSpA to refer to all patients.[7]

EPIDEMIOLOGY

AxSpA usually starts in the third decade of life with a male to female ratio of 2:1 for r-axSpA, and with an equal sex distribution among patients with nr-axSpA. The percentage of patients with nr-axSpA is increasing over time, which is partly due to its better recognition[8] (figure 1).

Most data on the prevalence of axSpA pertain to r-axSpA with a prevalence ranging widely from 0.1% to 1.4%. Differences in study design can explain some variability; however, it is well-known that the prevalence of the disease is highly affected by the background prevalence of the human leucocyte antigen (HLA)-B27, its major genetic association.[9]

Populations with high background prevalence of HLA-B27 show higher rates of axSpA, such as in Northern Europe and among the native peoples of the circumpolar arctic and subarctic regions of Eurasia and North America.[10] In contrast, the near absence of axSpA in southern Africa and the low rates in Japan is linked to low HLA-B27 prevalence. AxSpA prevalence (including both r-axSpA and nr-axSpA) varies between 0.3% and 1.4%,[11 12] and the estimates for the entire group of SpA (including axSpA and pSpA) from 0.3% to 1.9%, making it at least as prevalent as rheumatoid arthritis.[13]

PATHOGENESIS

The primary pathophysiology in axSpA occurs in the entheses and in the subchondral bone.[14 15] Although synovitis may also occur, it is a secondary process originating from signals in the enthesis.[14] Entheseal and bone pathology occurs in individuals with a specific genetic background. Genetic studies estimate a heritability greater than 90%. The most important genetic risk factor is HLA-B27, but other major histocompatibility complex (MHC) variants are also involved.[16] Two non-MHC genetic loci have also been associated with axSpA, the endoplasmic reticulum aminopetidase (ERAP) and the interleukin-23 (IL-23) receptor.[17] Polymorphisms on these loci have functional consequences and associate with disease manifestations.[18 19] Of note, ERAP1 associates with axSpA only in HLA-B27-positive cases, indicating the relevance of peptide presentation by HLA-B27.[20]

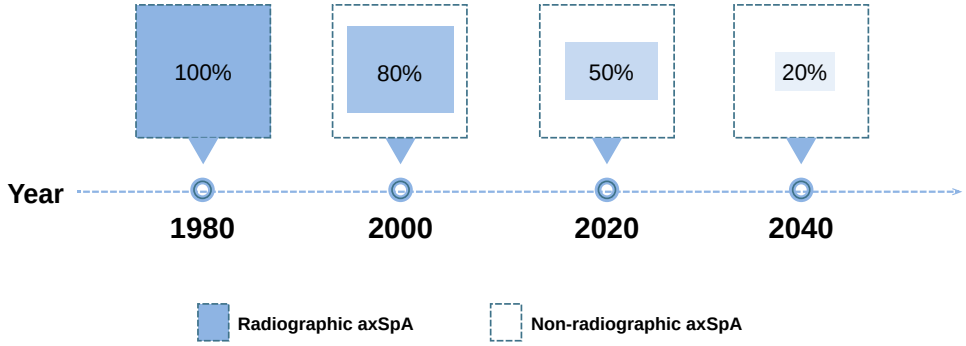

Figure 1 Distribution of axial spondyloarthritis subtypes over time. The graph represents an estimation of the prevalence ratio between non-radiographic and radiographic axial spondyloarthritis, showing the estimated percentage of patients with radiographic axial spondyloarthritis for each period at the time of diagnosis. Adapted from Benavent *et al*. Clin Rheumatol. 2021 Feb;40(2):501–512. axSpA, axial spondyloarthritis.

Entheses are load-bearing structures with a specific immune microenvironment that, in susceptible individuals, may be activated by mechanical and microbial triggers.[14] There is increasing evidence of the importance of mechanical stress in the onset and progression of axSpA.[21 22] In addition, damage to the skin, induced by psoriasis, and to the intestinal mucosa by inflammatory bowel disease (IBD), facilitate exposure to pathogens. This mechanism may happen even in asymptomatic patients. In fact, subclinical gut inflammation has been linked to earlier disease onset and worse prognosis.[23] Dysbiosis is thought to be of relevance in the link between the intestine and SpA pathogenesis[15] (figure 2).

Axial inflammation, bone destruction and new bone formation are key events in the pathophysiology of axSpA. Even though, it is yet to be fully clarified the mechanisms that govern their interplay, several studies using bone biopsies, animal models and imaging had already yielded important insights. Subchondral bone marrow oedema (BME) visible on MRI is the earliest detectable change in biopsy specimens.[24 25] BME is then replaced by an inflammatory granulation tissue, containing also adipocytes and fat vacuoles, that erodes the subchondral bone plate, but also has bone-forming capabilities.[26] Fatty lesions on MRI are thought to be the imaging translation of this repair tissue.[27]

Thus, inflammation can lead either to bone destruction or to bone formation. One hypothesis defends that bone destruction, driven by the contact between osteoblasts and osteoclasts (by receptor activator of NF-κB–receptor activator of NF-κB ligand interactions), prevails with sustained inflammation, while bone formation implies that inflammation subsides and the absence of osteoclasts.[28] In axSpA, inflammation is thought to fluctuate, which allows repair and an anabolic response driven by bone morphogenic proteins and Wnt proteins.[14] Several clinical studies corroborate the observation that inflammation leads to subsequent new bone in axSpA.[28–35] Whether or not a repair mechanism (fatty lesions) mediate this effect, remains an open question.[36]

Tumour necrosis factor (TNF)-α and IL-23/IL-17 are, thus far, identified as the major pro-inflammatory cytokine pathways in axSpA.[14] The pivotal role of TNF-α in the pathogenesis of axSpA is supported by the success of TNF inhibitors (TNFi) in controlling the symptoms of the disease,[37] More recently, IL-17-inihibitors (IL-17i) have also proved effective in axSpA, but not IL-23-inhibitors (IL-23i).[38] These results offer important clues on the role of the IL-23/IL-17 pathway in SpA. IL-17 is produced by T helper 17 (TH17) cells in response to IL-23, in a later stage of their differentiation. The inefficacy to IL-23i in controlling axial manifestations suggests, however, an uncoupling between the two cytokines. The fact that IL-17 secretion might take place in the absence of IL-23 and that cell types other than TH17, such as the type 3 innate lymphoid cells, produce IL-17 independently of IL-23 supports this claim.[38]

Both TNF-α and IL-17 induce bone destruction and cause a downregulation of osteoblast function when osteoblasts and osteoclasts interact. In absence of osteoclasts, as in axSpA, however, these cytokines lead to bone formation,[39] suggesting their inhibition can, potentially, interfere with the disease progression.

CLINICAL, LABORATORY AND IMAGING FEATURES

The most common, and often presenting, symptom of axSpA is chronic (lasting >3 months) almost daily back pain (CBP),

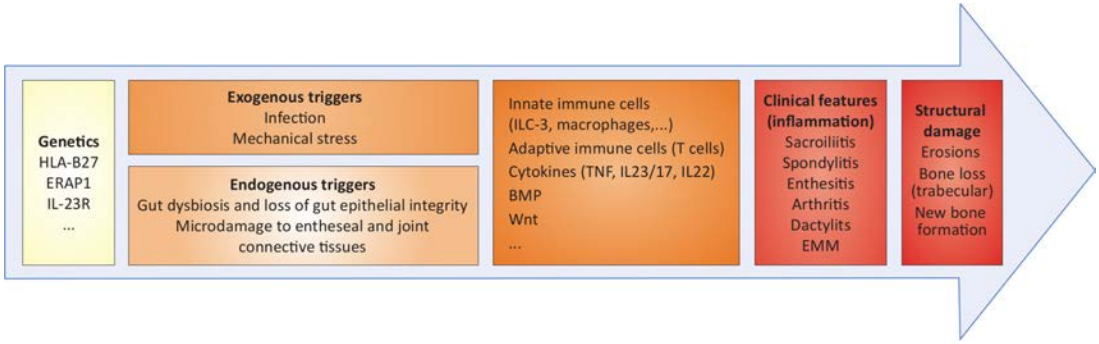

Figure 2 Pathogenesis scheme for axial spondyloarthritis. A schematic presentation of the various aspects that play a role in the pathogenesis of axial SpA. BMP, bone morphogenetic protein; EMM, extra-musculoskeletal manifestation; ERAP, endoplasmic reticulum aminopetidase; HLA, human leucocyte antigen; IL, interleukin; ILC, innate lymphoid cell; SpA, spondyloarthritis; TNF, tumour necrosis factor.

which is frequently accompanied by morning stiffness. Pain and stiffness usually involve the lower spine and the buttocks, but any level of the spine can be affected. CBP in axSpA typically has an insidious onset and has inflammatory characteristics: it is worse in the second part of the night and in the morning, it is relieved with activity and worsened by rest and usually improved by non-steroidal anti-inflammatory drugs (NSAIDs). Several criteria have been proposed to define inflammatory back pain (IBP).[40 41] Though typical, not all axSpA patients have IBP. In fact, up to one third of patients present with mechanical back pain,[42] also diseases other than axSpA may present with IBP.[43] In axSpA, axial inflammation (synovitis and enthesitis) leads to irreversible structural damage and both can limit the mobility of the spine.[44] However, limited spine mobility is usually a late disease manifestation and, although a characteristic feature of axSpA, it may also occur in patients with CBP from diseases other than axSpA.[45]

In addition to CBP, patients with axSpA can present peripheral manifestations. Arthritis and enthesitis are the most common peripheral manifestations in axSpA, each occurring in approximately 30% of the patients.[46] Peripheral arthritis, presenting as a swollen and painful joint, is usually an asymmetrical monoarthritis/oligoarthritis, and involves predominantly the lower extremities. Peripheral enthesitis usually manifests with pain, stiffness and/or tenderness. The most common affected entheses are at the insertion of the Achilles tendon and the plantar fascia. However, axial enthesitis (eg, at the insertion of the anterior longitudinal ligament) together with synovitis of the axial joints (costovertebral, costosternal and manubriosternal joints), can also be involved causing chest/back pain. Dactylitis (sausage digit), which is a swelling of a finger or toe as the consequence of a combination of synovitis, tenosynovitis and enthesitis is a typical feature of axSpA but it occurs in <10% of the patients.[46]

Patients with axSpA may present concomitant extramusculoskeletal manifestations (EMMs), that is, uveitis, IBD and psoriasis. Uveitis is associated with HLA-B27 positivity,[47] and is the most frequent EMM, occurring in approximately 25% of the patients.[46] Uveitis presents typically as unilateral acute anterior uveitis (AAU), and frequently alternates from one eye to the other. Psoriasis (10%) and IBD (5%–10%), including both ulcerative colitis and Crohn's disease, are less frequent EMM of axSpA.[46 48] In severe cases, patients might present with constitutional symptoms, such as low-grade fever and weight loss. Heart (eg, aortic valve insufficiency), lung (restrictive lung disease) and kidney (eg, IgA nephropathy) involvement can also occur in axSpA.

Different laboratory and imaging features are found in axSpA. The presence of HLA-B27, tested in peripheral blood samples, is positive in 70%–90% of patients. Inflammation can be quantified by measuring the levels of the C reactive protein (CRP) or the erythrocyte sedimentation rate (ESR). However, up to 60% of patients with axSpA have symptoms despite normal acute phase reactants.[49] Inflammatory lesions of the axial skeleton can be seen with MRI of the SIJ and the spine. The ASAS group define active sacroiliitis as the presence of BME on MRI in subchondral bone highly suggestive of SpA.[50] On the MRI of the spine, the presence of ≥5 corner inflammatory lesions discriminates well between axSpA and no axSpA.[51] Radiographs can detect structural abnormalities (sclerosis, erosions, joint space narrowing/widening or ankylosis) that occur in the SIJ and in the spine. The mNY grading system is traditionally used to quantify structural damage in the SIJ,[2] with a score of 0 (normal), 1 (suspicious changes), 2 (minimal abnormalities), 3 (unequivocal abnormalities) and 4 (total ankylosis) given to each joint. Definite structural changes (radiographic 'sacroiliitis') are defined as bilateral grade ≥2 or unilateral grade ≥3. Structural lesions can also be seen with MRI both in SIJ and in the spine (eg, erosions, sclerosis and fatty lesions). Definitions of each lesion have been published.[52 53]

There are gender differences in the presentation of axSpA. Male patients are more likely to be HLA-B27 positive,[54] a feature associated with imaging abnormalities typical of r-axSpA and with a higher likelihood of AAU.[47 55] Female patients, on the other hand, are less likely to show inflammation and structural damage on imaging studies, and to be positive for HLA-B27.[54] A lower prevalence of HLA-B27 is associated with a higher likelihood of peripheral features and EMM (especially psoriasis) in axSpA.[56–59]

DIAGNOSIS

The diagnosis of axSpA relies on recognising the pattern (the '*Gestalt*') of axSpA, taking into account all features that are present, as well as those that are absent and considering alternative diagnoses. SpA features are identified during the history taking (eg, family history, back pain characteristics, response to NSAIDs, history of enthesitis/arthritis/dactylitis or EMMs), physical examination (eg, arthritis) and in laboratory (eg, CRP and HLA-B27) and imaging (eg, MRI-SIJ) exams. Early diagnosis allows early treatment aiming at reducing the disease burden and improving long-term prognosis. However, the SpA-pattern is sometimes difficult to recognise, especially in early disease and in absence of objective findings leading to uncertainty. In clinical practice, clinicians may use diagnostic algorithms for guidance[42] (figure 3).

Diagnostic algorithms are based on probability rules. The probability of the disease is calculated considering each feature's ability to discriminate between axSpA and no axSpA. Positive and negative likelihood ratios (LR+ and LR−) are easy ways to quantify diagnostic value (table 1).

The higher the LR+ the more likely a diagnosis of axSpA if the feature is positive. Conversely, the lower the LR− the less likely the diagnosis if the feature is negative. The LR+ of present features and the LR− of absent features are multiplied to get the LR-product.[60] Clinicians may follow the diagram in figure 2 to guide their diagnostic reasoning. Importantly, the diagram assumes that the patient comes from a population with a 5% prevalence of axSpA (ie, patients with CBP in general practice).[4]

It should be kept in mind that some features, especially peripheral features and EMM, absent at presentation may occur later on.[58] On the other hand, a negative MRI of the SIJ for the presence of BME is unlikely to become positive within 1 year.[61] Thus, usually repeating the scan does not help in the diagnosis. Also, important to note that the diagnostic value of family history of axSpA is low when the HLA-B27 status is already known.[62] Moreover, IBP is an important feature for referring patients with suspicion of axSpA to the rheumatologist but does not add much diagnostic utility thereafter.[63]

Conventional radiography of the SIJ is usually used as the first imaging modality to identify the involvement of SIJ, mostly because of feasibility reasons. However, on top of the exposure to radiation, this method has major limitations. Damage in the SIJ only becomes visible in pelvic radiographs several years after the start of the symptoms.[64] In addition, the interpretation of radiographs of the SIJ is often challenging even among experienced readers.[65] MRI of the SIJ is recommended if the diagnosis cannot be made based on clinical features and conventional radiographs, but yet the clinical suspicion of axSpA remains high (figure 4).

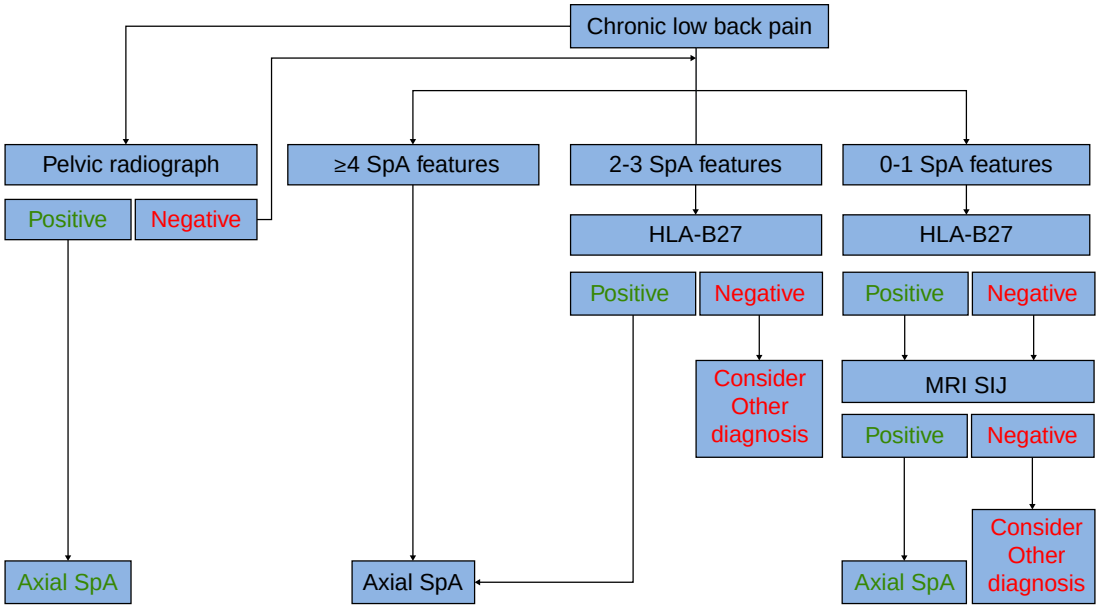

Figure 3 ASAS adaptation of the Berlin algorithm. ASAS, Assessment in SpondyloArthritis international Society; HLA, human leucocyte antigen; SIJ, sacroiliac joints; SpA, spondyloarthritis. SpA features: inflammatory back pain, alternating buttock pain, good response to non-steroidal anti-inflammatory drugs, peripheral arthritis, enthesis, dactylitis, psoriasis, inflammatory bowel disease, uveitis, elevated acute phase reactants, preceding infection, family history. Adapted from van den Berg *et al*.[42]

The presence of BME on MRI of the SIJ fulfilling the ASAS definition increases the likelihood of a diagnosis of axSpA, especially if structural changes are also present. However, clinicians should bear in mind that BME is less specific for axSpA than initially thought.[66] BME can also occur in patients with non-specific back pain, osteitis condensans, healthy individuals, post-partum women, recreational runners and athletes (although deep (extensive) lesions are exclusively found in axSpA patients).[3 67]

Table 1 Diagnostic value of SpA features

SpA feature	LR+	LR−
Inflammatory back pain*	3.1*	0.33
Heel enthesitis	3.4	0.71†
Peripheral arthritis	4.0	0.67†
Dactylitis	4.5	0.85†
Acute anterior uveitis	7.3	0.80†
Psoriasis	2.5	0.94†
IBD	4.0	0.97†
Positive family history	6.4‡	0.72
Good response to NSAIDs	5.1	0.27
Raised acute-phase reactants (CRP/ESR)	2.5	0.63
HLA-B27	9.0§	0.11
Sacroiliitis on MRI	9.0¶	0.11
Radiographic 'sacroiliitis' grade ≥3	20**	0.61

Adapted from Rudwaleit *et al*.[60]
*If the patient is referred based on the presence of IBP: LR+=1.4–1.7.
†Ignore if negative for the calculation of the LR− product (feature may develop later).
‡Lower if HLA-B27 status is known.
§Applies to European Caucasians, may differ in other ethnic groups.
¶LR varies with different definitions.
**Best estimation (poor reliability should be taken into account).
CRP, C reactive protein; ESR, erythrocyte sedimentation rate; HLA, human leucocyte antigen; IBD, inflammatory bowel disease; IBP, inflammatory back pain; LR−, negative likelihood ratio (LR−=(1−sensitivity)/specificity); LR+, positive likelihood ratio (LR+=sensitivity/1−specificity); LR, likelihood ratio; NSAIDs, non-steroidal anti-inflammatory drugs; SpA, spondyloarthritis.

Too much reliance on positive imaging findings can easily lead to overdiagnosis and overtreatment.[68] Likewise, the absence of inflammation on MRI does not, per se, rules out axSpA. Recent data suggest that in addition to the 'classical' axSpA phenotype, dominated by imaging findings, some axSpA patients, mostly women, have a high likelihood of peripheral features co-occurring with CBP but without axial imaging findings.[69]

MRI of the spine has little diagnostic value on its own, and there is conflicting data on the value of combining MRI of the spine with SIJ for diagnosis.[70] Also, abnormalities on spine radiographs do not always occur and when they do it is often too late in the disease course to be of use in early diagnosis. Other imaging modalities, such as skeletal scintigraphy, ultrasonography of the SIJ and positron emission tomography are not recommended for the diagnosis of axSpA.[71] The role of (low-dose) CT for diagnosis is yet to be defined.

A major delay of 5–7 years remains between the start of CBP and the diagnosis of axSpA. Despite a similar age of onset,

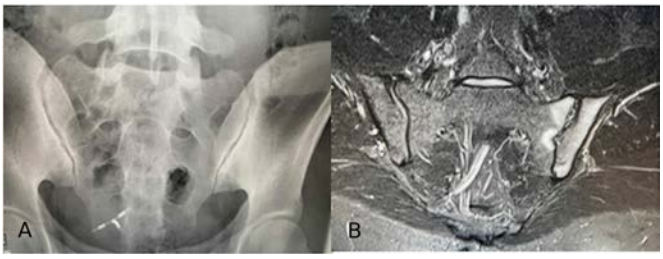

Figure 4 Imaging findings in non-radiographic axial spondyloarthritis. Clinical case: a female patient, 40 years old, complaining of back pain during the last 6 months with inflammatory characteristics (worsening with rest improving with exercise and awaking at second half of night) and morning stiffness of 1 hour. (A) On the pelvis radiograph, no changes are observed; (B) on MRI (fat suppressed sequence) of sacroilliac joints, deep bone marrow oedema suggestive of axial spondyloarthritis on the left sacroiliac bone (both at the sacral margin and iliac bone) is shown.

the diagnostic delay is larger in women (mean 8.8 years) than in men (6.5 years).[72] Differences in disease presentation, as described above, and physician bias may render the recognition of the SpA-pattern in women more difficult.[57 73] In addition, in both genders, too much reliance on the presence of radiographic changes can further delay the diagnosis of axSpA among patients with CBP in primary care. Several referral strategies have been proposed over the years.[74] More recently, ASAS has endorsed a screening method for early referral.[75] Patients with CBP starting before 45 years of age should be referred to the rheumatologist if ≥1 SpA feature (see next section) is present. The method is flexible to local conditions, such as limited availability of imaging and HLA-B27 typing (which are not mandatory) and can therefore be applied widely.

CLASSIFICATION CRITERIA

Given the limitations of the existing classification criteria in including earlier stages of the disease, the ASAS axSpA criteria were established in 2009 and later implemented in most studies.[5 6 76] The ASAS axSpA criteria are applicable to the entire spectrum of the disease (nr-axSpA and r-axSpA) and incorporate MRI of the SIJ.[50] The criteria are meant to be applied in patients with CBP and an onset before age 45 years old and have a diagnosis of axSpA. They have two possible entry arms: the 'imaging arm' (presence of sacroiliitis on radiography or MRI) and the 'clinical arm' (presence of HLA-B27). To classify as axSpA, patients must additionally have at least one (or two, in case of the clinical arm) typical characteristics of SpA, so-called SpA features: IBP, arthritis, enthesitis, dactylitis, uveitis, IBD, psoriasis, good response to NSAIDs, family history of SpA, presence of HLA-B27 and elevated CRP. Their implementation allowed the inclusion in clinical trials of patients covering the entire spectrum of the disease, especially those at an earlier stage, thus representing one of the major advances in the last decade.[77] These criteria have shown to perform well against the rheumatologist's diagnosis, with an overall sensitivity of 82% and specificity of 89%.[78]

However, the ASAS classification criteria for axSpA are not without criticism. They may lead to overdiagnosis in case they are misused for making a diagnosis. But this is a conceptual error, because similar to all classification criteria, the ASAS criteria should not be used to diagnose patients but to classify patients already diagnosed with axSpA (as described above) in order to be included in a study.[79] On the other hand, some experts argue that all features are given the same weight despite not having the same value (LR+ and LR−).[80] The main reason for assigning them the same weight was simplicity, favouring implementation. Nevertheless, ASAS in collaboration with SPondyloArthritis Research and Treatment Network is currently conducting a large prospective study, which will re-evaluate the criteria in an international cohort to provide further insight.

MONITORING

Numerous tools are now available to monitor axSpA. As the disease affects deep anatomical structures, it is difficult to make a proper assessment by physical examination. Therefore, most of the tools used in axSpA are based on laboratory or imaging findings and patient-reported outcomes.[81 82] Multiple patient-reported outcomes have been developed and validated to determine the disease status and impact. The use of one or the other depends on the disease domain to be explored and the setting (ie, clinical practice or research).[83]

To assess disease activity in clinical practice, the use of composite indices is preferred.[84] Currently, it is recommended to use the Ankylosing Spondylitis Disease Activity Score (ASDAS), which consists of four questions answered by the patient (axial pain, peripheral pain-inflammation, morning stiffness duration and global disease activity) and CRP value in mg/L (using 2 if below the threshold or <2 mg/L).[85–87] According to ASDAS, clinicians may classify disease activity as inactive (<1.3), low activity (<2.1), high activity (<3.5) and very high activity (>3.5).[86] In addition, clinically important improvement is considered if a decrease between two assessments of at least 1.1 is achieved, and major improvement if the decrease is 2.0. A flare of the disease is defined as an increase in the ASDAS ≥0.9 compared with the previous assessment.[88] Additionally, the Bath Ankylosing Spondylitis Disease Activity Index is also available as a valid alternative, preferably in combination with CRP. This is an older index composed of six questions that assesses the first three items of the ASDAS plus fatigue, enthesitis and severity of morning stiffness, ranging between 0 (no disease activity) and 10 (very high disease activity).[83] In the past, this index has been used extensively, however, the ASDAS has shown better psychometric properties and it is currently recommended as the preferred index in clinical practice.[89] For clinical trials, the instruments to assess disease activity recommended within the ASAS-Outcome Measures in Rheumatology (OMERACT) core set are usually employed.[76] Additionally, the ASAS clinical response criteria (ASAS20, ASAS40, ASAS5/6, ASAS partial remission) are commonly used in recent trials.[90] The ASAS-OMERACT core set already exists for at least two decades and is currently in an update process.[91]

It is common for axSpA to affect physical function and spinal mobility. To assess physical function, the Bath Ankylosing Spondylitis Functional Index is recommended, an index composed of 10 questions, with a total score between 0 (good physical function) and 10 (poor physical function).[92] To determine mobility impairment, usually the Bath Ankylosing Spondylitis Metrology Index is employed that encompasses several measurements of the axial skeleton.[82 93] Recently, the ASAS Health Index has been developed and validated to assess overall functioning and quality of life in patients with axSpA. This index, freely available in 15 languages, encloses 17 items addressing functional limitation in daily activities. A lower score indicates a better health status.[94]

The CRP and the ESR are the used laboratory parameters to monitor activity in axSpA. These parameters are raised in only 40% of patients with axSpA and therefore, per se, they are only useful in a minority of patients to monitor disease activity.[49] As mentioned, MRI can detect inflammatory signs in the SIJs and spine. However, their routine use in clinical practice to monitor axSpA is not recommended, as its additional value compared with more feasible tools remains to be elucidated.[71] For research studies, different scores have been developed to quantify inflammation in the SIJ and in the spine, which are frequently used to evaluate treatment response.[95–97]

If disease activity persists, it results in irreversible structural damage.[31 44] The recommended tool for evaluating damage is conventional radiography of the SIJ and spine, but there is no consensus on how to use it for monitoring in clinical practice.[71] For research, the modified Stoke Ankylosing Spondylitis Spinal Score is employed for most studies, with a total score ranging from 0 to 72.[3] First results of scoring low-dose CT

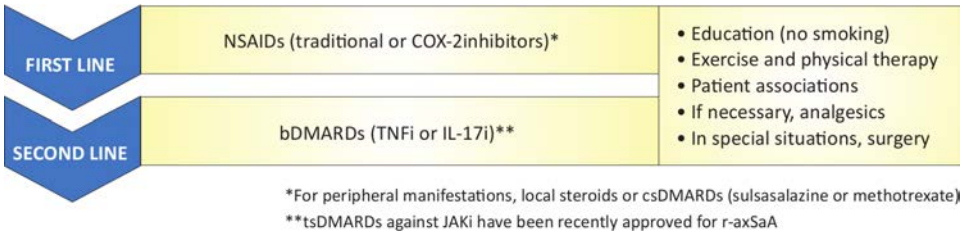

Figure 5 Recommendations to manage axial spondyloarthritis. bDMARDs, biolgical disease-modifying anti-rheumatic drugs; COX-2, cyclooxygenase-2; csDMARDs, conventional synthetic disease-modifying anti-rheumatic drugs; IL-17i, IL-17 inhibitors; JAKi, Janus kinase inhibitors; NSAIDs, non-steroidal anti-inflammatory drugs; r-axSpA, radiographic axial spondyloarthritis; TNFi, tumour necrosis factor inhibitors; tNSAIDs, traditional non-steroidal anti-inflammatory drugs; tsDMARDs, targeted synthetic disease-modifying anti-rheumatic drugs Adapted from van der Heijde *et al*.[101]

scans of the spine show a promising increase in sensitivity to change.

BURDEN OF THE DISEASE

Axial SpA usually begins in the third decade of life, which is a very active period in occupational, social and economic spheres.[98] Two thirds of active employed population with axSpA have work-related issues, leading to substantial direct and indirect costs to the society.[99] As a consequence, axSpA is associated with a high burden of the disease, which is comparable in patients with r-axSpA and nr-axSpA.[100] Older age, lower level of education, longer disease duration, higher disease activity (objective signs of inflammation), reduced physical functioning and employment in more physically demanding jobs are the major determinants of disease burden in axSpA.[22 29–31]

MANAGEMENT

The primary goal of treatment is to maximise health-related quality of life through control of symptoms and inflammation, prevention of progressive structural damage and preservation/normalisation of function and social participation.[101 102] Treatment should be guided through tight control according to a specific target, usually aiming at achieving sustained remission and, if not possible, low disease activity, employing the ASDAS as preferred instrument.[89] Importantly, the treatment in axSpA should be based on shared decisions between patients and rheumatologists and includes non-pharmacological and pharmacological therapies (figure 5).

Non-pharmacological therapies such as physical exercise and physiotherapy are recommended throughout the disease course.[101] Recent evidence has evaluated the paradoxical effect of exercise in axSpA.[103] On one hand, physical exercise is the cornerstone of treatment as it reduces disease activity and improves spinal function and quality of life. On the other hand, mechanical stress could contribute to inflammation and new bone formation at the entheseal and articular sites.[21] Further studies should address this possible paradox, but for the moment it is advisable to encourage patients to regular exercise.

Different types of pharmacological treatment are available for treating axial manifestations of axSpA ((figure 6)).

The first line are NSAIDs. Both traditional NSAIDs and cyclooxygenase-2 (COX-2)-selective inhibitors in full dose are efficacious in reducing the symptoms and signs of the disease.[104] However, drug pharmacokinetics, concomitant manifestations, comorbidities, pregnancy and potential adverse effects must be taken into account.[105 106] In patients with concomitant IBD in remission, the use of COX-2 inhibitors for a maximum period of 2 weeks may be preferred over traditional NSAIDs.[107] Usually, clinical response to full-dose NSAID is observed within 2 weeks. In case of insufficient response after this period, a second NSAID is recommended. To date, there is insufficient evidence to conclude whether switching between traditional NSAIDs and COX-2 inhibitors is more effective than treatment with a second NSAID of the same class.[108] A recent study suggested that switching NSAID classes may be more effective but further studies are needed to confirm these data.[109]

Biological disease-modifying antirheumatic drugs (bDMARD) are indicated as second-line treatment for axial manifestations. Currently, there are two classes of bDMARDs available: TNFi and IL-17i. bDMARDs are indicated if the target is not achieved after 4 weeks receiving at least two different NSAIDs.[110] In addition, patients must have at least one of the following three characteristics to be eligible: an elevated CRP value, inflammation on MRI or radiographic evidence of sacroiliitis.[101] TNFi for axSpA are classified into fusion protein (etanercept[111 112]) and monoclonal antibodies (adalimumab,[113 114] certolizumab pegol,[115] golimumab[116 117] and infliximab[118]). Among the IL-17i, secukinumab[119 120] and ixekizumab[121–123] are available. All bDMARDs except infliximab (intravenous) are for subcutaneous

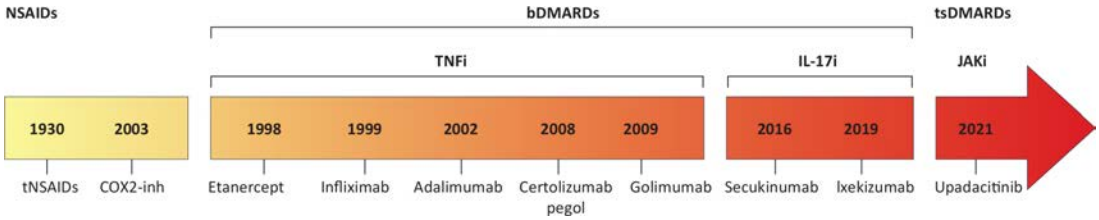

Figure 6 Evolution of pharmacological drugs available to treat axial spondyloarthritis. The figure shows the date (year) for first time approval of a new type of drug by regulatory agencies (European Medicines Agency or Food and Drug Administration) for radiographic axial spondyloarthritis. bDMARDs, biolgical disease-modifying anti-rheumatic drugs; COX2-inh: cyclooxygenase-2 inhibitor; IL-17i, IL-17 inhibitors; JAKi, Janus kinase inhibitors; NSAIDs, non-steroidal anti-inflammatory drugs; TNFi, tumour necrosis factor inhibitors; tNSAIDs, traditional non-steroidal anti-inflammatory drugs; tsDMARDs: targeted synthetic disease-modifying anti-rheumatic drugs.

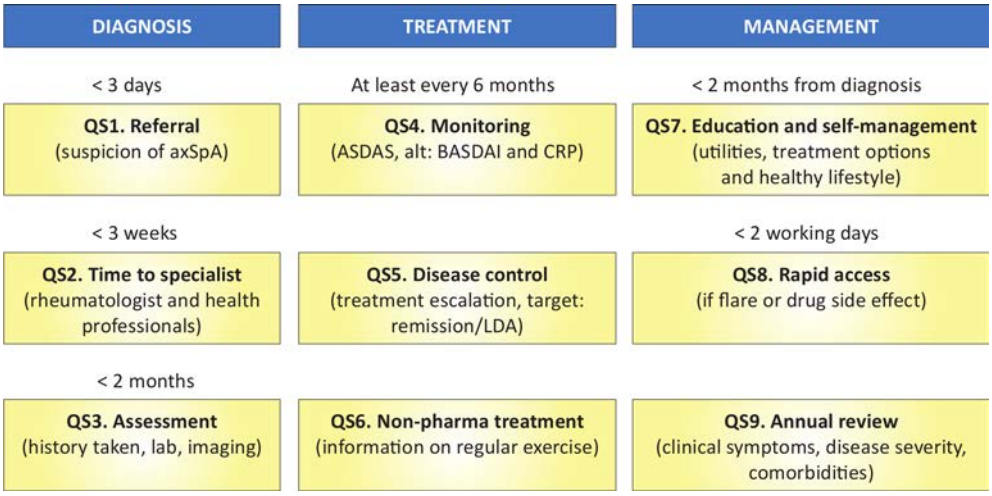

Figure 7 ASAS quality standards to improve the quality of health and care services for patients with axial spondyloarthritis. Adapted from Kiltz *et al*.[147] ASAS, Assessment in SpondyloArthritis international Society; axSpA, axial spondyloarthritis; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; CRP, C reactive protein; LDL, low disease activity.

administration. Overall, their efficacy in r-axSpA and nr-axSpA is comparable. All above-mentioned bDMARDs are approved by the European Medicines Agency (EMA) and Food and Drug Administration (FDA) for r-axSpA. For nr-axSpA, certolizumab is the only TNFi approved by FDA and EMA, while adalimumab, etanercept and golimumab are only approved by the EMA; infliximab is not approved by any regulator due to lack of data. Secukinumab and ixekizumab are approved for nr-axSpA by both agencies. Another IL-17i under investigation is bimekizumab.[124] Both TNFi and IL-17i relief symptoms and signs of the disease, with a good safety profile, but no evidence of superiority of one over the other is available. However, given the greater experience with TNFi, current practice is to start with a TNFi. Furthermore, in case of concomitant uveitis or IBD, a monoclonal antibody TNFi is recommended.[101]

Other bDMARDs are not effective in treating patients with axSpA. These treatments include abatacept,[125 126] IL-6 inhibitors[127 128] and IL-12/IL-23 inhibitors.[129] In patients with previous TNFi exposure, rituximab does not seem to be effective either. Similarly, there is also no evidence that conventional synthetic DMARDs such as methotrexate, sulfasalazine, leflunomide or hydroxychloroquine are effective for improving axial manifestations and therefore their use is not indicated in patients with purely axial disease.[108] Sulfasalazine may be considered in patients with peripheral arthritis. Local injection of glucocorticoids in peripheral (or more rarely in SIJ) may be considered too but the use of long-term treatment with glucocorticoids is not recommended for axSpA.

Recently, targeted synthetic DMARDs (tsDMARDs) against Janus kinase inhibitor (JAKi) have shown to improve axial manifestations in patients with r-axSpA. An advantage of these new therapies is that they are orally administrated. Upadacitinib has been recently approved as the first JAKi for patients with r-axSpA but no data on nr-axSpA are available yet.[130] Other tsDMARDs including tofacitinib[131] and filgotinib[132] have shown to be efficacious in phase II studies. Further approval for new JAKi and for nr-axSpA are expected.

Approximately 60% to 65% of patients achieve clinical response after a first bDMARD.[133] Some characteristics (male sex, no smoking, shorter disease duration, elevated CRP and inflammatory lesions on MRI) are associated with a better response (evidence available only for TNFi).[37] It is therefore advisable to encourage patients to stop smoking.[101] If the clinical response is sustained over time, tapering bDMARD (evidence available only for TNFi) can be considered to minimise side effects and costs. Tapering may be successful but stopping usually result in flares in a large proportion of patients.[134] The main factor determining the success of tapering is longer time in remission or low disease activity prior to dose reduction. However, discontinuation of bDMARDs is not recommended, as this leads to disease flare in most patients.[135] Nevertheless, if for any reason, such as surgery or pregnancy, discontinuation is temporarily required, evidence supports that the likelihood of achieving a similar response after restarting is very high.[136]

In case the first bDMARD fails, it is recommended to switch to a second bDMARD, either TNFi or IL-17i. No strategy (switching target or cycling) is preferred but so far, most studies included patients who switched either from a first TNFi to a second/third TNFi or from a TNFi to an IL-17i.[119 122 137] Further evidence is required to determine which is the best strategy. In addition, the place of JAKi in the management algorithm needs to be defined.

The effect of different therapies on structural damage progression (assessed by spinal radiographs) in axSpA is controversial. Initial studies showed that continuous administration of NSAIDs could slow the progression of structural damage, especially in patients with syndesmophytes and elevated CRP.[138 139] However, another trial has not confirmed these data.[140] With TNFi, the opposite was true: pivotal studies did not show inhibition of structural damage while later studies suggest they might have a protective effect, mainly after long-term treatment by controlling disease activity.[141–143] But this remains an open question.[144] Finally, recent trials with TNFi and IL-17i have shown that only a minority of patients progress in the short term.[119 122] However, to date, there is no head-to-head study to compare the results between the different drugs. Future studies are expected to clarify the effect of the different therapies on the progression of structural damage. But in the meantime, causal inference analysis using observational data may contribute to a better understanding of whether disease modification is possible in axSpA.[145]

Finally, when managing patients with axSpA, it is relevant to consider the ASAS Quality Standards.[146] These comprehend an initial and thereafter annual review of all aspects of the disease.

This includes assessment of the patients in terms of current disease management, and any further support they may need in the future, in order to maximise health, participation in society and life satisfaction. Focus should not only be on clinical symptoms and severity of disease but also on comorbidities such as cardiovascular risk management or osteoporosis, employment, psychological factors and lifestyle including physical activity. Ideally, this review is performed by a multidisciplinary team under the supervision of a rheumatologist (figure 7).

RESEARCH AGENDA

In recent years, enormous advances in the understanding and management of axSpA occurred but still relevant unmet needs are to be resolved.[147] In the future, further efforts should be made in identifying the disease at an early stage. This starts with increasing the awareness of primary care physicians and other specialists treating patients with CBP. The optimisation of the use of imaging and other biomarkers for early diagnosis is likely to also play a role. A better understanding of the overlap and differences between axSpA and other phenotypes of SpA such as pSpA and psoriatic arthritis should be clarified.

Efforts to further improve the standardisation of instruments to monitor the disease and treatment response are required. In clinical practice, the implementation of more recent developed instruments such as the ASDAS is needed. In addition, the employment of mobile devices to manage the disease should be explored. For research, the updated ASAS-OMERACT core set will consider the new advances.

The development of new drugs against known and new targets is also required to successfully treat those patients who fail to current available drugs. In this sense, the management recommendations should be updated in order to incorporate new drugs, especially JAKi.

SEARCH STRATEGY AND SELECTION CRITERIA

Data for this review were identified by searches of MEDLINE, PubMed and references from relevant articles using the search terms “spondyloarthritis” or “ankylosing spondylitis”, and “pathogenesis” or “diagnosis” or “classification” or “treatment” or “management” or “burden” or “work”. Articles published in English until February 2021 were included. We largely selected publications in the past 4 years, but did not exclude commonly referenced and highly regarded older publications. We also searched the reference lists of articles identified by the search strategy and selected those we judged relevant.

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CLINICAL SCIENCE

Short-term dose and duration-dependent glucocorticoid risk for cardiovascular events in glucocorticoid-naive patients with rheumatoid arthritis

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ABSTRACT

Objectives Rheumatoid arthritis (RA), along with glucocorticoid use, is associated with cardiovascular disease. Cardiovascular safety of glucocorticoids in RA is controversial and may be related to dose and duration of use. We determined if initiating glucocorticoids in steroid-naive RA patients would increase cardiovascular event (CVE) risk in a dose and duration-dependent manner over short-term intervals.

Methods Patients enrolled in CorEvitas (formerly Corrona) RA registry. Cox proportional-hazards models estimated adjusted HRs (aHR) for incident CVE in patients who initiated glucocorticoid treatment, adjusting for RA duration, traditional cardiovascular risk factors and time-varying covariates: Clinical Disease activity Index, disease-modifying antirheumatic drugs use and prednisone-equivalent use. Glucocorticoid use assessed current daily dose, cumulative dose and duration of use over rolling intervals of preceding 6 months and 1 year.

Results 19 902 patients met criteria. 1106 CVE occurred (1.66/100 person-years). Increased aHR occurred at current doses of ≥5–9 mg 1.56 (1.18–2.06) and ≥10 mg 1.91 (1.31–2.79), without increased risk at 0–4 mg 1.04 (0.55–1.59). Cumulative dose over preceding 6 months showed increased aHR at 751–1100 mg 1.43 (1.04–1.98) and >1100 mg 2.05 (1.42–2.94), without increased risk at lower doses; duration of use over preceding 6 months exhibited increased aHR for >81 days of use 1.54 (1.08–2.32), without increased risk at shorter durations. One-year analyses were consistent.

Conclusions Over preceding 6-month and 1-year intervals, initiating glucocorticoids in steroid-naïve RA patients is associated with increased risk of CVE at daily doses ≥5 mg and increased cumulative dose and duration of use. No association with risk for CVE was found with daily prednisone of ≤4 mg or shorter cumulative doses and durations.

Key message

What is already known about this subject?

- Both rheumatoid arthritis (RA) and glucocorticoid use increase risk for cardiovascular events. There is controversy regarding the cardiovascular safety and risk of glucocorticoid use in RA patients. Effects of short-term and low-dose use are not well understood.

What does this study add?

- In a large, real-word clinical registry of patients with long-standing disease, there is a daily dose, cumulative dose and duration of use glucocorticoid threshold for cardiovascular event risk in RA when analysed over short-term intervals of 6 months and 1 year.
- Relative cardiovascular safety was found with <5 mg of prednisone-equivalent daily dose and lower cumulative doses and durations of use.

How might this impact on clinical practice or future developments?

- Physicians should be aware of that low-dose and short-term use of glucocorticoids may increase risk of cardiovascular events when prescribing for treatment of RA in a treat-to-target approach.
- Patient education of this risk threshold is essential.

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterised by inflammatory destructive arthritis. Risk for cardiovascular disease (CVD) in RA is increased due to high prevalence of traditional risk factors, accelerated atherosclerosis and chronic inflammation.[1] Disease activity is directly related to cardiovascular risk.[2] Glucocorticoids (GCs) are commonly prescribed as initial, so-called bridge, treatment for RA but are often employed for intervals that extend beyond the onset of action of other conventional, targeted synthetic or biological disease-modifying antirheumatic drugs (cs/ts/bDMARDs).[3] It is often clinically challenging to taper GC. However, GCs are associated with CVD and may potentiate hypertension, hyperlipidaemia, diabetes mellitus, congestive heart failure and obesity.[4–6] Given that CVD is the major comorbidity of RA,[1 2] the juxtaposition of these circumstances presents a therapeutic dilemma.

Controversy exists regarding the risks and benefits of GC in RA patients. Previous small studies demonstrated an increased number of adverse events in RA patients over longer intervals with a daily prednisone-equivalent doses of >5 to 10 mg.[7–10] However, debate remains regarding the detrimental

cardiovascular effects of GC therapy in RA patients.[11–16] Relative cardiovascular safety is generally assumed with lower dose and shorter durations of use, especially over short-term intervals. However, little data has actually been reported regarding the temporal effects of short-term interval GC use preceding cardiovascular events (CVE).

The 2016 and 2019 European League Against Rheumatism and 2015 and 2021 American College of Rheumatology (ACR) recommend the use of 'low-dose' GC for 'the least amount of time' in combination with DMARDs for the treatment of RA.[3 17–19] Thus, it is important to determine the safety of initiating 'low-dose' GC in regard to the development of CVE. Furthermore, CVE in RA may be decreasing due to better control of disease activity following the widespread use of ts/bDMARDs, perhaps making the determination of the contribution of GC to CVE even more challenging in the present era.[20]

We, therefore, examined the CorEvitas (formerly Corrona) RA registry, a longitudinal database of RA patients, to determine whether there was a relationship between CVE in RA and use of GC in the dose ranges and duration of use that are consistent with published guidelines and routine clinical practice. We sought to determine the relative safety or risk for incident CVE in steroid-naïve patients who initiate 'low-dose' GC over short-term intervals of use based on real-world clinical observation, while adjusting for traditional cardiovascular risk factors, RA duration and disease activity and cs/ts/bDMARD use. Given the ubiquitous use of GC in this and other inflammatory conditions in a variety of dose ranges, including what is considered 'low-dose,' it was both timely and appropriate to reexamine this association.

METHODS

Study Cohort Entry Criteria

The CorEvitas (formerly Corrona) RA registry was previously described.[21 22] Inclusion criteria included age ≥18 years old and receiving a diagnosis of RA by a rheumatologist.[23] Data were collected between 1 October 2001 and 31 March 2018. During this period, 48 535 patients enrolled. Exclusion criteria included: any history of current or past GC therapy at or prior to enrolment; absence of a follow-up visit; missing data for either gender, age or duration of RA; or patients that had >15 months between visits. Patients were treated per their rheumatologist without treatment assignment.

Data collection

Observational data were collected from both treating physicians and patients at registry enrolment and at regular intervals consistent with the frequency of scheduled visits occurring every 2–9 months (median 4.6 months, IQR 3.60–6.24). At enrolment, detailed medical history was obtained from patients and review of medical records to accurately document lifetime comorbidities and prior treatments and medication use, including GC use.

Measure of GC use

GC use after entry into the registry was documented as equivalent milligrams of prednisone. Multiple measures of GC use were assessed. Current daily dose was defined as the most recent recorded dose at the time of a CVE or the most recent recorded dose in the registry for patients without an event. Cumulative total dose was defined as the summation of prednisone-equivalent dosage updated at each visit in a continuous, rolling manner over the preceding 6 months or 1 year (see online appendix efigure 1). Duration of use was defined as the summation of the absolute number of days a patient was treated with GC in a rolling manner over the preceding 6 months or 1 year (see online appendix efigure 1). Interval ranges for daily and cumulative dose were chosen based on equivalent quartiles of patient-time. Quartiles of duration of use were also chosen to have intervals with equal numbers of patient-time in each.

Event definition and documentation

For this study, CVE were defined as cardiac death, myocardial infarction, stroke, hospitalisation for hypertension, coronary revascularisation procedures, ventricular arrhythmia, unstable angina, congestive heart failure, transient ischaemic attacks, deep vein thrombosis (DVT), peripheral arterial thromboembolic event, urgent peripheral arterial revascularisation, peripheral arterial ischaemia, pulmonary embolism (PE), acute coronary syndrome or 'other' event. 'Other' events included complex or overlapping events, other arrythmias or conduction abnormality, cardiomyopathy, unspecified coronary artery disease, or events the reporting physician felt more comfortable categorising as 'other' if there was potential overlap with category choices provided.

At follow-up visits, both physician and patient-derived clinical data were updated in detail, including medication and dose changes for GC and cs/ts/bDMARDs.[22] Incident comorbidities and targeted medical events, including CVE, were specifically ascertained and collected at each visit by the treating rheumatologist (see online appendix file 1). After the receipt of a report of CVE on the registry form, the site then completed a separate e-form with deidentified primary hospital or cardiologist records confirming and validating the event with description of specific drugs and dose used for treatment (see online appendix efigure 2).[24] These forms were reviewed to confirm and validate the event, and ensure that it had not been previously reported, with any duplicate events excluded. In addition, a physician could report an event between formal registry visits. Finally, CVE, in particular CV death, were also reported on registry exit form. Any event that was not confirmed and validated was excluded.

Data analysis

The registry enrolment visit date was the index date. Only the first CVE following enrolment was used. Missing data for covariates were carried forward from the prior visit. If missing GC dose at a visit occurred, the prior dose was carried forward.

Time to first CVE was modelled using Cox proportional-hazards regression models to estimate unadjusted and adjusted HR and 95% CIs. Our model computed cumulative dose or duration of use over the preceding 6 months or 1 year at every daily time point from the index date to the last time point for each patient. This last time point could be a CVE, last registry visit, or dropout from the registry, whichever occurred first. At each time point, the model compared the risk of a CVE in patients at each quartile of prednisone use (current dose, cumulative dose, duration of use) to the risk in patients with no use.

For the adjusted analysis, baseline covariables in the model included age, sex, race, duration of RA, history of CV disease, diabetes mellitus, hyperlipidaemia, hypertension, statin use, NSAID use, tobacco use, year of enrolment, baseline modified Health Assessment Questionnaire score (mHAQ) and the baseline Clinical Disease Activity Index (CDAI) for RA. The CDAI is a validated disease activity metric that includes tender and swollen joints (28 joint count), as well as physician and patient evaluation of global arthritis activity on a 10-point Visual Analogue Scale.[25] Additionally, time-varying covariates in the

model included measures of prednisone use as described above, NSAID use, cs, b, tsDMARDS and CDAI, which were updated at each follow-up visit.

A sensitivity analysis excluded all venous thromboembolisms (DVT and/or PE) as CVE to determine if excluding venous events impacted risk. A different sensitivity analysis excluded all patients with prior history of a CVE to assess whether this comorbidity had influenced risk. Another sensitivity analysis excluded 'other' CVE to assess its influence on the outcomes of interest.

Student's t-test or χ^2 test compared data at baseline. All analyses were generated using Stata V.16.1 (StataCorp).

RESULTS

Sample characteristics

A total of 19 902 patients (41%) met entry criteria. Exclusions occurred as follow: 21 162 patients had prior history of prednisone use; 5059 patients had no follow-up; 42 patients were missing information regarding use of prednisone; 1672 patients had time between visits of >15 months; 743 patients had missing information for covariates (age, gender, duration of RA, smoking status, CDAI and/or mHAQ).

For the 19 902 who met criteria, the follow-up included 66 436 patient-years and 127 674 follow-up visits over >16 years. Of these patients, 2500 (12.6%) initiated GC during the follow-up. Median time to first use in the registry was 19 months (IQR: 9.1–38.4) after enrolment.

Assessment of CVE risk with initiating GC use

A total of 1106 CVE occurred, yielding a rate of 1.66 CVE per 100 patient-years (95% CI 1.57 to 1.77). As depicted in table 1 of unadjusted enrolment characteristics prior to any CVE and follow-up interval, patients who developed CVE had a greater prevalence of traditional CV risk factors, more severe RA, and were more likely to use csDMARD. Table 2 displays the frequency of each CVE.

Table 3 displays unadjusted and adjusted HR for daily and cumulative dose and duration of GC use over the preceding 6 month and 1 year intervals. Online appendix table 1 shows the number of patients contributing time to each category. Unadjusted current daily dose of <5 mg was not associated with increased risk, while doses ≥5 mg increased risk in a dose-response manner. Figure 1 demonstrates the adjusted risk of CVE based on daily prednisone-equivalent dose with similar findings.

As shown in table 3, cumulative doses of >750 mg over the preceding 6 months were associated with increased unadjusted risk for developing a CVE. Figure 2 shows the risk for developing a CVE remained for cumulative doses of >750 mg after adjustment for covariates. In both unadjusted and adjusted analyses, cumulative doses ≤750 mg were not associated with increased risk. Also shown in table 3, cumulative doses of >1110 mg over the preceding 1 year were associated with significant increased unadjusted risk for developing a CVE. Figure 2 shows this risk remained for cumulative doses of >1100 mg after adjustment for covariates. In both unadjusted and adjusted analyses, cumulative doses ≤1100 mg over the preceding 1-year were not associated with increased risk.

As shown in table 3, GC use for >80 days over the preceding 6 months interval was associated with increased unadjusted risk for developing a CVE. Shorter use than 80 days was not associated with increased risk. Figure 3 illustrates similar risk when adjusted for covariates. Over the preceding 1-year interval, a similar, if less smooth, increased unadjusted (table 3) and adjusted (figure 2) risk for a CVE after 100 days of use was found.

Sensitivity analyses

Online appendix table 2 shows the results of the sensitivity analysis when DVT and PE were excluded. With exclusion of DVT/PE, 1007 CVE occurred. Results were similar to the primary analysis.

Online appendix table 3 shows the results of the sensitivity analysis when a history of prior CVE was excluded. With this exclusion, the total number of patients was 18 168, with 2300 initiating prednisone. There were 829 CVE in this analysis. Results were similar to the primary analysis.

Online appendix table 4 shows the results of the sensitivity analysis when 'other' CVE were excluded. With this exclusion, 817 events occurred. The results were similar to the primary analysis.

DISCUSSION

We report, for the first time, that the relative cardiovascular safety or risk of initiating GC in a real-world clinical sample of steroid-naïve RA patients with longstanding disease at registry enrolment is associated with a threshold daily dose, cumulative dose, and duration of use when analysed over short-term intervals of the preceding 6 months or 1 year. The risk for CVE increased directly with increasing current daily dose, with the greatest estimated risk at ≥5–9 mg and ≥10 mg of prednisone-equivalents. Similarly, the risk for CVE increased in a dose-response manner with increasing cumulative dose over these short-term intervals of analysis. The risk for CVE based on duration of use found increased risk after 80-days of use over the preceding 6 months and 100-days over the preceding 1 year in the dose ranges reported. There is 'noise' in the duration of use data, especially over the preceding 1 year, and it is possible that this is due to a threshold effect related to dose. That is, the duration of use analysis does not necessarily account for dose; thus, similar durations of use may have different total doses, especially with longer use. Of additional clinical importance, we found no increased risk for CVE with current prednisone-equivalent daily doses of <5 mg or cumulative doses of ≤750 mg over the preceding 6 months or ≤1100 mg over the preceding 1 year. We found no increased risk with duration of use ≤80 days over the preceding 6 months or ≤100 days over the preceding 1 year. It is critically important to note that these findings remained after adjustment for established cardiovascular risk factors, RA duration, disease activity and cs/ts/bDMARDs.

These novel insights can be immediately employed in clinical practice. The methodology of our analysis allows the application to patients based on their most recent 6 months or 1 year GC use. We believe that our data demonstrate that GC use should be tapered to a dose of <5 mg prednisone-equivalents as expeditiously as possible, while being aware of duration of use and cumulative dose. Thus, clinicians should provide counselling and education of these findings when encountering a reluctance on the part of a pain-free patient to taper GC, or succumb to the temptation to simply increase the dose to make the patient feel better until their next visit.

Both the IMPROVED and CareRA trials established that GCs can be tapered in patients with early RA in a protocolised, supervised, investigational regimen.[26 27] Verschueren *et al* also reported on a supervised step-down GC taper in 19 patients with early RA.[28] The observational data we report on in patients

Table 1 Unadjusted sample characteristics stratified by development of a cardiovascular event (CVE) measured at registry enrolment*

	No CVE (N=18 796)		CVE (N=1106)		P value
Variable	**Mean**	**SD**	**Mean**	**SD**	
Age (years)	57.82	13.36	65.44	11.07	<0.001
Duration of rheumatoid arthritis (years)	8.84	9.42	11.56	10.76	<0.001
mHAQ	0.32	0.42	0.38	0.47	<0.001
CDAI	12.73	12.50	14.04	12.84	0.001
28 Joint Count: Tender	3.93	5.81	4.44	6.39	0.005
28 Joint Count: Swollen	3.81	5.39	4.18	5.33	0.028
Patient Global Assessment (0–100 scale)	28.95	26.08	31.71	26.44	0.001
Physician Global Assessment (0–100 scale)	21.76	20.38	23.75	20.53	0.002
Body mass index (kg/m^2)	29.44	7.14	29.60	7.00	0.468
	N	%	N	%	
Gender					<0.001
Male	4134	22.0	369	33.4	
Female	14 662	78.0	737	66.6	
Race					0.202
Asian	286	1.5	12	1.1	
Black	1184	6.3	55	5	
Mixed race	223	1.2	14	1.3	
Native American	122	0.6	6	0.5	
Other	105	0.6	3	0.3	
Pacific Islander	21	0.1	2	0.2	
Unknown	132	0.7	3	0.3	
White	16 723	89.0	1011	91.4	
History of cardiovascular disease					<0.001
Yes	1457	7.8	277	25.0	
History of diabetes					<0.001
Yes	1502	8.0	176	15.9	
History of hyperlipidaemia					<0.001
Yes	4154	22.1	333	30.1	
History of hypertension					<0.001
Yes	5583	29.7	511	46.2	
Smoking status					0.001
Never	11 330	60.3	604	54.6	
Previous	4946	26.3	339	30.7	
Current	2520	13.4	163	14.7	
Exercise					<0.001
None	5879	31.3	390	35.3	
1–2 times/week	5313	28.3	277	25.0	
3–4 times/week	4054	21.6	213	19.3	
5–6 times/week	1376	7.3	68	6.1	
Daily	1748	9.3	141	12.7	
Not sure	426	2.3	17	1.5	
Statin use					<0.001
Yes	3750	20.0	303	27.4	
NSAID use					0.024
Yes	10 272	54.6	643	58.1	
Analgesic use					<0.001
Yes	8516	45.3	565	51.1	
Prior or current biologic/targeted DMARD use					0.815
Yes	8633	45.9	504	45.6	
Prior or current conventional DMARDs					<0.001
Yes	16 447	87.5	1019	92.1	
Prior or current methotrexate use					<0.001
Yes	14 027	74.6	893	80.7	

*Patients were enrolled with prevalent disease.
CDAI, Clinical Disease Activity Index; DMARDs, disease-modifying antirheumatic drugs; mHAQ, modified Health Assessment Questionnaire; NSAID, non-steroidal anti-inflammatory drug.

Table 2 Frequency of each cardiovascular event

Events	Frequency	Per cent
Acute coronary syndrome	3	0.27
Cardiac arrest	19	1.72
Congestive heart failure	106	9.58
Cardiovascular death	8	0.72
Deep vein thrombosis	83	7.5
Hospitalisation for hypertension	16	1.45
Myocardial infarction	117	10.58
Other cardiovascular event*	289	26.13
Peripheral arterial event	4	0.36
Peripheral arterial intervention	3	0.27
Pulmonary embolism	26	2.35
Coronary revascularisation	204	18.44
Stroke	136	12.3
Transient ischaemic attack	51	4.61
Unstable angina	16	1.45
Urgent peripheral arterial revascularisation	1	0.09
Ventricular arrhythmia	24	2.17
Total events	1106	100

*Other events included the following: complex or overlapping events (eg, acute coronary syndrome with coronary revascularisation), atrial fibrillation, other supraventricular arrhythmia, unspecified bradycardia, other unspecified conduction abnormality, new but stable angina, unspecified coronary artery disease, cardiac syncope or orthostatic hypotension, cardiomyopathy, other cardiac interventional procedure, abdominal aortic aneurysm, other peripheral arterial disease or cardiac event not otherwise specified.

with longstanding disease are perhaps more representative of a general population of RA patients on GC.

It should also be noted that we stratified our subject sample to see how subjects who developed CVE differed at the baseline time of registry enrolment from those who did not (table 1). Those who developed CVE were older, had more traditional CVD risk factors, greater disease activity, longer disease duration, and more commonly were on non-bDMARDs. Again,

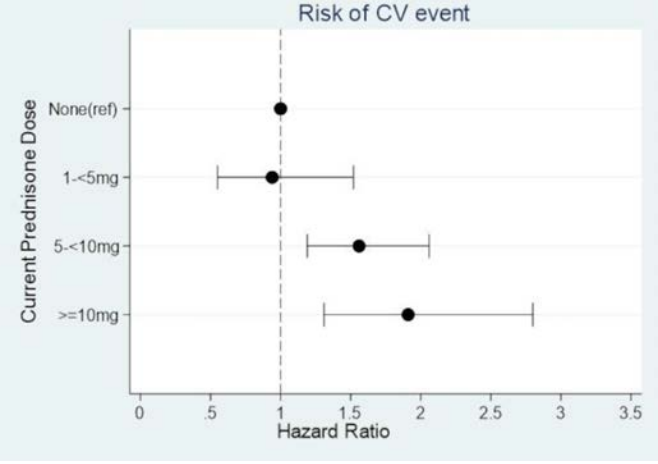

Figure 1 Adjusted risk of cardiovascular (CV) event associated with current daily prednisone-equivalent dose. There is a threshold for increased risk of an event. Prednisone-equivalent doses of 5–9 mg and ≥10 mg were associated with an increased risk. However, doses <5 mg were not associated with increased risk. The risk was adjusted for traditional CV risk factors, rheumatoid arthritis disease activity and duration, and cs/ts/bDMARD use. cs/ts/bDMARD, conventional, targeted synthetic or biological disease-modifying antirheumatic drugs.

Table 3 Unadjusted and adjusted HRs for cardiovascular (CV) event with initiating glucocorticoid use

Daily dose (mg)†	Unadjusted HR (95% CI)	Adjusted* HR (95% CI)
None	1 (ref)	1 (ref)
1-<5	1.04 (0.61 to 1.76)	0.94 (0.55 to 1.59)
≥5–9	1.78 (1.35 to 2.35)	1.56 (1.18 to 2.05)
≥10	2.09 (1.44 to 3.05)	1.91 (1.31 to 2.79)
Cumulative dose (mg)†	**Unadjusted HR (95% CI)**	**Adjusted* HR (95% CI)**
Over preceding 6 months:		
None	1 (ref)	1 (ref)
1–380	0.93 (0.56 to 1.50)	0.86 (0.53 to 1.40)
381–750	1.31 (0.88 to 1.95)	1.20 (0.81 to 1.79)
751–1100	1.62 (1.18 to 2.24)	1.43 (1.04 to 1.98)
>1110	2.25 (1.57 to 3.22)	2.05 (1.42 to 2.94)
Over preceding 1 year:		
None	1 (ref)	1 (ref)
1–500	0.99 (0.64 to 1.54)	0.93 (0.60 to 1.45)
501–1100	1.28 (0.89 to 1.83)	1.19 (0.83 to 1.70)
1101–2100	1.63 (1.18 to 2.25)	1.47 (1.06 to 2.03)
>2100	1.97 (1.41 to 2.74)	1.74 (1.25 to 2.43)
Duration of use (days)	**Unadjusted HR (95% CI)**	**Adjusted* HR (95% CI)**
Over preceding 6 months:		
None	1 (ref)	1 (ref)
1–80	0.77 (0.46 to 1.29)	0.72 (0.60 to 1.45)
81–160	1.66 (1.16 to 2.36)	1.54 (1.08 to 2.20)
161–181	1.71 (0.76 to 3.81)	1.56 (0.70 to 3.48)
>181	1.79 (1.38 to 2.35)	1.57 (1.20 to 2.05)
Over preceding 1 year:		
None	1 (ref)	1 (ref)
1–100	1.08 (0.72 to 1.62)	1.02 (0.68 to 1.53)
101–220	1.50 (1.10 to 2.05)	1.41 (1.03 to 1.93)
221–360	0.99 (0.60 to 1.62)	0.88 (0.54 to 1.44)
>360	2.15 (1.59 to 2.92)	1.88 (1.39 to 2.56)

*Adjusted for age, sex, race, duration of RA, history of CV disease, diabetes mellitus, hyperlipidaemia, hypertension, statin use, NSAID use, tobacco use, year of enrolment, baseline modified health assessment questionnaire score, CDAI and cs, b, tsDMARDS use.
†Prednisone-equivalents.
CDAI, Clinical Disease Activity Index; cs/ts/bDMARDs, conventional, targeted synthetic or biological disease-modifying antirheumatic drugs; NSAID, non-steroidal anti-inflammatory drug; RA, rheumatoid arthritis.

adjustment for these factors found an independent association with GC use.

The methodology of our investigation expands on the prior literature. Both Saag *et al* and Davis *et al* studied adverse events, including CVE, in RA patients receiving GC.[7 10] However, there are numerous differences in methodology of the present investigation including the historical therapeutic interval and duration of observation,[10] robustness of numbers, as well as specific focus on CVE. Our adjustment for multiple confounding variables further distinguishes our approach from prior studies that did not adjust for all these variables.[8 9 29–32] Our findings provide context beyond these prior studies by highlighting the relative cardiovascular safety of doses of GCs ≤4 mg daily over the 6-month interval described.[18 33] Our findings also add evidence to the EULAR and ACR task force recommendations for steroid taper.[19 34] Huscher *et al* also reported a threshold for GC side effects in RA patients without examining CVE.[35]

Others have looked at the effects of short-term GC use. George *et al* reported on the effect of dosages on serious infectious events (SIEs) using a Medicare claims database.[36] They found a

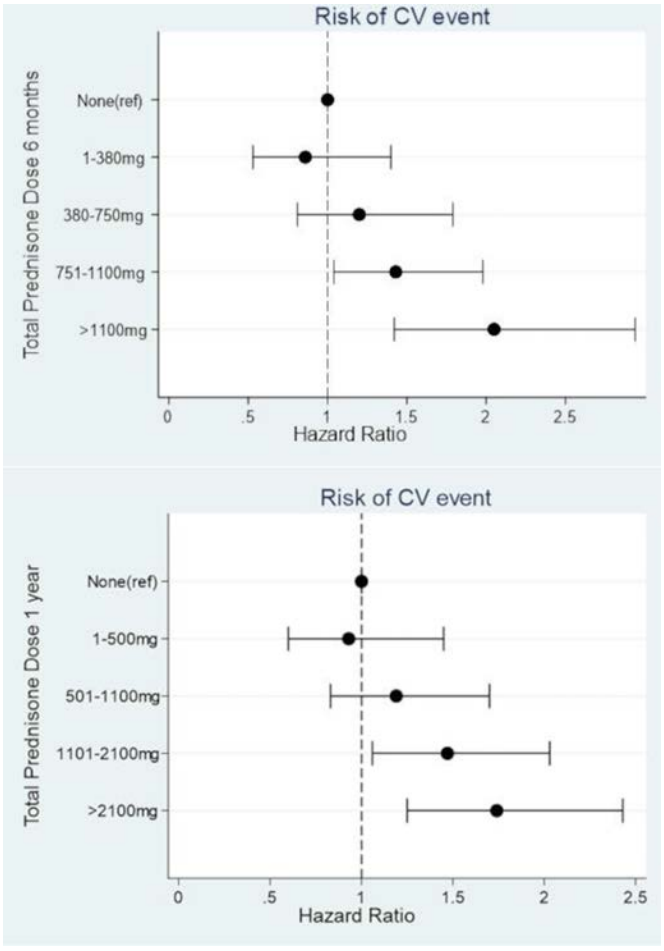

Figure 2 Top: adjusted risk of cardiovascular (CV) events associated with total glucocorticoid use over preceding 6 month interval. Bottom: adjusted risk of CV event associated with total glucocorticoid use over preceding 1-year interval. There was a dose-response increase in risk for CV event. Over the preceding 6 months of use, cumulative prednisone-equivalent doses of 751–1100 mg and >1100 mg were associated with increased risk for a CV event. Over the preceding 1 year of use, cumulative doses of 1101–2011 mg and >2100 mg were associated with increased risk for a CV event. This risk was adjusted for traditional CV risk factors, rheumatoid arthritis disease activity and duration, and cs/ ts/bDMARD use. cs/ts/bDMARD, conventional, targeted synthetic or biological disease-modifying antirheumatic drugs.

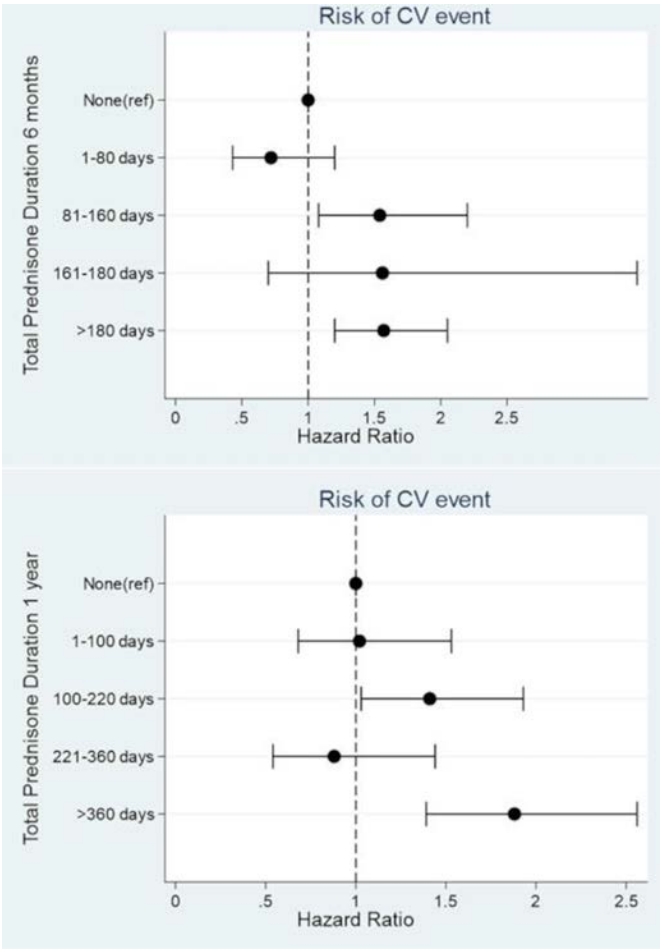

Figure 3 Top: adjusted risk of cardiovascular (CV) event associated with total duration of use of glucocorticoids over preceding 6-month interval. Bottom: adjusted risk of CV event associated with total duration of use of glucocorticoids over preceding 1-year interval. There was a duration of use threshold for increased risk. Over the preceding 6 months, prednisone-equivalent use for ≥81 days was associated with an increased risk for a CV event. Over the preceding 1 year, use for 101–220, and >360 days was associated with an increased risk for a CV event, with trend towards increased risk between 221 and 260 days. The risk was adjusted for traditional cardiovascular risk factors, rheumatoid arthritis disease activity and duration, and cs/ts/bDMARD use. cs/ts/bDMARD, conventional, targeted synthetic or biological disease-modifying antirheumatic drugs.

robust relationship of SIEs with increasing doses, although they were not able to determine the effect of actual disease activity. Similarly, Yao *et al* used a national insurance database to assess the effects of short 'burst' courses (≤14-days) of prednisone on incident adverse events of gastrointestinal (GI) bleeding, sepsis, and heart failure at 5–30 days and 31–90 days from use, finding a higher incidence rate of these events at both time points in the general population who used prednisone.[37] Together, the findings of these studies of predominantly non-cardiac adverse events support our conclusion that detrimental effects of GC are strongly associated with short-term intervals preceding the event.

Our study has several additional strengths. Our investigation spanned a 16-year period, while prior studies were based much shorter duration of observation.[8 9 29–32 38–40] The data were derived from over 700 participating sites of real-world clinical observation in the USA The protocol independently confirmed and validated events with hospital records. We then analysed reported events, not limiting to MACE, increasing the real-world clinical relevance for practising physicians. We excluded prevalent and past GC users, in an effort to minimise the effect of past use. Sensitivity analyses demonstrated that having a history of prior CVE, venous-related events, or the 'other' category of CVE did not influence the results, a particularly important design feature of our analysis.[41 42]

Our study is not without limitations. Patients in observational registries are treated without assignment of interventions. Although we adjusted for multiple confounding factors, there is still a risk of channelling bias or residual confounding. While GC dose and usage were updated in the registry at each visit, there is potential for patient reporting to be limited by recall bias. However, the percentage of patients we studied without prior GC use at the time of registry enrolment (41%) is similar to that reported in the ARAMIS registry, supporting our robust data collection methods.[43] It is not clear whether our findings are

applicable to early RA patients treated with GC since our cohort contained predominantly RA patients with longer disease duration. A similar observational analysis of the success of GC taper in early RA patients not participating in a supervised protocol would be of interest.

A possible limitation of this report is that we were not able to adjust our findings for time-varying changes in C reactive protein, which is a known risk factor for CVE, or for either rheumatoid factor or anticitrullinated protein antibody status as laboratory values are not mandated in this observational registry. However, we adjusted for other traditional cardiovascular risk factors, as well as time-varying changes in actual RA disease activity measured at the time of each registry visit. As is the case with long-term observational data, the cohort of patients at different time points may differ as patients enter and exit the registry. The risk was established with a very large number of patients over very long observational intervals that greatly increased our statistical power. Nevertheless, assignment of risk to a specific individual may not be appropriate. In addition, with any statistical association, we cannot determine causality with absolute certainty. While a prospective randomised controlled trial of GC dosing would be ideal, it is highly unlikely that this kind of trial will ever be conducted given the pragmatic challenges with the number of patients required, study duration, funding, ethics, and other challenges associated with the complete absence of steroid use in a control group.

In conclusion, we reported that daily doses of ≥5 mg of prednisone-equivalents, elevated cumulative dose and extended duration of use of GC over the preceding 6-month and 1-year intervals are associated with an increased risk for incident CVE in steroid-naïve patients with RA. We also emphasise the relative absence of CVE with dosing of ≤4 mg per day, lower cumulative dose and a duration of use of only 6 months prior to an event. Physicians treating patients with RA should consider these threshold ranges of GC use when prescribing prednisone as a part of a treat-to-target regimen.

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CLINICAL SCIENCE

Lifetime risk of rheumatoid arthritis-associated interstitial lung disease in *MUC5B* mutation carriers

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ABSTRACT

Objectives To estimate lifetime risk of developing rheumatoid arthritis-associated interstitial lung disease (RA-ILD) with respect to the strongest known risk factor for pulmonary fibrosis, a *MUC5B* promoter variant.

Methods FinnGen is a collection of epidemiological cohorts and hospital biobank samples, integrating genetic data with up to 50 years of follow-up within nationwide registries in Finland. Patients with RA and ILD were identified from the Finnish national hospital discharge, medication reimbursement and cause-of-death registries. We estimated lifetime risks of ILD by age 80 with respect to the common variant rs35705950, a *MUC5B* promoter variant.

Results Out of 293 972 individuals, 1965 (0.7%) developed ILD by age 80. Among all individuals in the dataset, *MUC5B* increased the risk of ILD with a HR of 2.44 (95% CI: 2.22 to 2.68). Out of 6869 patients diagnosed with RA, 247 (3.6%) developed ILD. In patients with RA, *MUC5B* was a strong risk factor of ILD with a HR similar to the full dataset (HR: 2.27, 95% CI: 1.75 to 2.95). In patients with RA, lifetime risks of ILD were 16.8% (95% CI: 13.1% to 20.2%) for *MUC5B* carriers and 6.1% (95% CI: 5.0% to 7.2%) for *MUC5B* non-carriers. The difference between risks started to emerge at age 65, with a higher risk among men.

Conclusion Our findings provide estimates of lifetime risk of RA-ILD based on *MUC5B* mutation carrier status, demonstrating the potential of genomics for risk stratification of RA-ILD.

Key messages

What is already known about this subject?

► Interstitial lung disease (ILD) is one of the most common extra-articular complications of rheumatoid arthritis (RA). The *MUC5B* promoter variant rs35705950 is an important genetic risk factor for ILD, and case–control studies have identified it to be a risk factor also for RA-ILD.

What does this study add?

► By integrating large-scale genotype data with clinical data from nationwide healthcare registries, we show that in patients with RA, *MUC5B* variation is strongly associated with a lifetime risk of RA-ILD.

How might this impact on clinical practice or future developments?

► This study highlights the importance of genetic predisposition on the development of RA-ILD. Further studies are needed to investigate the impact of *MUC5B* on outcomes of RA-ILD.

INTRODUCTION

Interstitial lung disease (ILD) is one of the most common extra-articular manifestations of rheumatoid arthritis (RA).[1] The cumulative risk of developing clinical ILD during the RA disease course has varied in different studies, ranging from 5.0% to 7.7% in long-term follow-up studies of RA cohorts[1–3] to up to 10% in a study using death records.[4] Even higher estimates for subclinical radiographic findings consistent with ILD have been observed in patients with RA, ranging from 19% to 33%.[5–7] Although the RA-ILD course can vary, the disease is associated with significantly increased mortality compared with patients with RA without ILD.[3 4 8]

Clinical risk factors for RA-ILD include older age, male gender, tobacco smoking, high levels of anticitrullinated protein antibodies and disease activity.[2 9] The strongest known genetic risk factor for idiopathic pulmonary fibrosis (IPF) is the common variant rs35705950, a promoter variant near the *MUC5B* gene.[10] A recent case–control study has demonstrated that the *MUC5B* promoter variation is associated with an increased risk of ILD among patients with RA.[11] The aim of this study was to evaluate the lifetime risk of ILD in patients with RA, comparing the risk to the population, and estimate how the *MUC5B* promoter variant modifies these risks in the real-world setting.

METHODS

FinnGen is a collection of prospective epidemiological and disease-based cohorts, and hospital biobank samples. The unique personal identification number links the genotypes to multiple nationwide registries, and cases were identified through the national hospital discharge registry (starting from 1968) including both inpatient and outpatient data, the national death registry (1969–) and the medication reimbursement registry (1964–).

RA was defined as patients having medication reimbursement for inflammatory rheumatic diseases (code 202), with an additional requirement of two contacts with the International Classification of Diseases, Tenth Revision (ICD-10) codes

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beginning with M05 (seropositive RA) or M06 (seronegative RA). In our recent validation study of RA diagnoses in Finnish biobank patients (unpublished), this combination resulted in a positive predictive value of 0.87 compared with chart review. Negative predictive value for any RA diagnosis was 1.0. Those without RA who had other inflammatory rheumatic diseases or inflammatory bowel disease were excluded.

ILD cases were identified with J84, M05.1/J99.0 (ICD-10), 515, 516 (ICD-9) or 484.99 or 517.01 (ICD-8) with following criteria: (1) the first and only record in the death registry or (2) after the initial diagnosis, a second contact (or death due to ILD) was required within 5 years, that is, we excluded individuals with no further healthcare contacts with ILD within 5 years. No exclusions were made based on temporality of RA and ILD. For both RA and ILD, age at onset was defined as age at first registered diagnosis.

For *MUC5B* (mucin 5B, oligomeric mucus/gel-forming), we studied carriers of the minor allele for the promoter variant rs35705950 (G>T) with minor allele frequency 0.1 (no enrichment compared with non-Finnish Europeans[12]) and mean INFO 0.948 indicating high imputation quality. Individuals homozygous for the variant were analysed jointly with the heterozygotes.

Start of follow-up was set at birth, with follow-up ending at the first record of the endpoint of interest, death, or at the end of follow-up on 31 December 2019, whichever came first. Using the Cox proportional hazards model, we estimated adjusted HRs and 95% CIs (CI). With age as time scale, all regression models were stratified by sex, adjusted for 10 principal components of ancestry, FinnGen genotyping array and cohort. We report cumulative incidences with 95% CIs by age 80. We used R V.3.6.3. Detailed information on genotyping, disease definitions and analyses are provided in online supplemental methods.

Patient and public involvement

This study was carried out without direct patient and public involvement.

RESULTS

Among 293 972 individuals (mean age at the end of follow-up: 59.8, SD: 17.3, 56.4% women), we identified 1965 patients (1172 men, 793 women) diagnosed with ILD by end of follow-up. Out of 6869 patients with RA (mean age at onset: 49.4, SD: 14.9, 71.1% women), 247 (3.6%) had been diagnosed with ILD. Out of these 247 individuals, 20 (8.1%) had been diagnosed with ILD >1 year before the earliest record of RA, 36 (14.6%) within a year prior to or after the earliest record of RA and 191 (77.3%) >1 year after. Out of patients without RA, 19.3% were *MUC5B* carriers, and out of patients with RA, 20.9%. Among all individuals in the dataset, the *MUC5B* promoter variant rs35705950 was associated with ILD with a HR of 2.44 (2.22–2.68, $p=3.87\times10^{-77}$), and among patients with RA, with a HR of 2.27 (1.75–2.95, $p=8.15\times10^{-10}$). In a formal test for interaction by introducing an interaction term in the regression model, we found no evidence of an interaction between *MUC5B* and RA (p=0.16). These interaction tests indicate that the effect of *MUC5B* is similar in the population and in patients with RA.

Next, we quantified the lifetime risk of ILD for four groups: (1) *MUC5B* non-carriers in the population, (2) *MUC5B* carriers in the population, (3) *MUC5B* non-carriers with RA and (4) *MUC5B* carriers with RA (figure 1, table 1). The corresponding lifetime risks were (1) 1.5% (95% CI: 1.3% to 1.6%), (2) 4.4% (95% CI: 4.1%–4.8%), 3) 6.1% (95% CI: 5.0%–7.2%) and (4) 16.8% (95% CI: 13.1%–20.2%). In sex-specific analyses, the lifetime risk was 20.9% (95% CI: 14.1%–27.1%) in men with RA who are *MUC5B* carriers, and the corresponding lifetime risk in women was 14.5% (95% CI: 10.2%–18.6%). Accounting for competing risks (non-ILD causes of death) yielded marginally lower estimates of lifetime risks, particularly in men (online supplemental table 1).

Lastly, we observed an association between *MUC5B* and risk of RA (HR: 1.10, 1.04–1.17, p=0.0009), with a somewhat larger association in men (HR: 1.17, 1.05–1.30, p=0.005) than in women (HR: 1.08, 1.01–1.16, p=0.04). The effects remained similar when excluding all 1172 men with ILD (HR: 1.13 in men, 1.01–1.26, p=0.03) and all 793 women with ILD (HR: 1.05 in women, 0.98–1.13, p=0.19). This observation was replicated in UK Biobank (1911 RA cases; see online supplemental methods for details) with a HR of 1.15 (1.03–1.28, p=0.01). Meta-analysing the effects from FinnGen and UK Biobank, the HR was 1.11 ($p=4.07\times10^{-5}$).

DISCUSSION

In this large observational cohort study, we demonstrate that a combination of RA and *MUC5B* variation confers a 10-fold elevated risk of ILD compared with the population. Every sixth

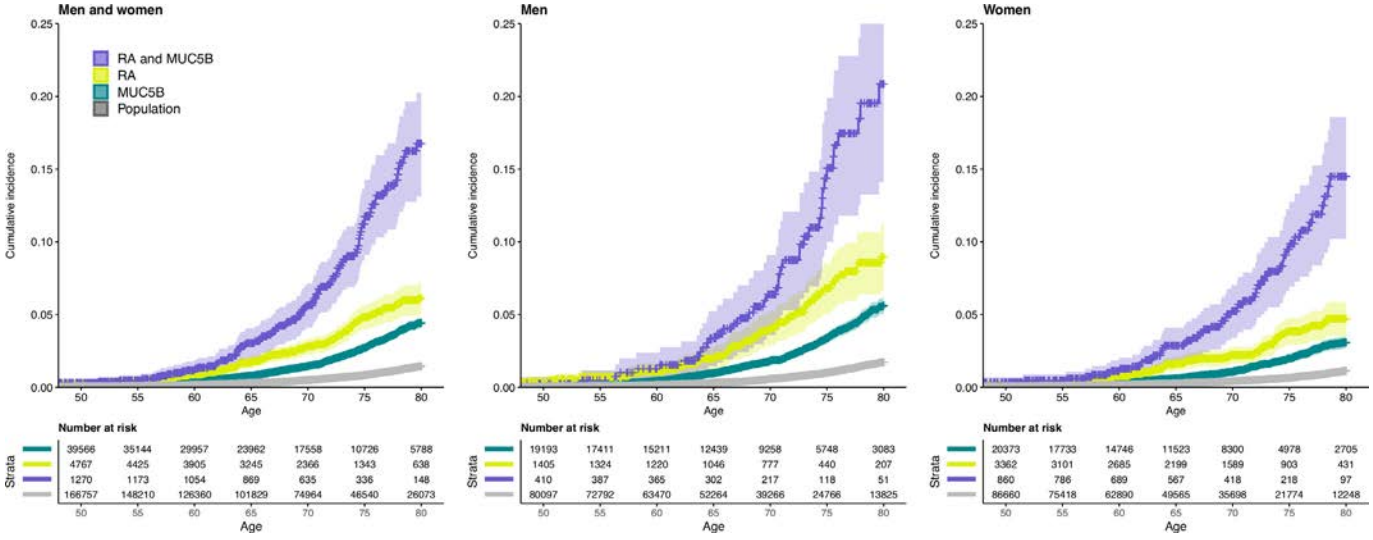

Figure 1 Lifetime risk of interstitial lung disease in the population for *MUC5B* carriers and non-carriers with respect to diagnosis of RA. The risks are shown for men and women both combined and individually. *MUC5B*=carriers of the minor allele for the promoter variant rs35705950. Sample size: 293 972 (128 233 men and 165 739 women). RA, rheumatoid arthritis.

Table 1 Data characteristics, and effect of RA and *MUC5B* on risk of ILD

	Individuals without RA		Individuals with RA	
	Non-carriers of *MUC5B* promoter variant	Carriers of *MUC5B* promoter variant	Non-carriers of *MUC5B* promoter variant	Carriers of *MUC5B* promoter variant
N	231 860	55 243	5431	1438
ILD cases	1007	711	151	96
ILD cases in men/women	600/407	461/250	70/81	41/55
Age at ILD onset, men/women (mean (SD))	66.9 (10.4)/63.0 (13.3)	67.9 (8.3)/65.3 (11.1)	65.6 (9.1)/64.1 (9.2)	68.5 (7.4)/66.9 (8.7)
Risk of ILD in women and men				
Lifetime risk, % (95% CI)	1.5 (1.3–1.6)	4.4 (4.1–4.8)	6.1 (5.0–7.2)	16.8 (13.1–20.2)
HR (95% CI)	Reference	2.49 (2.25–2.75)	4.99 (4.20–5.94)	9.84 (7.96–12.2)
P value	–	1.24×10^{-71}	2.67×10^{-74}	4.40×10^{-99}
Risk of ILD in men				
Lifetime risk, % (95% CI)	1.7 (1.6–1.9)	5.6 (5.1–6.2)	9.0 (6.7–11.2)	20.9 (14.1–27.1)
HR (95% CI)	Reference	2.63 (2.31–2.98)	5.72 (4.46–7.34)	8.23 (5.96–11.4)
P value	–	2.04×10^{-50}	6.81×10^{-43}	1.56×10^{-37}
Risk of ILD in women				
Lifetime risk, % (95% CI)	1.1 (1.0–1.3)	3.1 (2.6–3.5)	4.7 (3.6–5.9)	14.5 (10.2–18.6)
HR (95% CI)	Reference	2.26 (1.92–2.66)	4.49 (3.53–5.70)	11.9 (8.96–15.8)
P value	–	1.46×10^{-22}	1.31×10^{-34}	7.86×10^{-66}

ILD, interstitial lung disease; RA, rheumatoid arthritis.

patient with RA carrying the *MUC5B* risk allele was diagnosed with ILD by age 80, and the risk rapidly increased after age 65. A case–control study by Juge and colleagues recently demonstrated enrichment of *MUC5B* carriers in patients with RA-ILD, with supporting evidence from gene expression in lung parenchyma and high-resolution imaging.[11] Using large-scale biobank data, we now show how this finding translates to lifetime risks and demonstrate the potential of genomics for risk stratification of RA-ILD and early identification of patients.

Prevalence of RA-ILD shows high variability in the literature depending on the population, diagnostic methods and disease definitions used.[13] Our lifetime risks compare well with previous estimates of clinically significant disease, reported to occur in up to 5%–10% of patients with RA.[2–4] We show that the effect of *MUC5B* is similar in the population and in patients with RA, but as both *MUC5B* and RA are important risk factors of ILD, patients with RA who are *MUC5B* carriers are at a much higher risk of ILD than *MUC5B* carriers without RA.

The common variant rs35705950 in the *MUC5B* promoter is strongly associated with upregulation of *MUC5B* expression in the lungs, and the general association between the variant and ILD has been widely replicated.[10 11 14] In addition, evidence from fine-mapping indicates that rs35705950 might be a causal variant: Bayesian fine-mapping analyses of genome-wide association study (GWAS) results can be used for defining variant sets (credible sets), that with high probability contain one or several causal variants. Several sources report rs35705950 as the only variant in the credible sets for the locus in GWASs on ILD and IPF.[15 16]

We were unable to account for some important risk factors, such as smoking and disease activity, and did not consider other common or rare genetic risk factors,[14 17] all of which are likely to further contribute to the risk. We did not have information about histological or radiological patterns of ILD. The study was limited to individuals of European ancestry, but *MUC5B* may be a relevant risk factor also in other populations[11], although many have allele frequencies that are much lower.[12] With a prevalence of 2.3% for RA and 0.7% for ILD, our sample is slightly enriched in cases, which may affect our estimates. Although ILD was identified through healthcare registries, recurring healthcare encounters were required to reduce the proportion of false positives in our study, and the long-term risk of ILD in patients with RA was in line with previous studies.[1–4] Patients with RA might be exposed to more chest imaging as part of their standard care and due to increased awareness for the risk of ILD particularly during recent years, which could overestimate the risk difference between patients with and without RA. We also observed a modest association between *MUC5B* and RA, which was replicated in UK Biobank. This association was not detected in a previous study with a smaller sample size by Juge and colleagues.[11] This tentative finding, which was clearer in men, requires further replication with consideration of other important risk factors, such as smoking. As the effects remained similar when excluding all patients with ILD, we propose that the temporal sequence of ILD and RA is unlikely to impact the association.

In conclusion, the *MUC5B* promoter variant is a common risk factor for ILD in patients with RA and confers a significantly elevated lifetime risk of ILD. This study demonstrates the potential of genomics for risk stratification of RA-ILD and highlights the importance of genetic predisposition on the development of RA-ILD. Studies are needed to further investigate the interaction of clinical and genetic risk factors in the development of RA-ILD, and the impact of *MUC5B* on outcomes of RA-ILD.

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Patient consent for publication Not required.

Ethics approval Patients and control subjects in FinnGen provided informed consent for biobank research, based on the Finnish Biobank Act. Alternatively, separate research cohorts, collected prior the Finnish Biobank Act came into effect (in September 2013) and start of FinnGen (August 2017), were collected based on study-specific consents and later transferred to the Finnish biobanks after approval by Fimea, the National Supervisory Authority for Welfare and Health. Recruitment protocols followed the biobank protocols approved by Fimea. The Coordinating Ethics Committee of the Hospital District of Helsinki and Uusimaa (HUS) approved the FinnGen study protocol Nr HUS/990/2017. The FinnGen study is approved by Finnish Institute for Health and Welfare (permit numbers: THL/2031/6.02.00/2017, THL/1101/5.05.00/2017, THL/341/6.02.00/2018, THL/2222/6.02.00/2018, THL/283/6.02.00/2019, THL/1721/5.05.00/2019, THL/1524/5.05.00/2020 and THL/2364/14.02/2020), Digital and population data service agency (permit numbers: VRK43431/2017-3, VRK/6909/2018-3, VRK/4415/2019-3), the Social Insurance Institution (permit numbers: KELA 58/522/2017, KELA 131/522/2018, KELA 70/522/2019, KELA 98/522/2019, KELA 138/522/2019, KELA 2/522/2020, KELA 16/522/2020 and Statistics Finland (permit numbers: TK-53-1041-17 and TK-53-90-20).This analysis was conducted with the UK Biobank Resource under Application Number 22627. The Biobank Access Decisions for FinnGen samples and data utilized in FinnGen Data Freeze 7 include: THL Biobank BB2017_55, BB2017_111, BB2018_19, BB_2018_34, BB_2018_67, BB2018_71, BB2019_7, BB2019_8, BB2019_26, BB2020_1, Finnish Red Cross Blood Service Biobank 7.12.2017, Helsinki Biobank HUS/359/2017, Auria Biobank AB17-5154 and amendment #1 (August 17 2020), Biobank Borealis of Northern Finland_2017_1013, Biobank of Eastern Finland 1186/2018 and amendment 22 § /2020, Finnish Clinical Biobank Tampere MH0004 and amendments (21.02.2020 & 06.10.2020), Central Finland Biobank 1-2017, and Terveystalo Biobank STB 2018001. The UK Biobank analysis was conducted with the UK Biobank Resource under Application Number 22627.

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TRANSLATIONAL SCIENCE

B cell depletion impairs vaccination-induced CD8$^+$ T cell responses in a type I interferon-dependent manner

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ABSTRACT

Objectives The monoclonal anti-CD20 antibody rituximab is frequently applied in the treatment of lymphoma as well as autoimmune diseases and confers efficient depletion of recirculating B cells. Correspondingly, B cell-depleted patients barely mount de novo antibody responses during infections or vaccinations. Therefore, efficient immune responses of B cell-depleted patients largely depend on protective T cell responses.

Methods CD8$^+$ T cell expansion was studied in rituximab-treated rheumatoid arthritis (RA) patients and B cell-deficient mice on vaccination/infection with different vaccines/pathogens.

Results Rituximab-treated RA patients vaccinated with Influvac showed reduced expansion of influenza-specific CD8$^+$ T cells when compared with healthy controls. Moreover, B cell-deficient JHT mice infected with mouse-adapted Influenza or modified vaccinia virus Ankara showed less vigorous expansion of virus-specific CD8$^+$ T cells than wild type mice. Of note, JHT mice do not have an intrinsic impairment of CD8$^+$ T cell expansion, since infection with vaccinia virus induced similar T cell expansion in JHT and wild type mice. Direct type I interferon receptor signalling of B cells was necessary to induce several chemokines in B cells and to support T cell help by enhancing the expression of MHC-I.

Conclusions Depending on the stimulus, B cells can modulate CD8$^+$ T cell responses. Thus, B cell depletion causes a deficiency of de novo antibody responses and affects the efficacy of cellular response including cytotoxic T cells. The choice of the appropriate vaccine to vaccinate B cell-depleted patients has to be re-evaluated in order to efficiently induce protective CD8$^+$ T cell responses.

Key messages

What is already known about this subject?
- B cell-depleted individuals cannot mount antibody responses upon vaccination; hence protection against vaccination-preventable diseases depends on CD8$^+$ T cell responses.

What does this study add?
- We found that B cell depletion strongly impairs vaccination-induced CD8$^+$ T cell responses.
- Mechanistically, B cells promote CD8$^+$ T cell responses in a type I interferon-dependent manner.

How might this impact on clinical practice or future developments?
- Patients treated with rituximab should be vaccinated when B cells have repopulated in order to mount efficient CD8$^+$ T cell responses.
- Vaccines inducing a cytokine milieu that is not dominated by type I interferon could be beneficial for B cell-depleted patients.

INTRODUCTION

Antibody responses play a key role in mediating protection against severe infections and the efficacy of the majority of currently available vaccines relies on the induction of long-lasting antibody responses. In particular during the current SARS-CoV-2 pandemic, it is discussed to which extend antibody and T cell responses contribute to protection. In some convalescent patients, rapidly decreasing antibody titres were observed. The question arose, whether such patients are still protected from SARS-CoV-2 reinfection by long-lasting T cell memory.

B cell depletion using the anti-CD20 antibody rituximab is an effective treatment of lymphoproliferative diseases such as non-Hodgkin's lymphomas,[1] various autoimmune diseases, including immune thrombocytopaenia (ITP),[2 3] rheumatoid arthritis (RA),[4] anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis,[5] systemic lupus erythematosus,[6] multiple sclerosis,[7] and prevents graft failure after some solid organ transplantations.[8] Since B cell depletion massively reduces the formation of SARS-CoV-2-specific antibodies, it is intensively discussed whether B cell depleting therapy with rituximab and Ocrelizumab should be postponed until SARS-CoV-2 vaccination has been performed.[9] In the absence of antibody responses, CD8$^+$ cytotoxic T cells take over important functions in protection against pathogens. For B cell-depleted patients it is therefore of utmost importance to mount functional CD8$^+$ T cell responses upon vaccination.

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Recently, it became evident that B cell depletion influences $CD8^+$ T cell responses. In a murine model of ITP, rituximab treatment inhibited splenic $CD8^+$ T cell proliferation and thus protected against T cell-mediated autoimmune thrombocytopaenia.[10] Furthermore, it was reported that B cells promote survival of intra-islet $CD8^+$ T cells in NOD mice and that B cell deficiency significantly delayed diabetes development.[11] B cells also play a specific role in modulating the contraction of $CD8^+$ T cell responses following immunisation with *Listeria monocytogenes* and in establishing efficient $CD8^+$ T cell memory.[12] Furthermore, B cells were required to prevent virus-specific $CD8^+$ T cell memory exhaustion upon lymphocytic choriomeningitis virus infection.[13]

Whether B cells support T cell responses by direct cell-cell contact or via cytokine and chemokine expression is still largely unclear. A $CXCR5^+$ subset of $CD8^+$ T cells was shown to constitute early effector cells that migrate into B cell follicles and thus might be able to directly interact with B cells.[14] Several chemokines and cytokines such as type I interferon (IFN-I) were shown to orchestrate lymphocyte responses locally or via systemic inflammatory signals. In addition to direct anti-viral function, IFN-I directly triggers the IFN-I receptor (IFNAR) of $CD8^+$ T cells to promote their expansion.[15–17]

IFN-I are potent antiviral cytokines that are induced early upon various infections and thus are targeted by many viral evasion strategies. The poxvirus strains vaccinia virus (VACV) and modified vaccinia virus Ankara (MVA) are relevant vaccine models to study vaccination in vivo. In contrast to its parental strain VACV, MVA lost several IFN-I inhibitors during passaging on chicken embryo fibroblasts and therefore efficiently induces serum IFN-I responses in mice.[18]

Here, we studied the impact of B cell depletion on $CD8^+$ T cell expansion during immunisation with different viruses. We found massively reduced $CD8^+$ T cell responses in B cell-depleted RA patients upon influenza vaccination. $CD8^+$ T cell expansion was also strongly reduced in B cell deficient mice upon influenza and MVA infection, but not upon VACV infection. Direct IFNAR signalling of B cells was necessary to trigger proper T cell activation and MHC-I upregulation, thus licensing B cells to promote $CD8^+$ T cell expansion.

RESULTS

Patients suffering from rheumatic diseases are frequently treated with rituximab. Rituximab has a high depletion efficiency, which lasts for approximately 6 months (figure 1A). During a therapy cycle, vaccination against seasonal influenza is recommended, whereas the protective efficacy of influenza vaccination under conditions of B cell depletion is debated. To study the impact of B cell depletion on the induction of $CD8^+$ T cell responses, rituximab-treated RA patients and healthy controls were human leucocyte antigen (HLA)-typed and vaccinated with Influvac. Influenza-specific T cells were determined 7 days post vaccination (figure 1B online supplemental figure 1). An increase of influenza-specific $CD8^+$ T cells was observed in healthy individuals, but not in B cell deficient patients (figure 1C). To directly compare T cell responses of different donors, the fold induction of specific T cells post vaccination was calculated (figure 1D). Of note, the observed reduced T cell expansion in rituximab-treated patients was independent on other immunomodulatory comedication (online supplemental figure 2). Thus, B cell depleted RA patients show reduced $CD8^+$ T cell expansion upon anti-influenza vaccination. During the current SARS-CoV-2 pandemic, such patients are particularly vulnerable and bare an enhanced mortality risk.[19 20] COVID-19 vaccination of younger patients just started and is applied independently of the rituximab treatment cycle, as similarly done for influenza vaccination. One patient with granulomatosis and polyangiitis (GPA) was analysed 4 weeks after second BNT162b2 vaccination for anti-SARS-CoV-2 antibody titres (figure 1E). In contrast to healthy controls, who mount high anti-SARS-CoV-2 IgG responses, no antibody titre was detected in the serum of this patient. Of note, as SARS-CoV-2 specific HLA-multimers are not available yet, T cell expansion could not be tested.

Since the analysis of immune responses in RA patients is potentially confounded by generally impaired immune status due to primary diseases and concomitant immunomodulatory treatment, the molecular mechanism of how B cells affect $CD8^+$ T cell expansion was further addressed in B cell-deficient mice. To this end, JHT mice, in which the deletion of the J elements of the immunoglobulin heavy chain locus (JHT) resulted in a premature block of B cell development, were analysed. Upon infection with the mouse adapted influenza strain PR8, JHT mice showed significantly reduced expansion of nucleoprotein- and polymerase acidic protein-specific $CD8^+$ T cells when compared with wild type mice (figure 2A,B). Thus, B cells are needed to efficiently induce influenza-specific $CD8^+$ T cell responses in humans and mice.

To analyse whether the impact of B cells on T cell expansion is a unique feature on influenza infection, wild-type mice and JHT mice were infected with VACV, which is known to induce particularly strong T cell responses. The expansion of VACV-specific T cells was measured using an major histocompatibility complex (MHC)-I multimer loaded with the immune-dominant peptide B8. Upon VACV infection, wild type and JHT mice showed similar T cell expansion (figure 2C). Following MVA infection the expansion of B8-specific $CD8^+$ T cells was significantly increased in wild type mice compared with JHT mice (figure 2D). To analyse whether B cell reconstitution of B cell-deficient mice restored T cell responses, splenic B cells of wild type mice were adoptively transferred into JHT mice 1 day prior to MVA infection. In B cell-reconstituted JHT mice the expansion of B8-specific $CD8^+$ T cells was comparable with that in wild type mice (figure 2E), whereas adoptive transfer of serum from wild type mice, which contains natural antibodies but no B cells, had no impact (figure 2F). These data indicate that B cells support the induction of B8-specific $CD8^+$ T cell responses on MVA infection, whereas during VACV infection B cells are not needed. Thus, the capacity of B cells to modulate $CD8^+$ T cell responses is dependent on the properties of the pathogen/vaccine.

MVA and VACV induce distinct cytokine milieus upon infection: While MVA induces systemic IFN-I responses, VACV efficiently inhibits systemic IFN-I responses and rather induces an IL-12 dominated cytokine milieu.[15 18] To test whether IFN-I responses affect B and T cell responses, we made use of conditional CD19-$Cre^{+/-}$IFNAR$^{flox/flox}$ mice (IFNAR-B) in which the IFNAR is selectively deleted on B cells. Upon VACV infection, the expansion of B8-specific $CD8^+$ T cells was similar in IFNAR-B and wild type mice (figure 3A), whereas upon MVA infection the expansion of T cells was significantly reduced in IFNAR-B mice (figure 3B). To test whether B cells are directly triggered by IFN-I, B cells from Mx2-luc reporter mice expressing a luciferase reporter upon IFNAR triggering were adoptively transferred into albino C57BL/6 mice. Upon MVA infection, a strong luciferase signal was detected by in vivo imaging particularly in the spleen and lymph nodes, which declined within the following day (figure 4). These results indicated that B cells were directly

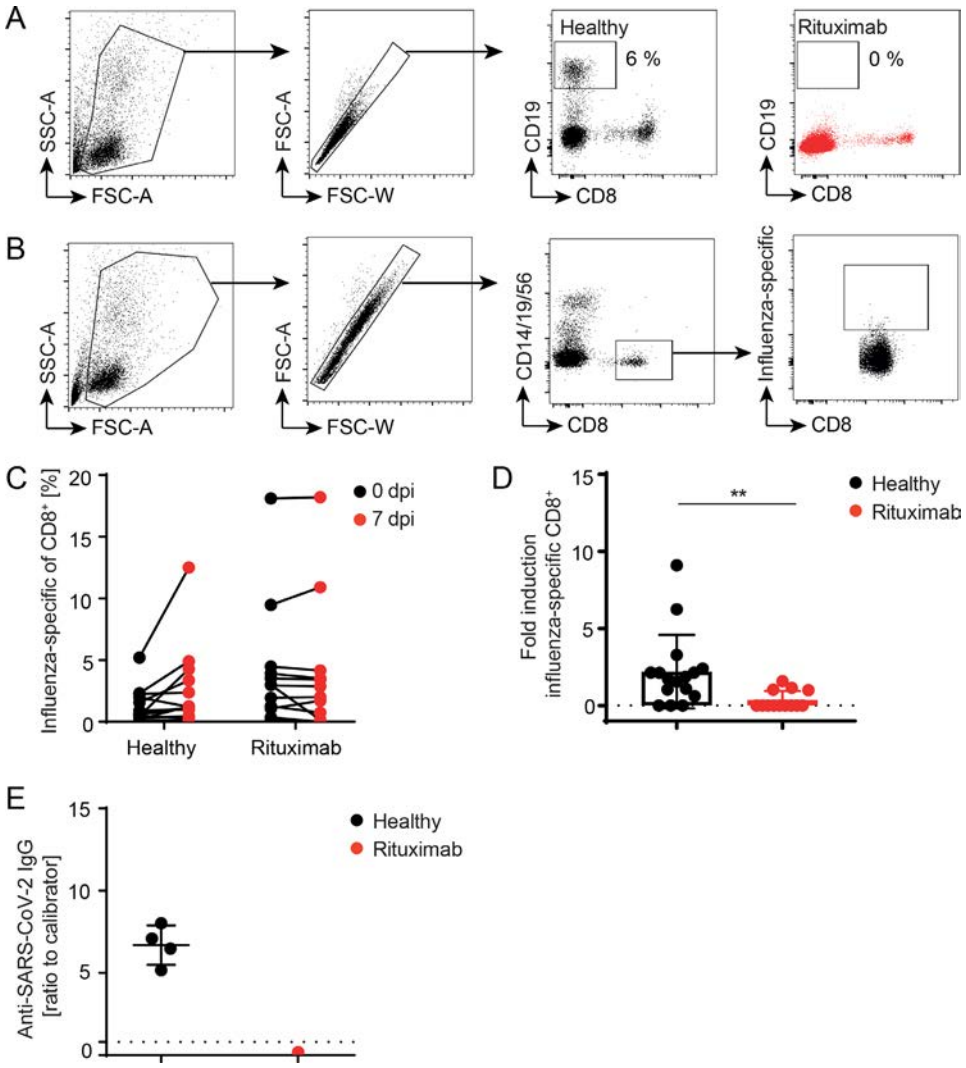

Figure 1 B cell depletion affects CD8$^+$ T cell response upon influenza vaccination. Healthy subjects and rituximab-treated RA patients were vaccinated against seasonal influenza. (A) Rituximab treatment efficiently depletes circulating B cells from blood. (B) Influenza-specific CD8$^+$ T cells were determined after excluding CD14$^+$/CD19$^+$/CD56$^+$ cells by using one or more personalised MHC-I multimers (left panels). B cell depletion efficiency was monitored using flow cytometry (right panel). (C) The frequency of influenza-specific T cells of CD8$^+$ T cells was monitored on day 0 and 7 post vaccination. (D) Fold induction was calculated for each MHC-I multimer measurement (n=10 healthy, n=5 rituximab). Healthy subjects and one rituximab-treated GpA patient were fully vaccinated against SARS-CoV-2. (E) Serum IgG against SARS-CoV-2 S1 was determined (n=4 healthy, n=1 rituximab). Titre was considered positive when >0.8 ratio to calibrator (dotted line). error bars indicate mean±SD; **p≤0.01; one-tailed Mann-Whitney U test. FSC-A, forward scatter-area; RA, rheumatoid arthritis; SSC-A side scatter-area.

triggered by IFN-I early after MVA infection, which is in accordance with the fast onset of MVA induced IFN-I responses.[21]

To study effects of direct IFNAR signalling, B cells were isolated from spleens of MVA-infected wild type and IFNAR-B mice and analysed for differential gene expression by RNA sequencing. B cells of wild type mice expressed higher messenger RNA (mRNA) levels of MHC-I, β−2-microglobulin, and Ly6C than B cells of IFNAR-B mice (figure 5A). Furthermore, IFNAR-deficient B cells highly upregulated many chemokine receptors as well as CXCL1, CXCL9, and CXCL13, while CXCL10 was down-modulated when compared with wild type B cells (figure 5B). Thus, direct IFNAR-triggering of B cells modulates pathways involved in antigen presentation and tissue homoeostasis.

To test whether virus-specific CD8$^+$ T cells showed distinct chemokine receptor expression, MVA-specific T cells were sorted by fluorescence activated cell sorting (FACS) using an MHC-I multimer and mRNA was sequenced. Of note, no differences in chemokine receptor expression were found comparing B8-specific CD8$^+$ T cells of wild type and IFNAR-B mice (figure 5C). Even being less frequent, B8-specific CD8$^+$ T cells showed very similar gene expression profiles when compared with T cells from wild type mice.

In accordance with sequencing data, B cells' surface expression of MHC-I and the B8 presenting haplotype H2-K^b was significantly increased upon direct IFNAR triggering, while MHC-II expression was upregulated upon infection IFNAR-independently (figure 6A–C). In addition, MVA infection induced CD86 and CD69 expression on wild type B cells, which was significantly reduced on IFNAR-deficient B cells (figure 6D–E). Thus, direct IFNAR signalling activates B cells and induces the expression of MHC-I as well as costimulatory molecules, and thus has a major impact on the capacity for antigen presentation of B cells.

DISCUSSION

Here, we report that B cell depletion can affect the expansion of virus-specific CD8$^+$ T cells, depending on the T cell stimulating

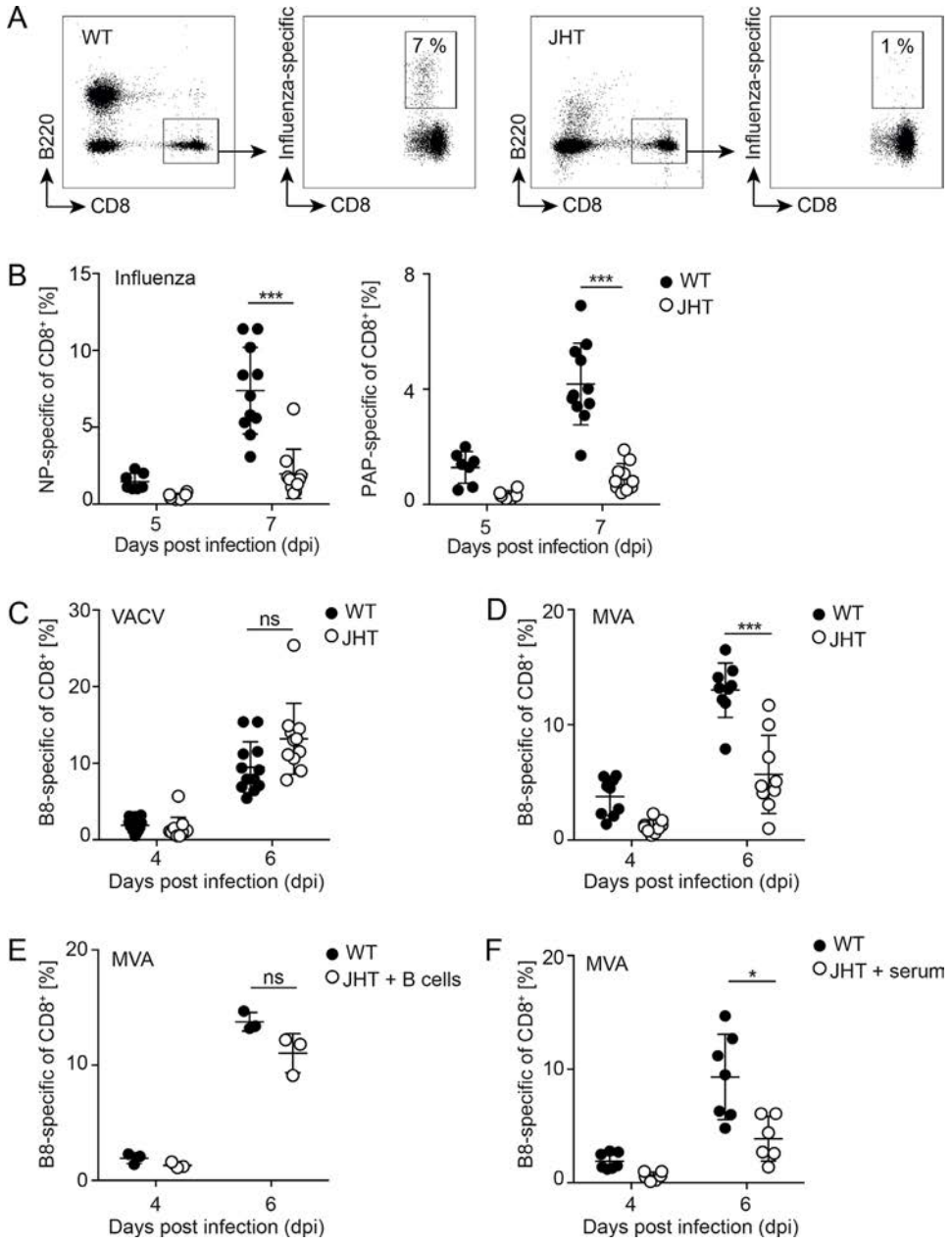

Figure 2 B cell deficient mice show reduced virus-specific CD8$^+$ T cell response upon influenza and MVA, but not VACV infection. (A) Wild type (WT) and JHT mice were infected with 5×10^3 ffu mouse adapted influenza virus for 7 days. (B) Influenza-specific CD8$^+$ T cells were determined by using nucleoprotein (NP) or polymerase acidic protein (PAP) specific MHC-I multimers. WT and JHT mice were infected with 10^5 pfu of (C) VACV or (D) MVA and B8-specific CD8$^+$ T cells were determined by using a MHC-I multimer. Data shown are pooled from 2 to 3 experiments with n=3–4. JHT mice were reconstituted with (E) 10^7 B cells or (F) 300 µL serum of WT mice 1 day prior to MVA infection and B8-specific T cell expansion was monitored. One out of two independent experiments is shown. Error bars indicate mean±SD; *p≤0.05, ***p≤0.001; one-tailed Mann-Whitney U test. MVA, modified vaccinia virus Ankara; ns, not significant; VACV, vaccinia virus.

pathogen/vaccine. The underlying mechanism is mediated via direct IFNAR signalling of B cells, which showed enhanced MHC-I, CD69, and CD86 expression, increased activation, and a distinct chemokine expression profile.

Most RA patients treated with rituximab received an immunomodulatory comedication and thus are therapeutically immunosuppressed. Since rituximab is not licensed as first-line RA treatment, the patients received other immunomodulatory treatments earlier. Additionally, RA patients were recently shown to harbour exhausted CD4$^+$ T cells,[22] which might influence the outcome of CD8$^+$ T cell responses as well. Furthermore, patients are not immunologically naïve, since they were previously vaccinated against seasonal influenza virus or were in contact with the pathogen itself. The analysis of T cell expansion upon vaccination reflects a reactivation of memory CD8$^+$ T cells rather than a primary response. The question remains, whether upon other diseases than RA B cell depletion influence CD8$^+$ T cells responses as well. To prove that reduced expansion of CD8$^+$ T cells in patients treated with rituximab was not caused by such secondary effects, we studied the result of B cell depletion on T cell responses in mice.

Here, we report a reduced in vivo expansion of antigen-specific CD8$^+$ T cells in B cell-deficient mice upon infection with different viruses, suggesting the presence of a species-independent

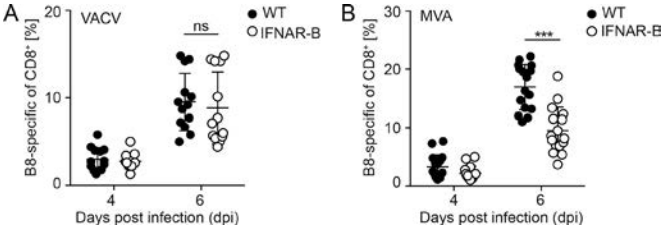

Figure 3 IFNAR depletion on B cells affects B8-specific CD8$^+$ T cell responses upon MVA, but not VACV infection. Wild type (WT) and CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ (IFNAR-B) mice were infected with 10^5 pfu (A) VACV or (B) MVA. B8-specific CD8$^+$ T cells were determined by using an MHC-I multimer. Data shown are pooled from 3 to 4 experiments with n=3–4. Error bars indicate mean±SD; ***p≤0.001; one-tailed Mann-Whitney U test. IFNAR, type I interferon receptor; MVA, modified vaccinia virus Ankara; ns, not significant; VACV, vaccinia virus.

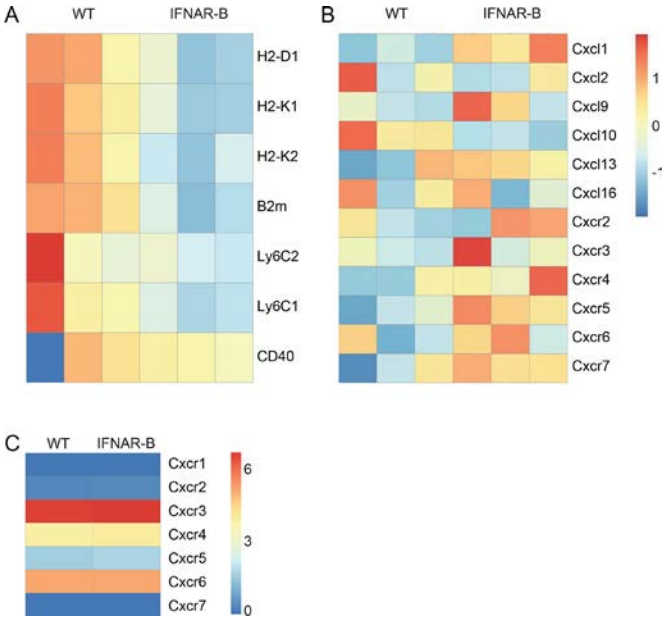

Figure 5 MVA-induced IFN-I responses activate B cells, but do not affect CXCR5$^+$CD8$^+$ T cell responses. Wild type (WT) and IFNAR-B mice were infected with 10^5 pfu MVA and B cells were isolated 1 day post infection via untouched magnetic cell separation and prepared for mRNA sequencing. Differentially regulated (A) surface molecules and (B) chemokine as well as chemokine receptor expression profiles are shown. n=3 (C) WT and IFNAR-B mice were infected with 10^5 pfu MVA and B8-specific CD8$^+$ T cells were FACS-sorted six days post infection from spleens using a B8-specific MHC-I multimer. RNA sequencing samples were pooled from three different mice and chemokine expression profiles were analysed. IFN-I, type I interferon; IFNAR, IFN-I receptor; MVA, modified vaccinia virus Ankara.

mechanism of immune cell cross-talk. This phenomenon is remarkable, as dendritic cells (DCs) are broadly accepted to be the main APC responsible for T cell priming.

Guo *et al* showed that on anti-CD20 treatment, splenic CD8$^+$ T cell proliferation was inhibited in a murine model of ITP.[10] In that study, B cell depletion led to increased numbers of FOXP3$^+$, CD4$^+$, and CD8$^+$ T cells within the spleen and lymph nodes, while splenic CD8$^+$ T cells showed a reduced proliferation upon in vitro stimulation.[10] In our experiments, the impaired T cell expansion was restored by adoptive transfer of B cells. B1 cell-derived natural antibodies, which are present in the serum of naïve mice, were shown to decorate antigen rather unspecifically and to enhance antigen presentation by antigen trapping.[23] However, we found that serum transfer was not effective in restoring the deficit in CD8$^+$ T cell expansion in B cell deficient mice.

Upon MVA infection, the lack of IFNAR expression exclusively on B cells resulted in reduced T cell expansion as similarly detected in B cell deficient mice. Thus, besides serving as a direct third signal for T cell responses[15] IFN-I can also increase CD8$^+$ T cell responses indirectly via B cells. IFN-I responses were shown to critically modulate the overall cytokine milieu and in particular, to inhibit IL-12 responses.[15 24 25] Furthermore, IL-12 was shown to serve as third signal in T cell activation as well,[26 27] which might explain why in the absence of IFN-I responses B cells are dispensable for CD8$^+$ T cell expansion. Direct IFNAR triggering on B cells induced the activation of the STAT1 pathway and enhanced the expression of Ly6C and CD69. Moreover, MHC-I and CD86 were induced, thus facilitating adequate antigen presentation. Interestingly, B cells were described before to cross-present MHC-I restricted antigen, although less efficiently than DC.[28] Thus, IFN-I is a key mediator to promote efficient interaction between B cells and CD8$^+$ T cells. Of note, virus-induced IFN-I was also reported to confer disintegration of B cell follicles[29] and to drive B cell reduction by differentiating B cells into short-lived antibody-secreting cells.[30] This mechanism called 'B cell decimation' was independent of B cell-intrinsic IFN-I sensing.[30]

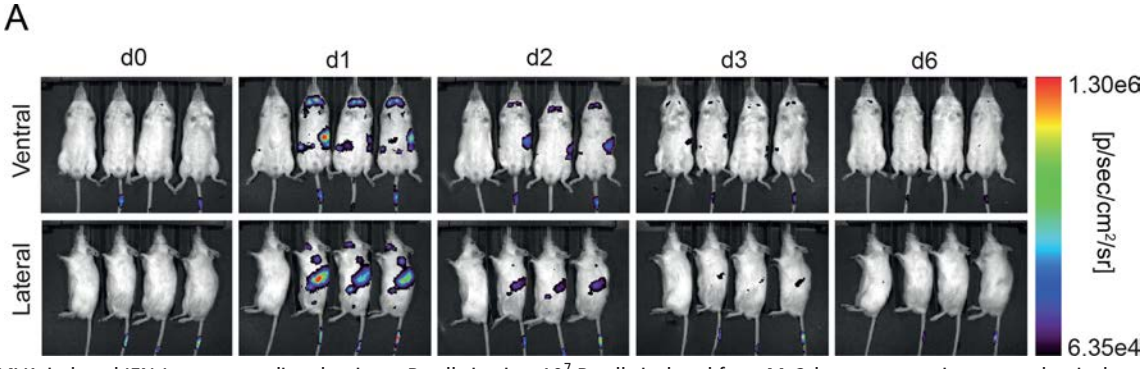

Figure 4 MVA-induced IFN-I responses directly trigger B cells in vivo. 10^7 B cells isolated from Mx2-luc reporter mice were adoptively transferred into albino C57BL/6 wild type mice 1 day prior to infection. Upon treatment with phosphate buffered saline (PBS) (first mouse per row) or infection with 10^5 pfu MVA (mouse 2–4 per row), luciferase reporter expression in adoptively transferred B cells was monitored after luciferin administration by in vivo imaging at different days (d) postinfection (scale=p/sec/cm^2/sr). one out of two independent experiments is shown. IFN-I, type I interferon; MVA, modified vaccinia virus Ankara.

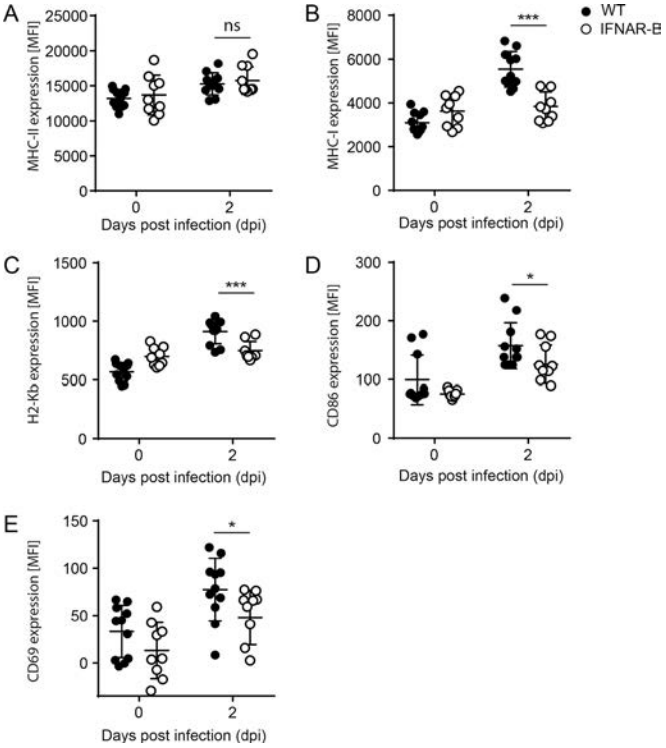

Figure 6 MVA-induced IFN-I responses modulate antigen presentation in B cells. Wild type (WT) and IFNAR-B mice were infected with 10^5 pfu MVA and splenocytes were isolated 48 hours post infection. Expression of (A) MHC-II, (B) MHC-I, (C) H2-kb, (D) CD86, and (E) CD69 was analysed by flow-cytometry. Data shown are pooled from 2 to 3 experiments with n=2–4. Error bars indicate mean±SD; *p≤0.01; ***p≤0.001; one-tailed Mann-Whitney U test. DPI, days post infection; IFN-I, type I interferon; IFNAR, IFN-I receptor; MVA, modified vaccinia virus Ankara; MFI, mean fluorescence intensity; NS, not significant.

Whether B cells and $CD8^+$ T cells are in direct contact within secondary lymphoid organs has been discussed controversially. B cell follicles and T cell zones are organised in separate compartments in secondary lymphoid organs. In human (HIV) and simian immunodeficiency virus (SIV) infection, B cell follicle sanctuaries were shown to permit a persistent infection reservoir due to the absence of protective $CD8^+$ T cell responses.[31–33] Quigley *et al* showed that a $CXCR5^+$ subset of $CD8^+$ T cells infiltrates B cell areas of tonsils.[14] During chronic viral infection with lymphocytic choriomeningitis virus or SIV, $CXCR5^+CD8^+$ T cells migrate into B cell follicles and critically contribute to the control of viral replication.[34–36] Upon MVA infection, IFNAR deficient B cells showed enhanced expression of CXCL13, which was previously shown to attract $CXCR5^+CD8^+$ T cells.[14] Of note, CXCR5 expression of sorted MVA-specific $CD8^+$ T cells was very similar in wild type and IFNAR-B mice. These data suggest that in IFNAR-B mice, $CXCR5^+CD8^+$ T cells initially infiltrate B cell follicles, but cross-talk with B cells may be reduced. $CD4^+$ T cells can directly interact with B cells, critically increase $CD8^+$ T cell responses by providing help,[37 38] and are activated in a spatially distinct compartment of lymph nodes before encountering $CD8^+$ T cells.[39] Thus, $CD4^+$ T cells might function as a link between B cell and $CD8^+$ T cell responses.

Of note, rituximab treatment of RA patients not only depletes recirculating B cells, but also a $CD20^+$ terminally differentiated T cell subset with immune-regulatory and proinflammatory function.[40] Nevertheless, the frequency of $CD20^+CD8^+$ T cells is very low in humans and might not be the primary cause for reduced T cell expansion in rituximab-treated patients.

Here, we studied the immune response against an influenza vaccine in B cell depleted RA patients. It is possible that antigen-specific T cell responses are also reduced in rituximab-treated patients after vaccination against other diseases. Of note, SARS-CoV-2 infection induces only mild IFN-I responses due to active IFN-I blockade[41 42] and patients with severe COVID-19 displayed a highly impaired IFN-I response when compared with patients with moderate COVID-19 courses.[43 44] Among the available COVID-19 vaccines, the mRNA-based vaccines induce IFN-I dominated cytokine milieus.[45] In contrast, for adenovirus-based vaccines it was shown that excessive IFN-I responses rather inhibit transgene expression, and as a consequence, vectors inducing only minor IFN-I responses were chosen for the development of an immunogenic vaccine.[46 47] Among SARS-CoV-2 adenoviral vectors, HAd5-based vaccines most likely induce less IFN-I compared with ChAdOx1-based vaccines. Considering a reduced $CD8^+$ T cell responses in the presence of IFN-I with simultaneous absence of B cells, the non-IFN-I inducing adenovirus based vaccines could be even better suited to induce decent $CD8^+$ T cell responses in B cell-depleted patients compared with mRNA-based vaccines.[46 48]

Patients treated with rituximab were reported to bare an enhanced mortality risk if infected with SARS-CoV-2.[19 20] With regard to COVID-19 disease, it appears therefore not advisable to delay vaccination of such patients a few months after rituximab suspension, when naïve B cells have repopulated. In contrast to other vaccines, COVID-19 vaccine should rather be administered as soon as available. In order to induce at least protective $CD8^+$ T cell responses, the usage of vaccines inducing a cytokine milieu that is not dominated by IFN-I could be beneficial for such patients.

MATERIAL AND METHODS

Patients and healthy controls

After immunisation withInfluvac season 2012/2013 or 2013/2014 (Mylan Healthcare) PBMC were isolated on day 0 and 7, and frozen at −80°C. The frequency of influenza virus specific $CD8^+$ T cells was determined using HLA matched pentamers (Proimmune) (online supplemental table 1). Five RA patients (one male, four female, average age 63 years) and 10 healthy controls (five male, five female, average age 31 years) were identified with one or more matching HLA subtypes. After BNT162b2 vaccination, 1 GPA patient (female, age 20 years) and four healthy controls (two female, 1 male, average age 33 years) were recruited. Characteristics of patients are indicated (online supplemental table 2).

Mice

C57BL/6 (wild type) and albino C57BL/6BrdCrHsd-Tyrc (C57BL/6 albino) mice were purchased from Harlan Winkelmann or Envigo. $IFNAR^{-/-}$, [49] JHT,[50] $CD19\text{-}Cre^{+/-}IFNAR^{flox/flox}$ (IFNAR-B),[51] and Mx2-luc reporter mice[52] were described before. All mice were bred under specific pathogen free conditions at the central animal facility of TWINCORE and the Helmholtz Centre for Infection Research, Brunswick, Germany, or the Paul-Ehrlich-Institut, Langen, Germany. Mouse experimental work was carried out using 8 to 16 week old mice in compliance with regulations of the German animal welfare law (F107/64, 09/1655, 10/0265, 10/0266, 11/0367, 12/0939, 13/1073).

Viruses and infection

MVA and VACV strain Western Reserve (originally provided by Bernard Moss, NIH, Bethesda, Maryland, USA)[53] were propagated and titrated on chicken embryonic fibroblasts and purified by sucrose density gradient centrifugation. Mouse-adapted influenza A/PR/8/34 (H1N1 PR8)[54] was propagated in the chorioallantoic fluid of 10 days old pathogen free embryonated chicken eggs at 37°C[55] and was kindly provided by Dr. P. Blazejewska, Dr. K. Schughart, and Carlos A. Guzmán (Helmholtz Centre for Infection Research Brunswick, Germany). In all infection experiments, mice were treated with 10^5 pfu MVA/VACV, or 5×10^3 ffu influenza virus dissolved in PBS intravenously.

Adoptive cell and serum transfer experiments

B cells were isolated from spleens, via untouched magnetic B cell separation kit (Miltenyi). 10^7 B cells with a purity of 90%–98% were adoptively transferred into recipient mice. For serum transfer, 300 µL serum pooled from different wild-type animals was injected 1 day prior to infection.

In vivo imaging

Reporter mice were intravenously injected with 3 mg of D-luciferin (PerkinElmer) diluted in PBS and anaesthetised using 2.5% isoflurane (Abbott). The emitted light signals were measured in the in vivo imaging system IVIS SpectrumCT (Calliper) and analysed with Living Image 4.5 software (Calliper).

Flow cytometric analysis and cell sorting

All antibodies were purchased from eBioscience or BD-Pharmingen. Cells were measured using flow cytometry (LSR II, BD) and data were analysed by FlowJo software. FACS sorting was conducted using a MoFlo XDP cell sorter (Becton Dickinson).

ELISA

Anti-SARS-CoV-2 IgG antibody titres were determined from serum using an ELISA (Euroimmun AG, EI 2606–9601 G) according to the manufacturer's instructions. The ratio of the optical density to the calibrator was used to classify the samples as negative (ratio <0.8) or positive (ratio ≥1.1).

Deep sequencing and pathway analysis

After 24 hours of MVA infection, B cells were isolated from spleens of C57BL/6 and IFNAR-B mice using the untouched magnetic B cell separation kit (Miltenyi). FACS sorting of B8-specific $CD8^+$ T cells from spleens was conducted using a MoFlo XDP cell sorter (Becton Dickinson). After RNA isolation using tNucleoSpin RNA kit (Macherey-Nagel) mRNA sequencing was performed at TRON (Translational Oncology Mainz, Germany). Pathway analysis was performed as described in online supplemental methods section.

Statistical analysis

Statistical analyses were performed using GraphPad Prism V.6 software as indicated.

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Contributors TG, UK, TW and RES planned the study and TG and UK designed experiments. TG, KB, MK, and MB performed and analyzed experiments. LG and TW were involved in treatment and recruitment of the patients. HM and LV analyzed sequencing data and performed statistics. MV and MH performed HLA-typing of patients. CAG and GS provided virus stocks. TG, UK, and TW wrote the manuscript. All authors critically reviewed the manuscript.

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Competing interests None declared.

Patient consent for publication Not required.

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Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information. All data relevant to the study are included in the article or uploaded as supplementary Information.

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EPIDEMIOLOGICAL SCIENCE

Impaired fertility in men diagnosed with inflammatory arthritis: results of a large multicentre study (iFAME-Fertility)

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ABSTRACT

Objectives The impact of inflammatory arthritis (IA) on male fertility remains unexplored. Our objective was to evaluate the impact of IA on several male fertility outcomes; fertility rate (number of biological children per man), family planning, childlessness and fertility problems.

Methods We performed a multicentre cross-sectional study (iFAME-Fertility). Men with IA 40 years or older who indicated that their family size was complete were invited to participate. Participants completed a questionnaire that included demographic, medical and fertility-related questions. To analyse the impact of IA on fertility rate, patients were divided into groups according to the age at the time of their diagnosis: ≤30 years (before the peak of reproductive age), between 31 and 40 years (during the peak) and ≥41 years (after the peak).

Results In total 628 participants diagnosed with IA were included. Men diagnosed ≤30 years had a lower mean number of children (1.32 (SD 1.14)) than men diagnosed between 31 and 40 years (1.60 (SD 1.35)) and men diagnosed ≥41 years (1.88 (SD 1.14)).This was statistically significant (p=0.0004).The percentages of men diagnosed ≤30 and 31–40 years who were involuntary childless (12.03% vs 10.34% vs 3.98%, p=0.001) and who reported having received medical evaluations for fertility problems (20.61%, 20.69% and 11.36%, p=0.027) were statistically significant higher than men diagnosed ≥41 years.

Conclusions This is the first study that shows that IA can impair male fertility. Men diagnosed with IA before and during the peak of reproductive age had a lower fertility rate, higher childlessness rate and more fertility problems. Increased awareness and more research into the causes behind this association are urgently needed.

Key messages

What is already known about this subject?
- Inflammatory arthritis (IA) is associated with male infertility, erectile dysfunction and hypogonadism.

What does this study add?
- The diagnosis of IA before or during the peak of the male reproductive age was associated with a lower fertility rate, higher rates of involuntary childlessness and fertility problems.

How might this impact on clinical practice or future developments?
- Rheumatologists should be aware that IA and/or the pharmacological treatment associated with IA may impair male fertility.
- Multiple biological and non-biological mechanisms can be responsible for this association and more research is urgently needed.

INTRODUCTION

Spondyloarthritis (SpA) and rheumatoid arthritis (RA) are frequent causes of inflammatory arthritis (IA) that can affect men before or during the peak of their reproductive age.[1–4] Even though IA is associated with male infertility, erectile dysfunction and hypogonadism[5 6] the impact of IA on male fertility remains largely unexplored. This is even more striking if we consider that several frequently prescribed anti-rheumatic drugs have been associated with reversible or irreversible testicular toxicity.[7]

The majority of people aspire to have children and it is known that men desire parenthood as much as women do.[8–10] Nonetheless, the impact of IA on one of the most important markers of fertility, the male fertility rate (total number of children per man),[11–13] has never been studied before.

Childbearing decisions and reproductive potential are strongly influenced by multiple psychosocial, demographic and biological factors.[9 14] Furthermore, it has been demonstrated that men diagnosed with chronic diseases are exposed to additional factors that have an effect on their childbearing decisions and their reproductive potential.[15 16]

In women diagnosed with IA, several factors related to IA have been associated with lower fertility rates.[17–19] It can be expected that some of these factors could also influence the fertility rate of men diagnosed with IA, such as impaired sexual function, lower intercourse frequency, deciding not to have a family or to have smaller

families due to concerns about the impact of IA or antirheumatic treatment.

Therefore, we aimed to evaluate the impact of IA on relevant markers of male fertility. Our primary objective was to compare the fertility rate of men diagnosed with IA based on their age at diagnosis. Additionally, we compared the fertility rate of men diagnosed with IA with the general male population of the Netherlands. To further evaluate the impact of IA on male fertility, as secondary objectives we compared the total number of pregnancies per man, desired family size (family planning), the proportion of childless men and fertility outcomes based on the results from medical evaluations for fertility problems.

METHODS

Study design and patient selection

We conducted a multicentre cross-sectional study in eight Dutch hospitals (iFAME (Inflammunity and Fertility in Men)-Fertility study). In the Netherlands, most men become a father between the age of 30 and 40 years and this period is considered to be the peak of reproductive age.[20] Therefore, men who were diagnosed with IA based on the expert opinion of their rheumatologists (RA, juvenile idiopathic arthritis (JIA) and SpA (ankylosing spondylitis (AS), psoriatic arthritis (PsA), reactive arthritis, enteropathic arthritis), who at the time of inclusion were 40 years or older and who indicated that their 'family size' was completed were included. Men who were still planning on having biological children in the future were excluded.

To evaluate the impact of IA on male fertility we considered the age at diagnosis of IA and divided participants into three study groups: diagnosis ≤30 years (before the peak of reproductive age), diagnosis between 31 and 40 years (during the peak of reproductive age) and diagnosis ≥41 years (after the peak reproductive age).

We estimated the mean number of children number per men without IA in their reproductive lifespan at 1.7 (SD: 1.0) and estimated a mean number of 1.4 children as significantly different. Using data simulation that accounted for dispersion and under-dispersion, to reject the null hypothesis with a 80% power (alpha=0.05; two sided), it was estimated that 548 men were needed to be included in the study (n=137, n=137 and n=274 per group, respectively).

Data collection

A self-reported questionnaire developed for this study was used. The design of this questionnaire was based on the 'fertility experiences questionnaire (FEQ)'. The FEQ was validated in women with subfertility and when compared with medical records it was proven to be over 90% sensitive for fertility outcomes.[21] In addition, we adapted the questionnaire to our population using previous questionnaires that have evaluated fertility outcomes in male kidney transplant recipients[22] and in women with rheumatic diseases.[23 24] Our questionnaire was divided into four sections: general demographic information, medical history, family planning and fertility outcomes (online supplemental 1). The digital version of the questionnaire that was distributed to participants was built using the survey software GemsTracker/LimeSurvey (LimeSurvey, Hamburg, Germany).

Men who fulfilled the inclusion criteria of being 40 years or older and diagnosed with IA were invited to participate in the study. These men received a letter from their hospital that included information about the study. To ensure the protection of privacy data, the letter included a personalised link to complete the digital questionnaire. To increase the number of responders, a second letter was sent to all non-responders.

Our primary outcome, the male fertility rate, was calculated using the answers to the question 'How many biological children did you have?'. This is a validated method that has been used to evaluate fertility. For secondary outcomes, other collected data include, but are not limited to, total number of pregnancies, desired family size, satisfaction with final family size and relevant medical history regarding fertility and pregnancy outcomes. A pregnancy was defined as 'any positive pregnancy test (even if it did not result in a live born child)' and time to pregnancy (TTP) was determined with the answers provided to the question 'How many months did it take for your partner to get pregnant?'.

A Likert scale questionnaire (scale ranging from completely disagree (0) to completely agree (10)) was used to evaluate the impact of IA on family planning/desired number of children.

Statistical analysis

Comparisons between the three groups and between the groups and the general population were tested. Categorical variables were presented as number (percentage), and continuous variables are reported as mean±SD or median ±IQR, as appropriate. Continuous variables were compared using a one-way analysis of variance, Tukey post hoc test, paired t-test and Wilcoxon rank. Categorical variables were compared using χ^2 tests and Fisher's exact tests. To control for confounders, multivariate regression model (analysis of covariance) was used. All potential confounders were fitted into the model. The level of significance was set as a two-tailed $p \leq 0.05$, and statistical analyses were completed using Stata V.15 (StataCorp).

Patient and public involvement

Six male patients diagnosed with IA and who are active members of the research advisory board from the Department of Rheumatology of the Erasmus University Medical Center were involved in the design of the questionnaire and the invitation letter. We carefully assessed the burden on participating patients. We intend to share the results to participating patients and will appropriately disseminate the results.

RESULTS

Between September 2019 and January 2021, a total of 1841 men were invited to participate in the study. All hospitals invited men from the three study groups using a 1:1:2 ratio until the necessary number of patients per group to achieve statistical power was reached. In total, 628 men agreed to participate (response rate of 34.1%). A detailed description of the demographics characteristics of these men is presented in table 1. Due to current privacy regulations that are applicable in the Netherlands, it was not possible to describe the demographic characteristics of the non-responders.

Total number of biological children (fertility rate)

Men diagnosed ≤30 years had a lower number of children (1.32 (SD 1.14)) than men diagnosed between 31 and 40 years (1.56 (SD 1.27)) and men diagnosed ≥41 years (1.88 (SD 1.14)) (see figure 1). There was a statistically significant difference between groups (p=0.0004). The total number of children was statistically significant lower in men diagnosed <30 years and in men diagnosed 31–40 years compared with men diagnosed >41 years (p<0.001 and p=0.020, respectively). The difference between men diagnosed <30 and 31–40 years was not statistically significant (p=0.264).

Table 1 Demographic characteristics

	All patients (N=628)	IA diagnosed ≤30 years (N=137)	IA diagnosed 31–40 years (N=149)	IA diagnosed ≥41 years (N=342)	P value
General information					
Age at inclusion in the study, mean (SD)	57.17 (9.98)	53.01 (9.96)*	52.76 (7.35)*	61.06 (9.47)	0.001
Born in the Netherlands, n (%)	531 (94.48)	117 (92.13)	132 (94.96)	277 (95.19)	0.143
Education Bachelor degree or higher, n (%)	223 (35.51)	61 (44.53)*	51 (34.23)	111 (32.46)	0.048
Currently in a relationship, n (%)	423 (67.36)	89 (64.96)	100 (67.11)	234 (68.42)	0.765
Inflammatory arthritis					
Diagnosis, n (%)					
RA	297 (47.29)	42 (30.66)*†	67 (44.97)	188 (55.32)	0.001
JIA	10 (1.59)	10 (6.45)	0	0	–
SpA (incl. PsA)	320 (50.96)	90 (65.69)*	83 (55.70)	147 (42.98)	0.001
Age at diagnosis, mean (SD)	41.30 (13.08)	23.76 (6.17)*†	36.52 (2.48)*	51.25 (7.77)	0.001
Disease duration, mean (SD)	15.89 (11.88)	29.51 (11.30)*†	16.30 (8.29)*	9.68 (7.77)	0.001
Concerning your IA, have you ever received information about your desire to have children? Yes, n (%)	139 (22.13)	45 (33.83)*	36 (24.66)*	37 (11.31)	0.001
Comorbidities					
Type 2 diabetes mellitus, n (%)	54 (8.60)	13 (9.49)	10 (6.71)	31 (9.06)	0.635
Cardiovascular disease,‡ n (%)	98 (15.61)	17 (12.41)	13 (8.72)*	68 (19.88)	0.006
Inflammatory bowel disease, n (%)	21 (3.34)	5 (3.65)	7 (5.04)	7 (2.05)	0.278
Urogenital comorbidities,§ n (%)	27 (4.30)	6 (4.38)	3 (2.01)	18 (5.26)	0.264

*P≤0.05 compared with those diagnosed age ≥41 years.
†p≤0.05 compared with those diagnosed age ≥31–40 years.
‡Arterial hypertension, angina pectoris, myocardial infarction, heart failure, stroke, peripheral vascular disease and dyslipidaemia.
§Urogenital infection, sexually transmitted disease, cryptorchidism, varicocele, testicular torsion, epididymitis, prostatitis, inguinal hernia, urogenital surgery, urogenital trauma and exposure to chemicals or radiation that can result in DNA damage.
IA, inflammatory arthritis; JIA, juvenile idiopathic arthritis; PsA, psoriatic arthritis ; RA, rheumatoid arthritis; SpA, spondyloarthritis.

After adjusting for potential confounders (current age, education level, history of cardiovascular disease, diagnosis of infertility in partner and diagnosis of RA, JIA and SpA) and considering the total number of children of men diagnosed ≥41 years as our reference group, we observed a statistically significant negative effect on the total number of children of men diagnosed ≤30 years (p=0.002) (see table 2). Furthermore, the total number of children per disease was not statistically significant between diseases.

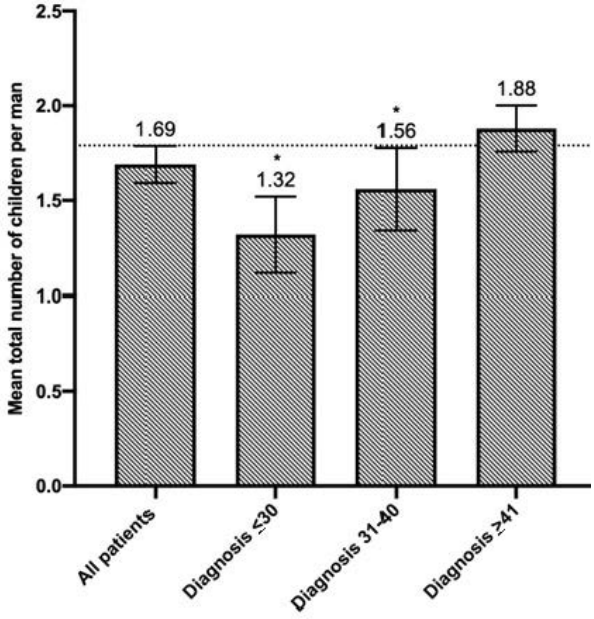

Figure 1 Mean total number of children per man for all participants and per group. Error bars represent 95% CI. The dotted line represents the mean number of children per man for men older than 40 years in the Netherlands. *Statistically significantly different compared with men diagnosed ≥41 years.

Lastly, we compared the fertility rate of the study groups with the fertility rate of all men living in the Netherlands who at the time of our last inclusion were 40 years or older (1.79, Statistics Netherlands (CBS),personal communication, 18 August 2020). Compared with the fertility rate of men ≥40 years from the general population, the fertility rate of men diagnosed ≤30 and 31–40 years was statistically significant lower (1.32, p=0.001 and 1.56 p=0.03, respectively). The fertility rateof men diagnosed ≥41 years was not statistically significant different (1.88, p=0.128).

Total number of pregnancies per man

In contrast to the fertility rate, where only live births are taken into account, the total number of pregnancies per man includes any positive pregnancy test independent of the final pregnancy outcome. Men diagnosed ≤30 years had a lower total number of pregnancies (1.45 (SD 1.37)) than men diagnosed between 31 and 40 years (1.73 (SD 1.69)) and men diagnosed ≥41 years (1.98 (SD 1.45)). There was a statistically significant difference between groups (p=0.0023). The total number of pregnancies was statistically significant lower in men diagnosed ≤30 years compared with men diagnosed ≥41 years (p=0.002). There were no statistically significant differences between men diagnosed <30 and 31–40 years (p=0.261) and between men diagnosed 31–40 and ≥41 years (p=0.219).

Childlessness

In the Netherlands, the percentage of childless men ranges between 20% and 25%.[25] In total, 143 men (22.27%) were childless most of whom were voluntary childless (n=99 (69.23%)).

Table 2 Analysis of covariance: effect of dichotomised age at diagnosis of IA (based on our study groups) on total number of children per man and considering the total number of children of men diagnosed ≥41 years as our reference group

	Crude (n=615)		Adjusted* (n=609)	
	B (95% CI)	P value	B (95% CI)	P value
31–40 years	−0.398 (−0.624 to −0.171)	0.001	−0.207 (−0.455 to 0.040)	0.101
≤30 years	−0.517 (−0.744 to −0.291)	0.000	−0.406 (−0.660 to −0.152)	0.002

*Adjusted for confounders (age at inclusion in the study, education level, cardiovascular disease, diagnosis of infertility in partner and diagnosis of RA, JIA and SpA).
IA, inflammatory arthritis; JIA, juvenile idiopathic arthritis; RA, rheumatoid arthritis; SpA, spondyloarthritis.

The percentage of childless men was significantly higher in men diagnosed ≤30 years (n=45 (33.83%)) and in men diagnosed 31–40 years (n=39 (26.90%)) compared with men diagnosed ≥41 years (n=59 (17.25%), p=0.001).

In addition, we compared the percentages of voluntary and involuntary childlessness between the groups. The proportion of men who were voluntary childless was statistically significant different (29 (24.79), 24 (18.32) and 46 (14.64), p=0.048). The proportion of men who were involuntary childless was also statistically significant different between our groups (16 (12.03%), 15 (10.34%) and 13 (3.98%), p=0.001). Among childless men, the percentage of men who were involuntary childless was statistically significant between our groups (35.56% vs 38.46% vs 22.03%, p=0.046).

Desired number of children and family planning

The desired number of children was not statistically different between the three groups (1.75 (SD 1.32) vs 1.86 (SD 1.22) vs 2.03 (SD 1.18), p=0.083). Statistically significant more men diagnosed ≤31 years and 31–40 years reported feeling unsatisfied with their final number of children than men diagnosed ≥41 years (n=22 (16.67%), n=14 (9.66%) and n=18 (5.50%), p=0.010). Approximately one-third of these men reported that the diagnosis of IA and/or the medical treatment associated with it, were the main reason to have less children (31% and 28%, respectively).

The difference between desired and final number of children was significantly wider in men diagnosed ≤30 years (0.41 (SD 0.98)) compared with men diagnosed ≥41 years (0.14 (SD 0.77), p=0.003). Compared with men diagnosed 31–40 years, the difference between desired and final number of children was not statistically significant different (0.29 (SD 0.74), p=0.181) (see figure 2).

Furthermore, to analyse the impact of IA on the fertility rate of men who wanted to become a father, we conducted a subgroup analysis where all men who were voluntary childless were excluded (see table 3).

Using a Likert scale questionnaire, a significant negative effect of IA on family planning was reported by men diagnosed ≤30 and 31–40 years (see figure 3). Statements such as 'I was concerned that my medications would harm my child' or 'I was afraid that my child would get the same disease as me' were graded with a significantly higher degree of agreement among men diagnosed ≤30 and 31–40 years.

Moreover, among men who remained voluntary childless, the statement 'My disease reduced my desire to have children' was graded higher by men diagnosed ≤30 years (5.93 (2.42)) than by men diagnosed 31–40 years (3.73 (1.91)) and by men diagnosed ≥41 years (1.35 (1.14)). This was statistically significant different (p=0.001).Among men who remained involuntary childless and compared with men diagnosed ≥41 years, the statement 'Stopping of weaning off my medication because of my desire to have children was not possible because my disease was too active' was graded statistically significant higher by men diagnosed ≤30 years (see figure 4).

Fertility

Statistically significantly more men diagnosed ≤30 and 31–40 years reported having received medical evaluations for fertility problems, compared with men diagnosed ≥41 years (n=27 (20.61%), n=30 (20.69%) and n=35 (11.36%), p=0.027) and ultimately receiving a diagnosis of low sperm quality (n=9 (6.57%), n=12 (8.05%) and n=12 (3.51%), p=0.086). Statistically significant more female partners of men diagnosed ≤30 years received a diagnosis of infertility secondary to an unknown cause (see table 4).

In men who achieved a pregnancy, TTP was statistically significant higher in men diagnosed 31–40 years (6.74 (SD 11.12) months) compared with men diagnosed ≤41 years (4.77 (SD 8.47) months, p=0.045) and not statistically significantly different when compared with men diagnosed ≤30 years (5.69 (SD 10.93), p=0.623).

DISCUSSION

Our study is the first of its kind to demonstrate that IA can significantly impair male fertility. The diagnosis of IA before or during the peak of the male reproductive age was associated with a lower fertility rate, lower number of pregnancies, higher rates of involuntary childlessness and fertility problems.

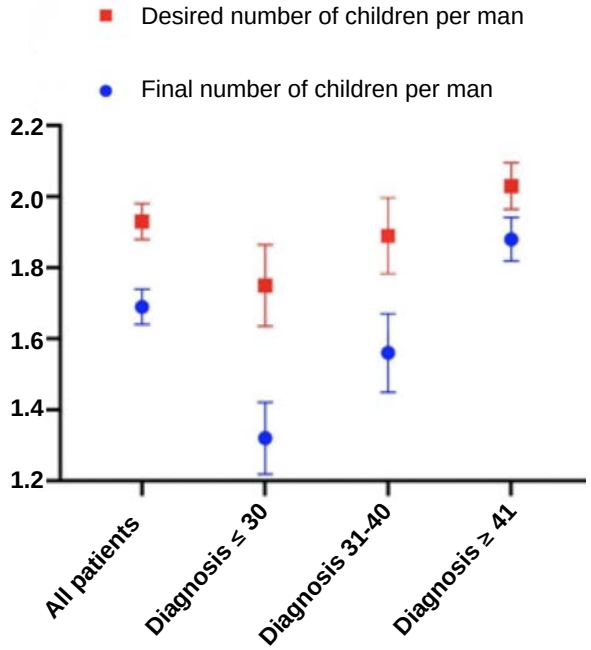

Figure 2 Comparison of the desired and final number of children per man for all participants and per group (mean+95% CI).

Table 3 Analysis of covariance: effect of dichotomised age at diagnosis of IA (based on our study groups) on total number of children per man (excluding men who were voluntary childless) and considering the total number of children of men diagnosed ≥41 years as our reference group

	Crude (n=507)		Adjusted* (n=501)	
	B (95% CI)	P value	B (95% CI)	P value
31–40 years	−0.279 (−0.501 to −0.058)	0.013	−0.205 (−0.434 to 0.022)	0.078
≤30 years	−0.474 (−0.702 to −0.246)	0.000	−0.352 (−0.550 to −0.113)	0.004

*Adjusted for confounders (age at inclusion in the study, education level, cardiovascular disease, diagnosis of infertility in partner and diagnosis of RA, JIA and SpA).
IA, inflammatory arthritis; JIA, juvenile idiopathic arthritis; RA, rheumatoid arthritis; SpA, spondyloarthritis.

Respecting family planning we observed that the number of desired children per man was lower in men diagnosed before and during the peak of male reproductive age. Nonetheless, this was not statistically significant different between our groups and it was similar to the number of desired children per man reported for the general population of the Netherlands (1.81–2.29).[26] Conversely, the difference between the desired and final number of children was significantly larger in men diagnosed before and during the reproductive age, indicating that the lower fertility rates are primarily affected by reduced fertility potential and not by a reduced desire for parenthood.

In this regard, men diagnosed with IA before and during the peak of their reproductive age were two times more likely to remain involuntary childless (12% and 10%). To put this into perspective, it is estimated that around 4% of healthy couples who want children remain involuntary childless.[27]

Moreover, it was shown that the diagnosis of IA may have a major impact on family planning. Not only did IA significantly reduce the desire to have children of men diagnosed before and during the peak of reproductive age who remained voluntary childless but also concerns or difficulties with regard to pharmacological treatment were larger in men diagnosed with IA before the peak of reproductive age who remained involuntary childless.

Lastly, the diagnosis of IA before and during the peak of reproductive age is associated with male fertility problems. These men were twice as likely to be evaluated for fertility problems and being subsequently diagnosed with abnormal sperm quality. In this regard, it has been estimated that abnormal sperm quality affects 2% of adult men.[28] This estimation is considerably lower compared with the 6.5% and 8% reported by men diagnosed with IA before and during the peak of reproductive age.

Similar to our results, Uzunaslan *et al* reported that, compared with healthy men, men diagnosed with AS had statistically significant fewer children (1.9 vs 2.5) and a higher rate of infertility (9.1 vs 2.9%).[29] These findings could be in part explained by the high incidence of varicocele and sperm abnormalities that have been reported for men diagnosed with AS.[6 30 31] Nonetheless, this study was primarily designed to study the impact of Behçet's syndrome on male fertility and only included 79 male patients diagnosed with AS.

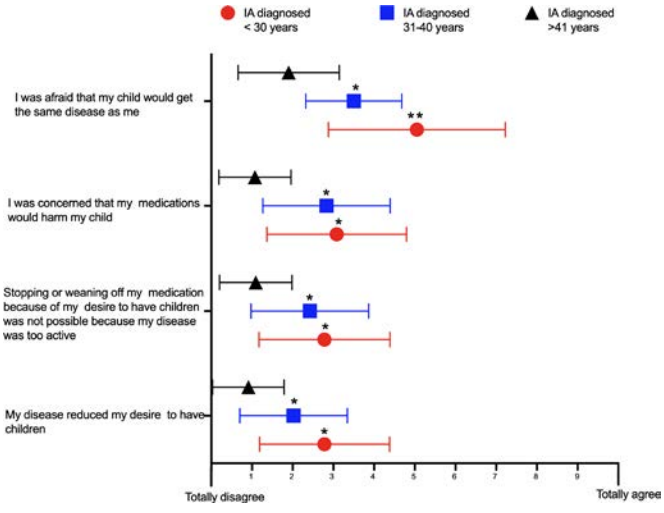

Figure 3 Likert scale questionnaire regarding the influence of Ia on family planning. men answered the questions using a 0–10 scale where 0 meant 'totally disagree' and 10 'totally agree' (mean with SD) *P≤ 0.05 compared with those diagnosed age ≥41 years. **P≤0.05 compared with those diagnosed 31–40 years and ≥41 years. IA, inflammatory arthritis.

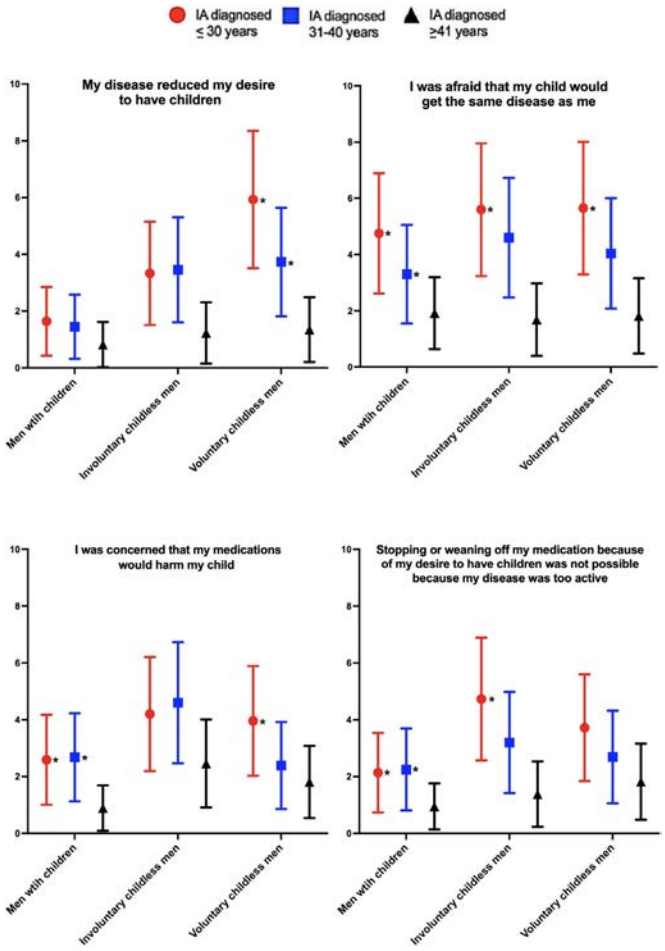

Figure 4 Comparison of the reported impact of Ia on different aspects of family planning in men with children, involuntary and voluntary childless men. A Likert scale with 0 meaning 'totally disagree' and 10 'totally agree' was used (mean with SD). *p≤ 0.05 compared with those diagnosed age ≥41 years. IA, inflammatory arthritis.

Table 4 Fertility evaluation

	All patients (N=628)	IA diagnosed ≤30 years (N=137)	IA diagnosed 31–40 years (N=149)	IA diagnosed ≥41 years (N=342)	P value
Fertility					
Male fertility evaluation, n (%)	93 (15.74)	27 (20.61)*	30 (20.69)*	35 (11.36)	0.027
Female fertility evaluation (partner), n (%)	71 (15.04)	18 (18.56)	24 (20.69)	29 (11.42)	0.069
Male fertility evaluation outcome					
No male fertility problem identified, n (%)	47 (7.48)	14 (10.22)	14 (9.40)	19 (5.56)	0.129
Low sperm quality, n (%)	33 (5.45)	9 (6.77)	12 (8.22)	12 (3.67)	0.086
Infertility secondary to unknown cause, n (%)	7 (1.16)	3 (2.26)	3 (2.05)	1 (0.31)	0.105
Female fertility evaluation outcome					
No female fertility problem identified, n (%)	34 (5.41)	8 (6.02)	11 (7.53)	15 (4.59)	0.066
Female infertility secondary to known cause‡, n (%)	24 (3.96)	6 (4.51)	9 (6.16)	9 (2.75)	0.199
Female infertility secondary to unknown cause, n (%)	7 (1.16)	4 (3.01)*	2 (1.37)	1 (0.31)	0.047

*P≤0.05 compared with those diagnosed age ≥41 years.
†P≤0.05 compared with those diagnosed age ≥31–40 years.
‡Endometriosis, fallopian tube obstruction, polycystic ovary syndrome, uterine abnormality, early menopause.
IA, inflammatory arthritis.

Multiple mechanisms can be responsible for our findings. Biological mechanisms, namely inflammation, may contribute to the impaired fertility in men with IA. Several cytokines that are characteristic of the immune response associated with IA, such as tumour necrosis factor (TNF), play important roles in modulating testicular homoeostasis and regulating spermatogenesis.[32 33] Increased expression of messenger RNA for interleukin-1-beta, TNF and interferon-gamma has been observed in testicular tissue of men with disturbed spermatogenesis.[34] Correspondingly, inflammation may impair normal reproductive development before or during puberty, or have a direct negative impact on the spermatogenesis during the reproductive age.[35–40]

Beyond inflammation, pharmacological treatment associated with IA can also result in damage to the male reproductive axis.[41 42] Moreover, side effects such as hypogonadism and low sperm quality have been associated with frequently used immunosuppressive agents.[13] It has been estimated that among involuntary childless men that present to infertility clinics, 25% take drugs that have the potential to negatively impact male sexual function and 10% take drugs associated with male fertility impairment.[42]

Furthermore, several psychosocial factors, associated with a diagnosis of IA, may have contributed to the lower fertility rate as observed in this study.[43] In our study, due to problems or concerns associated with IA and its treatment and based on medical advice (or the lack of), men with IA and their partners decided to become voluntarily childless or to delay their plans to become parents. These psychosocial factors were of special importance for men diagnosed before the peak of reproductive age. Moreover, some of these psychosocial factors could be associated with psychological comorbidities that are highly prevalent in patients diagnosed with IA such as depression and anxiety. These comorbidities have also been associated with sexual health problems.[44–46]

Our study has several strengths. It is the first large study (≥600 participants) specifically designed to detect statistically significant differences in a robust outcome measure (fertility rate). In addition, we used an extensive questionnaire to gain insight into most of the factors that might have influenced our primary outcome measure. Our study has important limitations. First, our response rate was low. However, the response rate is comparable to similar studies that explored male fertility rate in chronic diseases.[22] Second, men diagnosed with chronic diseases and especially those who use pharmacological therapy are more aware of potential fertility problems[47 48] and it can be expected that these men are more likely to seek fertility evaluation. Furthermore, men who experience fertility problems might be more willing to participate in these type of studies. Both factors are potential sources of selection bias in our study. In this respect, in the Netherlands, strict healthcare policies and referral guidelines reduce the possibility of self-referrals or unnecessary fertility evaluations. It is also reassuring that the response rates were similar between the three groups of men and that the results from our control group, men diagnosed ≥41 years, were strikingly similar to the data available in the general population further strengthening our comparisons. Lastly, this was a retrospective study. Recently, it has been shown that the sperm quality of male patients diagnosed with AS improved after being treated with TNF-α inhibitors.[49 50] Furthermore, to get approval, new drugs are facing more strict protocols with regard to testicular toxicity. Therefore, the current conditions for men with IA, regarding treatment options and treatment strategies (biological therapy, shared-decision process, treat to target strategies), might be different than they were when our participants were in the peak of their reproductive age.

The results of this study may have several implications. In the clinical setting, rheumatologists should be aware that IA and/or the pharmacological treatment associated with IA may impair male fertility. Accordingly, they should discuss this with their patients, inform them about the impact of IA on male fertility and if indicated, adjust treatment aiming at low disease activity with the safest treatment strategy possible.[6 50] For research purposes, basic, translational and epidemiological studies are needed to understand the impact of inflammation, pharmacological treatment and psychosocial factors associated with IA on male fertility. To corroborate our findings and to further describe the magnitude of the impact of IA on male fertility, large prospective studies are strongly recommended.

In conclusion, the diagnosis of IA before or during the peak of reproductive age can result in impaired male fertility. Rheumatologists should be aware of this novel association and approach their patients accordingly. Multiple biological and non-biological mechanisms can be responsible for this

association and more research is urgently needed to improve the quality of care for men diagnosed with IA and a desire for parenthood.

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CLINICAL SCIENCE

Role of ultrasound for assessment of psoriatic arthritis patients with fibromyalgia

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ABSTRACT

Objective To investigate whether ultrasonography (US), as an objective imaging modality, can optimise the evaluation of disease activity in psoriatic arthritis (PsA) patients with concomitant fibromyalgia syndrome (FMS).

Methods The study population included 156 consecutive PsA patients who were recruited prospectively and fulfilled the ClASsification criteria for Psoriatic ARthritis criteria. The patients underwent complete clinical evaluation including assessment of fulfilment of the 2016 fibromyalgia classification criteria. All of the patients underwent US evaluation including 52 joints, 40 tendons and 14 entheses. The US score was based on the summation of a semiquantitative score (including synovitis, tenosynovitis and enthesitis). Scoring was performed by a sonographer blinded to the clinical data. Spearman's correlation coefficient and multivariate linear regression models were used to examine the association of FMS with clinical and the US scores.

Results Forty-two patients (26.9%) with coexisting PsA and FMS were compared with 114 (73.1%) PsA patients without FMS. Patients with PsA and FMS had significantly increased scores for clinical composite indices, including non-Minimal Disease Activity, Composite Psoriatic Disease Activity Index (CPDAI), Disease Activity for Psoriatic Arthritis (DAPSA) and Psoriatic Arthritis Disease Activity Score (PASDAS) ($p<0.001$). In contrast, the total US score and its subcategories were similar for those with and without FMS. The total US score significantly correlated with CPDAI, DAPSA and PASDAS ($p<0.001$) in the PsA without FMS but not in the PsA with FMS group. FMS was significantly associated with higher clinical scores ($p<0.001$) but not with the US score (multivariable linear regression models).

Conclusions US has significantly greater value than composite clinical scores in the assessment of disease activity in PsA patients with FMS.

Key messages

What is already known about this subject?

- Patients with psoriatic arthritis (PsA) and concomitant fibromyalgia syndrome (FMS) have significantly increased scores for clinical composite indices compared to PsA without FMS.

What does this study add?

- The ultrasonography (US) score did not demonstrate differences between PsA patients with and without FMS.
- The presence of FMS was associated with higher clinical scores but not with US scores.

How might this impact on clinical practice or future developments?

- US has significantly greater value than composite clinical scores in the assessment of disease activity in PsA patients with coexisting FMS.
- US can serve as an objective tool for assisting in PsA evaluation by reflecting disease activity regardless of the presence of FMS.

INTRODUCTION

Psoriatic arthritis (PsA) is an inflammatory musculoskeletal (MSK) disease affecting up to one-third of psoriasis patients.[1] PsA may involve the peripheral MSK system as well as the axial skeleton. The peripheral involvement includes synovitis, dactylitis, tenosynovitis and enthesitis.[1] In line with the concept of treat to target, clinical assessment is the recommended way to evaluate PsA patients, and it is based on the evaluation of tender and swollen joints, enthesitis and patient-reported outcomes. Treatment options range from non-steroidal anti-inflammatory drugs (NSAIDs) to small molecules and biological drugs.[1]

Fibromyalgia syndrome (FMS) is a chronic pain syndrome that can present in isolation or concomitantly with inflammatory joint disease.[2] Several studies on PsA showed a prevalence of coexisting FMS ranging between 18% and 25%.[3–5] Those studies demonstrated higher clinical scores in patients that had both PsA and FMS compared with those with PsA alone.

Clinical enthesitis is a hallmark of PsA.[6 7] It is routinely evaluated by applying pressure on accessible entheseal points.[8] Similarly, the evaluation of FMS includes applying pressure on pre-defined fibromyalgia tender points, and some entheseal points are located close to the FMS tender points, making the differentiation between these disease entities challenging.[9]

Ultrasonography (US) is an imaging modality that is gaining increasing popularity in rheumatology due to its bedside utilisation, ability to assess different sites at a single evaluation and affordable price.[10 11] Several studies have shown the greater value of US over physical examination.[10–13] Moreover, studies on enthesitis demonstrated better sensitivity and specificity of US compared with physical examinations.[14 15] Accordingly, the EULAR (European League against Rheumatism) recommendations for imaging in spondyloarthritis emphasised that US can be used for diagnosis and disease monitoring of peripheral involvement.[16] However, there is only one study that examined the use of US in PsA patients with concomitant FMS.[17] That study included a relatively small number of patients and evaluated only enthesitis but no other important features of PsA, such as synovitis or tenosynovitis. The aim of this study was to examine whether US is superior to composite clinical scores for the evaluation of disease activity in PsA patients with concomitant FMS by serving as an objective tool that is not influenced by the presence of FMS.

METHODS

Patients and setting

The study population included consecutive PsA patients that were recruited prospectively between July 2018 and July 2020. All of the patients fulfilled the ClASsification criteria for Psoriatic ARthritis (CASPAR).[18] The study was conducted at the Department of Rheumatology of the Tel-Aviv Medical Center (Tel-Aviv, Israel) which serves as a primary, secondary, and tertiary referral centre providing medical service to PsA patients with a wide range of disease activity and severity.

Clinical assessment

All of the study patients underwent a complete clinical assessment by two experienced rheumatologists (OE and VF) according to a standardised protocol that included demographics and disease characteristics. The physical examination included evaluation of the body mass index (BMI), 66/68 joint count, presence of dactylitis, count of enthesitis by the Leeds and Spondyloarthritis Research Consortium of Canada (SPARRC) enthesitis indices, body surface area and psoriasis severity area (PASI) for psoriasis evaluation. Physician and patient global assessment (PhGA, PGA) and pain assessment were evaluated by a Visual Assessment Scale (VAS) of 0–10. The patients filled in the Health Assessment Questionnaire-Disability Index (HAQ-DI) and the Short-Form Health Survey (SF-36), the Dermatology Life Quality Index (DLQI), the ankylosing spondylitis quality of life (ASQOL) questionnaire, the Beck questionnaire for assessment of depression and the Functional Assessment of Chronic Illness Therapy (FACIT) questionnaire for evaluation of fatigue. Blood tests for C reactive protein (CRP) and erythrocyte sedimentation rate (ESR) were performed as well.

Four clinical disease activity indices were used as follows (1) Minimal Disease Activity (MDA) in PsA was calculated based on fulfilment of at least five out of the seven following criteria: tender joint count (TJC) ≤1, swollen joint count (SJC) ≤1, PASI ≤1, patient pain VAS ≤15, patient global disease activity VAS ≤20, HAQ-DI ≤0.5 and tender entheseal points ≤1.[19] (2) Disease Activity for Psoriatic Arthritis (DAPSA) was calculated based on the calculation of the sum of the TJC, SJC, CRP (mg/dL), patient assessment of pain VAS and PGA VAS.[20] (3) The Composite Psoriatic Disease Activity Index (CPDAI) was based on five domains (joints, skin, entheses, dactylitis and axial disease) that were evaluated by TJC, SJC, HAQ-DI, PASI, DLQI, dactylitis and enthesitis count and Bath Ankylosing Spondylitis Disease Activity Index and ASQOL.[21] (4) The Psoriatic Arthritis Disease Activity Score (PASDAS) was based on a formula that included TJC, SJC, CRP (mg/L), Leeds Enthesitis Index (LEI), dactylitis, patient global VAS, physician global VAS and the SF-36 physical component summary score.[22 23] The scoring methodology is provided in detail in the supplement.

Fibromyalgia assessment included the tender point count, Widespread Pain Index (WPI) and Symptom Severity Scale (SSS) score. Patients were classified as having fibromyalgia according to the 2016 fibromyalgia classification criteria[2] and filled in the Fibromyalgia Impact Questionnaire.

US assessment

On the same day of the clinical assessment, all of the patients underwent US evaluation by a single rheumatologist (AP) with 5 years of experience in MSK US. The scanning was performed with the Affinity 50 US device (Philips Healthcare, Washington state, USA), equipped with a high frequency, 5–18 MHz, linear transducer for superficial structures. Power Doppler (PD) settings were standardised with a Doppler frequency of 8 MHz (gain was adjusted until the background signal was removed), a pulse repetition frequency (PRF) of 700 Hz and a low wall filter. For deeper structures, an additional linear transducer with a 5–12 MHz frequency, a Doppler frequency of 6.7 MHz and a PRF of 700 Hz was used. The patients were asked to stop NSAIDs 3 days before the clinical and US assessments. No patient was treated with glucocorticoids. Analgesics were permitted, 10 (6.3%) patients used paracetamol, 6 (3.8%) tramadol and 6 (3.8%) medical cannabis (these drugs are not expected to affect the sonographic findings).

The US scanning was performed in a darkened room and both B-Mode (grey scale) and Doppler were used according to a standardised protocol that included 52 joints, 40 tendons and 14 entheses points. The scanned joints included: wrist, radio-ulnar, metacarpophalangeal, proximal phalangeal and distal phalangeal, elbow, knee (supra-patellar recess), ankle, talo-navicular, subtalar and metatarsophalangeal. The scanned tendon included: the six wrist extensor compartments, 5 extensor tendons of the

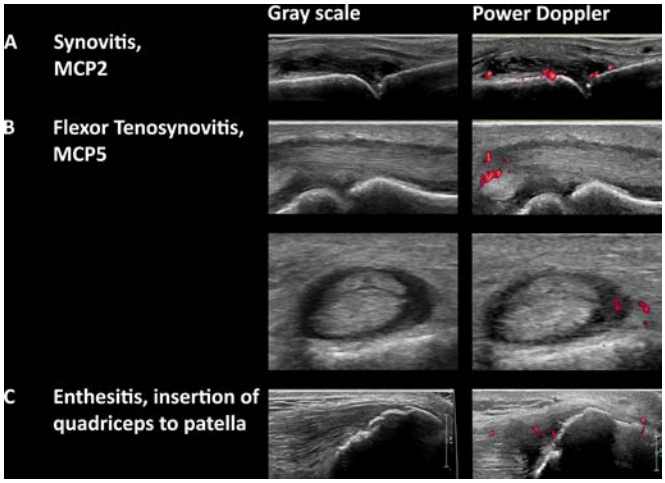

Figure 1 US scan of synovitis, tenosynovitis and enthesitis. (A) Grey scale and PD of MCP2 synovitis (grade three synovial hypertrophy and grade 2 PD). (B) Grey scale and PD of MCP5 flexor tenosynovitis (grade 2 tenosynovitis and grade 1 PD). (C) Enthesitis at the insertion of the quadriceps to the proximal patella (evidence of hypoechogenicity, thickening, MCP, metacarpophalangeal; PD, power Doppler; US, ultrasonography

Table 1 Demographics and clinical characteristics

Characteristics	PsA without FMS n=114 (73.1%)	PsA with FMS n=42 (26.9%)	P value
Age, mean (±SD)	51.1 (13.5)	54.4 (12)	0.17
Sex, female, n (%)	60 (52.6)	28 (66.7)	0.17
BMI, mean (±SD)	27.3 (5.1)	28.7 (5.1)	0.14
Smoking history, n (%)	42 (37.8)	20 (37.7)	0.22
Employed, n (%)	92 (80.7)	15 (36.6)	<0.001
Education, academic, n (%)	95 (83.3)	27 (64.3)	0.025
PSO duration, mean (±SD)	19.0 (14.6)	17.7 (12.3)	0.61
PsA duration, mean (±SD)	11.1 (11.9)	11.7 (11.4)	0.75
TJC, mean (±SD)	5.6 (6.1)	16.8 (13.6)	<0.001
SJC, mean (±SD)	1.1 (2.5)	1.5 (3.6)	0.49
Leeds enthesitis, mean (±SD)	0.6 (1.1)	2.5 (1.9)	<0.001
SPARCC enthesitis, mean (±SD)	1.4 (1.9)	6.3 (4.3)	<0.001
Dactylitis (≥1) (%)	11 (9.6)	3 (7.1)	0.86
PASI, mean (±SD)	1.8 (5.1)	1.3 (2.2)	0.57
GPhA, mean (±SD)	1.6 (1.8)	3.2 (2.4)	<0.001
PGA, mean (±SD)	4.3 (2.7)	8.1 (2.1)	<0.001
Pain, mean (±SD)	4.0 (2.9)	7.8 (1.9)	<0.001
CRP mg/L, mean (±SD)	7.7 (15.7)	9.8 (9.2)	0.43
ESR, mm/hour, mean (±SD)	20.3 (15.0)	29.1 (18.7)	0.007
HAQ, mean (±SD)	0.56 (0.64)	1.76 (0.6)	<0.001
SF36 (PCI), mean (±SD)	69.4 (17.8)	42. (23.8)	<0.001
SF36 (MCI), mean (±SD)	66.1 (25.3)	22.7 (17.3)	<0.001
FACIT, mean (±SD)	34.8 (10.6)	15.2 (8.4)	<0.001
Depression, mean (±SD)	8.6 (7.4)	24.8 (12.1)	<0.001
FIQ, mean (±SD)	37.6 (24.9)	91.2 (24.7)	<0.001
MDA, n (%)	52 (45.6)	1 (2.4)	<0.001
CPDAI, mean (±SD)	6.8 (3.7)	11.6 (1.2)	<0.001
DAPSA, mean (±SD)	15.9 (11.3)	35.1 (17.2)	<0.001
PASDAS	2.7 (1.8)	5.8 (1.7)	<0.001
WPI	1.9 (2.5)	11.5 (4.5)	<0.001
SSS	2.4 (2.3)	8.6 (2.8)	<0.001
Tender points, mean (±SD)	1.4 (2.7)	9.5 (5.5)	<0.001
Treatment			
csDMARDs, n (%)	48 (42.1)	19 (45.2)	0.87
Otezla, n (%)	3 (2.6)	1 (2.4)	1
Biologics, n (%)	62 (54.4)	23 (54.8)	1

BMI, body mass index; CPDAI, Composite Psoriatic Disease Activity Index; CRP, C reactive protein; csDMARDS, classical disease-modifying antirheumatic drugs; DAPSA, Disease Activity for Psoriatic Arthritis; ESR, erythroctye sedimentation rate; FIQ, Fibromyalgia Impact Questionnaire; FMS, fibromyalgia syndrome; GPhA, global physician activity; HAQ, Health Assessment Questionnaire; MDA, minimal disease activity; PASDAS, Psoriatic Arthritis Disease Activity Score; PASI, Psoriasis Area Severity Index; PGA, patients global activity; PsA, psoriatic arthritis; PSO, psoriasis; SF-36, Short-Form Health Survey; SJC, swollen joint count; SSS, Symptom Severity Index; TJC, tender joint count; WPI, Widespread Pain Index.

fingers, 5 flexor tendons of the fingers, peroneal tendons and the tibialis posterior, flexor digitorum longus and flexor halluces longus in the medial aspect of the ankle. The scanned entheses included 12 sites according to the modified MAdrid Sonographic Enthesis Index (MASEI)[24]: triceps insertion to olecranon, quadriceps insertion to proximal patella, patellar tendon insertion to distal patella and tibial tuberosity, Achilles and plantar fascia insertions to calcaneus and common extensor tendon to lateral epicondyle. All the mentioned above joints, tendon and enthuses were scanned bilaterally.

Synovitis was defined according to the European League against Rheumatism-Outcome Measures in Rheumatology (EULAR-OMERACT) definition as a hypoechoic intracapsular area regardless of the presence of effusion and with or without PD (figure 1A).[25] Tenosynovitis was defined according to the OMERACT US working group definitions as an anechoic or hypoechoic tendon sheath widening around the flexor tendon with or without PD (figure 1B).[26] Extensor paratenonitis of the fingers was defined as anechoic or hypoechoic thickened tissue surrounding the extensor tendon with or without PD.[27] Enthesitis (both inflammatory and structural lesions) was analysed according to the MASEI system (figure 1C).[24] The techniques of scanning and grading of each MSK structure are described in detail in the supplement.

The US scans were saved and scored within 1 week of assessment. The US reader (AP) was blinded to the clinical data. The total US score (including both grey and Doppler) could range between 0–659 and was based on the summation of synovitis (0–312), tenosynovitis (0–200) and enthesitis (0–147). An intra-reader agreement analysis was performed by reading and scoring the scans of 10 patients after 3 months from the initial reading. The intrareader agreement value was 0.95 with a prevalence-adjusted biased-adjusted kappa (PABAK) of 0.9 for all the grey scale MSK lesions, and 0.99 with a PABAK of 0.99 for all the Doppler. The detailed intra-agreement according to the different lesions (synovitis, tenosynovitis and enthesitis) at the different locations is provided in the supplement.

Statistical analysis

Descriptive statistics for the data included mean and SD for continuous variables and frequencies and relative frequencies for categorical variables. Continuous variables were compared between the PsA with and without FMS groups using the independent sample t-test, and the χ^2 test for independence for categorical variables.

Association between clinical activity indices and sonographic scores was tested using Spearman's correlation coefficient for each study group. Differences between Spearman's coefficients among patients with and without FMS were tested with the relevant z-test after applying Fisher's r-to-z transformation.

Multivariate linear regression models were constructed to predict various clinical activity indices and the total US score, with the presence of FMS as the studied predictor, adjusted for known confounders. Values were expressed by regression coefficient and their 95% CI. Inter-rater agreements were calculated by both Cohen's Kappa and PABAK.

The study had a power of approximately 80% with a 2-sided type I error to 0.05 to detect a mean difference of 7 points (a minimal clinically significant difference) in the total US score in favour of the FMS group. Where the assumption was of mean of 30 points for the non-FMS group and 37 points for the FMS group, each with an SD of 10 points, corresponding with an effect size of 0.47 (Cohen's d) and allocation ratio of 2:1 (non-FMS:FMS). All analyses were performed by RStudio Version 1.2.5001. A two-sided $p<0.05$ was considered statistically significant.

RESULTS

Demographics and clinical disease characteristics

One hundred and fifty-six patients that completed the study were divided into 114 (73.1%) with PsA without FMS and 42 (26.9%) PsA with FMS. Both groups were similar in demographic variables, with the exceptions of lower working class and education status in the PsA with FMS group compared with the PsA without FMS group ($p<0.001$ and $p=0.025$, respectively)

Table 2 Sonographic scores of psoriatic arthritis (PsA) patients without fibromyalgia syndrome (FMS) versus PsA patients with FMS

Score	PsA without FMS n=114	PsA with FMS n=42	P value
Total US score*, mean (±SD)	35.9 (22.9)	37.6 (19.1)	0.68
Total Grey scale score, mean (±SD)	32.3 (19.8)	33.7 (16.9)	0.69
Total power Doppler score, mean (±SD)	4.9 (6.3)	5.3 (6.1)	0.79
Synovitis* score, EULAR-OMERACT score, mean (±SD)	12.2 (10.2)	11.6 (8.9)	0.73
Synovitis Grey scale score, mean (±SD)	12.0 (10.0)	11.9 (8.8)	0.94
Synovitis power Doppler score, mean (±SD)	1.4 (2.2)	1.5 (2.9)	0.8
Tenosynovitis score, mean (±SD)	3.6 (4.9)	4.0 (4.5)	0.63
Tenosynovitis Grey scale score, mean (±SD)	2.5 (3.8)	2.8 (3.4)	0.63
Tenosynovitis power Doppler score, mean (±SD)	1.1 (1.9)	1.2 (1.7)	0.77
Enthesitis score, mean (±SD)	20.4 (14.5)	21.3 (11.6)	0.71
Enthesitis Grey Scale score, mean (±SD)	17.8 (12.6)	18.7 (10.1)	0.71
Enthesitis power Doppler score, mean (±SD)	2.6 (3.7)	2.6 (3.3)	0.9

*Synovitis was based on the EULAR-OMERACT score.
US, ultrasonography.

(table 1). Several clinical variables, such as TJC, enthesitis count, PGA, GPhA, pain level, as well as disease activity indices (including non-MDA, CPDAI, DAPSA and PASDAS) were significantly higher in the PsA with FMS group compared with the PsA without FMS group (p<0.001). In addition, outcome as patient-reported HAQ, SF36, pain, fatigue and depression were significantly higher in the PsA with FMS group compared with the PsA without FMS group (p<0.001).

Comparison of sonographic findings of PsA without FMS to PsA with FMS

Comparisons of all the US scores were similar for PsA patients with or without FMS, including the total US score and its subcategories of synovitis, tenosynovitis and enthesitis scores and their breakdown to grey scale and Doppler scores (table 2).

Correlation of US scores with clinical activity indices in PsA with and without FMS

The total US score and its components (grey scale and Doppler) correlated significantly with several clinical activity indices, including CPDAI, DAPSA and PASDAS (p<0.01) in the PsA without FMS group (table 3). In contrast, only the grey scale US score correlated with the DAPSA in the PsA with FMS group (p<0.05), while all the other correlations were non-significant.

Table 3 Correlations of US score with clinical activity indices in psoriatic arthritis (PsA) with and without fibromyalgia syndrome (FMS)

Scores and indices	PsA without FMS	PsA with FMS
US SCORE and DAPSA		
Total US score and DAPSA	0.37*	0.31
GS US score and DAPSA	0.34*	0.31**
PD US score and DAPSA	0.35*	0.24
US SCORE and CPDAI		
Total US score and CPDAI	0.39*	−0.06
GS US score and CPDAI	0.37*	−0.04
PD US score and CPDAI	0.31*	−0.06
US SCORE and PASDAS		
Total US score and PASDAS	0.41*	0.2
GS US score and PASDAS	0.38*	0.26
PD US score and PASADS	0.34*	0.08

*P<0.001, **p<0.05.
CPDAI, Composite Psoriatic Disease Activity Index; DAPSA, Disease Activity for Psoriatic Arthritis; GS, grey scale; PASDAS, Psoriatic Arthritis Disease Activity Score; PD, power Doppler; US, ultrasonography.

Association of fibromyalgia with various clinical activity indices and total US score

A multivariable linear regression model showed that PASI (p=0.03) and the presence of FMS (p<0.001) were associated with the DAPSA (table 4). Another similar model showed that SJC and FMS (p<0.001) were associated with the CPDAI. In addition, BMI (p=0.03), SJC and the presence of FMS (p<0.001) were associated with the PASDAS. Finally, age, SJC and CRP (p<0.001) were associated with the total US score but the presence of FMS was not.

DISCUSSION

The evaluation of PsA patients with concomitant FMS may pose a substantial challenge to the rheumatologist.[28 29] The clinical impression might be misleading, attributing active PsA to FMS-related symptoms, or attributing inactive PsA to active disease based on widespread pain and tenderness secondary to FMS. Accordingly, the consequences of these situations could lead to an unjustified continuation of the same treatment in the former setting or unnecessary switch to a different one in the latter.[28 29] The current study demonstrated that US can serve as an objective tool for assisting in the evaluation of PsA by reflecting disease activity regardless of the coexistence of FMS.

The present study highlighted the problem of evaluating disease activity of PsA patients with FMS by showing significantly fewer patients in MDA and increased scores for clinical composite indices, including CPDAI, DAPSA and PASDAS as well as more fatigue and depression and worse patients reported outcome as pain, patient global, HAQ and SF-36 compared with PsA without FMS. Similarly, Brikman *et al*'s cross-sectional study of 73 PsA patients showed that those with both PsA and FMS never achieved MDA and had significantly higher disease activity indices, such as DAPSA and CPDAI, compared with those without FMS.[3] Iannone *et al*' recent report on a longitudinal cohort that included 238 patients showed that those with the combination of PsA and FMS had significantly higher disease activity, with higher TJC and DAPSA and more functional disability according to HAQ, compared with the PsA group without FMS.[4] In addition, rates of remission and MDA were

Table 4 Multivariable linear regression model for association with Clinical Activity Indices* and total ultrasound score

Variables	DAPSA* regression coefficient (95% CI)	P value	CPDAI Regression coefficient (95% CI)	P value	PASDAS regression coefficient (95% CI)	P value	US Score regression coefficient (95% CI)	P value
Age	0.1 (–0.08 to 0.29)	0.3	0.02 (–0.02 to 0.07)	0.26	0.01 (–0.01 to 0.03)	0.33	0.47 (0.22 to 0.73)	<0.001
Sex	–2.61 (–6.96 to 1.74)	0.25	0.69 (–0.37 to 1.74)	0.2	0.04 (–0.47 to 0.56)	0.87	0.92 (–5.17 to 7.00)	0.77
BMI	0.19 (–0.23 to 0.61)	0.37	0.07 (–0.03. 0.17)	0.14	0.05 (0.006 to 0.10)	0.03	0.41 (–0.17 to 0.99)	0.16
Psoriasis duration	–0.03 (–0.19 to 0.14)	0.75	0.03 (–0.001 to 0.07)	0.13	0.003 (–0.02 to 0.02)	0.97	0.18 (–0.04 to 0.40)	0.11
FMS	19.50 (14.73 to 24.27)	<0.001	4.40 (3.21 to 5.56)	<0.001	2.93 (2.37 to 3.49)	<0.001	–2.9 (–9.60 to 3.77)	0.39
PASI	0.52 (0.06 to 0.99)	0.03	0.11 (–0.02 to 0.25)	0.11	0.05 (–0.02 to 0.12)	0.16	0.13 (–0.64 to 0.91)	0.74
CRP			0.16 (–0.28 to 0.61)	0.47	0.20 (–0.02 to 0.42)	0.08	5.30 (2.80 to 7.72)	<0.001
SJC			0.37 (0.19 to 0.55)	<0.001	0.31 (0.22 to 0.40)	<0.001	2.96 (1.91 to 4.01)	<0.001
Current sDMARDs	–2.31 (–6.76 to 2.13)	0.3	–0.96 (–2.03 to 0.11)	0.07	–0.29 (–0.80 to 0.23)	0.27	–3.79 (–9.90 to 2.32)	0.22
Current Biologics	–4.67 (–4.67 to 3.88)	0.85	–0.02 (–1.10 to 1.03)	0.97	0.04 (–0.47 to 0.55)	0.88	–0.04 (–6.00 to 5.90)	0.99

Clinical Activity Indices—DAPSA, CPDAI, PASDAS.

*The DAPSA includes SCJ and CRP and hence these variables were not included in this model

BMI, body mass index; CPDAI, Composite Psoriatic Disease Activity Index; CRP, C reactive protein; DAPSA, Disease Activity for Psoriatic Arthritis; FMS, fibromyalgia syndrome; PASDAS, Psoriatic Arthritis Disease Activity Score; PASI, psoriasis severity area; SCJ, swollen joint count; sDMARD, classical disease-modifying antirheumatic drugs.

significantly lower in the FMS and PsA group compared with PsA and no FMS group at 3, 6, 12 and 24 months. Interestingly, drug survival that was measured as persistence on treatment was significantly lower in the FMS and PsA group compared with the PsA and no FMS group, and that FMS was a strong negative predictor of persistence on therapy.

Several studies in PsA pointed at the discrepancy between US and clinical assessment findings, including physical examination and clinical disease activity indices. Wiell *et al* reported that US had higher sensitivity and specificity for synovitis in hand joints of PsA patients compared with the physical examination with MRI as gold standard.[30] Husic et al's study on 70 PsA patients demonstrated low to moderate correlations between the global US total score and DAPSA and CPDAI, and that none of the composite scores correlated with sonographic synovitis, enthesitis and tenosynovitis.[12] Michelsen *et al* conducted a study on 141 PsA patients and reported a correlation between US and DAPSA but not between US and CPDAI or PASDAS.[13] None of those studies examined the influence of the presence of FMS on the relationship between composite clinical indices and US. The present study showed that the total US score significantly correlated with clinical activity indices, including CPDAI, DAPSA and PASDAS in patients with PsA and no FMS but not in PsA patients with coexisting FMS. Furthermore and importantly, all of the clinical activity indices were significantly associated with the presence of FMS, while the US findings were not associated with FMS.

Enthesitis is an important feature of PsA.[6 7] The common method for evaluating enthesitis is based on applying local pressure and assessing tenderness at enthesis points. Similarly, evaluation of FMS includes the examining of tenderness at nearby tender points. As such, differentiating between these two pathologies could be difficult and frustrating,[28] and US could serve as a valuable modality for providing definitive information, with a number of studies having demonstrated its advantage over physical examination in the setting of enthesitis.[14 15] The current study showed similar grey scale and Doppler scores in PsA with and without FMS. Macchioni *et al*'s cross-sectional study compared clinical and sonographic enthesitis in 3 groups of patients comprised of 141 with PsA, 51 with psoriasis and 51 with FMS.[31] Those authors reported significantly more clinical enthesitis in the FMS group, while sonographic enthesitis was significantly more frequent in the PsA and psoriasis groups compared with the FMS group. Fiorenza *et al* also conducted a cross-sectional study in which they compared three groups of patients comprised of 39 with PsA, 23 with FMS and 39 with both PsA and FMS.[17] The results of that study showed that clinical enthesitis was similar and more common in the FMS and concomitant PsA and FMS groups, while entheseal abnormalities were detected similarly and significantly more on US in the PsA and PsA and concomitant FMS compared with the FMS group.

This study has some limitations that bear mention. First, the cross-sectional design reflected a single time point without examining the predictors of clinical outcome and prognosis over time in each group. Second, the cohort in this study had a long mean PsA duration, possibly reducing disease activity findings. Lastly, the semiquantitative US score used in this study has not been validated. However, there is no consensus on a single US index for disease activity assessment in PsA. For this reason, a comprehensive scanning protocol that included numerous relevant MSK structures, including joints, tendons and entheses in both lower and upper limbs was used, thus ensuring accurate assessment of disease activity state.

The strengths of this work are its being what we believe to be the largest US PsA-FMS study and the first to include not only enthesitis but also other US MSK lesions, such as synovitis and tenosynovitis. In addition, the cohort was well phenotyped both clinically and sonographically, which enabled assessment of different variables and controlling for multiple confounders. Finally, the internal validity was very good since all of the patients were diagnosed with PsA by fulfilling the CASPAR criteria, the diagnosis of FMS was based on the 2016 FMS criteria and the US assessor was blinded to the clinical data.

SUMMARY

Patients with coexisting PsA and FMS had increased scores of clinical measures compared with patients with PsA and no FMS. US scores were similar between the groups, independently of the presence of FMS. FMS was significantly associated with higher clinical indices scores but not with the US score. We therefore conclude that US has a significantly greater value than composite clinical scores in the assessment of disease activity in PsA patients with FMS.

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TRANSLATIONAL SCIENCE

Ex vivo mass cytometry analysis reveals a profound myeloid proinflammatory signature in psoriatic arthritis synovial fluid

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ABSTRACT

Objectives A number of immune populations have been implicated in psoriatic arthritis (PsA) pathogenesis. This study used mass cytometry (CyTOF) combined with transcriptomic analysis to generate a high-dimensional dataset of matched PsA synovial fluid (SF) and blood leucocytes, with the aim of identifying cytokine production ex vivo in unstimulated lymphoid and myeloid cells.

Methods Fresh SF and paired blood were either fixed or incubated with protein transport inhibitors for 6 hours. Samples were stained with two CyTOF panels: a phenotyping panel and an intracellular panel, including antibodies to both T cell and myeloid cell secreted proteins. Transcriptomic analysis by gene array of key expanded cell populations, single-cell RNA-seq, ELISA and LEGENDplex analysis of PsA SF were also performed.

Results We observed marked changes in the myeloid compartment of PsA SF relative to blood, with expansion of intermediate monocytes, macrophages and dendritic cell populations. Classical monocytes, intermediate monocytes and macrophages spontaneously produced significant levels of the proinflammatory mediators osteopontin and CCL2 in the absence of any in vitro stimulation. By contrast minimal spontaneous cytokine production by T cells was detected. Gene expression analysis showed the genes for osteopontin and CCL2 to be among those most highly upregulated by PsA monocytes/macrophages in SF; and both proteins were elevated in PsA SF.

Conclusions Using multiomic analyses, we have generated a comprehensive cellular map of PsA SF and blood to reveal key expanded myeloid proinflammatory modules in PsA of potential pathogenic and therapeutic importance.

Key messages

What is already known about this subject?

► The pathogenesis of psoriatic arthritis (PsA) is incompletely understood with T cells implicated; the role of myeloid populations has not yet been explored to the same extent.

What does this study add?

► Mass cytometry technology provides a more complete picture of the cellular composition of the inflamed joint fluid.
► CD14+ myeloid populations are enriched in PsA joints and monocytes/macrophages spontaneously produce substantial levels of proinflammatory proteins including osteopontin and CCL2.

How might this impact on clinical practice or future developments?

► Our data suggest that osteopontin and CCL2 have potential for both PsA diagnosis and treatment. Therapies to inhibit monocyte/macrophage activation and function also merit investigation as these cells may be driving inflammation in PsA.

INTRODUCTION

Psoriatic arthritis (PsA) is an immune-mediated inflammatory arthritis which forms part of the spondyloarthropathy (SpA) spectrum. Histopathological characteristics of PsA include enthesitis, synovitis, erosions and new bone formation. The pathogenesis of joint inflammation in PsA is poorly understood;[1] roles for tumour necrosis factor (TNF), interleukin (IL)-17A and IL-23 have been demonstrated with clinical efficacy of neutralising therapies against these cytokine targets.[2–4] Previous studies, which have predominantly focused on specific immune cell types within PsA synovial fluid (SF), have established potential roles for CD8 T cells,[5] particularly those producing IL-17,[6] natural killer (NK) cells[7] and dendritic cells (DCs).[8] Other myeloid populations have been relatively understudied in PsA but research points to the importance of this compartment in PsA pathogenesis. Within the synovium, CD163+ macrophages are increased in SpA compared with rheumatoid arthritis (RA) despite similar total CD68+ macrophage numbers; and blood-derived CD163+ cells have been shown to produce TNF (following in vitro LPS stimulation).[9]

Until recently, comprehensive characterisation of the cellular composition in the psoriatic joint has been technically difficult to achieve. In this study, we use mass cytometry (CyTOF) to simultaneously measure over 30 parameters in PsA SF and blood leucocytes directly ex vivo and then visualise the inflammatory cellular architecture of PsA using unsupervised clustering. We identify inflammatory proteins including osteopontin and CCL2 spontaneously produced by PsA SF myeloid cells without any in vitro stimulation. We combine this with both bulk and single-cell transcriptomic analyses and SF

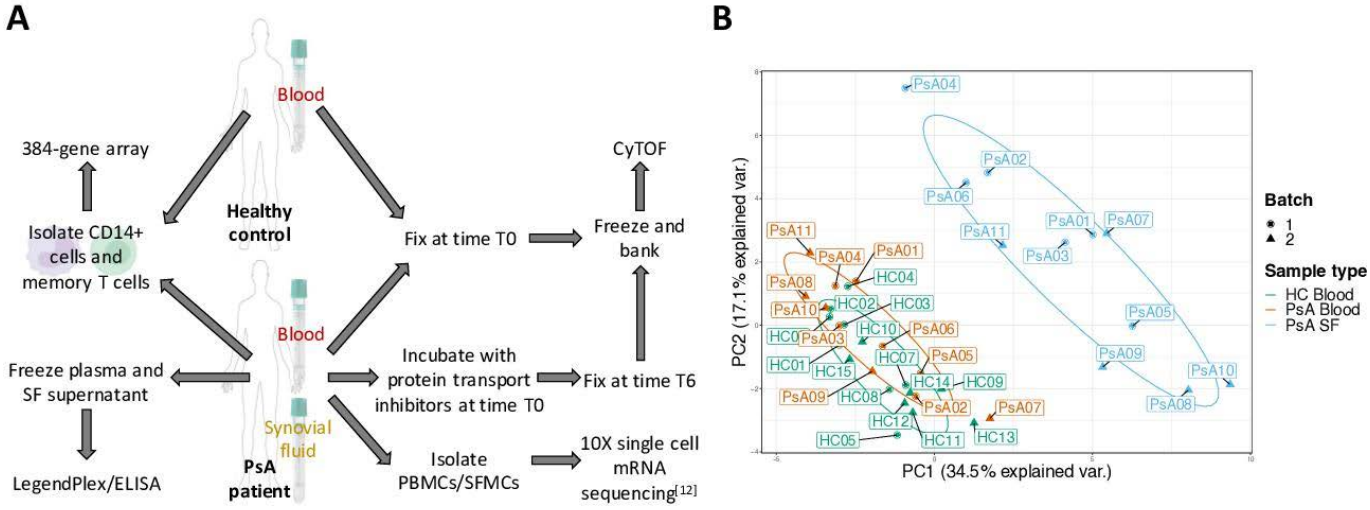

Figure 1 Overview of the experimental workflow and clear distinction of PsA synovial fluid (SF) and blood by principal component analysis. (A) Experimental workflow. Fresh peripheral blood and SF samples were split and either formaldehyde-fixed immediately following collection (time T0), or after incubation at 37°C with protein transport inhibitors for 6 hours (time T6). These samples were used for phenotyping and intracellular CyTOF analysis. In addition, CD14+ cells, memory CD8+ T cells and memory CD4+ T cells were sorted and extracted RNA used in a 384-gene array; plasma and SF supernatant frozen; and PBMCs and SFMCs freshly isolated for 10× (previously described in reference 12). (B) Unsupervised principal component analysis using the mean expression of lineage markers of CyTOF leucocyte samples at time T0 resolves PsA SF from matched and healthy control (HC) blood samples. CyTOF, mass cytometry; PsA, psoriatic arthritis.

protein quantification to identify a prominent role for myeloid-derived mediators in the pathogenesis of PsA.

METHODS

Study subjects and patient involvement

All PsA patients met Classification Criteria for Psoriatic Arthritis (CASPAR) criteria.[10] Blood and SF samples were collected from 11 consecutive patients not receiving biological DMARDS or steroids (6 males, 5 females, mean age 43.8±13.5 years; 4 patients on methotrexate) with large-joint oligo PsA undergoing intra-articular knee aspiration at Oxford University Hospitals; three of these were assessed by PCR array and three for single-cell (sc)RNA sequencing (scRNAseq). An additional three patients were included for targeted SF and plasma protein analysis. Blood from 15 anonymous healthy donors (10 males, 5 females, mean age 45.1±10.4 years) was also collected. Full informed consent was obtained from all subjects. Demographics for all study subjects are listed in online supplemental table S1.

For full details on experimental methods, see online supplemental material.

RESULTS

PsA SF shows marked increases in specific myeloid populations compared with PsA blood

CyTOF analysis of matched SF and blood from 11 PsA patients was performed, together with 15 blood samples from healthy donors (workflow outlined in figure 1A). We used a phenotyping panel containing 36 markers to enable identification of all major immune cell populations together with their activation status when samples were fixed immediately (T0). Principal component analysis clearly distinguished PsA SF samples from blood; while blood samples from PsA patients and healthy donors were interspersed (figure 1B).

We next carried out an in-depth analysis using the unbiased clustering algorithm FlowSOM.[11] Identified clusters were then manually annotated (online supplemental table S2), merged and visualised using t-distributed stochastic neighbor embedding (tSNE) (figure 2A and online supplemental figure S1A). Expression of key phenotypic markers of the different populations are represented as a heat map in figure 2B. The proportions of cell populations per patient are shown in figure 2C. We observed clear differences between the cell populations present in the SF compared with blood. Figure 2D shows that intermediate monocytes, macrophages, cDC1, cDC2, CD206+ cDC2, pDC, memory CD8 T cells, memory CD4 T cells and CD56bright NK cells were significantly increased in PsA SF (compared with PsA blood, all adjusted $p<0.01$). Nonclassical monocytes, basophils, naïve CD8 T cells, naïve CD4 T cells, B cells, CD56dim CD16+ NK cells and NKT cells were significantly decreased in PsA SF (all adjusted $p<0.01$).

Given that 4 of the 11 PsA patients were on methotrexate, we questioned whether this impacted cell population abundance. Methotrexate treatment did not affect monocyte or macrophage populations but increased cDC1, CD206+ cDC2 and CD56dim CD16+ NK cell populations, and reduced activated memory CD8 T cells (online supplemental figure S2A). These methotrexate effects did not impact the overall increases/decreases seen in PsA SF compared with blood. We also compared healthy blood to PsA blood, finding a reduced frequency of pDC and MAIT cells in PsA blood (online supplemental figure S2B).

Multiple memory T cell subsets are expanded in PsA SF

Given the expansion of memory T cells in PsA SF, we asked if any specific subpopulations were involved. Following data preprocessing and gating of CD3+ cells (online supplemental figure S3A), FlowSOM was used to cluster the cell populations, which were then manually annotated and merged (online supplemental figure S3B,C). PD1hi memory CD4 T cell, PD1mid memory CD4 T cell, CD25hi memory CD4 T cell and CD49a+ memory CD8 T cell populations were significantly increased in PsA SF compared with blood (online supplemental figure S3D).

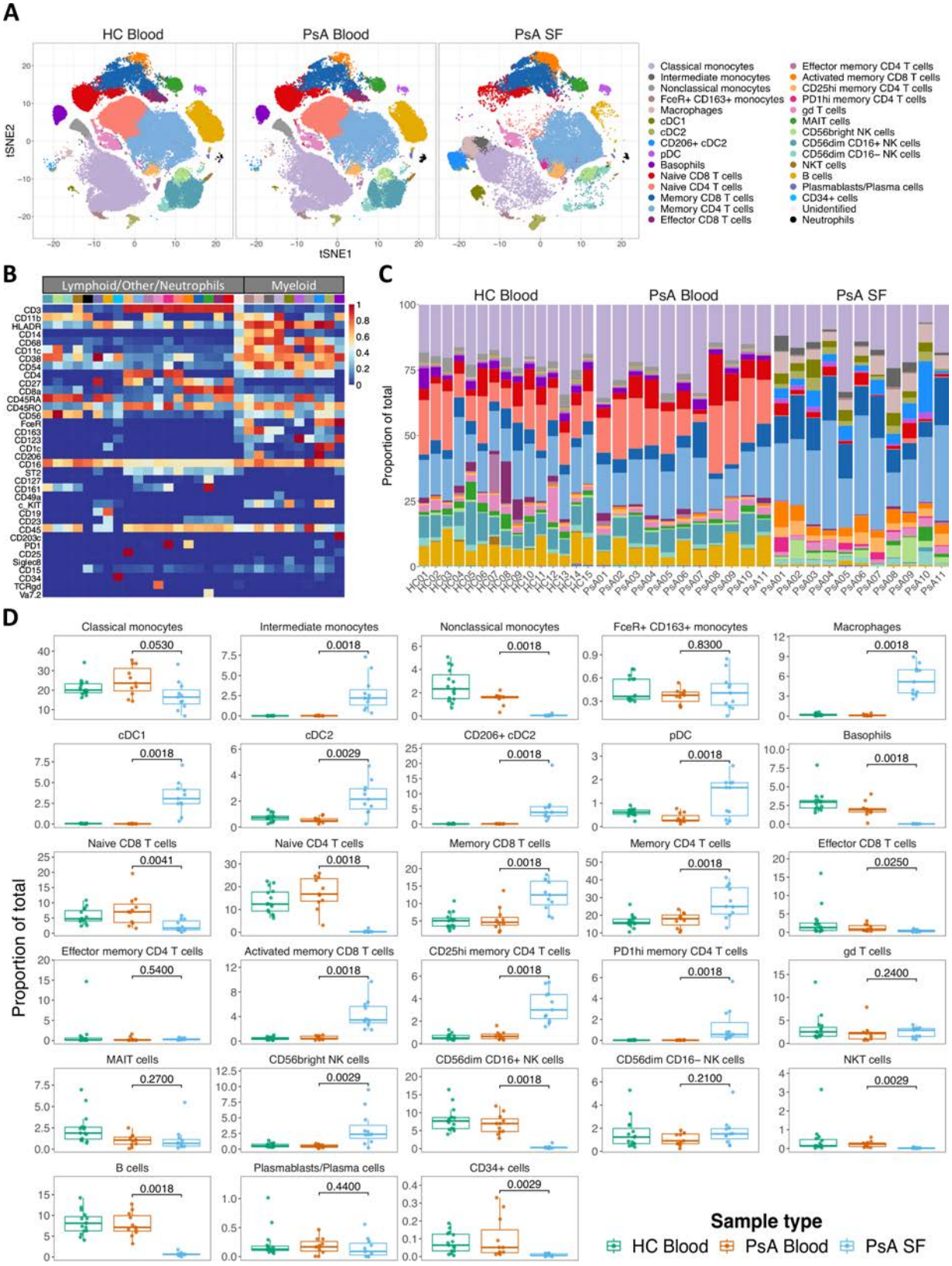

Figure 2 PsA SF CyTOF analysis shows expansion of multiple adaptive and innate cell populations compared with matched blood samples. (A) t-SNE plots showing leucocyte populations in 185 000 single cells (HC blood, n=15; PsA blood, n=11; PsA SF, n=11, 5000 randomly selected cells from each sample). Cells are coloured according to the annotated and merged clusters, and stratified by sample type. (B) Heatmap of the median arcsinh-transformed marker intensity normalised to a 0–1 range of the 36 phenotyping panel markers across the 30 annotated clusters. (C) Cell composition for each individual studied stratified by sample type; neutrophils that were still present were omitted from this analysis. (D) Comparison of cluster frequencies in PsA blood and PsA SF. All p values were calculated using paired Wilcox test and were corrected for multiple comparisons using the Benjamini-Hochberg adjustment at 5%. HC blood data are included for visualisation only. All samples were downsampled to an equal number of events (15 510 events) prior to clustering. CyTOF, mass cytometry; HC, healthy control; NK, natural killer; PsA, psoriatic arthritis; SF, synovial fluid.

PsA SF myeloid cells spontaneously release proinflammatory proteins on ex vivo incubation

To detect active protein production, we incubated matched SF and blood from 10 PsA patients for 6 hours ex vivo (without any stimulation but in the presence of protein transport inhibitors to abrogate protein secretion) and compared these (T6) with matched T0 samples (figure 1A). In addition to 18 cell surface lineage markers, 18 intracellular markers were included to detect cytokines, chemokines and other secreted proteins, including interferon γ (IFNγ), IL-4, IL-10, IL-17 and IL-21 (predominantly secreted from T cells) and IL-8, CCL2, CXCL10, osteoactivin and osteopontin (predominantly myeloid). FlowSOM was again used to cluster the cell populations in an unsupervised manner, followed by manual annotation of clusters (online supplemental table S2), merging and visualisation using tSNE (figure 3A and online supplemental figures S1B and S4A). In SF, osteopontin, CCL2 and IL-8 production were all significantly increased at T6 by at least 25% (with T6–T0>0.05%) in classical monocytes, intermediate monocytes and macrophages (mean expression osteopontin adjusted p=1.37e-06, 1.99e-03 and 1.31e-04, respectively, CCL2 adjusted p=3.31e-03, 1.34e-05 and 1.65e-04, respectively, and IL-8 adjusted p=5.05e-04, 4.45e-08 and 2.83e-04, respectively) (figure 3B and online supplemental table S3; other intracellular markers shown in online supplemental figure S5). In addition, CXCL10 increased at T6 in intermediate monocytes when examining 95th percentile expression (adjusted p=6.32e-03) (online supplemental figure S6).

In PsA blood, there were too few intermediate monocytes to analyse, however, in classical monocytes we observed at least a 25% increase in mean expression at T6 (with T6–T0>0.05%) in CCL2, IL-8, IFNγ and IL-4 (adjusted p=2.54e-09, 2.61e-05, 2.75e-07 and 5.33e-05) (online supplemental figures S4B, S7 and table S3).

Following our unsupervised analysis, we reverted back to a manual biaxial analysis for visualisation and confirmation. Figure 3C shows production of osteopontin, CCL2 and IL-8 by PsA SF CD11c+CD14+CD123- cells over 6 hours. Minimal osteopontin was detected in blood, while CCL2 was significantly higher in SF monocytes and IL-8 higher in blood monocytes (figure 3D). In addition, CXCL10 and osteoactivin production was higher in SF monocytes (online supplemental figure S8).

In terms of T cell cytokine release over the 6 hour period, the only significant finding we identified was an increase in 95th percentile expression of IL-10 in SF MAIT cells (adjusted p=6.68e-03) (online supplemental figure S6). A positive control using PMA/ionomycin stimulation was included in all three batches and demonstrated clearly the recall response of T cells and their ability to produce cytokines (online supplemental figure S9).

Gene expression analysis of SF T cells and monocytes shows upregulation of *SPP1* and *CCL2* compared with matched PBMCs

Next we sought to understand the ex vivo transcriptomic signature of the key immune cell populations which differed in PsA SF compared with blood. Gene expression analysis of freshly cell sorted CD14+ cells, memory CD8 T cells and memory CD4 T cells from matched peripheral blood mononuclear cell (PBMC) and SF samples (n=3) was performed using a targeted array of 370 genes involved in inflammation or autoimmunity. As expected, the CD14+ cells clustered separately from the T cells across the PsA patients (figure 4A and online supplemental figure S10; full fold change data in online supplemental table S4). For all three cell types, the majority of significantly dysregulated genes were upregulated in SF compared with blood (figure 4B). The gene for osteopontin, *SPP1*, was the highest upregulated gene (17.12 log2 fold change compared with blood) in SF CD14+ cells. *CCL2* and *CXCL10* were also upregulated in CD14+ cells, but not *CXCL8* (IL-8 gene). Despite significant upregulation of *OLR1* and *TNF*, their corresponding proteins were not significantly increased in the CyTOF dataset (figure 4C), although TNF protein was detected at time T6 in SF CD14+ cells in some patients (online supplemental figure S8).

Gene expression in PsA PBMCs was also compared with three healthy controls (online supplemental figure S11A). *SPP1* and *CCL2* are only upregulated in PsA SF vs PsA blood, with no significant difference when comparing PsA blood to healthy blood. *CXCL10* is upregulated in PsA SF but downregulated in PsA blood compared with healthy blood (online supplemental figure S11B). To confirm the presence of secreted cytokines and chemokines in SF, PsA plasma and SF supernatant were analysed. Figure 4D shows that osteopontin, CCL2 and IL-8 were all enriched in SF compared with plasma. CXCL10 was previously shown to be increased in PsA SF.[12]

To confirm these findings, we next interrogated scRNAseq data from a recently described study of ex vivo PsA blood and SF.[12] Unsupervised clustering of combined PBMC and synovial fluid mononuclear cell (SFMC) data identified two clusters of monocytes/macrophages as defined by lineage markers *CD14*, *FCGR3A* (gene for CD16), *LYZ*, *MS4A7*, *CD163* and *MRC1* (gene for CD206); the smaller cluster was defined by increased expression of *APOE* (figure 5A). We observed strongest expression of *SPP1* and *CCL2* in the myeloid clusters (figure 5A). In this new analysis, we performed differential gene expression analysis between blood and SF in the monocyte/macrophage cluster and found *SPP1* to be the most selectively upregulated SF gene and *CCL2* the third most upregulated gene (adjusted p=3.46e-239 and 8.08e-167, respectively, figure 5B). Both *SPP1* and *CCL2* were expressed by multiple cell populations in the SF, although at a lower level, including cDCs, NK cells and CD4 and CD8 T cells. Almost no expression of *SPP1* and *CCL2* was observed in the blood of any of the cell populations (figure 5C). For genes that had their corresponding proteins included in the CyTOF dataset, figure 5D shows that the monocyte/macrophage populations have the highest upregulation within the SF of a broad range of cytokine and chemokine genes.

DISCUSSION

In this study, we use multiple complementary approaches to characterise the cellular and inflammatory landscape in PsA directly ex vivo and identify expansions of immunologically active myeloid populations within the joints. In order to minimise artefact and best capture the in vivo environment, we utilised fresh whole SF and blood[13] for our CyTOF assays, with a 6 hour incubation window to allow interrogation of the intrinsic cytokine/chemokine secretion profile.[14] Here, we show expansion of myeloid populations within the PsA joints which spontaneously produce proinflammatory cytokines and chemokines.

We observed a marked expansion of intermediate monocytes in PsA SF. These cells exhibit features in common with macrophages,[15] are likely proinflammatory,[16] and resemble those seen in RA and inflammatory osteoarthritis, where they correlate with disease activity.[17 18] Interestingly, while the total number of monocytes/macrophages are similar in PsA and RA SF, significantly higher numbers of intermediate monocytes have been recently reported in PsA compared with RA.[19] Our unsupervised analysis

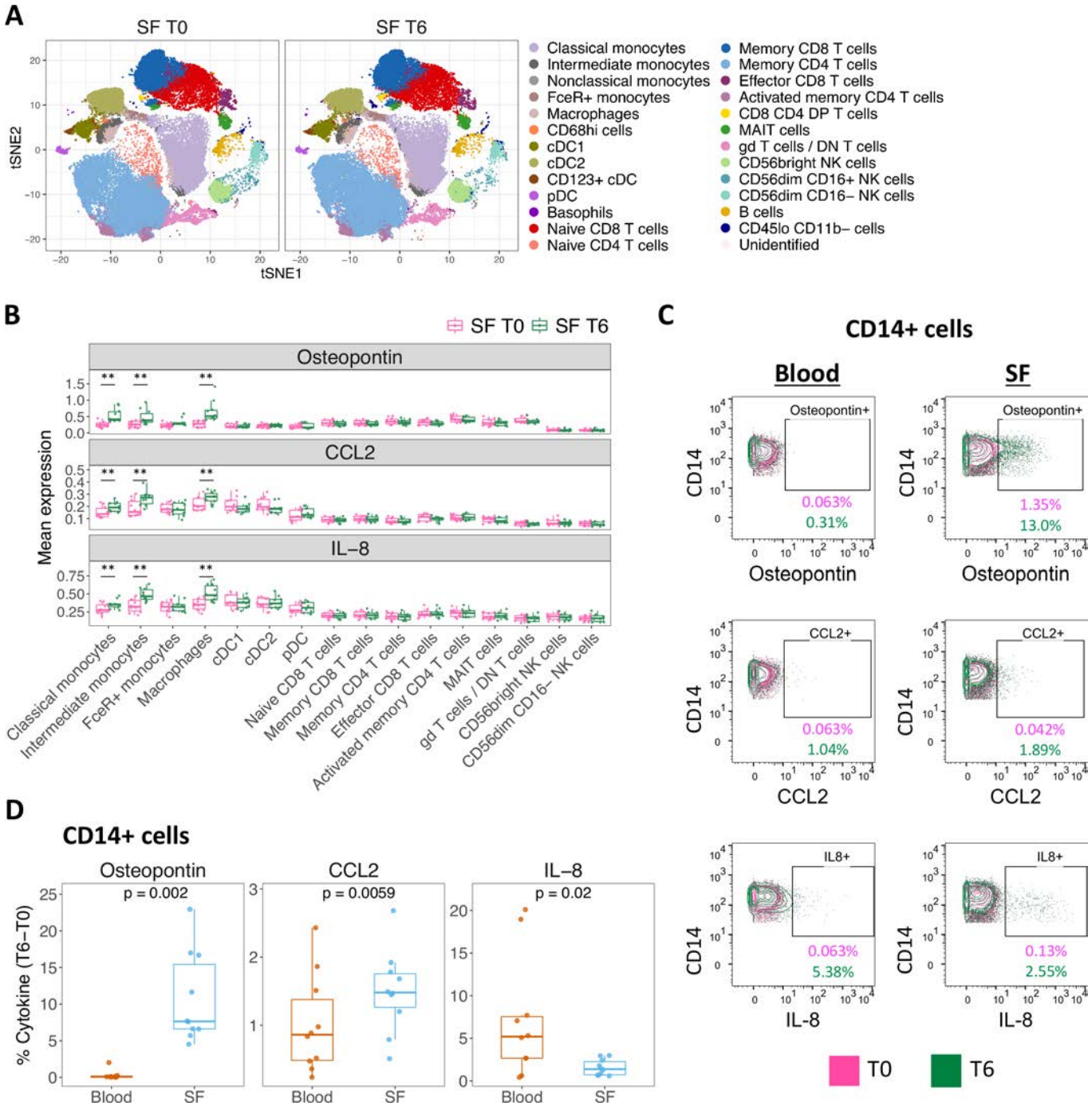

Figure 3 Osteopontin, CCL2 and IL-8 (CXCL8) are spontaneously produced by PsA SF monocytes/macrophages over 6 hour ex vivo. (A) t-SNE plots based on the arcsinh-transformed expression of 18 markers in 5000 randomly selected cells from each sample (n=10, only SF shown). Cells are coloured according to the annotated and merged clusters. Stratified by time. (B) Mean expression of osteopontin, CCL2 and IL-8 across the SF cell populations; any cell population containing <50 cells was omitted. **Indicates an overall increase in expression of at least 25% from T0 to T6 (with T6-T0>0.05%), and a false discovery rate (FDR) <0.01. (C) Manual analysis of intracellular CyTOF data from a representative patient using FlowJo. Following data preprocessing, FCS files were gated on CD3-CD19-CD11c+CD14+CD123- cells. The percentage of positive cells in each gate is shown. (D) Comparison of CD14 frequency of osteopontin, CCL2 and IL-8 production between SF and blood from manual analysis. The percentage of intracellular protein was calculated by subtracting the amount at time T0 from time T6 per patient sample for both blood and SF. All p values were calculated using paired Wilcox test. CyTOF, mass cytometry; FCS, flow cytometry standard; IL-8, interleukin 8; PsA, psoriatic arthritis; SF, synovial fluid.

identified spontaneous high levels of production of osteopontin, CCL2 and IL-8 by SF intermediate monocytes, classical monocytes and macrophages. CXCL10 production by SF intermediate monocytes was also observed. CD14+ cells in SF produced significantly more osteopontin, CCL2 and CXCL10 than blood, and our findings indicate that they are almost certainly the main cellular source of these factors which are enriched in the SF.

We were able to confirm our protein findings at the gene level, with *SPP1* (the gene for osteopontin) being the highest upregulated gene both in freshly sorted PsA SF CD14 monocytes/

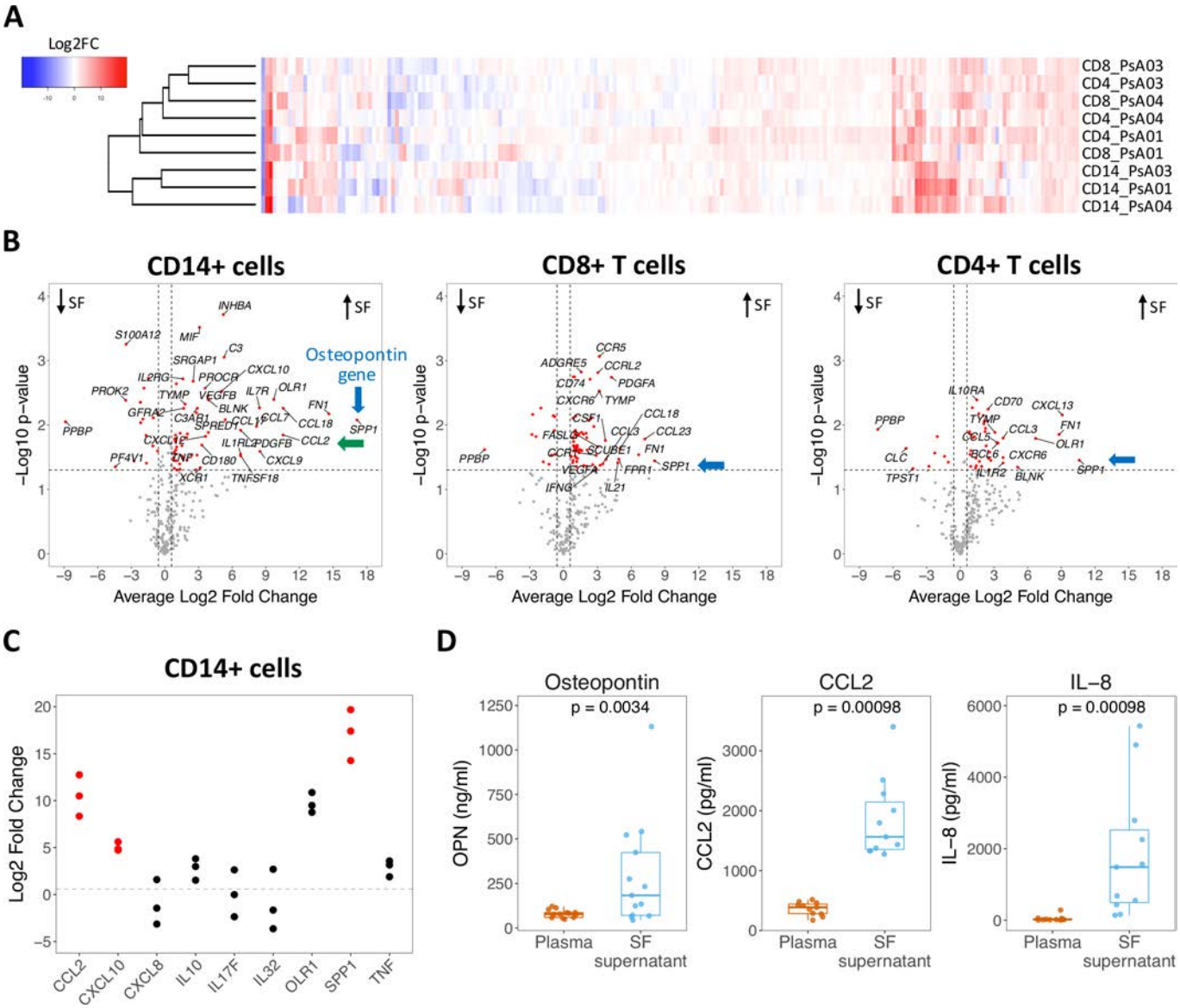

Figure 4 Gene expression analysis of isolated CD14+ cells, memory CD8+ T cells and memory CD4+ T cells from PsA SF compared with matched PBMCs (n=3). (A) Heatmap of log2 gene expression fold change (FC) between SF and PBMCs for all genes that were detected. (B) Volcano plots showing differences in gene expression between SF and PBMC for CD14, CD8 and CD4 cells. The significance of the modulation in gene expression between the two tissues (y-axis) is plotted against the log2 of the mean FC (x-axis) across the three PsA patients. Genes showing p<0.05 (one sample t-test) and mean FC >1.5 are coloured in red. Black arrows indicate the direction of upregulation and downregulation of transcripts in SF; blue arrows point to *SPP1*, the gene for osteopontin, and green arrow points to *CCL2*. (C) Log2 FC in CD14+ cells for the genes that had their proteins included in the CyTOF intracellular panel. Genes that have corresponding proteins that were significantly increased in SF after 6 hours as determined by CyTOF are coloured in red. (D) Osteopontin, CCL2 and IL-8 protein quantification in paired plasma and SF. CCL2 and IL-8 were measured by LegendPlex (n=11); osteopontin was measured by ELISA (n=13); p values were calculated using paired Wilcox test. CyTOF, mass cytometry; IL-8, interleukin 8; PsA, psoriatic arthritis; SF, synovial fluid.

macrophages (compared with PsA blood CD14+ cells) and in synovial monocytes/macrophages in a large scRNAseq dataset. *CCL2* and *CXCL10* upregulation also matched protein expression in myeloid populations. Interestingly osteopontin was not detected by CyTOF in SF T cells, nor CXCL13 in SF CD4 T cells, despite their genes being significantly upregulated, emphasising the importance of quantifying protein expression.[20] When comparing PsA SF and blood to healthy blood, *CXCL10*, *TNF* and *CCL5* were upregulated in PsA SF CD14+ cells but downregulated in PsA blood compared with healthy blood, indicating potential trafficking of these cells into the SF.

A caveat of our current study is that we do not have matched samples from other inflammatory joint diseases. By exploring the scRNAseq dataset from the Accelerating Medicines Partnership phase I project, *SPP1* and *CCL2* expression can be observed in RA synovial tissue monocyte populations.[21] A detailed comparison across different inflammatory diseases would make for an interesting future study.

Our study suggests a potential important role for myeloid cell production of osteopontin in PsA pathogenesis. Osteopontin showed the greatest increase in PsA SF CD14+ cells in independent bulk and scRNAseq datasets, was spontaneously produced

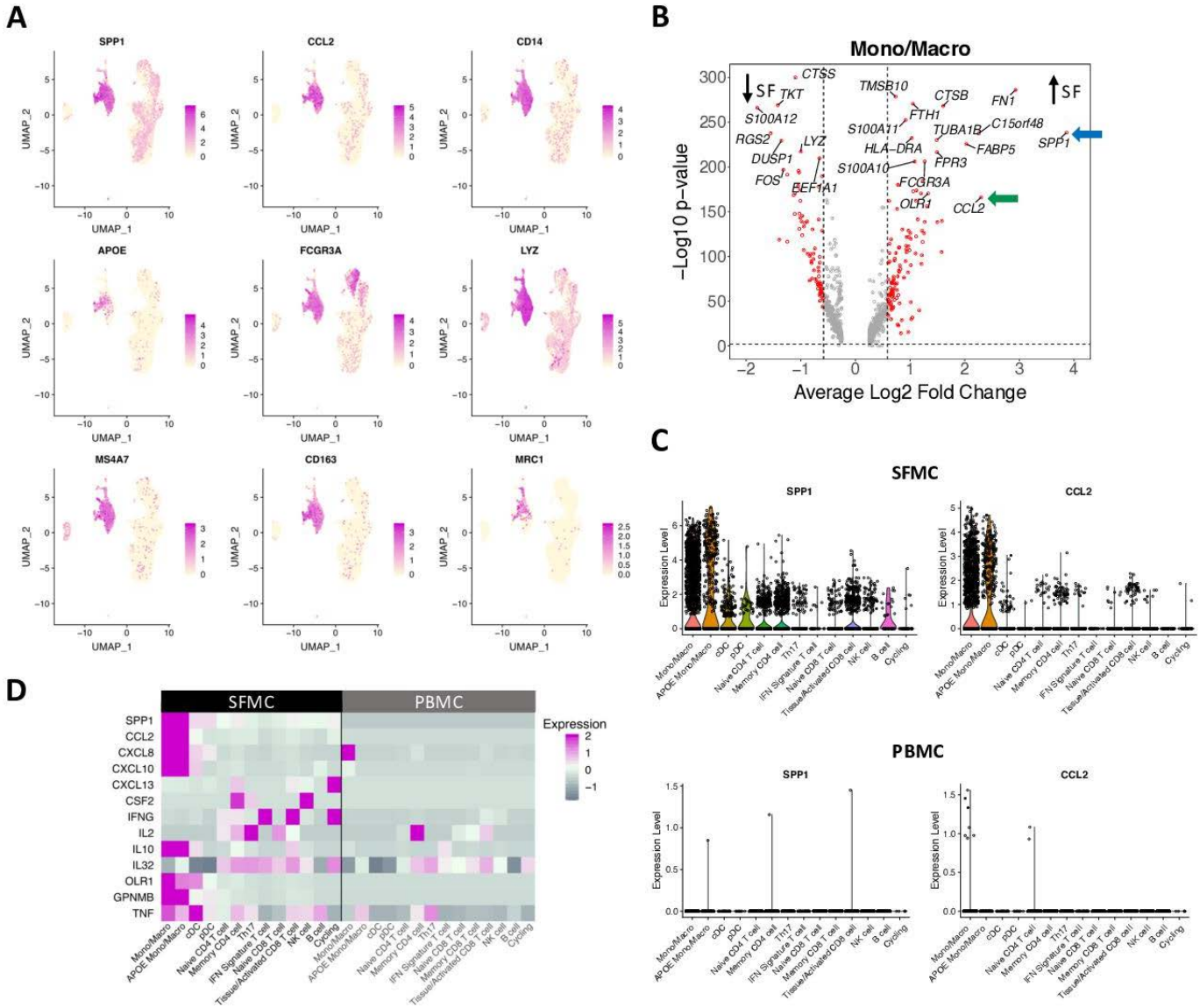

Figure 5 The genes for osteopontin and CCL2 are highly upregulated in monocytes/macrophages in a scRNA sequencing dataset of PsA SFMCs compared with matched PBMCs. (A) UMAPs of integrated PsA paired SFMC and PBMCs generated by 10× 3′ sequencing showing the relative expression of key annotated genes (n=3). (B) Volcano plot showing differences in gene expression. The significance of the modulation in gene expression between the two tissues (y-axis) is plotted against the log2 of the mean FC (x-axis) across the three PsA patients. Genes showing corrected p<0.01 and mean FC >1.5 are coloured in red. Black arrows indicate the direction of upregulation and downregulation of transcripts in SF; blue arrow points to *SPP1*, the gene for osteopontin, and green arrow points to *CCL2*. (C) Violin plots of *SPP1* and *CCL2* across all clusters based on log normalised RNA. (D) Heatmap of gene expression across all clusters for genes that had their proteins included in the CyTOF intracellular panel; any gene that was not expressed in any cluster has been omitted. CyTOF, mass cytometry; FC, fold change; PsA, psoriatic arthritis; SF, synovial fluid; UMAP, uniform manifold approximation and projection.

by PsA SF (not blood) myeloid populations and was present at increased levels in PsA SF. Osteopontin induces chemotactic migration of both macrophages and T cells,[22 23] stimulates Th1 and Th17 cytokine release and downmodulates IL-10.[24–27] Upregulated serum levels of osteopontin are associated with PsA compared with healthy controls, indicating osteopontin as a potential biomarker of PsA.[28] Previous work has shown that osteopontin serum and SF levels correlate with C reactive protein in RA patients.[29 30] Although we did not observe this correlation in our PsA patients (data not shown), a larger patient cohort would be required to form a conclusion. In addition, osteopontin has been shown to induce the expression of CCL2 and CCL4 in monocytes.[29] In both our CyTOF and gene expression assays, osteopontin and CCL2 were increased in the monocyte/macrophage population, demonstrating they may be intrinsically linked. Osteopontin has been reported as upregulated in PsA synovial biopsies,[31] and here we show that it is highly expressed in PsA SF, with by far the greatest production from the myeloid compartment. In patients with RA, serum levels of osteopontin predict effectiveness of tocilizumab,[32] although osteopontin neutralisation failed to induce clinical improvement,[33] perhaps due to rapid turnover.[34]

Both CXCL10 and CCL2 have been reported as upregulated in PsA serum[35] with CXCL10 increased in PsA SF[36] and CCL2 upregulated in RA SF.[37] We have previously suggested a potential role for the CXCL10 receptor CXCR3 on CD8 T cells in PsA pathogenesis,[12] and here we show that PsA SF monocytes are a key cellular source of CXCL10, likely contributing to

the recruitment of these cells. A clinical trial of RA patients that combined a monoclonal antibody targeting CXCL10 with methotrexate had a modest clinical effect.[38] The best described function of CCL2 is monocyte recruitment,[39] however, it has pleiotropic effects on myeloid cells[40] and is capable of recruiting other cell types including T cells.[41] Therefore, it may play a central role in both myeloid and T cell recruitment in PsA and may represent an opportunity to intervene therapeutically. CCL2 inhibition has shown efficacy in rat adjuvant arthritis,[42] but not RA.[43] CCL2 inhibition has not been tested in PsA, and our data would support such study.

Although we detected IL-8 (CXCL8) production by PsA monocytes/macrophages, this was greater in blood than SF and it is likely that synovial tissue cells or neutrophils are the dominant source for this cytokine in PsA joints. Previous studies have shown IL-8 to be present in the synovium of PsA patients,[44] and synovial neutrophils have been shown to produce more IL-8 than peripheral blood neutrophils in RA.[45] Given that SpA synovitis can be characterised by an infiltration of neutrophils,[46] it is reasonable to speculate they may be contributing to IL-8 production. Our study did not look at these cell types and this will be important to examine in future.

Our CyTOF approach allowed detailed study of both myeloid and lymphoid populations present in PsA SF and blood. We here confirm previous findings where technical considerations frequently only allowed focus on a particular cell type.[8 12 47–49] We observed SF expansion of PD1hi memory CD4 T cells, representing T follicular helper cells/peripheral T helper cells, similar to that seen in RA,[50 51] and of tissue-resident CD49a+ memory CD8 T cells[6] that we previously showed to be clonally expanded in PsA.[12] Interestingly, these cells are phenotypically similar to the integrin-expressing cells recently described in the joints in related SpA ankylosing spondylitis.[52 53] Although PsA SF T cells have the capability to produce cytokines such as IFNγ, IL-17 and GM-CSF upon in vitro stimulation,[54] we did not see any significant T cell cytokine production in our ex vivo unstimulated CyTOF assay. The ability to capture the exact moment when these cells are stimulated in vivo and exit dormancy may require a longer period of incubation or may be beyond current detection capabilities.

In summary, we have used direct ex vivo CyTOF analysis, validated by gene expression analysis to identify expanded SF monocytes/macrophage populations that are actively and spontaneously producing cytokines. These may be of diagnostic, prognostic and/or therapeutic importance.

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Contributors HA-M and PB conceived the project. NY and HA-M processed the blood and synovial fluid samples. HA-M, AM, NY and SC designed the CyTOF experiments; NY and SC performed the CyTOF assays; NY conducted the CyTOF analysis. NY, HA-M and PB analysed the data. SC performed the PCR array experiment; ALL and NY conducted the PCR array analysis. NY performed the ELISA. FP and HA-M performed the scRNAseq analysis. NY wrote the manuscript; PB and HA-M reviewed the manuscript. PB, JCK and HA-M supervised the study.

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Competing interests SC and AM are employees of UCB. HA-M received unrestricted research support from UCB. PB has received research support from Regeneron, Benevolent AI and GSK.

Patient consent for publication Not required.

Ethics approval Venous blood and knee synovial fluid were obtained under protocols approved by the Oxford Research Ethics committee (Ethics reference number 06/Q1606/139). Healthy venous blood was obtained under UCB Celltech UK HTA licence number 12504.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data relevant to the study are included in the article or uploaded as online supplemental information. Additional data that support the findings of this study are available on reasonable request.

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CLINICAL SCIENCE

Systemic Lupus Erythematosus Disease Activity Score (SLE-DAS) enables accurate and user-friendly definitions of clinical remission and categories of disease activity

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ABSTRACT

Objectives There is an unmet need for accurate and user-friendly definitions of systemic lupus erythematosus (SLE) disease activity and remission. We aimed to derive and validate the SLE Disease Activity Score (SLE-DAS) definitions for disease activity categories and clinical remission state.

Methods Derivation was conducted at Padova Lupus Clinic (Italy). Validation was prospectively performed at Cochin Lupus Clinic (France) and by post hoc analysis of BLISS-76 trial. At each clinic, an expert classified patients in three categories: remission, mild or moderate/severe activity. The SLE-DAS cut-offs were derived using the receiver operating characteristic curve analysis in Padova cohort; its performance was assessed against expert classification in Cochin cohort and British Isles Lupus Assessment Group (BILAG) index in BLISS-76. Gold standard for clinical remission state was the fulfilment of Definition Of Remission In SLE. A Boolean and an index-based definitions of remission were sustained by chi-square automatic interaction detection algorithm. An SLE-DAS online calculator was developed and tested.

Results We included 1190 patients with SLE: 221 in the derivation cohort and 969 in the validation cohorts (150 from Cochin; 819 from BLISS-76). Derived cut-offs were: remission, SLE-DAS ≤2.08; mild activity, 2.08<SLE-DAS≤7.64; moderate/severe activity, SLE-DAS >7.64. Regarding validation in Cochin cohort, sensitivity and specificity are above 90%, 82% and 95% for remission, mild and moderate/severe activity, respectively. The SLE-DAS Boolean-based and index-based remission showed sensitivity of 100% and specificity above 97%.

Conclusion The SLE-DAS is an accurate and easy-to-use tool for defining SLE clinical remission state and disease activity categories, validated against expert assessment and BILAG.

Key messages

What is already known about this subject?

- There is an unmet need for accurate and user-friendly definitions of systemic lupus erythematosus (SLE) disease activity categories and remission.
- The SLE Disease Activity Score (SLE-DAS) is a composite index with continuous measurement properties that includes important disease activity features absent from SLE Disease Activity Index 2000 (SLEDAI-2K) and presents higher predictive value for damage accrual as compared to SLEDAI-2K.

What does this study add?

- Derivation and validation of two SLE-DAS-based definitions of clinical remission presenting very high performance, consistent with Definition of Remission in SLE clinical remission criteria; derivation and validation of SLE-DAS cut-offs for defining categories of active disease presenting a high performance, consistent with expert clinician judgement and British Isles Lupus Assessment Group index; and development of the SLE-DAS online calculator, available at http://sle-das.eu/.
- These SLE-DAS definitions enables clinical interpretation of the SLE-DAS scores.

How might this impact on clinical practice or future developments?

- The SLE-DAS may facilitate treat-to-target management of patients with SLE, providing a useful instrument for guiding treatment strategies, for outcomes research and identifying candidates for clinical trials.

INTRODUCTION

Management of patients with systemic lupus erythematosus (SLE) should aim to achieve remission and to improve long-term patient outcomes.[1–7] Additionally, treatment of SLE should be tailored according to the intensity of SLE global disease activity, usually differentiated in mild, moderate and severe.[6 8]

However, the proposed definitions of remission and SLE disease activity categories are mostly based on the SLE Disease Activity Index 2000 (SLEDAI-2K), which has limitations in accurately defining clinical remission and especially the categories of disease activity.[9 10] To compensate the gaps in the SLEDAI-2K, the current definitions of remission

include additional items, such as the physician global assessment (PGA), disease features and the ongoing treatment.[2 3 11–13] As a result, these definitions are not practical for the use in daily clinical practice. Furthermore, discrimination of disease activity categories based on the SLEDAI-2K score is limited because it dichotomically scores (present/absent) disease activity in each organ domain, not graduating the extent of disease activity within each organ system. In addition, SLEDAI-2K does not include some important lupus features. Categorisation of disease activity applying the British Isles Lupus Assessment Group disease activity index (BILAG) was also proposed, but this is time-consuming and provides organ-based categories and not a global score of disease activity.[6 8 14] Furthermore, the BILAG might classify the same level of disease activity in different categories, as this classification is based on the change from the previous month and not strictly on the actual activity at the time of the visit. Hence, there is an important unmet need for more reliable and user-friendly definitions of SLE clinical remission and disease activity categories.

The SLE Disease Activity Score (SLE-DAS) is a validated instrument for measuring global disease activity, with 17 weighted clinical and laboratory parameters, including continuous measures for arthritis, proteinuria, thrombocytopaenia and leucopenia, with the other items scored dichotomously. Differently from SLEDAI, the SLE-DAS gives lower scores to mucocutaneous than systemic vasculitis and to localised skin rash than to generalised skin rash, hence improving the weighting system. Importantly, infrequent but important SLE manifestations absent from SLEDAI are included in SLE-DAS, namely ophthalmological (in neuropsychiatric item), cardiac/pulmonary involvement, gastrointestinal (in systemic vasculitis item) and haemolytic anaemia. Scoring of SLE-DAS with its online calculator is user-friendly. In longitudinal follow-up of patients in two clinical cohorts, we previously assessed the performance of SLE-DAS to discriminate a clinically significant change in SLE disease activity and found in the validation cohort that a score change ≥1.72 had a 95.5% sensitivity and 98.2% specificity for worsening, and an 89.5% sensitivity and 100% specificity for improvement.[15–18] As compared to SLEDAI-2K, the SLE-DAS had a higher accuracy in measuring SLE disease activity, a better sensitivity-to-change and a higher predictive value for damage accrual.[15]

In this study, we aimed to: (1) define SLE clinical remission state based on SLE-DAS; (2) derive and validate the SLE-DAS cut-off values in defining SLE disease activity categories; (3) develop an SLE-DAS online calculator and test its reliability.

PATIENTS AND METHODS

Study populations

In this observational multicentre study, we included consecutive SLE patients fulfilling the American College of Rheumatology (ACR'97) and/or Systemic Lupus International Collaborating Clinics (SLICC'12) classification criteria and followed at the Padova Lupus Clinic (Division of Rheumatology, University of Padova, Italy) or the Cochin Lupus Clinic (Internal Medicine Department, Cochin Hospital, France).[3 19 20] Additionally, we analysed data from the phase III, 76-week BLISS-76 (NCT00410384) trial.[21] All patients gave informed consent before inclusion.

Patient's assessments

The assessment was performed at the first visit occurring from 1 March 2018 to 30 June 2018 in Padova and from 1 June 2020 to 30 October 2020 in Cochin Lupus Clinics. At the inclusion visit, the attending clinician evaluated the SLE disease activity (in the preceding 30 days) using PGA (0–3 points, 10 cm Visual Analogue Scale), SLEDAI-2K and SLE-DAS.[15 22] The fulfilment of two definitions of clinical remission was also assessed, that is, the Definition Of Remission In SLE (DORIS) and the definition proposed by Zen *et al* (Doria).[2 3] Both definitions allow ongoing prednisone ≤5 mg/day.[2 3] At each centre, the senior clinician expert in SLE, blinded to the DAS and remission states and with the knowledge of laboratory results, classified each patient in one of three categories, according to clinical judgement[6 8]: (1) remission, (2) mild disease activity and (3) moderate/severe disease activity. As a guiding principle, the expert classified patients according to the organ system with the highest level of disease activity.

Data from intention-to-treat population of the BLISS-76 trial at the time of the baseline study visit were analysed in a post hoc study. For inclusion in BLISS-76, patients were required to have active disease (SLEDAI ≥6). We used BILAG data, assessed at the time of the study visits and scored SLE-DAS retrospectively from the study database (as detailed in online supplemental file).

Data analysis and statistics

For derivation analyses, we used data from the patients enrolled in the Padova Lupus Cohort. We derived the SLE-DAS cut-off values for defining mild and moderate/severe disease activity categories. In addition, we used two alternative approaches to derive definitions of SLE clinical remission state: an index-based and a Boolean-based definition.

Classification performance was evaluated using robust measures against imbalanced data: sensitivity, specificity and Youden index, which are well-known evaluation metrics and also the geometric mean (G-Mean), which considers the product of sensitivity and specificity, giving the balance between classification performance in both groups, and the discriminant power (DP), which evaluates how well the classification rule distinguishes both groups.[23–25]

All the cut-offs were derived from receiver operating characteristic (ROC) analysis using bootstrap as a way to evaluate the out-of-sample performance of the cut-off estimation method, simulating its variability. One thousand bootstrap samples were considered and for each bootstrap sample the cut-off was determined maximising one of the criteria: the Youden index, the DP or the G-Mean. The R package cutpointr[26] was used. The median and 95% bootstrap CIs for the cut-off values are presented. The area under the ROC curve (AUC) was also considered as a measure of discriminatory ability.

Derivation of the SLE-DAS cut-off values for disease activity categories

We applied ROC curves against the expert clinical judgement for defining the SLE-DAS cut-offs for (1) remission, (2) mild and (3) moderate/severe disease activity. In the ROC analysis, the cut-off values were sequentially determined according to the following dichotomisations: remission versus non-remission and remission/mild disease activity vs moderate/severe disease activity.[27]

Derivation of SLE-DAS definitions for clinical remission state

Following the suggestions proposed by the DORIS project group,[2] we tested two definitions of SLE clinical remission based on SLE-DAS:

A. Index-based definition, applying: (1) the SLE-DAS upper threshold for remission, derived by ROC curve against the expert clinical judgement and (2) prednisone ≤5 mg/day.

B. Boolean-based definition of remission: (1) all clinical items of SLE-DAS were required to be absent (allowing to be present only the items for anti-double stranded DNA (dsDNA) antibodies and hypocomplementaemia) and (2) prednisone ≤5 mg/day.

For remission state, DORIS definition was used as gold standard. Agreement between the definitions of remission was measured using Cohen's kappa coefficient. Additionally, the two definitions were substantiated by applying decision trees, using the chi-square automatic interaction detection (CHAID) algorithm. Decision trees are often used in machine learning as classification procedures. The CHAID algorithm generates a decision tree that provides a classification rule to identify homogeneous mutually exclusive subgroups in relation to a criterion variable (dependent variable), and in accordance with the combination of a range of independent variables (predictors). Decision trees were generated using IBM SPSS Statistics, V.26.

External validation of the SLE-DAS definitions for disease activity categories and clinical remission state

Using data from the patients enrolled in the Cochin Lupus Cohort, performance measures were calculated for: (1) the SLE-DAS index-based and Boolean-based definitions of remission, using the DORIS definition as gold standard; (2) the SLE-DAS cut-off values for defining disease activity categories, using the clinical classification from the expert as gold standard. Agreement between the definitions of remission was measured using Cohen's kappa coefficient.

Post hoc analysis of the BLISS-76 population at study baseline

We performed a post hoc analysis of the BLISS-76 trial, assessing data from the baseline study visit, where patients were expected to have a high level of disease activity. We analysed the performance of the SLE-DAS cut-off value for moderate/severe disease activity (prospectively validated in the Cochin Lupus Cohort) using the BILAG index as gold standard. Patients were considered to be in moderate/severe disease activity when presenting moderate or severe disease in at least one BILAG domain (≥1 B and/or ≥1 A).

IBM SPSS Statistics, V.26 and R software were used, and $p<0.05$ was considered statistically significant.

Development and reliability assessment of the SLE-DAS online calculator

Our information technology engineering team developed the SLE-DAS online calculator (http://sle-das.eu/) and we tested its reliability by comparing in each patient the SLE-DAS score calculated using a Microsoft Excel spreadsheet with the SLE-DAS formula[15] and applying the SLE-DAS calculator.

RESULTS

Characteristics of the patients

Overall 1190 patients were evaluated: 371 in the prospective clinical cohorts (221 and 150 from Padova Lupus Clinic and Cochin Hospital, respectively), and 819 patients from the retrospective analysis of the BLISS-76 population (table 1).[21]

Table 1 Characteristics of the patients included (n=1190)

	Padova cohort (n=221)	Cochin cohort (n=150)	BLISS-76 (n=819)
Patients characteristics			
Gender (female), n (%)	186 (84.2)	148 (98.7)	764 (93.3)
Ethnicity, n (%)*			
White/Caucasian	208 (94.1)	84 (56.0)	569 (69.5)
Black/African/African-American	3 (1.4)	32 (21.3)	118 (14.4)
North African	0 (0.0)	12 (8.0)	0 (0.0)
Hispanic/Latin	2 (0.9)	4 (2.7)	173 (21.1)
Asian	8 (3.6)	5 (12.0)	28 (3.4)
Age (years), mean±SD	45.44±13.47	35.57±9.94	40.17±11.49
Duration of SLE (years), mean±SD	15.36±9.51	12.77±9.27	7.52±7.10
SLEDAI (0–105), mean±SD	3.34±3.49†	2.62±2.55†	9.66±3.76‡
SLE-DAS, mean±SD	3.64±4.51	2.55±3.52	12.80±6.53
PGA (0–3), mean±SD	0.41±0.45	0.20±0.34	1.44±0.50
Disease activity categories, n (%)§			
Remission	151 (68.3)	117 (78.0)	NA
Mild disease activity	31 (14.0)	23 (15.3)	63 (7.7)
Moderate/severe disease activity	39 (17.6)	10 (6.7)	756 (92.3)
Active lupus manifestations			
Haematological abnormalities, n (%)	15 (6.8)	9 (6.0)	140 (17.1)
Immunological abnormalities, n (%)	151 (68.3)	102 (68.0)	606 (74.0)
Arthritis, n (%)	15 (6.8)	7 (4.7)	605 (73.9)
Mucocutaneous abnormalities, n (%)	13 (5.9)	13 (8.7)	665 (81.2)
Serositis, n (%)	3 (1.4)	0 (0.0)	71 (8.7)
Lupus nephritis, n (%)	35 (15.8)	8 (5.3)	91 (11.1)
Cardiopulmonary, n (%)	1 (0.5)	0 (0.0)	5 (0.6)
Systemic vasculitis, n (%)	1 (0.5)	0 (0.0)	0 (0.0)
Lupus treatment			
Antimalarials, n (%)	179 (81.0)	143 (95.3)	519 (63.4)
Immunosuppressants, n (%)	119 (53.8)	71 (47.3)	455 (55.6)
Prednisone, n (%)	137 (62.0)	56 (37.3)	623 (76.1)
Prednisone dose (mg/day), mean±SD	3.73±5.33	2.28±4.35	8.82±8.16

*Patients could be categorised in more than one race subgroup.
†SLEDAI-2K.
‡SELENA-SLEDAI.
§Disease activity categories according to physician's classification in the Padova and Cochin cohorts and to BILAG scores in the BLISS-76 population.
¶Includes mycophenolate mofetil, azathioprine, cyclosporin A, tacrolimus, methotrexate, cyclophosphamide, rituximab and belimumab.
BILAG, British Isles Lupus Assessment Group; NA, not applicable; PGA, physician global assessment; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; SLE-DAS, Systemic Lupus Erythematosus Disease Activity Score.

Derivation and validation of the SLE-DAS cut-offs for SLE disease activity categories

In the derivation cohort, the best cut-off values of SLE-DAS to define each SLE disease activity category were: SLE-DAS≤2.08 for remission; 2.08<SLE-DAS≤7.64 for mild disease activity; SLE-DAS>7.64 for moderate/severe disease activity (table 2). AUC values are high (≥0.9) supporting an outstanding discrimination.[28] Furthermore, the DP values associated with the cut-off points 2.08 (remission vs non-remission) and 7.64 (remission/mild vs moderate/severe) are, respectively, 4.71 and 3.70, which indicate a good DP.[25]

Sensitivity, specificity and G-Mean are above 90% for remission, above 80% for mild and above 95% for moderate/severe disease activity, both in Padova and Cochin cohorts (table 3). Characteristics of the discordant cases are shown in online supplemental tables S2 and S3.

Definition and validation of SLE clinical remission state according to SLE-DAS

SLE-DAS Boolean-based definition of clinical remission

The proportion of patients in clinical remission as defined by the DORIS and the Doria criteria sets was 64.7% and 74.0% in Padova and Cochin cohorts, respectively. Patients fulfilling the SLE-DAS Boolean-based remission (all clinical items of SLE-DAS=0 and prednisone dose ≤5 mg/day) exactly matched those defined by both DORIS and Doria definitions, without discordant cases.

Table 2 Derivation of the SLE-DAS cut-offs to discriminate dichotomous disease activity categories, using ROC analysis

	SLE-DAS cut-off	AUC
Remission versus non-remission		
Bootstrap results:		
Youden, median (95% CI)	2.08 (2.08 to 2.67)	0.990 (0.971 to 1.000)
DP, median (95% CI)	2.08 (1.32 to 2.21)	0.989 (0.969 to 1.000)
Remission/mild disease activity versus moderate/severe disease activity		
Bootstrap results:		
Youden, median (95% CI)	6.27 (5.82 to 8.33)	0.997 (0.992 to 1.000)
DP, median (95% CI)	7.64 (6.48 to 8.33)	0.997 (0.991 to 1.000)

DP: cut-off according to
DP(DP = $\sqrt{3}/\pi$(log X + log Y), X = sensitivity/(1 — sensitivity), Y = specificity/(1 — specificity)).
Youden: cut-off according to the Youden index criterion (Youden index= sensitivity + specificity - 1).
AUC, area under the curve; DP, discriminant power; ROC, receiver operating characteristic; SLE-DAS, Systemic Lupus Erythematosus Disease Activity Score.

SLE-DAS index-based definition of clinical remission

All patients in remission according to both DORIS and Doria also fulfilled the SLE-DAS cut-off for remission (SLE-DAS ≤2.08). When the condition of prednisone dose ≤5 mg/day was added to the SLE-DAS index-based cut-off for remission, discordance with DORIS was 0.9% in Padova cohort (ie, one patient with leucopenia of 2.9×10^9/L and one patient with thrombocytopaenia of 89×10^9/L, without any other active lupus manifestation) and 0.7% in Cochin cohort (ie, one patient with leucopenia of 2.9×10^9/L, without any other lupus manifestation).

Performance of Doria, SLE-DAS Boolean-based and SLE-DAS index-based definitions of clinical remission are shown in table 4. The agreement between DORIS and Doria criteria, and between DORIS and SLE-DAS Boolean-based definitions were perfect (κ=1, for both) and almost perfect between DORIS and SLE-DAS index-based remission (κ=0.98, p<0.0001) either in Padova or in Cochin cohort. For the derivation cohort, decision trees generated by CHAID are presented in figure 1. Considering as independent variables the prednisone dose, SLEDAI-2K and the SLE-DAS score of clinical items (irrespectively from anti-dsDNA antibodies and hypocomplementaemia), the CHAID classification rule is the same specified in the Boolean-based definition of remission. Using the SLE-DAS score with all its items, the decision tree classification rule also matches the index-based definition of remission. The CHAID algorithm identified SLE-DAS as the variable most significantly associated with remission (DORIS definition); the second one was the prednisone dose ≤5 mg/day. SLEDAI-2K was not incorporated in any of the trees, which means that its importance in predicting remission is superseded by the other two variables.

Table 3 Performance of SLE-DAS cut-offs for disease activity categories, as compared to physician's classification in the derivation and validation clinical cohorts, and to BILAG scores in the BLISS-76 population

	Disease activity category	Sensitivity%	Specificity%	G-Mean* %
Derivation Padova Cohort	Remission (SLE-DAS ≤2.08)	99.3	97.1	98.2
	Mild Disease Activity (2.08<SLE-DAS≤7.64)	80.7	98.4	89.1
	Moderate and Severe Disease Activity (SLE-DAS >7.64)	94.9	97.8	96.3
Validation Cochin Cohort	Remission (SLE-DAS ≤2.08)	99.1	93.9	96.5
	Mild Disease Activity (2.08<SLE-DAS≤7.64)	82.6	99.2	90.5
	Moderate and Severe Disease Activity (SLE-DAS >7.64)	100.0	98.6	99.3
Validation BLISS-76 Cohort	Remission and Mild Disease Activity† vs Moderate and Severe Disease Activity‡ (SLE-DAS ≤7.64 vs >7.64)	88.6	84.1	90.8 (88.7–92.6)

*G-Mean= $\sqrt{Sensitivity \times Specificity}$.
†No BILAG A or B.
‡≥1 BILAG A or B.
BILAG, British Isles Lupus Assessment Group; SLE-DAS, Systemic Lupus Erythematosus Disease Activity Score.

Table 4 Performance of Doria, SLE-DAS index-based and Boolean-based definitions of clinical remission compared to DORIS clinical remission criteria

	Definition of clinical remission	Sensitivity %	Specificity%	G-Mean* %
Derivation Padova Cohort	Doria	100.0	100.0	100.0
	SLE-DAS Boolean based	100.0	100.0	100.0
	SLE-DAS index based	100.0	97.4	98.7
Validation Cochin Cohort	Doria	100.0	100.0	100.0
	SLE-DAS Boolean based	100.0	100.0	100.0
	SLE-DAS index based	100.0	97.4	98.7

DORIS clinical remission: score of 0 in all clinical items of Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2K) and physician global assessment <0.5 (0–3 points) and prednisone dose ≤5 mg/day; Doria clinical remission: score of 0 in all clinical items of SLEDAI-2K and absence of haemolytic anaemia, myelitis and gastrointestinal lupus involvement and prednisone dose ≤5 mg/day; Boolean-based clinical remission: score of 0 in all clinical items of SLE-DAS and prednisone dose ≤5 mg/day; SLE-DAS index-based clinical remission: SLE-DAS ≤2.08 and prednisone dose ≤5 mg/day.
*G-Mean= $\sqrt{Sensitivity \times Specificity}$.
DORIS, Definition Of Remission In SLE; SLE-DAS, Systemic Lupus Erythematosus Disease Activity Score.

Evaluation of SLE-DAS cut-offs for SLE disease activity categories in post hoc analysis of the BLISS-76 population

In the BLISS-76 trial, sensitivity, specificity and G-Mean of the SLE-DAS cut-off to identify moderate/severe disease activity defined by the BILAG score was, respectively, 88.6%, 84.1% and 90.8% (table 3).

The ROC curve and the AUC value of SLE-DAS to detect ≥1 BILAG B are presented in figure 2. Performance of SLE-DAS and SELENA-SLEDAI to identify disease activity categories according to BILAG index and characteristics of the discordant cases are shown in online supplemental tables S1 and S4, respectively.

SLE-DAS online calculator

SLE-DAS scoring using the SLE-DAS online calculator took 1–2 min for each patient (depending on the positive items to enter), and there was no discrepancies with the SLE-DAS score calculated using the Microsoft Excel spreadsheet. The http://sle-das.eu/ website layout of the SLE-DAS online calculator is presented in figure 3.

DISCUSSION

In this study, we derived two SLE-DAS-based definitions of SLE clinical remission, one index-based and another Boolean, both showing in external validation a very high performance, consistent with the DORIS clinical remission criteria. Additionally, we derived the SLE-DAS cut-offs for defining categories of active disease, that showed in the validation cohorts a high performance, consistent with the expert clinician judgement and the BILAG index. These definitions can provide guidance to the clinicians using SLE-DAS in the treat-to-target strategy and adjusting treatment according to the level of disease activity in the management of patients with SLE (figure 4).

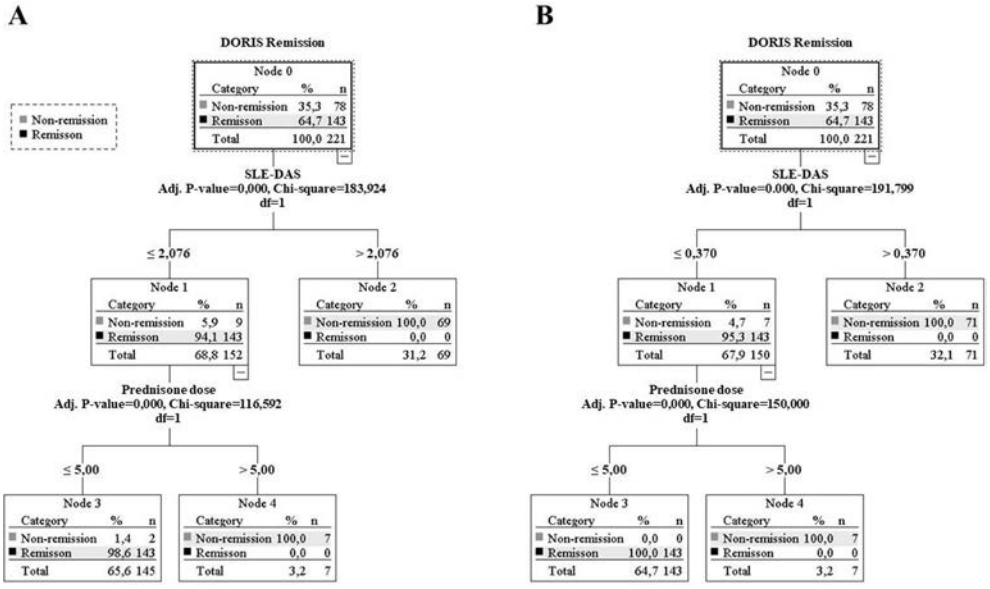

Figure 1 Decision tree for predicting remission according to DORIS clinical remission: (A) based on SLE-DAS score and prednisone daily dose; (B) based on clinical SLE-DAS (excluding anti-dsDNA antibodies and hypocomplementaemia) and prednisone daily dose—Padova Lupus Cohort. DORIS, definition of remission In SLE; SLE-DAS, systemic lupus erythematosus disease activity score.

The remission state is the preferred target in the management of patients with SLE; however, low disease activity is a good alternative goal if remission cannot be achieved.[6 12] Applying the SLE-DAS clinical remission definition, this target was fulfilled by most patients from the clinical cohorts in this study, hence suggesting it is an achievable target in clinical practice. The SLE-DAS definitions of clinical remission do not require the PGA (included in the DORIS definition), that needs standardisation to improve its reliability.[29] The SLE-DAS index-based definition was discordant with the DORIS criteria in just three cases (<1% of cases), that only presented mild cytopaenias with normal serology, not requiring specific treatment.[6 8] Conversely, the DORIS and Doria clinical remission criteria also allow ongoing mild leucopenia ($3.1–3.9\times10^9$/L), despite this is counted as lupus manifestations in the SLE classification criteria sets.[19 20 30 31] In the SLE-DAS clinical remission definitions, as in the DORIS and Doria criteria, we included the requirement of a prednisone dose ≤5 mg/day. This is important in order to ensure that disease activity is not masked by a high dose of glucocorticoids, as well as because prednisone >5 mg/day is an independent predictor of poor long-term outcome.[2 32–34]

Accurate definition of active SLE categories is useful as inclusion criteria in clinical trials, in guiding treatment strategies and in defining disease outcomes. Previous attempts to categorise the extent of SLE activity were based either on SLEDAI, BILAG or type of active organ manifestations.[6] However, SLEDAI-based categorisation is hampered by the dichotomous nature of its scoring instrument and the weights of the different items which can be inaccurate in defining the actual disease activity. The EULAR recommendations further propose to categorise

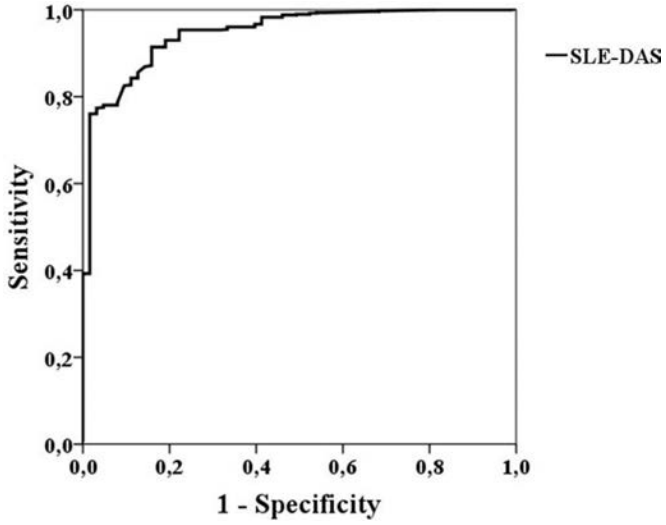

Figure 2 Receiver operating characteristics curve showing the performance of the Systemic Lupus Erythematosus Disease Activity Score (SLE-DAS) to detect ≥1 BILAG B organ domain score in the BLISS-76 population, AUC=0.948 (95% CI 0.923 to 0.973), p<0.0005. AUC, area under the curve; BILAG, British Isles Lupus Assessment Group.

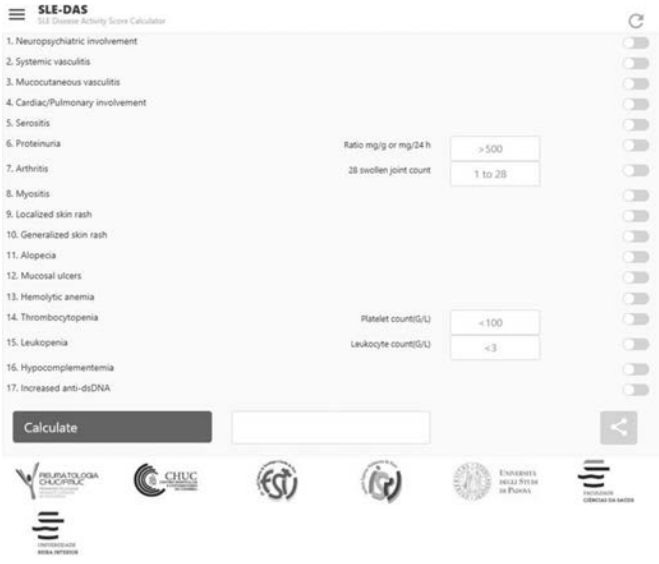

Figure 3 Compact layout of the SLE-DAS online calculator, freely available at http://sle-das.eu/. SLE-DAS, Systemic Lupus Erythematosus-Disease Activity Score.

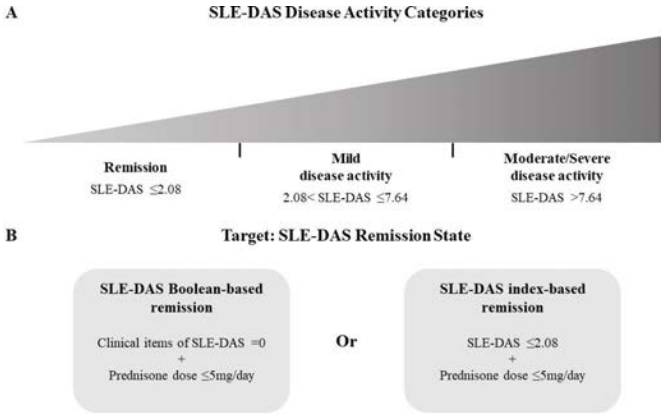

Figure 4 Cut-offs of SLE-DAS for (A) disease activity categories and (B) SLE-DAS remission state definitions. SLE-DAS, Systemic Lupus Erythematosus-Disease Activity Score.

patients with SLE according to the type and severity of organ involvement, such as classifying localised lupus rashes as mild, while generalised rashes are classified as moderate; thrombocytopaenia is categorised as mild, moderate or severe according to the platelet count.[6] There are also some controversial issues with BILAG classification, such as scoring a BILAG-defined severe arthritis variably as 'B' or 'A' depending on whether the clinician judges it as improving or not. By contrast, SLE-DAS allows a continuous and more objective measure of a core of disease activity manifestations, such as nephritis, arthritis, leucopenia and thrombocytopaenia. Thus, SLE-DAS was expected to allow a more appropriate categorisation of SLE activity. This was confirmed by our study, where the SLE-DAS cut-offs showed an excellent discrimination among the disease activity categories defined according to the expert judgement in a clinical setting or the BILAG index in a clinical trial setting.

Importantly, the online SLE-DAS calculator showed to be reliable and user-friendly. Scoring of SLE-DAS should be feasible in daily clinical practice, as it requires a similar workup time to that of SLEDAI-2K. Notably, applying the SLE-DAS is practical to identify patients in clinical remission state, being consistent with the DORIS and Doria definitions of remission.

Limitations of our study include the low prevalence of patients with high disease activity in the clinical cohorts, hindering the possibility to distinguish between moderate and severe activity categories in these settings. Furthermore, as there is no gold-standard definition for categories of active disease, we used as comparator the expert clinician's categorisation, which can be subjective. Also the DORIS criteria require better validation. However, we showed that the SLE-DAS categories perform equally well when compared with BILAG, which is a standardised measure. The SLE-DAS was retrospectively scored from the BLISS-76 database, using all the information available from SELENA-SLEDAI, BILAG and laboratory results, inferring the number of swollen joints and skin rashes extension.

Strengths of this study include its large multicentre and multiethnic population from both clinical practice and clinical trial settings, the blinding of the experts' clinical judgement to the DAS, the comparison against the BILAG for validation of active disease categories and the DORIS criteria for clinical remission state, and the excellent performance in the SLE-DAS definitions of both clinical remission and disease activity categories.

In conclusion, the SLE-DAS is an accurate and user-friendly instrument for classifying SLE in clinical remission state or in different categories of disease activity. The SLE-DAS may facilitate the treat-to-target strategy in the management of patients with SLE, providing a useful instrument in guiding disease treatment, defining new SLE outcomes, and identifying candidates for clinical trials.

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TRANSLATIONAL SCIENCE

Metagenome-wide association study revealed disease-specific landscape of the gut microbiome of systemic lupus erythematosus in Japanese

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ABSTRACT

Objective Alteration of the gut microbiome has been linked to the pathogenesis of systemic lupus erythematosus (SLE). However, a comprehensive view of the gut microbiome in SLE and its interaction with the host remains to be revealed. This study aimed to reveal SLE-associated changes in the gut microbiome and its interaction with the host by a comprehensive metagenome-wide association study (MWAS) followed by integrative analysis.

Methods We performed a MWAS of SLE based on shotgun sequencing of the gut microbial DNA from Japanese individuals (N_{case}=47, $N_{control}$=203). We integrated the result of the MWAS with the genome-wide association study (GWAS) data and plasma metabolite data.

Results Via species level phylogenetic analysis, we identified and validated increases of *Streptococcus intermedius* and *Streptococcus anginosus* in the patients with SLE. Microbial gene analysis revealed increases of *Streptococcus*-derived genes including one involved in redox reaction. Additionally, microbial pathways related to sulfur metabolism and flagella assembly were altered in the patients with SLE. We identified an overlap in the enriched biological pathways between the metagenome and the germline genome by comparing the result of the MWAS and the GWAS of SLE (ie, MWAS-GWAS interaction). α-diversity and β-diversity analyses provided evidence of dysbiosis in the metagenome of the patients with SLE. Microbiome-metabolome association analysis identified positive dosage correlation of acylcarnitine with *Streptococcus intermedius*, an SLE-associated taxon.

Conclusion Our MWAS followed by integrative analysis revealed SLE-associated changes in the gut microbiome and its interaction with the host, which contribute to our understanding of the relationship between the microbiome and SLE.

Key messages

What is already known about this subject?

- Alteration of the gut microbiome has been linked to the pathogenesis of systemic lupus erythematosus (SLE), but a comprehensive view of the gut microbiome in SLE and its interaction with the host remains to be revealed.
- Whole metagenome shotgun sequencing technology has many advantages over conventional 16S ribosomal RNA sequencing such as higher taxonomic resolution and applicability for the functional analysis. However, evaluation of the microbiome-disease association based on shotgun sequencing is still incomplete for SLE.

What does this study add?

- Our shotgun sequence-based metagenome-wide association study (MWAS) newly identified two bacterial species (*Streptococcus anginosus* and *Streptococcus intermedius*), eight bacterial genes and seven biological pathways which were significantly different between the healthy controls and the patients with SLE.
- Integrative analysis with the genome-wide association study (GWAS) result and the plasma metabolite data revealed the interactions between the gut microbiome and the host mediated by biological pathways or plasma metabolites.

How might this impact on clinical practice or future developments?

- Our shotgun sequencing-based MWAS and integrative analysis with the GWAS data and plasma metabolite profiles revealed an SLE-specific microbial landscape and its association with the host. Our analysis contributes to our understanding of the relationship between the gut microbiome and SLE.

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease, which is characterised by overactivation of the immune system and involvement of various organs such as kidney and brain. Despite advances in treatment, standardised mortality rates in patients with SLE were three times higher than in the general populations because of poor control of the disease activity or infection due to immunosuppressive treatment.[1] SLE results from a complex interplay of multiple genetic and environmental factors; however, much of the aetiology of SLE

remain to be elucidated. Therefore, extensive efforts have been spent to reveal the pathogenesis of SLE for the development of better clinical care.

Microbiome, which refers to the microbial communities inhabiting the human body, has a remarkable effect on our body by modulating our immune system or taking a part of our metabolic network.[2] The largest community of the human microbiota resides within the gut. Involvement of the gut microbiome is reported for various diseases, such as type 2 diabetes,[3] colorectal cancer,[4] rheumatoid arthritis (RA),[5 6] inflammatory bowel disease (IBD)[7], and multiple sclerosis.[8] Accompanied by the great progresses in high-throughput sequencing technology and success of the treatment such as faecal microbiome transplantation and probiotics, characterisation of the gut microbiome has become a major research area in human diseases.

Recently, relationship between the gut microbiome and SLE was studied to reveal an unexplained part of the SLE aetiology. Faecal microbiome from an SLE model mouse has capacity to induce SLE-like phenotype in a healthy mouse underlying the non-trivial relationship between the gut microbiome and the SLE pathogenesis.[9] Mechanistic insights of association between the gut microbiome and SLE have been obtained by mouse experiments (e.g, activation of the immune system caused by bacterial translocation from gut to liver[10] or microbe derived metabolites[11]). In human, SLE-associated taxa were searched through amplicon sequencing of 16S ribosomal RNA (rRNA) genes.[12–16] Although the findings were not universally consistent, reflecting difference in ethnicities and lifestyles, several SLE-associated clades were identified. However, 16S rRNA sequencing typically provides phylogenetic abundance at up to the genus level, making it suffer from low taxonomic resolution. In addition, 16S rRNA sequencing technology can only be used for phylogenetic analysis; thus, functional aspect of disease-specific microbial environment is overlooked.

In gut microbiome case-control study, metagenome-wide association studies (MWAS) based on whole-genome shotgun sequencing are replacing case-control comparison with 16S rRNA sequencing technology. Shotgun sequencing has a potential to detect the genomic composition of microbes at the species level, achieving higher taxonomic resolution than 16S rRNA sequencing technology. Furthermore, shotgun sequencing can be used for analysing microbial gene and pathway and is therefore useful for surveying the functional aspect of microbial environment. However, shotgun sequencing requires much larger sequencing cost and machine resource than 16S rRNA-sequencing. Additionally, analytic methods applied to shotgun sequencing data are usually complicated. For these reasons, evaluation of microbiome-disease association based on shotgun sequencing is still incomplete for many diseases including SLE.[17] Furthermore, insufficient number of shotgun sequencing studies in non-European population is problematic given the significant impact of ethnicity and lifestyle on the microbial landscape.[18 19]

Even with shotgun sequencing analysis, microbiome-host interaction is hardly evaluated unless performing integrative analysis with other modality data such as metabolic profiles. A large part of microbiome-host interaction is estimated to be mediated by metabolic signals.[2] Multiomics analysis based on microbial and metabolic data was performed in not many but several diseases such as IBD[7] and has revealed the involvement of metabolites in microbiome-disease association. Integrative analysis with the genome-wide association study (GWAS) is also useful for revealing a link between the gut microbiome and the host genome, namely MWAS-GWAS interaction.[5] Nonetheless, microbiome-host interaction in SLE has been never evaluated, hindering us from the comprehensive understanding of the microbiome-associated SLE pathology.

In this study, we carried out shotgun sequencing of faecal samples from 250 Japanese subjects, composed of 47 patients with SLE and 203 healthy controls (HCs). To identify SLE-associated microbes, we performed phylogenetic case-control comparison. We also performed microbial gene analysis followed by pathway analysis for revealing functional differences of the gut metagenome between the HCs and the patients with SLE. To reveal the microbiome-host interaction in SLE, we performed a combined biological pathway analysis of MWAS and GWAS. In addition, we performed an integrative analysis using plasma metabolite profiles obtained through the non-targeted metabolomics approach. The joint study of microbiome and metabolome can identify the functional readouts of disease-specific microbial activity which mediates the microbe-host interaction.

METHODS

Methods are provided in the online supplemental information.

RESULTS

High abundance of *Streptococcus anginosus* and *Streptococcus intermedius* in the SLE gut microbiome

We performed whole-genome shotgun sequencing analysis of a total of 250 faecal DNA samples (47 individuals with SLE and 203 HC subjects) derived from three sequencing groups (online supplemental table 1), which passed stringent quality control (QC) for sequence reads and samples. Procedures of sample QC and definition of the sample sets in each analysis are described in online supplemental figure 1. Then, we obtained phylogenetic relative abundances (online supplemental figure 3). For case-control comparison, we performed clade QC. After clade QC, we had 774 clades for case-control association test, including 12 phyla (L2), 25 classes (L3), 36 orders (L4), 72 families (L5), 178 genera (L6) and 451 species (L7).

We performed case-control comparison for each clade and identified that *Streptococcus anginosus* and *Streptococcus intermedius* significantly increased in SLE (effect size=0.617 and $P_{\text{microbe}}=3.7\times10^{-5}$ for *Streptococcus anginosus*, effect size=0.579 and $P_{\text{microbe}}=7.5\times10^{-5}$ for *Streptococcus intermedius*; figure 1A,B and table 1), satisfying an empirically estimated Bonferroni threshold ($\alpha=0.05$; $P_{\text{microbe}}<8.2\times10^{-5}$). As illustrated in a phylogenetic tree indicating the case-control association results of multilayered taxonomic levels (figure 1C), both of the clades with case-control discrepancy were species (L7) level. Since it was difficult to detect the species-level clades using classical 16S rRNA sequencing, our results underlay the strength of MWAS approach with shotgun sequencing to identify disease-associated microbial taxa.

As medication of the patients with SLE and male-female imbalance due to sex biased prevalence could be a confounding factor, we performed subanalysis (online supplemental table 2). Effect sizes were almost similar among subanalyses for the *Streptococcus anginosus* and *Streptococcus intermedius* ($0.487\leq$effect size≤0.647 for *Streptococcus anginosus*, $0.463\leq$effect size≤0.654 for *Streptococcus intermedius* (online supplemental figure 4 and table 2) after removing male subjects or those who took medications such as proton pump inhibitor, antibiotics or therapeutics for SLE. These results suggested that inclusion of the male subjects or those who took medications such as proton pump inhibitor, antibiotics or therapeutics minimally confounded the result but increased statistical power of the MWAS. The abundance of these two clades was not significantly different between the newly onset patients and the other patients, the patients with lupus nephritis (LN) and without

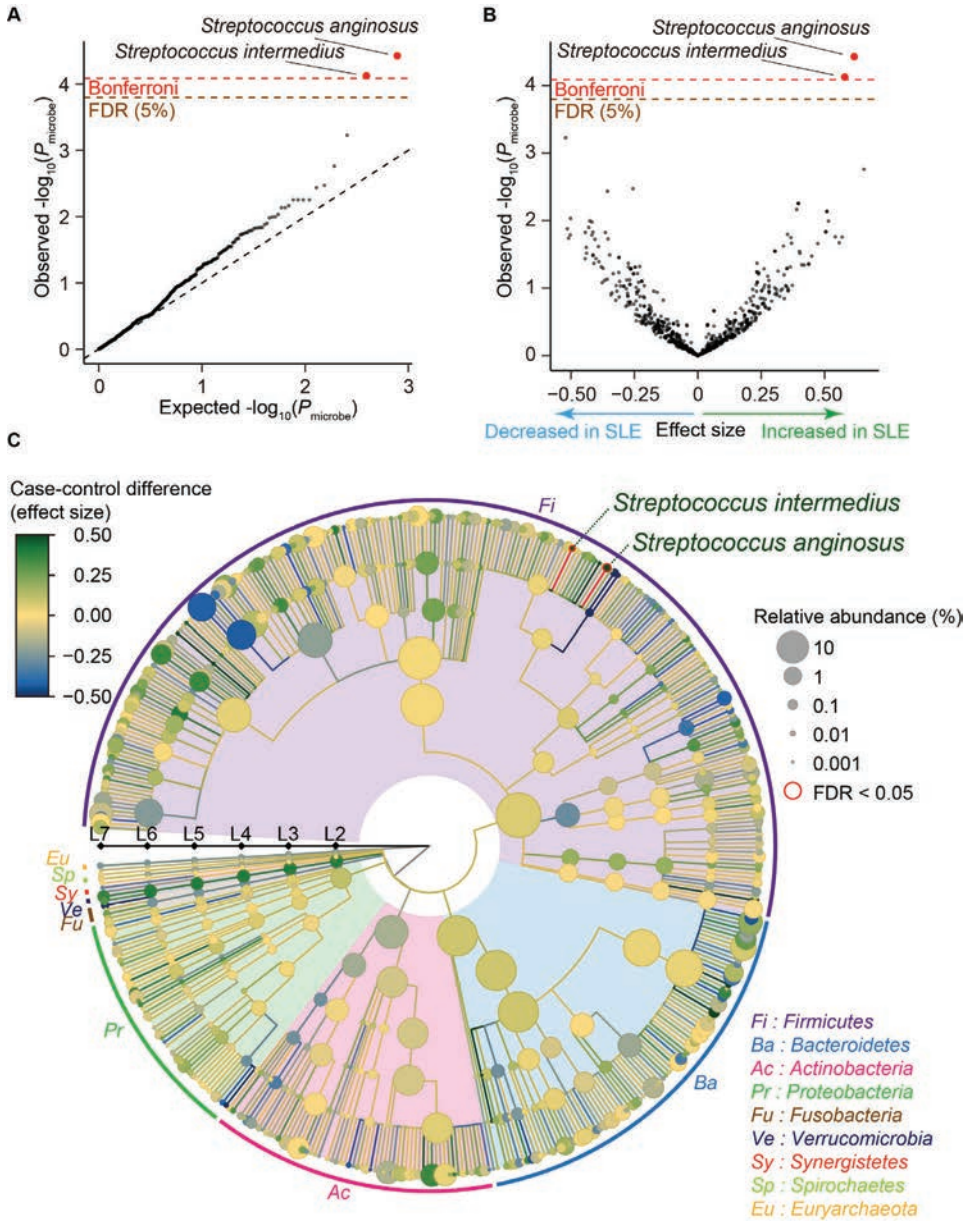

Figure 1 Result of the SLE MWAS based on the phylogenetic abundance data. (A) A quantile-quantile plot of the phylogenetic MWAS p values ($P_{microbe}$) of the clades. The x-axis indicates log-transformed empirically estimated median $P_{microbe}$. The y-axis indicates observed $-\log_{10}(P_{microbe})$. The diagonal dashed line represents y=x, which corresponds to the null hypothesis. The horizontal red line indicates the empirical Bonferroni-corrected threshold (α=0.05), and the brown line indicates the empirically estimated FDR threshold (FDR=0.05). Clades with $P_{microbe}$ less than the Bonferroni thresholds are plotted as red dots, and other clades are plotted as black dots. (B) A volcano plot. The x-axis indicates effect sizes in linear regression. The y-axis, horizontal lines and dot colours are the same as in (A). (C) A phylogenetic tree. Levels L2–L7 are from the inside layer to the outside layer. The size and the colour of dots represent relative abundances and effect sizes, respectively. The two clades with significant case-control associations (FDR<0.05) are outlined in red. FDR, false discovery ratio; MWAS, metagenome-wide association study; SLE, systemic lupus erythematosus.

LN or the patients with high SLE Disease Activity Index (SLE-DAI) and low SLE-DAI ($P_{microbe}$ >0.12; online supplemental table 2). We performed replication analysis for *Streptococcus anginosus* and *Streptococcus intermedius* by using a previously published shotgun sequencing study.[17] Association of *Streptococcus anginosus* and *Streptococcus intermedius* was significantly replicated (same effect direction and $P_{microbe}$ <0.05/2=0.025), confirming the associations identified by our SLE MWAS.

Table 1 Clades with significant case-control discrepancy in the SLE MWAS

		This study (Japanese, *N*=250)			Chen *et al* (Chinese, *N*=232)	
Microbe	**Level**	**Effect size**	**SE**	$P_{microbe}$	**Effect direction**	$P_{microbe}$
Streptococcus anginosus	Species (L7)	0.617	0.146	3.7×10^{-5}	Positive	1.9×10^{-6}
Streptococcus intermedius	Species (L7)	0.579	0.143	7.5×10^{-5}	Positive	9.1×10^{-4}

MWAS, metagenome-wide association study; SLE, systemic lupus erythematosus.

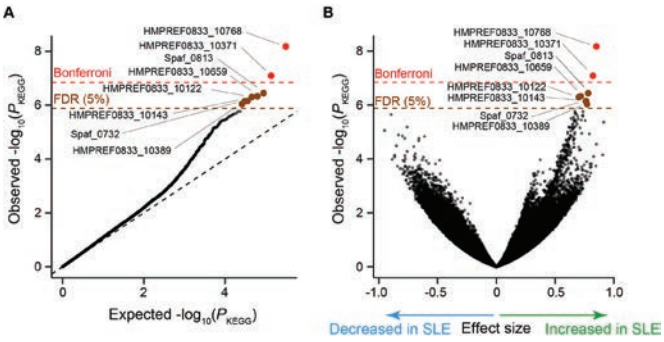

Figure 2 Result of the SLE MWAS based on the microbial gene abundance data. (A) A quantile-quantile plot of the MWAS p values of the genes (P_{KEGG}). The x-axis indicates log-transformed empirically estimated median P_{KEGG}. The y-axis indicates observed $-\log_{10}(P_{KEGG})$. The diagonal dashed line represents y=x, which corresponds to the null hypothesis. The horizontal red line indicates the empirical Bonferroni-corrected threshold (α=0.05), and the brown line indicates the empirically estimated FDR threshold (FDR=0.05). Genes with P_{KEGG} less than the Bonferroni thresholds are plotted as red dots. Genes with FDR<0.05 are plotted as brown dots, and other clades are plotted as black dots. (B) A volcano plot. The x-axis indicates effect sizes in linear regression. The y-axis, horizontal lines and dot colours are the same as in (A). FDR, false discovery ratio; KEGG, Kyoto Encyclopedia of Genes and Genomes; MWAS, metagenome-wide association study; SLE, Systemic lupus erythematosus.

High abundance of *Streptococcus*-derived genes in the gut metagenome of patients with SLE

We next performed a gene level MWAS. We obtained microbial gene abundance data based on Kyoto Encyclopedia of Genes and Genomes (KEGG) database.[20] After gene level QC, we retained 240,544 genes for case-control comparison. We performed case-control comparison for each gene and identified eight genes which significantly increased in SLE (empirically estimated false discovery ratio (FDR)=0.05; figure 2A,B, table 2). As conducted in the phylogenetic analysis, we performed subanalysis (online supplemental table 3). For the eight-genes increased in SLE, effect sizes were almost similar among subanalyses (online supplemental figure 5). These results suggested that inclusion of the male subjects or those who took medications such as proton pump inhibitor, antibiotics or therapeutics did not confound the result. The abundance of these eight genes was not significantly different between the newly onset patients and the other patients, the patients with LN and without LN or the patients with high SLE-DAI and low SLE-DAI (online supplemental table 3).

All of these eight genes were registered as *Streptococcus parasanguinis ATCC 15912* or *Streptococcus parasanguinis FW213* derived. In our metagenome data, although major derivation of these genes were *Streptococcus parasanguinis*, reference genomes of other *Streptococcus* such as *Streptococcus sanguinis* or unclassified *Streptococcus* were linked to these genes. *Streptococcus parasanguinis* was not significantly increased in the gut metagenome of the patients with SLE in our phylogenetic analysis (effect size=0.122, $P_{microbe}$=0.35), indicating the possibility of collective enrichment of the multiple species of *Streptococcus* which had the several genes in common or difference in the composition of genes among *Streptococcus parasanguinis* strains. Among the eight genes which significantly increased in the patients with SLE, Spaf_0732 was a glutaredoxin-like protein. Some glutaredoxin-like protein was involved in reactive oxygen metabolism.[21] As previously described, gut redox environment has substantial effect on the host's immune system,[22] and its alteration in the gut microbiome of autoimmune diseases such as RA was reported.[5 6]

Identification of metagenomic biological pathways altered in the patients with SLE

Using the result of the gene level MWAS, we performed a gene set enrichment analysis to evaluate the case-control discrepancy of the gut metagenome at pathway level. We evaluated 126 QC-passed pathways registered in KEGG database. We found that genes differentially abundant between case and control were significantly enriched on seven pathways (FDR<0.05; figure 3A,B, online supplemental table 4). One of the significant pathways was sulfur metabolism and sulfur was associated with redox reaction,[6] suggesting that altered redox reaction was associated with the pathology of SLE. Enrichment of flagellar assembly might result from bacteria-host interaction mediated by strong immune reaction to bacterial flagellar.[23]

SLE-specific biological pathways shared between metagenome and human genome

We integrated the result of the current SLE MWAS data and the previously published SLE GWAS data (4,943 cases and 8,483 controls)[24] for assessing the sharing of biological pathways between the gut microbiome and the host. We used PASCAL[25] for pathway analysis of the GWAS summary statistics. A total of 94 pathways registered in KEGG database were commonly evaluated for MWAS and GWAS. We compared the p values of the each KEGG pathway ($P_{pathway}$) between the SLE MWAS and the SLE GWAS. We found a significant overlap between the pathways enriched both in the SLE MWAS ($P_{pathway}$ for metagenome <0.05) and in the SLE GWAS ($P_{pathway}$ for SLE GWAS<0.05; P_{Fisher}=0.041; figure 3C). To check whether the overlap of the enriched pathways between the metagenome and the host genome truly reflected the SLE-specific changes in the biological pathways, we performed the same experiment

Table 2 Genes with significant case-control discrepancy in the SLE MWAS.

KEGG gene	Effect size	SE	P_{KEGG}	Gene name, definition	Organism
HMPREF0833_10768	0.850	0.141	6.7×10^{-9}	udk; uridine kinase	*Streptococcus parasanguinis ATCC 15912*
HMPREF0833_10371	0.821	0.148	8.1×10^{-8}	Hypothetical protein	*Streptococcus parasanguinis ATCC 15912*
Spaf_0813	0.781	0.149	3.6×10^{-7}	Hypothetical protein	*Streptococcus parasanguinis FW213*
HMPREF0833_10659	0.716	0.138	4.7×10^{-7}	Methyltransferase small domain protein	*Streptococcus parasanguinis ATCC 15912*
HMPREF0833_10122	0.701	0.135	5.0×10^{-7}	Membrane protein	*Streptococcus parasanguinis ATCC 15912*
HMPREF0833_10143	0.762	0.149	7.0×10^{-7}	Hydrolase	*Streptococcus parasanguinis ATCC 15912*
Spaf_0732	0.760	0.149	7.1×10^{-7}	nrdH; Glutaredoxin-like protein	*Streptococcus parasanguinis FW213*
HMPREF0833_10389	0.770	0.152	9.1×10^{-7}	Hypothetical protein	*Streptococcus parasanguinis ATCC 15912*

KEGG, Kyoto Encyclopedia of Genes and Genomes.;

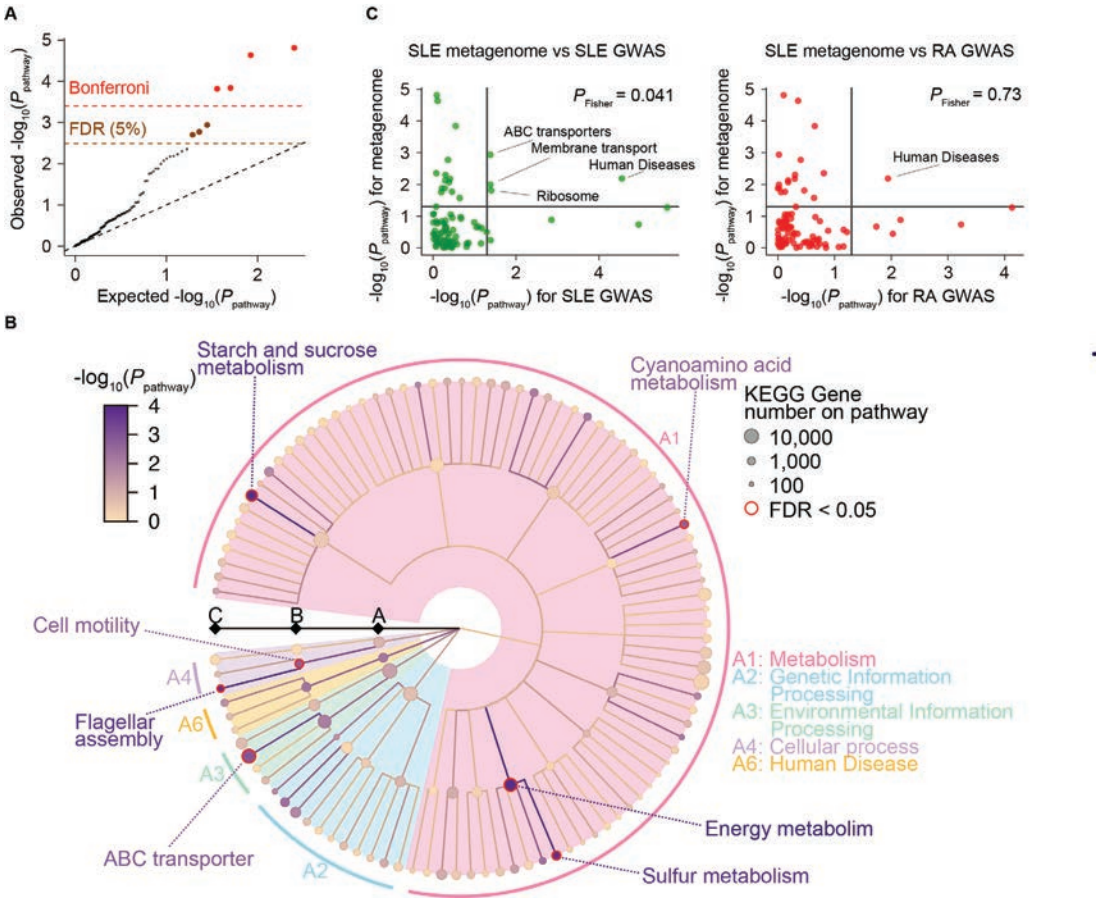

Figure 3 MWAS results of the SLE case-control pathway association tests. (A) A quantile-quantile plot of the MWAS p values of pathways based on KEGG pathways ($P_{pathway}$). The x-axis indicates log-transformed empirically estimated median $P_{pathway}$. The y-axis indicates observed $-\log_{10}(P_{pathway})$. The diagonal dashed line represents y=x, which corresponds to the null hypothesis. The horizontal red dashed line indicates the Bonferroni-corrected threshold (α=0.05), and the brown dashed line indicates the FDR threshold (FDR=0.05) calculated with Benjamini-Hochberg method. Pathways with p values less than the Bonferroni thresholds are plotted as red dots. Pathways with FDR<0.05 are plotted as brown dots, and other pathways are plotted as black dots. (B) System diagram of KEGG pathways. The three levels are defined as A, B and C and described from the inside layer out. The size and the colour of dots represent set sizes and $P_{pathway}$, respectively. The seven pathways with significant enrichment (FDR<0.05) are outlined in red. (C) Comparison of $P_{pathway}$ between the SLE MWAS and GWAS data. The x-axis indicates the $P_{pathway}$ of the GWAS data (left, SLE GWAS; right, RA GWAS). The y-axis indicates the $P_{pathway}$ of the SLE MWAS. The horizontal and vertical black lines indicate $P_{pathway}$ of 0.05. The overlap of the pathway enrichment was evaluated by classifying the pathways based on the significance threshold of $P_{pathway}$<0.05 or $P_{pathway}$≥0.05 and using Fisher's exact test. FDR, false discovery rate; GWAS, genome-wide association study; KEGG, Kyoto Encyclopedia of Genes and Genomes; MWAS, metagenome-wide association study; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.

with the SLE MWAS data and RA GWAS data (14,361 cases and 43,923 controls).[26] When using the RA GWAS data, the overlap of the enriched pathways between the metagenome and the host genome was not significant (P_{Fisher}=0.73; figure 3C). Therefore, our results suggested that there was a commonality in the enriched biological pathways between human genome and metagenome in SLE, namely MWAS-GWAS interaction.

Dysbiosis in the gut microbiome of the patients with SLE

Dysbiosis refers to an unbalanced microbiota, which is usually harmful for us. Decrease in the α-diversity (ie, within individual diversity) of microbiome was one of the most constant findings of the gut dysbiosis[27] and reported in many disease conditions including IBD.[28] As for SLE, decrease in α-diversity of the gut microbiome was still controversial.[12–17] Therefore, we performed case-control comparison of α-diversity in the phylogenetic data (L2–L7) and the gene abundance data based on KEGG database. Significant decreases of α-diversity in the low taxonomic level phylogenetic data (L5–L7; $P_{\alpha\text{-diversity}}$<0.05/6=0.0083) and the gene abundance data were observed ($P_{\alpha\text{-diversity}}=7.9\times10^{-5}$; figure 4A,B, online supplemental tables 5 and 6). In subanalysis, significant decreases of α-diversity in the phylogenetic data at L5 and L6 levels and the gene abundance data were still observed ($P_{\alpha\text{-diversity}}$<0.05/6=0.0083 for phylogenetic data and $P_{\alpha\text{-diversity}}$<0.05 for gene abundance data; online supplemental tables 5 and 6). Although decrease of α-diversity in the phylogenetic data at L7 level was not significant when removing patients with antibiotics usage ($P_{\alpha\text{-diversity}}$=0.052), direction of the effect size was consistent. Microbial α-diversity was not significantly different between the newly onset patients and the other patients, the patients with and without LN or the patients with high SLE-DAI and low SLE-DAI ($P_{\alpha\text{-diversity}}$ > 0.05/6 in phylogenetic analysis and $P_{\alpha\text{-diversity}}$ >0.05 in functional analysis; online supplemental tables 5 and 6).

Next, we performed a β-diversity analysis for checking whether SLE affected the overall microbial composition or

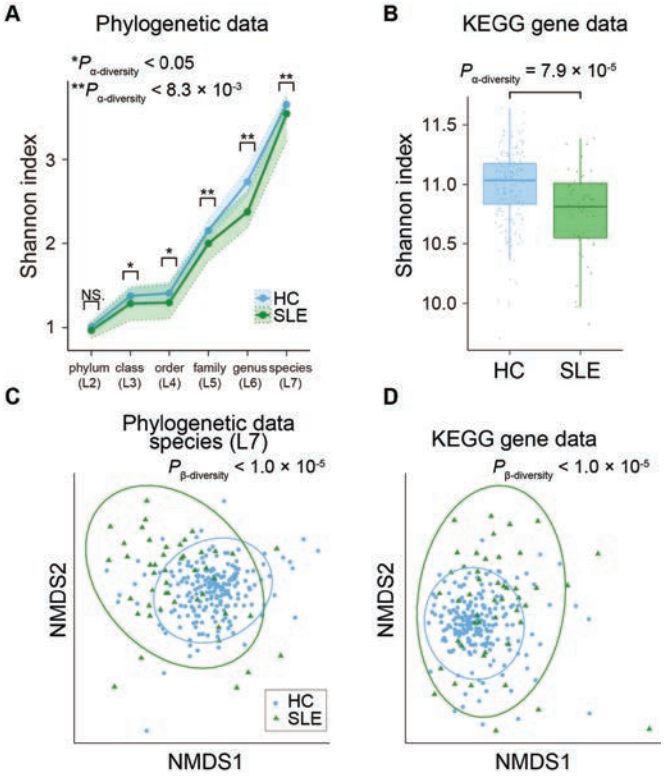

Figure 4 Case-control comparison of the microbial diversities in SLE. (A) α-diversities of the phylogenetic relative abundance data for the six taxonomic levels. Blue and green dots represent the median Shannon index of the HC and SLE subjects. Upper and lower dashed lines indicate the first and third quantile of Shannon index for the HC and SLE subjects. (B) α-diversities of the gene abundance based on KEGG gene databases. Boxplots indicate the median values (centre lines) and the IQRs (box edges), with whiskers extending to the most extreme points within the range between (lower quantile − [1.5×IQR]) and (upper quantile + [1.5×IQR]). (C) β-diversities of the phylogenetic relative abundance data at the species level. Result of NMDS based on Bray-Curtis distance is represented. Blue and green dots represent the HC and SLE subjects. (D) β-diversities of the gene abundance based on KEGG gene database. Result of NMDS based on Bray-Curtis distance is represented. Blue and green dots represent the HC and SLE subjects. $*P_{\alpha\text{-diversity}}<0.05$; $**P_{\alpha\text{-diversity}}<0.0083$. HC, healthy control; KEGG, Kyoto Encyclopedia of Genes and Genomes; NMDS, non-metric multidimensional scaling; SLE, systemic lupus erythematosus.

not. We performed PERMANOVA,[29] based on Bray-Curtis distance calculated from the phylogenetic data (L2–L7) and the gene abundance data. Significant differences were detected in the phylogenetic data (L2–L7) and the gene abundance data with consistency in subanalysis ($P_{\beta\text{-diversity}}<0.05/6=0.0083$ for phylogenetic data and $P_{\beta\text{-diversity}}<0.05$ for gene abundance data; figure 4C,D, online supplemental figure 6, tables 7 and 8). There was no significant difference in the overall microbial composition between the newly onset patients and the other patients, the patients with and without LN or the patients with high SLE-DAI and low SLE-DAI ($P_{\beta\text{-diversity}}>0.05/6$ in phylogenetic analysis and $P_{\beta\text{-diversity}}>0.05$ in functional analysis; online supplemental tables 7 and 8). Collectively, diversity analysis provided evidence of the dysbiosis observed in the gut microbiome of the patients with SLE.

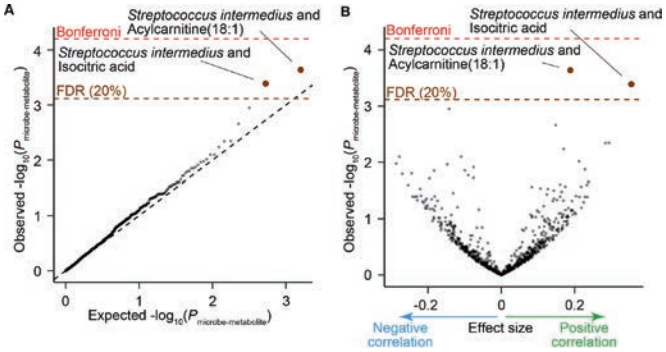

Figure 5 Result of the microbe-metabolite association analysis. (A) A quantile-quantile plot of the p values from the microbe-metabolite association analysis ($P_{\text{microbe-metabolite}}$). The x-axis indicates log-transformed empirically estimated median $P_{\text{microbe-metabolite}}$. The y-axis indicates observed $-\log_{10}(P_{\text{microbe-metabolite}})$. The diagonal dashed line represents y=x, which corresponds to the null hypothesis. The horizontal red dashed line indicates the Bonferroni-corrected threshold (α=0.05), and the brown dashed line indicates the FDR threshold (FDR=0.20) calculated with Benjamini-Hochberg method. The microbe-metabolite pairs with FDR<0.20 are plotted as brown dots, and the other microbe-metabolite pairs are plotted as black dots. (B) A volcano plot. The x-axis indicates effect sizes in linear regression. The y-axis, horizontal dashed lines and dot colours are the same as in (A). FDR, false discovery rate; HC, healthy control; SLE, systemic lupus erythematosus.

Association between plasma metabolite and the gut microbiome of the patients with SLE

Gut microbiome can affect our body by changing the profiles of circulating metabolites.[30–32] To assess the association between the SLE-associated taxa and plasma metabolites, we integrated the phylogenetic data and plasma metabolite profiles which were previously obtained from a part of the participants of this study.[33] As the focus of this analysis is not the case-control discrepancy but the microbe-metabolite association, we combined 94 HC subjects and 9 patients with SLE, which resulted in 103 participants. Abundance of the two metabolites, acylcarnitine(18:1) and isocitric acid were significantly positively correlated with the abundance of *Streptococcus intermedius* ($P_{\text{microbe-metabolite}}<4.1\times10^{-4}$; FDR<0.20; figure 5A,B, table 3). We performed a replication analysis using another dataset composed of 75 HC subjects.[8] Positive correlation between acylcarnitine(18:1) and *Streptococcus intermedius* was successfully replicated ($P_{\text{microbe-metabolite}}=0.0080$). Acylcarnitine is formed by carnitine and acyl-coenzyme A (CoAs) derived from fatty acids. Acylcarnitine was reported to be one of the main components of faecal bacteria-metabolite network and associated with the numerous dysbiosis associated species.[7] Acylcarnitine can work as an inflammatory

Table 3 Microbe-metabolite pairs with significant association

		Discovery (*N*=103)				Replication (*N*=75)		
Microbe	Metabolite	Effect size	SE	$P_{\text{microbe-metabolite}}$	*q*	Effect size	SE	$P_{\text{microbe-metabolite}}$
Streptococcus intermedius	Acylcarnitine(18:1)	0.188	0.049	2.3×10^{-4}	0.16	0.166	0.061	0.0080
Streptococcus intermedius	Isocitric acid	0.355	0.097	4.1×10^{-4}	0.16	0.155	0.119	0.19

signal,[34] suggesting that the gut microbiome of the patients with SLE is associated with the overactivation of the immune system in SLE via acylcarnitine.

DISCUSSION

In this study, we conducted a MWAS of Japanese patients with SLE using whole-genome shotgun sequencing. Our study revealed following biological features associated with the SLE gut metagenome (online supplemental figure 7): (1) *Streptococcus anginosus* and *Streptococcus intermedius* were increased in the SLE metagenome; (2) eight genes derived from *Streptococcus* including a gene related to redox reaction increased in the SLE metagenome; (3) various biological pathways, including those related to sulfur metabolism and flagella assembly were enriched among genes differentially abundant between case and control; (4) there existed an SLE-specific link between biological pathway of the gut microbiome and the host genome, namely MWAS-GWAS interaction; (5) the features of dysbiosis, decreases in α-diversity and changes in the overall composition of the gut microbiome, were observed among the patients with SLE; (6) plasma acylcarnitine(18:1) level was positively associated with the abundance of *Streptococcus intermedius*.

One of the principal findings of our study was increase of *Streptococcus anginosus* and *Streptococcus intermedius* in the SLE metagenome. Because it was difficult to detect these species level clades using classical 16S rRNA sequence analysis, these results demonstrate the value of metagenome shotgun sequencing in identifying disease-associated taxa. Considering the clinical features of SLE such as female-biased prevalence and frequent prophylactic antibiotics usage before renal biopsy or during immunosuppressive treatment, we evaluate the effect of these factors on the result of MWAS. Although the findings of the MWAS were often not replicated across studies due to the difference in ethnicity and lifestyle, our results were validated in the independent SLE metagenome dataset from the Chinese cohort,[17] suggesting that our statistical analysis robustly identified the taxa specifically abundant in the SLE metagenome. *Streptococcus anginosus* and *Streptococcus intermedius* belong to *Streptococcus anginosus* group and are parts of normal flora of the oral cavity and gastrointestinal tracts. Involvement of oral-gut interaction mediated by microbes was reported in several diseases,[5 35] suggesting the possibility of association between the oral-gut axis and the SLE pathology. Liu *et al* reported that *Streptococcus intermedius* produced and secreted a histone-like DNA binding protein, which induced proinflammatory cytokine production of macrophage derived cell line.[36] Therefore, interaction between *Streptococcus intermedius* and the host immune system could be related to the pathology of SLE.

Although the *Streptococcus anginosus* and *Streptococcus intermedius* consistently increased in the existing two shotgun sequencing studies (Chen *et al*[17] and our study), some previous findings in Chen *et al* were not replicated in this study. Although Chen *et al* reported that *Ruminococcus gnavus* increased in the patients with SLE, especially those with LN, it did not increase in our study (effect size=−0.001, SE=0.155, P_{microbe}=1.00 in case-control comparison and effect size=0.195, SE=0.355, P_{microbe}=0.59 in comparison between patients with SLE with LN or without LN). Additionally, among the 74 species which increased in Chen *et al* and evaluated in our study, two species, *Streptococcus anginosus* and *Streptococcus intermedius*, were replicated with $P_{\text{microbe}} < 0.05/74 = 6.8 \times 10^{-4}$. Among the 74 species, 38 species have the same directional effects between the studies. This heterogeneity might reflect the effects of geography and lifestyle on the gut microbiome, while differences in the analytic methods could not be rejected. Thus, further studies in the different countries or global meta-analysis will be warranted to further clarify the difference in the gut microbiome of the patients with SLE among different countries.

Our gene analysis revealed that eight genes including a glutaredoxin-like protein gene were increased in the SLE gut metagenome. Since all of these genes were derived from *Streptococcus,* there might be a possibility that some of these genes were enriched simply because of coabundance with *Streptococcus* genes truly relevant to the SLE condition. Further functional validation of each gene would be warranted. Subsequent pathway analysis based on the result of the gene analysis revealed an alteration of various biological pathways including sulfur metabolism and flagella assembly. Sulfur metabolism is reported to be altered in the metagenomes of other diseases such as RA,[6] and it is related to redox environment. Together with the result of the gene level analysis, our results suggested that alteration of redox environment was associated with the pathology of SLE. Flagellar is known to elicit strong immune response. Zeevi *et al* reported that gut bacteria which had structural variant in flagellar protein had higher growth ratio, implying the loss-of-function adaptation to the host's immune system.[23] There was an interaction between the gut bacteria and the immune system via flagellar, and the alteration of flagellar-related pathways in the SLE gut metagenome could be associated with changes in the host's immune system. Through the MWAS-GWAS integrative analysis, we showed that there was a biological pathway level commonality between the host genome and the metagenome in among the patients with SLE. Although biological pathway level commonality between host genome and metagenome was evaluated in other autoimmune diseases,[5 8] disease specificity of the commonality had not been evaluated. In this study, we showed that there was no pathway level commonality between the result of the SLE MWAS and the RA GWAS. Pathway level microbe-host interaction detected from the SLE MWAS and the SLE GWAS should reflect an SLE-specific disease mechanism.

Decrease in α-diversity of the gut microbiome, which is one of the major characteristics of dysbiosis, in patients with SLE had been controversial; some reported significant decreases in α-diversity in the SLE gut microbiome,[13 14 17] and others showed no differences.[12 15 16] This might be due to the difference in study design, such as sample number, country, medication and treatment of the patients with SLE. In our analysis, α-diversity of the gut microbiome in the patients with SLE significantly decreased. Additionally, we certified the robustness of our result by subanalysis. Diversity of the gut microbiome is considered as important for the homeostasis of the host's immune system, and decrease in α-diversity is reported in autoimmune diseases such as type 1 diabetes and IBD.[27] Observed decrease of α-diversity in the gut microbiome of SLE could be associated with abnormal activation of the immune system in the patients with SLE. Through β-diversity analysis, we found that SLE condition significantly affected the overall microbial composition. As recently suggested by Ma *et al*,[37] heterogeneity of the human microbiome among individuals tended to increase in the disease condition (Anna Karenina principle; AKP), and there is a possibility that AKP is also applicable to the case of SLE.

In this study, we identified a positive correlation between *Streptococcus intermedius* and acylcarnitine(18:1) followed by replication in another dataset. Acylcarnitine is known to form complex network with various microbes,[7] association between *Streptococcus intermedius* and acylcarnitine(18:1) could be mediated by both direct interaction and indirect interaction mediated by other microbes. Rutkowsky *et al* reported that acylcarnitine induced inflammation

in macrophage derived cell line by cyclooxygenase-2 dependent manner.[34] Our microbe-metabolome association analysis revealed a functional readout from the SLE gut microbiome, which could be associated with the pathology of SLE. Further analysis including case-control comparison with greater number of cases and in vivo validation would be warranted.

Our SLE MWAS had a few limitations. First, our study had only a moderate sample size compared with other studies on more common diseases such as colorectal cancer[38 39] and type 2 diabetes[3] due to the relatively rare prevalence of SLE. Although we robustly detected SLE-associated taxa and genes, there may exist other taxa and genes with smaller effect size. Thus, future large-scale studies such as cross-cohort meta-analysis are needed to detect the evidence of such weaker associations (online supplemental figure 8), where our study will contribute. Second, some of the patients in out cohort were under treatment or antibiotics, which could be potential confounding factors. However, the stable consistency of the effect sizes among the subanalyses indicated that these factors might not confound the result of the MWAS. Replication by independent cohort further supported the robustness of the result. Third, it is still challenging to reveal mechanistic insights into disease biology from MWAS. Pathway and bacteria-metabolite analysis in our study provided potential causal mechanisms as well as those suggested previously.[9–11 40] However, biological overview is still elusive due to the low throughput of mice experiment and technical and ethical difficulty in intervention to the human subjects. Future studies involving the latest technologies such as organoids and organs-on-chips technology,[41] would be promising for studying the mechanistic insights into the relationship between the gut microbiome and SLE.

In conclusion, our shotgun sequencing-based MWAS and integrative analysis with GWAS and plasma metabolite profiles revealed the altered gut microbiome in SLE and its association with the host. Our analysis contributes to the understanding of the relationship between the gut microbiome and SLE and provides useful resources for future research such as in vivo functional investigation or large-scale meta-analysis.

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Contributors YT, TK and YO designed the study and conducted the data analysis. YT and YO wrote the manuscript. YT, YMae, EO-I, TK, DM, YMat, TN and SN conducted the experiments. YT, YMae, EO-I, TK, KY, KS, HM, MYo, MYa, TN and SO collected the samples. HI, KT, AK and YO supervised the study. All authors contributed to the article and approved the submitted version.

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Competing interests None declared.

Patient consent for publication Not required.

Ethics approval This study was approved by the ethical committee of Osaka University (ID: 734–12).

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available in a public, open access repository. Data are available on reasonable request. The whole-genome shotgun sequencing data are deposited in National Bioscience Database Center (NBDC) Human Database (http://humandbs.biosciencedbc.jp/) with the accession number of hum0197.

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TRANSLATIONAL SCIENCE

Molecular basis for clinical diversity between autoantibody subsets in diffuse cutaneous systemic sclerosis

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ABSTRACT

Objectives Clinical heterogeneity is a cardinal feature of systemic sclerosis (SSc). Hallmark SSc autoantibodies are central to diagnosis and associate with distinct patterns of skin-based and organ-based complications. Understanding molecular differences between patients will benefit clinical practice and research and give insight into pathogenesis of the disease. We aimed to improve understanding of the molecular differences between key diffuse cutaneous SSc subgroups as defined by their SSc-specific autoantibodies

Methods We have used high-dimensional transcriptional and proteomic analysis of blood and the skin in a well-characterised cohort of SSc (n=52) and healthy controls (n=16) to understand the molecular basis of clinical diversity in SSc and explore differences between the hallmark antinuclear autoantibody (ANA) reactivities.

Results Our data define a molecular spectrum of SSc based on skin gene expression and serum protein analysis, reflecting recognised clinical subgroups. Moreover, we show that antitopoisomerase-1 antibodies and anti-RNA polymerase III antibodies specificities associate with remarkably different longitudinal change in serum protein markers of fibrosis and divergent gene expression profiles. Overlapping and distinct disease processes are defined using individual patient pathway analysis.

Conclusions Our findings provide insight into clinical diversity and imply pathogenetic differences between ANA-based subgroups. This supports stratification of SSc cases by ANA antibody subtype in clinical trials and may explain different outcomes across ANA subgroups in trials targeting specific pathogenic mechanisms.

Key messages

What is already known about this subject?

- Linking skin and protein expression to clinical differences between subgroups in systemic sclerosis (SSc) has been challenging.
- The hallmark antinuclear autoantibodies (ANA) used to diagnose SSc also predict clinically important differences in skin and internal organ disease.

What does this study add?

- This study uses clinical and ANA heterogeneity across a well-characterised broad SSc cohort to better understand the molecular architecture of early diffuse cutaneous SSc.
- We demonstrate for the first time striking differences in longitudinal patterns of serum protein markers between ANA subgroups in SSc.
- High-dimensional analysis of skin gene expression with patient-level pathway analysis suggests biological basis for differences between ANA-based subgroups.

How might this impact on clinical practice or future developments?

- Defining the molecular basis for clinical diversity gives insight into SSc disease biology relevant to clinical practice and trial design.

INTRODUCTION

Systemic sclerosis (SSc) patients are characterised by antinuclear autoantibodies (ANA), including antitopoisomerase-1 antibodies (ATA), Scleroderma (Scl)-70, anticentromere antibodies or anti-RNA polymerase III antibodies (ARA).[1] Different major organ-based complications link with ANA. For example, ATA is associated with significant interstitial lung fibrosis,[1 2] while ARA carries a tenfold increased risk of scleroderma renal crisis.[3] These strong associations with specific disease manifestations suggest that there are pathobiological differences beyond ANA underlying diverse clinical outcomes.

The skin and blood are readily accessible to compare gene and protein expression in SSc subgroups to better understand molecular correlates of clinical phenotypes. Skin analysis may be especially informative to understand differences between ANA subgroups because skin changes over time have been linked to ANA reactivities. ARA generally has a higher peak skin score than ATA in early diffuse cutaneous SSc (dcSSc) but faster improvement, whereas ATA may show slower regression.[4 5]

With the objective of understanding the molecular basis for heterogeneity in SSc, we undertook a detailed longitudinal analysis of skin and blood samples from a cohort of early-stage dcSSc followed over 12 months. This included measurement of serum proteins reflecting pathogenesis or

extracellular matrix turnover and with genome-wide assessment of gene expression. To put our findings in the broader context, we also studied late-stage dcSSc and limited cutaneous systemic sclerosis (lcSSc) and have compared our findings with matched healthy control subjects. We have specifically tested the hypothesis that hallmark ANA specific to SSc associate with different patterns of gene expression and proteins reflecting fundamental differences in pathogenesis in dcSSc. Our results strongly suggest that ANA specificity defines distinct biological subgroups within SSc with implications for case selection for clinical trials and therapeutic strategies in clinical practice.

METHODS

This was a single-centre, prospective observational study comprising four distinct participant cohorts: early dcSSc (<5-year duration), established dcSSc (>5-year duration), lcSSc and healthy volunteers (HC). Blood samples for serum and plasma and in PAXtubes were collected with concomitant 4 mm skin biopsies in RNAlater.

The early dcSSc cohort were reviewed every 3 months for a 12-month period, with blood sample collection and clinical assessment at each visit, and a 4 mm skin biopsy at baseline, month 3 and month 12.

Serum was analysed for soluble markers associated with collagen synthesis and degradation and fibrosis, including the constituents of the enhanced liver fibrosis (ELF) test. RNA expression analysis was performed on the skin and whole blood.

Additional methodology is described in the online supplemental material.

Statistical analysis

The prospective cohort was assigned the status of 'improver', 'progressor' or 'stable' based on change in Modified Rodnan Skin Score (MRSS) of greater than or equal to four points AND ≥20% change from baseline at the 12-month time point. For soluble markers, analysis of variance (ANOVA) with Tukey post hoc analysis or Kruskal-Wallis with post hoc Mann-Whitney U test was used. The Benjamini-Hochberg false discovery rate (FDR) was used for multiple comparisons. All statistical analysis was performed using the software R. Gene set enrichment analysis (GSEA) was used for pathway analysis.

Table 1 Clinical and demographical features of the BIOPSY cohort

	Early dcSSc at baseline	Early dcSSc at 12 months	Established dcSSc	lcSSc	HC
Total (n)	21	20	15	16	16
F (%)	12 (57.1)	11 (55)	12 (80)	12 (75)	9 (56.3)
Age (yrs)	52 (23–75)	54 (24–76)	56.9 (24–73)	52.5 (27–85)	43.3 (28–81)
Disease duration (yrs)	1.75 (0.5–4.9)	2.6 (1.5–5.9)	13 (5–20.8)	9 (3.5–30.4)	
MRSS	18 (7–39)	16 (2–38)	10 (2–36)	4 (2–12)	
Antibody					
ATA (%)	8 (38.1)	7 (35)	4 (26.7)	2 (12.5)	
ARA (%)	6 (28.6)	6 (30)	6 (40)	0	
ACA (%)	0	0	0	10 (62.5)	
ANA neg (%)	2 (9.5)	2 (10)	1 (6.7)	1 (6.3)	
Others (%)	5 (23.8)	5 (25)	7 (46.7)	3 (18.8)	
Organ involvement					
Lung (%)	6 (28.6)	7 (35)	8 (53.3)	0	
Muscle (%)	6 (28.6)	6 (30)	1 (6.7)	0	
Kidney (%)	3 (14.3)	4 (20)	1 (6.7)	0	
PAH (%)	1 (4.8)	1 (5)	1 (6.7)	0	
Cardiac (%)	3 (14.3)	3 (15)	1 (6.7)	1 (6.3)	
GI (%)	3 (14.3)	3 (15)	4 (26.7)	1 (6.3)	
Overlap conditions					
RA (%)	1 (4.8)	3 (15)	0	1 (6.3)	
PM/DM (%)	6 (28.6)	6 (30)	3 (20)	0	
Sjogren's (%)	0	0	1 (6.7)	2 (12.5)	
Immunosuppression					
MMF (%)	9 (42.9)	17 (85)	9 (60)	0	
MTX (%)	7 (33.3)	5 (25)	2 (13.3)	3 (18.8)	
HCQ (%)	5 (23.8)	5 (25)	1 (6.7)	5 (31.3)	
Azathioprine (%)	1 (4.8)	0	0	0	
Tocilizumab (%)	1 (4.8)	3 (15)	0	0	
Cyclophosphamide (%)	1 (4.8)	0	0	0	
IvIG (%)	0	2 (10)	1 (6.7)	0	
Untreated (%)	3 (14.3)	0	5 (33.3)	9 (56.2)	

Results presented as median and range unless otherwise stated.

ACA, anticentromere antibody; ANA, antinuclear autoantibody; ARA, anti-RNA polymerase III antibody; ATA, antitopoisomerase-1 antibody; BIOPSY, BIOlogical Phenotyping of diffuse SYstemic sclerosis; DM, dermatomyositis; dsSSc, diffuse cutaneous systemic sclerosis; F, female; GI, gastrointestinal; HC, healthy volunteer; HCQ, hydroxychloroquine; IvIG, intravenous immunoglobulin; lcSSc, limited cutaneous systemic sclerosis; MMF, mycophenolate mofetil; MRSS, Modified Rodnan Skin Score; MTX, methotrexate; PAH, pulmonary arterial hypertension; PM, polymyositis; RA, rheumatoid arthritis.

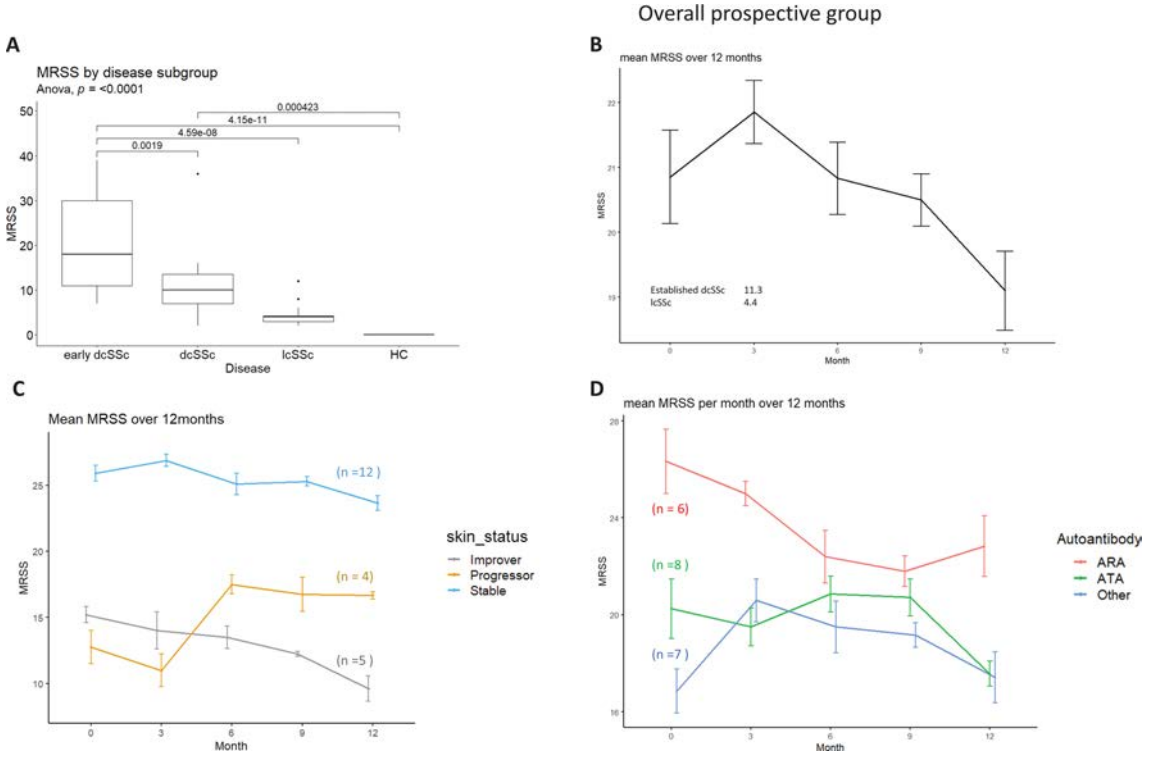


Figure 1 Baseline and longitudinal change in Modified Rodnan Skin Score (MRSS) in the **BIO**logical **P**henotyping of diffuse **SY**stemic sclerosis (BIOPSY) cohort. (A) Median MRSS across the BIOPSY cohort (Tukey post hoc p values <0.05 included) of early diffuse cutaneous systemic sclerosis (dcSSc) (n=21), established dcSSc (n=15), lcSSc (n=16) and healthy volunteer (HC) (n=16). (B) Mean MRSS and SEM of early dcSSc during prospective follow-up. (C) Mean MRSS and SEM based on skin status from baseline to 12 months. (D) Mean MRSS and SEM by autoantibody subset in early dcSSc cohort. ANOVA, analysis of variance; ARA, anti-RNA polymerase III antibody; ATA, antitopoisomerase-1 antibody; lcSSc, limited cutaneous systemic sclerosis.

Patient involvement

Patients and HC provided informed consent and attended visits as part of routine care or for purposes of research sampling.

RESULTS

Patient characteristics

The **BIO**logical **P**henotyping of diffuse **SY**stemic sclerosis (BIOPSY) dataset was generated to provide a platform for the integrated analysis of skin and blood samples, together with detailed clinical phenotyping (online supplemental figure 1). The study recruited 52 patients with SSc (21 early dcSSc, 15 established dcSSc and 16 lcSSc) and 16 gender-matched HC to the early dcSSc cohort. Thirty-six (69%) of the patients with SSc are women. Baseline characteristics are summarised in table 1. Mean disease duration in the early dcSSc cohort was 24 months (SD 12 months). ANA frequency in BIOPSY reflected the overall dcSSc population: ATA n=14 (27%), ARA n=12 (23%) and others n=26 (50%), which is aligned with those of other recent large SSc cohorts.[1 5]

One patient died during the study period from cardiac complications. These cases were managed in line with current treatment guidelines in the UK.[6] As expected, all patients with early dcSSc were on immunosuppression by 12 months, most often (85%) including mycophenolate mofetil (MMF). The doses of corticosteroids used in the prognostic dcSSc group did not exceed 10 mg prednisolone a day, and patients on corticosteroids were evenly distributed between the different autoantibody subtypes.

MRSS for early dcSSc was 18 (IQR 19). At a group level, MRSS peak was 21 (22) at 3 months and fell to 16 (14.25) at 12 months (figure 1). The median MRSS for the established patients with dcSSc was 10 (6.5) and for the lcSSc was 4 (1.25). Lower skin scores were seen in subjects with more established disease of greater than 36-month duration and in cases of early disease with less than 20-month duration. There was no significant relationship between disease duration and baseline MRSS (r=0.133, p=0.575).

For around half of the BIOPSY cohort, MRSS was clinically stable over 12 months. The remaining cases split between those that are significantly worsening (n=4) and those that show clinically significant improvement (n=5). Prospective dcSSc cases were divided into the three most recognised ANA-based subgroups, namely, ARA, ATA or 'others' for the purposes of analysis (which includes ANA positive, extractable nuclear antigen (ENA) negative or alternative ENAs). Group-level change in MRSS for the ANA subgroups is shown in figure 1. There was equal distribution of autoantibody subsets (specifically ATA and ARA) in each of the skin trajectory cohorts.

There was no significant difference between group-level skin score change between different immunosuppressive treatments or between those that were already on immunosuppression and those that started during the first 3 months of follow-up.

Differential longitudinal change in serum protein markers between ANA subgroups

Baseline serum protein marker analysis

At baseline, markers of collagen synthesis discriminated early dcSSc from HCs (online supplemental table 1 and figure 2). Composite fibrotic indices (C3 fibrotic index and C6 fibrotic index) did not outperform the markers of protein synthesis. The

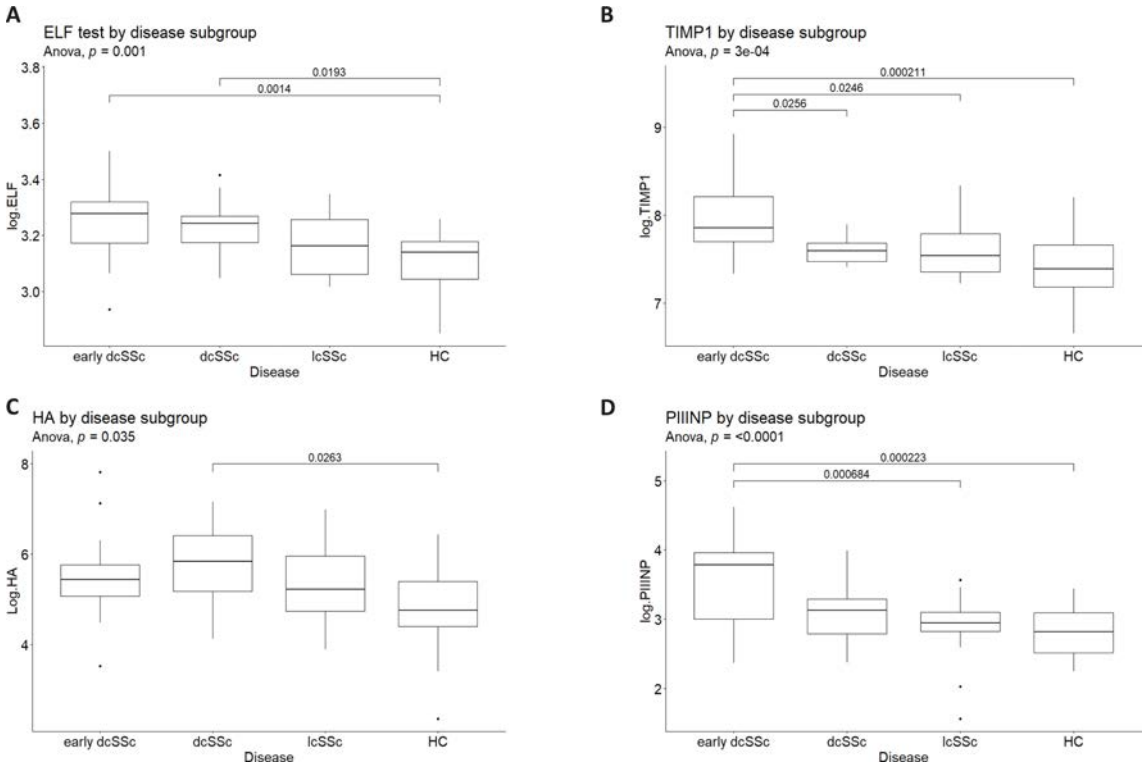


Figure 2 Baseline scores for enhanced liver fibrosis (ELF) and constituents in **BIO**logical **P**henotyping of diffuse **SY**stemic sclerosis (BIOPSY) cohort. (A–D) ELF test and constituents at baseline (Tukey post hoc p values included). (A) ELF at baseline. (B) Tissue inhibitor of metalloproteinases-1 (TIMP-1). (C) Hyaluronic acid (HA). (D) Type III procollagen peptide (PIIINP). ANOVA, analysis of variance; dcSSc, diffuse cutaneous systemic sclerosis; HC, healthy volunteer; lcSSC, limited cutaneous systemic sclerosis.

ELF composite score discriminated early dcSSc from HCs and was driven largely by type III procollagen peptide (PIIINP) and tissue inhibitor of metalloproteinases-1 (TIMP-1) (figure 2).

Longitudinal serum protein marker analysis in the early dcSSc cohort

Longitudinal changes in serum proteins over 12 months in early dcSSc in serum proteins explored differences based on skin score trajectory and ANA-defined subgroups.

Only ProC1 showed association longitudinally with skin progression (online supplemental figure 3). There were consistent and remarkable differences in the change in serum proteins between the major ANA-based subgroups (figure 3 and online supplemental figure 3). This was most evident for ELF, and the three constituents (PIIINP, hyaluronic acid and TIMP-1) and ProC1, where there is a linear increase overall for both ARA and 'other' groups whereas ATA shows decline over time from baseline values.

Integrated transcriptional analysis of the skin

Baseline transcriptional analysis of the skin

To better understand the molecular basis for longitudinal, clinical and serum protein differences between subgroups of SSc, a detailed analysis of global gene expression was undertaken across the BIOPSY cohort for skin and blood RNA.

There was clear differentiation between early dcSSc and HC by principal component analysis (PCA) and unsupervised clustering of significantly differentiated genes (731 genes; FDR <0.001) on baseline samples (figure 4B,C), with established dcSSc and lcSSc having a more similar transcriptional phenotype in the skin.

A focused analysis of early dcSSc and HC baseline skin biopsy samples identified 491 differentially expressed genes (fold change (FC) ≥1.5 and FDR<0.001) that separated these subpopulations and indicated a very distinct molecular signature shared by most cases of early dcSSc (online supplemental figure 4A,B).

Next, we explored differences in skin gene expression within the patients with early dcSSc based on ANA status. PCA and unsupervised cluster analysis of differentially expressed genes (n=384, p<0.01; FDR 0.4) clearly separating ARA and ATA patients (online supplemental figure 4C,D) with 'other ANA' patients being intermediate between ARA and ATA in some cases.

Analysis of ARA and ATA patients with early dcSSc revealed 61 differentially expressed genes at baseline (FC ≥1.5 and FDR<0.1) that fully differentiated these ANA subgroups (figure 4D and online supplemental table 3). These include genes previously associated with fibrosis and SSc showing significant difference between autoantibodies within the early dcSSc subgroup and across the whole SSc spectrum. Examples include inhibin subunit beta A (INHBA), interleukin 6 signal transducer (IL6ST), apelin (APLN) and complement 6 (C6) (figure 5D–G).

Similar analysis was performed on whole blood baseline samples, although we could not identify any genes that would directly differentiate ARA+ and ATA+ cases (online supplemental figure 4E,F).

Longitudinal transcriptional analysis of paired early dcSSc samples at 3 months and 12 months

Longitudinal sampling of the early dcSSc cases at 3 and 12 months showed stability of the gene expression profiles in the skin and blood over time (online supplemental figure 5) suggesting that gene-expression-based classification is a robust

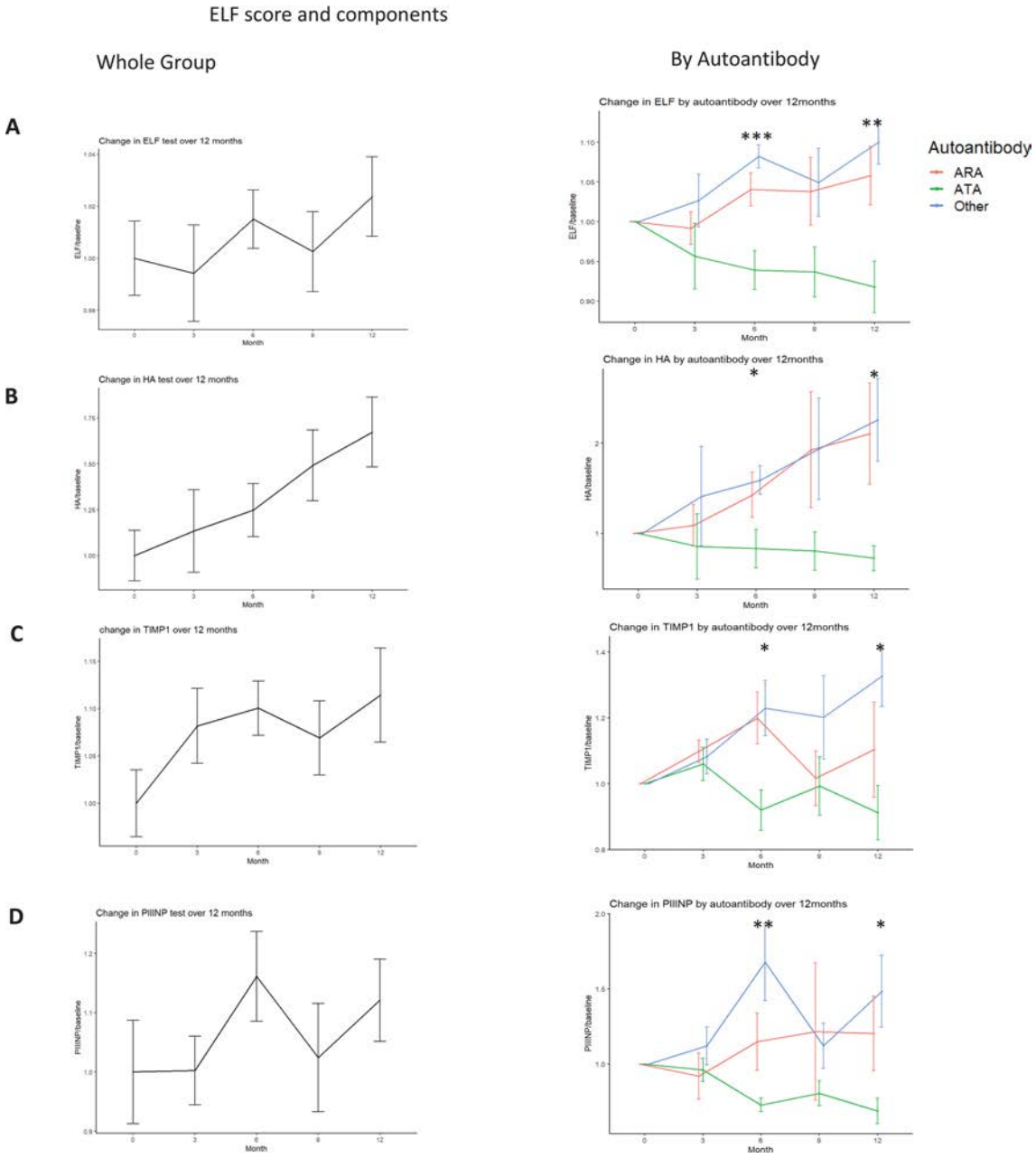


Figure 3 Longitudinal analysis of circulating proteins by group and autoantibody subset in the **BIO**logical **P**henotyping of diffuse **SY**stemic sclerosis (BIOPSY) cohort. Results of enhanced liver fibrosis (ELF) test and constituents and mean change (±SEM) as fraction over time on a group level and by antibody subtype. (A) ELF test. (B) Hyaluronic acid (HA). (C) Tissue inhibitor of metalloproteinases-1 (TIMP-1). (D) Type III procollagen peptide (PIIINP). *P<0.05; **p<0.01; ***p<0.001. ARA, anti-RNA polymerase III antibody; ATA, antitopoisomerase-1 antibody.

assessment that changes relatively little at a global level over 12 months.

SSc-specific gene expression in the skin shows relevant changes across the disease spectrum

To compare our findings with previously reported SSc-associated gene expression signatures in the skin, we used a robust SSc-associated composite signature of SSc-specific genes identified from publicly available gene expression datasets for whole skin.[7–11] Our analysis replicated this SSc-associated signature across different time points for the BIOPSY cohort of early dcSSc and showed consistent relevant differences across the BIOPSY cohort for both upregulated and downregulated SSc signature scores (figure 4A). Both the upregulated and downregulated genes of the SSc signature showed differences from healthy controls for all SSc subgroups. The global differences reflected a spectrum of the disease, with greatest difference observed in the baseline early dcSSc and least in established dcSSc and lcSSc. Notably, the signature became attenuated at later time points in the longitudinal cohort and in late-stage dcSSc and lcSSc, in contrast to the relative stability of overall gene expression in BIOPSY for individual patients. This suggests that the global expression signature of SSc reflects stage and severity of skin disease. Overall, the composite disease-associated signature analysis provides strong external validation of our cohort compared with other datasets although likely to be less informative about patient-level MRSS change than our analysis of the prospectively collected and rigorously phenotyped BIOPSY dataset.

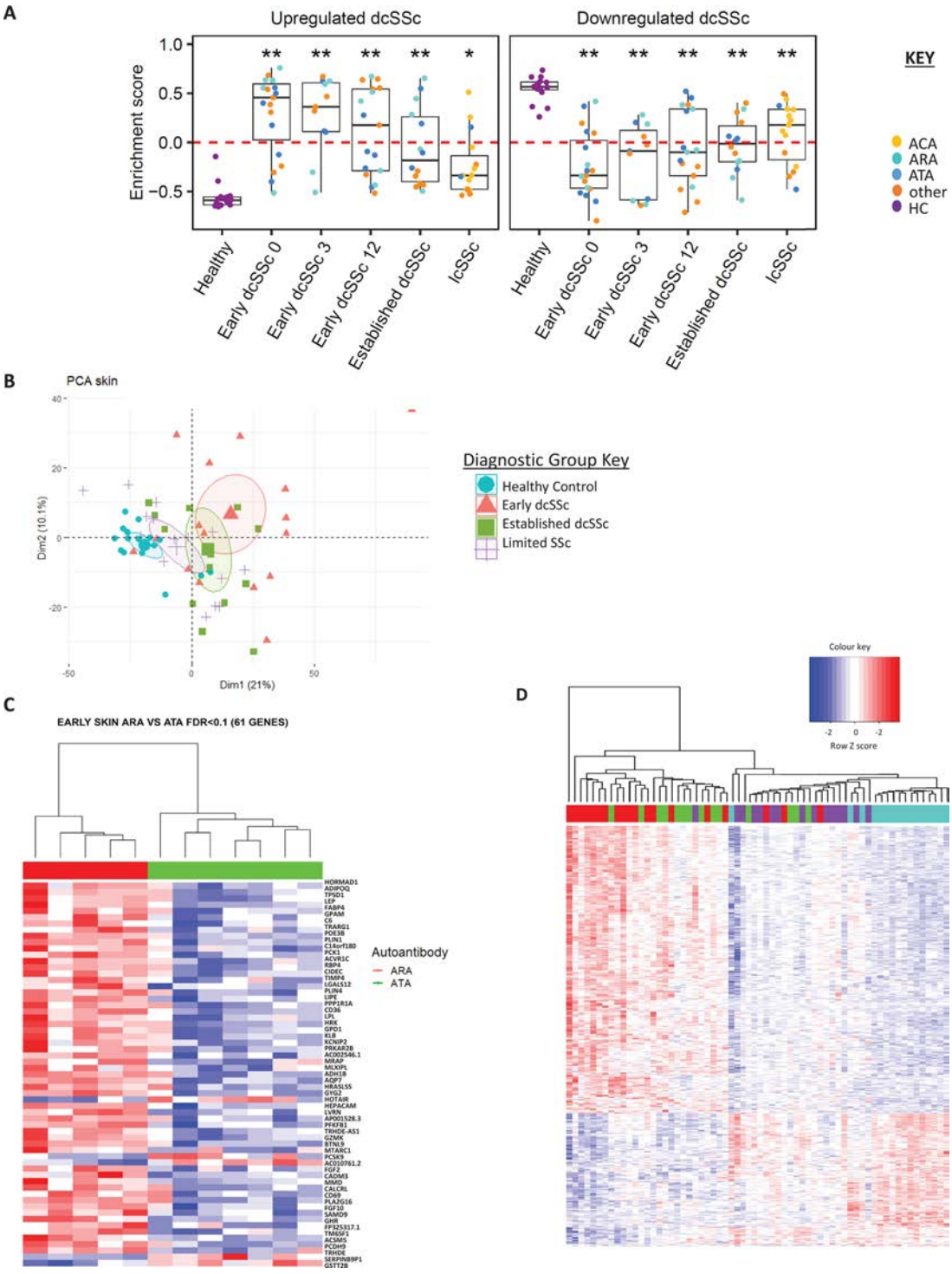
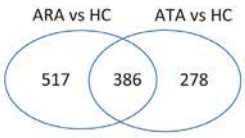


Figure 4 Whole skin transcriptomic analysis for the **BIO**logical **P**henotyping of diffuse **SY**stemic sclerosis (BIOPSY) cohort differentiates autoantibody subsets. (A) Overall view of enrichment scores of genes upregulated in diffuse cutaneous systemic sclerosis (dcSSc) and downregulated in dcSSc across BIOPSY cohort and time points. The SSc-specific composite signature is derived from multiple publicly available datasets and reflects those genes that are consistently upregulated or downregulated in SSc skin biopsies. *P<0.05; **p<0.001. (B and C) Principal component analysis (PCA) and unsupervised hierarchical clustering of all baseline BIOPSY cohort skin samples based on 731 differentially expressed genes (false discovery rate (FDR) <0.001). Disease subtype indicated by colour bar (red=early dcSSc, green=established dcSSc, purple=lcSSc and turquoise=healthy volunteer (HC)). (D) Hierarchical clustering based on 61 significantly differing gene expressions (FDR <0.1) from the skin comparing anti-RNA polymerase III antibody (ARA)-positive (red) and antitopoisomerase-1 antibody (ATA)-positive (green) early dcSSc. ACA, anticentromere antibodies; lcSSC, limited cutaneous systemic sclerosis.

Differences in gene expression for ARA-positive and ATA-positive dcSSc compared with healthy controls

To explore similarities and differences between gene expression profiles for the two major ANA antibody subtypes of early dcSSc, we compared the baseline differences between ARA and ATA subgroups and HC in the skin. In the skin, 664 and

A



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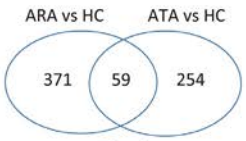

C

SKIN - upregulated genes											
Upregulated in ARA vs HC			Upregulated in both ARA vs HC			Upregulated in both ATA vs HC			Upregulated in ATA vs HC		
Gene	F.C	FDR	FDR	FC	Gene	FC	FDR	Gene	F.C	FDR
PLA2G2D	5.789	0.000	0.000	6.313	CXCL9	1.845	0.001	MT-TC	6.013	0.013
CXCL13	4.357	0.000	0.000	5.686	TNFSF18	2.063	0.000	PRR33	5.893	0.021
CXCL10	4.088	0.000	0.000	5.288	SFRP4	4.243	0.000	MTND1P23	5.885	0.023
SAA1	3.457	0.001	0.000	5.034	COMP	4.018	0.000	CICP14	2.565	0.012
C6	3.255	0.000	0.000	4.930	COL4A4	3.939	0.000	C22orf23	2.374	0.024
PRR9	2.659	0.036	0.001	4.815	LTF	2.716	0.012	CD177	2.277	0.009
HAPLN1	2.607	0.001	0.000	4.509	ADAMDEC1	2.595	0.000	APLN	2.214	0.002
PCK1	2.461	0.005	0.000	4.426	ADAM12	3.303	0.000	SPINK7	2.128	0.008
HORMAD1	2.449	0.020	0.000	4.362	COL11A1	3.176	0.000	MT-ND6	2.107	0.017
INHBA	2.438	0.000	0.000	4.249	SULF1	2.759	0.000	PERM1	2.105	0.030
C14orf180	2.435	0.004	0.000	4.242	THBS4	2.861	0.001	GP1BA	1.991	0.000
CXCL11	2.384	0.001	0.001	4.082	SERPINE1	2.193	0.009	CRMP1	1.946	0.004
IDO1	2.381	0.000	0.000	3.994	HP	1.999	0.022	TMPRSS4	1.925	0.006
ADIPOQ	2.298	0.002	0.000	3.694	SCG2	2.868	0.000	GSTT2B	1.918	0.031
DGKI	2.241	0.000	0.000	2.972	CPXM1	3.684	0.000	MTCO1P12	1.915	0.027
PLA2G2A	2.218	0.007	0.000	2.609	LAMP5	3.673	0.000	COL1A1	1.915	0.002
TIMP4	2.178	0.001	0.000	3.618	TNFSF4	2.117	0.000	APLNR	1.901	0.001
MMP9	2.134	0.001	0.000	3.568	TIMD4	1.707	0.003	CYTL1	1.894	0.000
CIDEC	2.123	0.002	0.000	3.477	PENK	1.931	0.000	GPX2	1.873	0.003
SLAMF8	2.109	0.000	0.000	3.387	COL8A1	2.303	0.000	GPR68	1.864	0.000

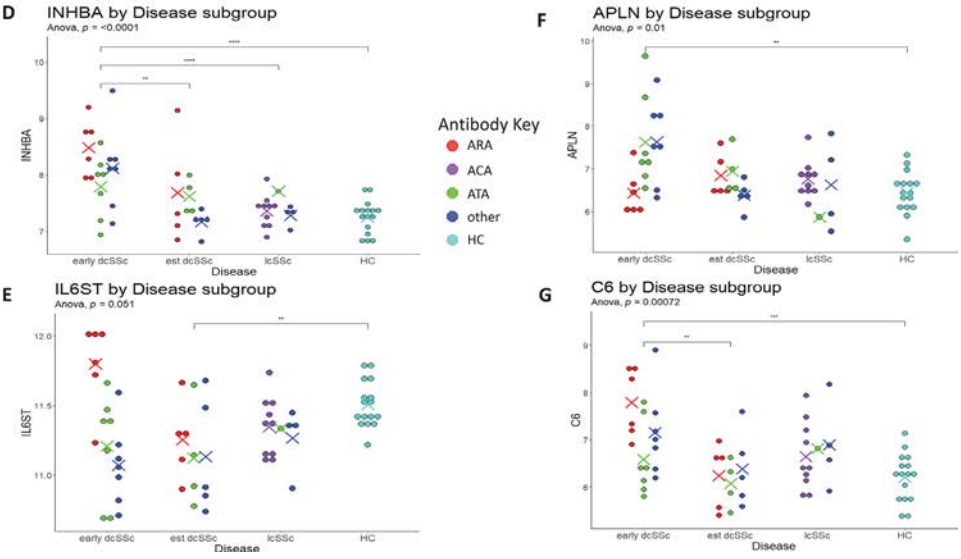

Figure 5 Results from analysis of gene expression of early diffuse cutaneous systemic sclerosis (dcSSc) autoantibody subgroups compared with healthy volunteer (HC). (A) Venn diagram of number of significantly differently expressed genes in the skin in anti-RNA polymerase III antibody (ARA) compared with HC and antitopoisomerase-1 antibody (ATA) compared with HC and those significantly differentially expressed in both. (B) Venn diagram to show number of significantly differentially expressed genes in blood by autoantibody comparisons. (C) Table showing top 20 significantly differentially expressed genes (false discovery rate (FDR) <0.05) with highest fold change (FC) between autoantibody and healthy control and corrected p value found in the skin. (D–G) Scatter plots of select genes associated with fibrosis. Genes selected from online supplemental table 3, colour defined by autoantibody state. Mean value of each disease subgroup and autoantibody state indicated by 'X'. Tukey post hoc p value across disease subgroups included (*p<0.05; **p<0.01; ***p<0.001). Gene expression expressed in log format: (D) inhibin subunit beta A (INHBA), (E) interleukin 6 signal transducer (IL6ST), (F) apelin (APLN) and (G) complement 6 (C6). ACA, anticentromere antibodies; ANOVA, analysis of variance.

903 differentially expressed genes were identified between ATA versus HC and ARA versus HC, respectively, with only 386 transcripts shared between the two disease subpopulations (figure 5A–C). This further suggests meaningful differences between gene expression profiles in the skin of the two ANA-based subgroups.

The same analysis was performed on the transcripts from blood, with 430 differentially expressed genes between ARA and HC, and 313 genes were significantly differentially expressed between ATA and HC. Only 59 genes were shared between the two disease subpopulations. Unlike the direct comparison between the autoantibody subsets in blood, we were able to appreciate shared upregulated genes when the analysis included HC in blood.

Patient-level pathway analysis differentiates autoantibody subsets

To better understand the functional significance of differentially expressed genes in skin at baseline, GSEA was used for

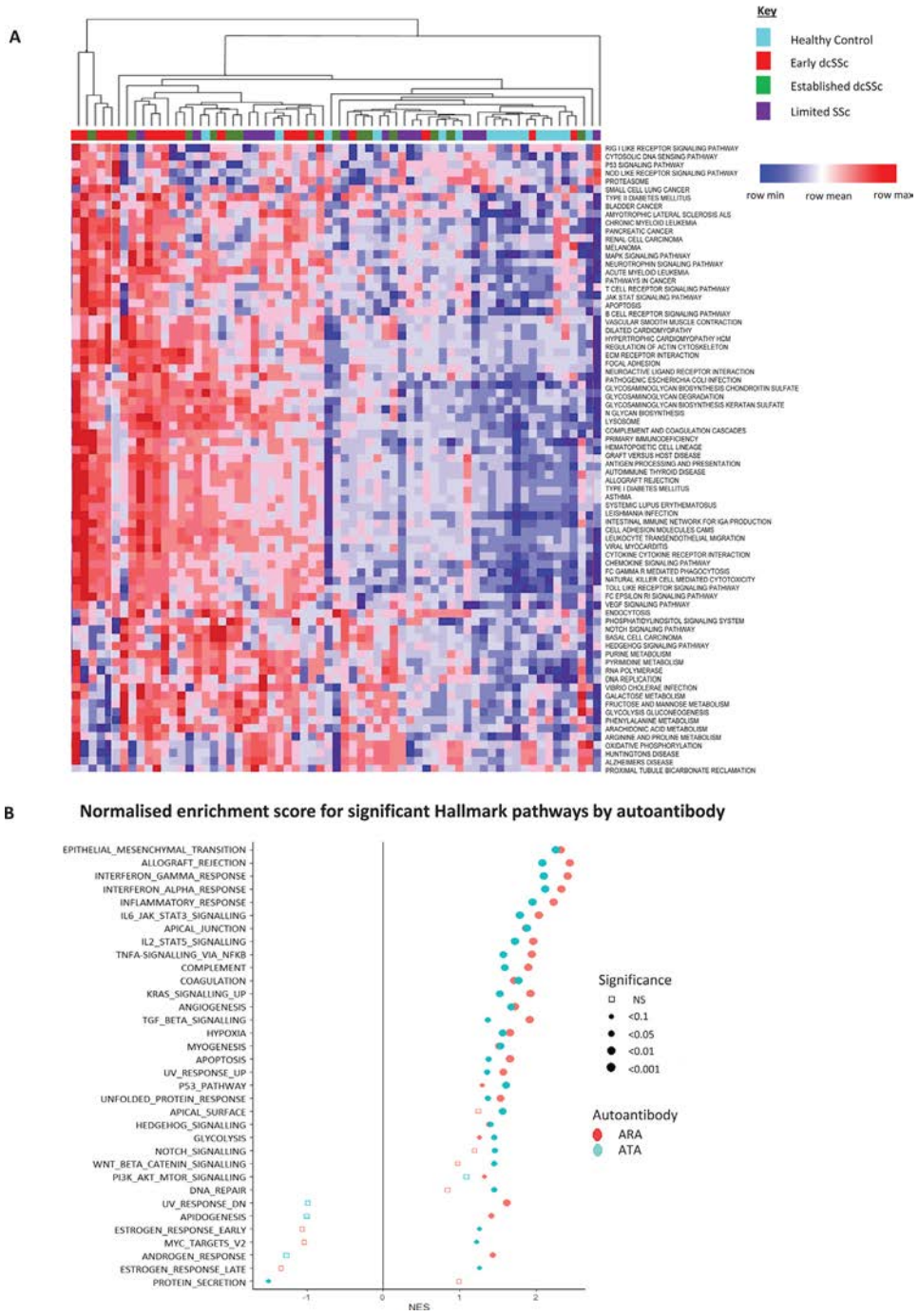

Figure 6 Pathway analysis for differentially expressed pathways across the **BIO**logical **P**henotyping of diffuse **SY**stemic sclerosis (BIOPSY) cohort. Hierarchical clustering of single-sample GSEA (ssGSEA) using significantly differentially expressed pathways. (A) ssGSEA of significantly differentially expressed KEGG pathways across whole systemic sclerosis (SSc) spectrum and healthy controls (colour bar). Patient with SSc subgroups highlighted with early diffuse cutaneous SSc (dcSSc) (red), established dcSSc (green), lcSSc (purple) and healthy volunteer (HC) (turquoise). (B) Cleveland dot plot demonstrating the normalised enrichment score for Hallmark pathways in anti-RNA polymerase III antibody (ARA) compared with HC (red) and antitopoisomerase-1 antibody (ATA) compared with HC (green) and significance of pathway. GSEA, gene set enrichment analysis; KEGG, kyoto encyclopedia of genes and genomes; lcSSc, limited cutaneous systemic sclerosis; NES, normalised enrichment score; NS, not significant.

individualised pathway analysis across the BIOPSY cohort (figure 6A).

The comparison of differentially expressed Hallmark pathways for ATA and ARA versus healthy controls for the skin suggested overlapping differential pathway expression, with clear differences between the two major ANA subgroups as well as overlap (figure 6B and online supplemental figure 6A–C). None of the gene sets for the parallel whole blood analysis passed the threshold for difference on GSEA. Overlapping pathways using Hallmark are linked to aspects of SSc pathobiology that are likely to be shared across dcSSc cases. These pathways include allograft rejection, inflammation, IL6 signalling, transforming growth factor (TGF)-beta signalling, angiogenesis and complement as well as upregulation of interferon (IFN) alpha response. Conversely, oestrogen response and Myc targets are increased for ATA-positive skin but downregulated in ARA

compared with HC, while adipogenesis, ultraviolet response down and androgen are increased in ARA but downregulated in ATA. These data provide insight into differences that could be highly relevant to the clinical, biomarker and gene expression features of these ANA-based subgroups.

DISCUSSION

In the present study, we have used the intrinsic clinical diversity across the SSc spectrum to help interpret molecular phenotypes and elucidate differences in potential transcriptional drivers in different stages and subsets of disease. This has important implications for both clinical practice and research, especially early-stage drug trials that will necessarily include relatively small numbers of patients, and risk being confounded by clinical and molecular imbalance between treatment arms. By demonstrating for the first time clear differences in serum proteins and skin gene expression between ANA subgroups of early dcSSc, our findings begin to explain how ANA reactivities are such strong predictors of clinical outcome and internal organ involvement.[1]

The results of serum protein analysis provide an anchor for our findings. We show that serum markers that have been validated as cross-sectional markers of skin fibrosis[8] have remarkably different trajectories of change between ANA subgroups, specifically the two dominant ANA reactivities, ATA and ARA. Given our findings, despite the well-established correlation of the ELF test with MRSS and forced vital capacity (FVC),[12] interpretation on a group level in early dcSSc with a mixed ANA profile, and especially over time, may be misleading. Unlike previous work on circulating markers of collagen turnover,[13 14] we did not identify clear differences between markers of collagen degradation (C1M, C4M and C6M) between disease subgroups.[15] One explanation is that while ELF reflects important pathological events in the skin that drive fibrosis, skin score trajectory is also influenced by processes that resolve fibrosis and that are not captured by ELF. Alternatively, it may be that ELF levels in blood reflect multisystem disease outside the skin compartment that is not captured by serial measurement of skin score. At a practical level, our findings highlight how important it therefore is to take the antibody subtypes into consideration when interpreting potential biomarkers, as the natural trajectory may be intrinsically different.

Taken together, whole skin gene expression analysis differentiates stage and subset of SSc and gives robust insight into the differential gene expression between SSc and HC. Differential gene expression resulted in complete separation of early dcSSc and HC (similar to Skaug *et al*[16]), with limited and established dcSSc also forming moderately distinctive subgroups. As previously reported,[8 9] we observed relative stability in gene expression profiles over 3 months and 12 months. Skin transcriptomic differences between ATA and ARA patients with early dcSSc are especially relevant in the context of the contrasting longitudinal changes in serum markers of fibrosis observed in the BIOPSY Study. This implies fundamental differences in skin biology and possibly pathogenic mechanism between ARA and ATA subgroups. This is supported by a recently published analysis of data from the Genetics vs Environment in Scleroderma Outcome Study (GENISOS) cohort, which suggests distinct gene expression differences between major ANA reactivities.[17]

Our data suggest that a relatively small number of transcripts clearly separate ARA and ATA skin gene expression. Many of these genes have already been identified to show altered expression in SSc (IL6ST (gp130), APLN and C6[18–21]) or other fibrotic diseases (INHBA[22]). We found shared signatures across these autoantibody subsets, as well as differences that likely contribute to the distinctive clinical phenotype of these autoantibody profiles.

The fact that there were no differentiating transcriptomes in the blood between ARA+ and ATA+ patients suggests that these key differences are important in the skin pathology and clinical diversity of skin disease notable in these autoantibodies.

Hallmark ANA-associated differences may offer insight into diversity in outcome and treatment response within early dcSSc, including clinical trials. Some recent studies have analysed intrinsic subset gene sets, which found patients who responded to MMF or abatacept (a CTLA4-Ig fusion protein) tended to be in the inflammatory subset[11 23] whereas those who responded to dasatinib (a tyrosine kinase inhibitor with antifibrotic potential) were in the fibroproliferative group.[24] However, these studies did not look at the differential response to targeted therapies based on antibody subtype. It is possible that the intrinsic gene subsets[9] are differentially represented between hallmark ANA subgroups in early-stage SSc and that future classification approaches incorporating both molecular and serological aspects may provide further opportunities for case stratification.

However, molecular differences between ATA and ARA identified in the present study may have relevance to treatment response for skin or internal organ disease in SSc based on other recent trial data. For example, subgroup analysis of recent phase two and phase three trials of tocilizumab in dcSSc suggests treatment benefit was much more marked in ATA-positive patients, where prevention of decline in lung function on tocilizumab was highly significant in ATA-positive subjects but not statistically significant in ATA negative.[25–27] In contrast, the RIociguat Safety and Efficacy in patients with diffuse cutaneous Systemic Sclerosis (RISE-SSc) trial of riociguat showed a major benefit preventing MRSS progression in the ARA subgroup and no benefit for the ATA subgroup.[28] Finally, the large SENSCIS trial of nintedanib showed a numerically greater preservation of lung function in ATA-negative compared with ATA-positive cases. This is notable because the ATA-negative group also demonstrated numerically greater improvement in MRSS.[29] These are consistent with our hypothesis that ANA subgroups may respond differently to therapies targeting specific pathogenic mechanisms in the skin and lung.

These clinical associations raise the possibility that some of the SSc-specific autoantibodies may have a direct role in pathogenesis and that it may differ between ARA and ATA. The strongest evidence is for ATA, where ATA immune complexes (ICs) have greater effect on the IFN mRNA signature in fibroblasts compared with ARA-ICs and controls,[30 31] a key cell type mediating skin fibrosis in SSc and contributing to the heterogeneity seen in SSc.

Taken together, our findings support the overarching hypothesis that there are distinct but overlapping pathogenic processes linking immunity and fibrosis in the skin in all dcSSc, especially the interplay between adipocyte function, immunity and fibrosis. Thus, in ARA-positive cases, local connective tissue/adipocyte biology may be key to the severity and progression of skin change, and this may be independent of immune cell drive. In contrast, ATA-positive dcSSc may reflect more persistent or refractory immune-cell-driven skin fibrosis that is less dependent on local factors and adipocyte biology. In addition, these observations may fit with novel mechanisms proposed by Lerbs *et al*[32] linking fibrosis to failed elimination of myofibroblasts. It is plausible that this mechanism is more relevant in ARA-positive cases of dcSSc than ATA.

There are notable strengths to this study. First, this is a well-characterised cohort of patients, prospectively collected with only two assessors performing MRSS (minimising interobserver variability). We present a real-life treated cohort of patients with dcSSc who, as would be expected, developed complications during the study period and had medications changed. By including a broad spectrum of patients with SSc, we can interpret any findings in the context of all patients with SSc.

There are also limitations. Being a single-centre study requiring significant time commitment of subjects meant that it comprised a relatively small cohort of patients. Within the prospective cohort of patients, there are only small numbers of progressors or improvers, so these findings should be interpreted with caution. There were also some missing samples, due to patient refusal or technical difficulties. Although we have speculated about treatment effects, this was an observational study, unable to formally compare treatments between patients.

In conclusion, BIOPSY provides a template for translational research that can integrate clinical observation and modern integrative molecular methods. In this way, we have been able to better understand biological differences between subsets of SSc and the relationship between skin disease, autoantibody subgroup and candidate molecular markers. Our results have implications for clinical practice, trial design and basic science studies of SSc.

Contributors All authors contributed significantly to the study design and manuscript and reviewed and edited the final manuscript. Individual contribution as set out below. Conceptualisation: CPD and NW. Methodology: ED-S, NW, CPD, JS and YVT. Investigation: KENC, CC, JS and YVT. Data analysis: KENC, AT, NG and YVT. Supervision: KENC, CC and CPD. Writing—original draft: KENC and CPD. Writing—review and editing: KENC, CC, EC, AT, KN, NG, MAM, JS, YVT, VO, ED-S, NW, SMF and CPD.

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Data availability statement Data are available upon reasonable request. All data, code and materials used in the analysis are available to any researcher for purposes of reproducing or extending the analysis upon request to the corresponding author.

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TRANSLATIONAL SCIENCE

TGFβ promotes low IL10-producing ILC2 with profibrotic ability involved in skin fibrosis in systemic sclerosis

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ABSTRACT

Objective Innate lymphoid cells-2 (ILC2) were shown to be involved in the development of lung or hepatic fibrosis. We sought to explore the functional and phenotypic heterogeneity of ILC2 in skin fibrosis within systemic sclerosis (SSc).

Methods Blood samples and skin biopsies from healthy donor or patients with SSc were analysed by immunostaining techniques. The fibrotic role of sorted ILC2 was studied in vitro on dermal fibroblast and further explored by transcriptomic approach. Finally, the efficacy of a new treatment against fibrosis was assessed with a mouse model of SSc.

Results We found that ILC2 numbers were increased in the skin of patients with SSc and correlated with the extent of skin fibrosis. In SSc skin, KLRG1^{-} ILC2 (natural ILC2) were dominating over KLRG1^{+} ILC2 (inflammatory ILC2). The cytokine transforming growth factor-β (TGFβ), whose activity is increased in SSc, favoured the expansion of KLRG1^{-} ILC2 simultaneously decreasing their production of interleukin 10 (IL10), which regulates negatively collagen production by dermal fibroblasts. TGFβ-stimulated ILC2 also increased myofibroblast differentiation. Thus, human KLRG1^{-} ILC2 had an enhanced profibrotic activity. In a mouse model of SSc, therapeutic intervention-combining pirfenidone with the administration of IL10 was required to reduce the numbers of skin infiltrating ILC2, enhancing their expression of KLRG1 and strongly alleviating skin fibrosis.

Conclusion Our results demonstrate a novel role for natural ILC2 and highlight their inter-relationships with TGFβ and IL10 in the development of skin fibrosis, thereby opening up new therapeutic approaches in SSc.

Key messages

What is already known about this subject?

- Type 2 innate lymphoid cells (ILC2) have emerged as a player in inflammatory and fibrotic processes.
- Research to date on innate lymphoid cells in systemic sclerosis (SSc) was descriptive suggesting a potential role in the disease development.
- Transforming growth factor-β (TGFβ) pathway is important in SSc pathophysiology notably through its direct role on fibroblasts.

What does this study add?

- We deeply described ILC2 presence and localisation in fibrotic skin.
- We depicted a new indirect mechanism by which TGFβ could lead to fibrosis, triggering the switch from an 'inflammatory' phenotype (KLRG1^{high}) to a 'natural' phenotype (KLRG1^{low}) ILC2.
- These TGFβ-activated ILC2, characterised by a diminished interleukin 10 (IL10) production, promote collagen synthesis by fibroblasts.
- Using both in vitro and in vivo models, we established the importance of the combined role of TGFβ and IL10 in the fibrotic process.

How might this impact on clinical practice or future developments?

- These data provide important support for the use of combination therapies in SSc.
- The combined use of an antifibrotic drug such as pirfenidone and IL10 could be a new therapeutic approach in this very complicated disease.

INTRODUCTION

Systemic sclerosis (SSc) is a systemic autoimmune disorder characterised by a dysregulated extensive fibrotic process that impacts epithelial barriers, within the gut, lung and skin.[1] Its pathogenesis remains poorly understood, and treatments for disease progression are limited. While the adaptive immune system has long been considered to be involved in SSc development, recent observations have established an important role of the innate immune system.[2 3] As an example, a type 2 macrophage signature has been identified in both skin and lung from patients with SSc.[4]

Interestingly, innate lymphoid cells (ILCs) that patrol environmental interfaces to defend against

infection and protect barrier integrity have emerged as crucial effectors in inflammatory and fibrotic diseases.[5 6] Their cytokine production and transcription factor expression allow the identification of three distinct subsets. Type 1 ILC (ILC1) are Tbet expressing cells that produce interferon-γ and tumour necrosis factor, and are dependent on IL12 and IL18 for their generation. GATA3 expressing type 2 ILC (ILC2), which are dependent on thymic stromal lymphopoietin (TSLP), IL25 and IL33 release IL5 and IL13 whereas type 3 ILCs (ILC3) express the transcription factor RORγt, release IL17 and IL22 and are generated after IL23 and IL1β stimulation.[7]

The role of ILC2 was first highlighted in allergic reactions.[8 9] More recently, murine studies have shown the role of ILCs in hepatic and pulmonary fibrosis, with a major effect of IL13 production. Interestingly in the context of SSc, TGFβ is another important profibrotic factor[10–12] and an essential cytokine for the development of ILC2,[13] suggesting a potential role of ILC2 on SSc pathogenesis.

To add another level of complexity, ILC2 constitute a heterogeneous population of cells and at least two separate clusters are described based on their differential responses to microenvironment. Inflammatory ILC2 (iILC2) respond to IL25 and produce IL17 in addition to IL13, whereas natural ILC2 (nILC2) respond to IL33 and release high levels of IL13. The differential expression of killer cell lectin-like receptor G1 (KLRG1) has been identified as a marker, with iILC2 being KLRG1$^+$ and nILC2 being KLRG1$^-$. iILC2 can be considered a transient progenitor based on its ability to migrate to tissue and then differentiating into nILC2 in response to activation signals.[14 15] However, the relevance of this plasticity in human pathologies remains to be established.

In patients with SSc, we and others have demonstrated increased levels of homeostatic cytokines for ILC2, such as IL25, IL33 and TSLP.[16–18] Furthermore, our group found that TSLP is increased in the blood and skin of patients with SSc, with levels correlating to skin fibrosis.[18] In human SSc, the role of ILC2 remains elusive as only one observational study showed an increased proportion of circulating ILC2 at the blood and tissue level,[19] thus questioning the potential implication of ILC2 in the fibrotic process.

In our study, we demonstrate the potential role of ILC2 in the establishment of fibrosis in human SSc. We showed that KLRG1 expression on ILC2 was linked to the fibrotic stage of the disease. Mechanistically, in vitro and in vivo studies revealed that this switch operates in a TGFβ-dependent manner, leading to a decrease in IL10 production and a profibrotic phenotype. Interestingly, while pirfenidone alone (acting partly by the inhibition of TGFβ-induced effects) failed to significantly affect the fibrotic process in vivo, the addition of IL10 synergistically alleviated fibrosis. Overall, this study unravels a new role for ILC2 in fibrotic diseases and paves the way for new therapeutic strategies for human SSc.

METHODS

Materials and methods are described in the online supplemental file.

RESULTS

Number of circulating ILC2 is decreased in human SSc with the extent of skin fibrosis

To investigate the potential contribution of ILC2 in SSc pathogenesis, we first monitored the total ILC population and subpopulations in the whole blood of patients with SSc (SSc, n=73) and age-matched and sex-matched healthy donors (HDs) (n=59) (table 1).

The gating strategy to identify ILCs in the peripheral blood is described in the online supplemental figure 1A. The frequency and absolute numbers of ILCs (defined as Lin$^-$ CD45$^+$CD127$^+$, figure 1A) were lower in SSc compared with HDs (0.04%±0.02% vs 0.09%±0.07%, p<0.0001 and 0.0009±0.0003 vs 0.002±0.001, p=0.0004; figure 1B and online supplemental figure 1AC, respectively), with ILC1, ILC2 and ILC3 being 74%, 15% and 11% in SSc and 55%, 18% and 27% in HDs, respectively (figure 1C). When focusing on ILC2 defined as Lin$^-$CD45$^+$CD127$^+$CRTH2$^+$, their frequency and absolute numbers were approximately three times lower in SSc

Table 1 Demographic, clinical and biological characteristics of the SSc population

	Patients with lcSSc (n=50)	Patients with dcSSc (n=23)	All patients with SSc (n=73)	P value*
Female (%)	39 (78)	9 (40)	48 (65.8)	
Age at onset, mean±SD years†	48.7±13.2	49.9±14.5	49±13.5	ns
Disease duration, mean±SD years†	10.7±6.0	11.9±9.47	11.5±8.6	ns
RP (%)	50 (100)	23 (100)	73 (100)	ns
Digital ulcers (%)	20 (40)	7 (30.8)	27 (37)	0.02
mRSS, mean±SD	5.9±5.5	24.6±12.7	11.1±11.6‡	<0.0001
PAH (%)	7 (14)	1 (4.3)	8 (11)§	ns
Interstitial lung disease (%)	11 (22)	13 (56.5)	24 (32.9)¶	ns
Lung fibrosis (%)	7 (14)	1 (4.3)	8 (11)¶	ns
Renal crisis (%)	1 (2)	0 (0)	1 (1.4)¶	ns
Antinuclear autoantibody-positive	50 (100)	23 (100)	73 (100)	ns
Anticentromere antibody-positive	25 (50)	1 (4.34)	26 (35.6)	ns
Antitopoisomerase antibody-positive	4 (8)	11 (47.8)	15 (20.5)	ns
Anti-ARNIII polymerase antibody-positive	1 (2)	1 (4.34)	2 (2.7)	ns
Immunomodulatory agents	44 (22)	60.8 (14)	49.3 (36)	ns

Except where indicated otherwise, values are the number (%).
*lcSSc versus dcSSc.
†Age at onset of symptoms other than RP and disease duration since symptoms other than RP.
‡Data were available for 34 patients.
§Data were available for 36 patients.
¶Data were available for 35 patients.
dcSSc, diffuse cutaneous SSc; lcSSc, limited cutaneous systemic sclerosis; mRSS, modified Rodnan skin thickness score; ns, not significant; PAH, pulmonary hypertension; RP, Raynaud's phenomenon; SSc, systemic sclerosis.

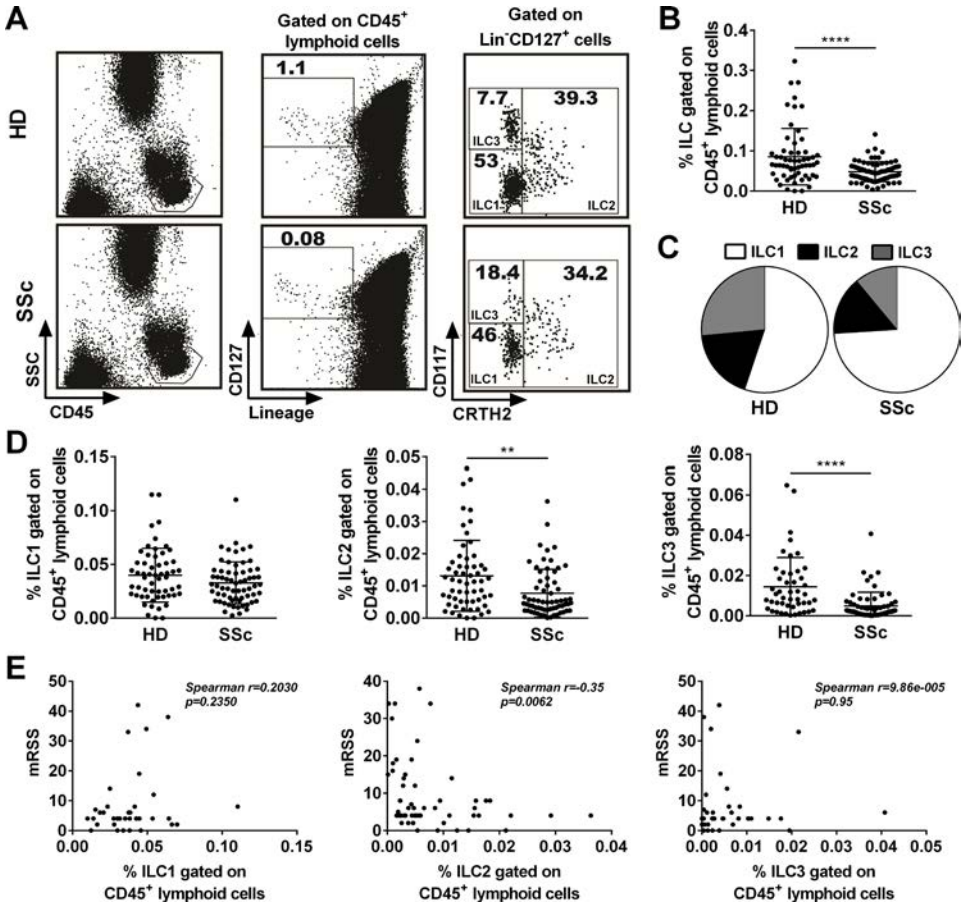

Figure 1 Characterisation of innate lymphoid cells (ILCs) in the blood of patients with systemic sclerosis (SSc) and healthy donors (HDs). (A) Representative dot plot of circulating ILCs in the HD and SSc blood samples and (B) ILC frequency quantification. (C) Proportion of ILC subsets in the blood from HDs and patients with SSc. (D) Percentage of circulating ILC1, ILC2 and ILC3 in the HD and SSc blood. (E) Correlations between circulating ILC1, ILC2 and ILC3 with the extent of cutaneous fibrosis (modified Rodnan skin thickness score (mRSS)). Data are the mean±SEM (n=59 and 73 for HDs and patients with SSc, respectively). Comparisons between groups were calculated using Mann-Whitney U test. **P<0.01; ****p<0.0001.

compared with HDs (figure 1D and online supplemental figure 1BD, respectively). However, no differences in the frequency of KLRG1$^+$ ILC2 were found (online supplemental figure 1CE). Decreased were also the frequency and absolute numbers of ILC3, but not of ILC1 when SSc and HDs were compared (figure 1D and online supplemental figure 1BD).

To evaluate the clinical relevance of these observations, we analysed the ILC numbers to different relevant clinical parameters. We specifically observed a correlation between the quantity of circulating ILC2 and the modified Rodnan skin thickness score (mRSS), showing that lower is the amount of circulating ILC2 and higher is the cutaneous fibrosis (figure 1E and online supplemental figure 1DF). Of note, the ILC1 and ILC3 frequencies were not correlated with the mRSS. When comparing patients with or without interstitial lung disease, we found no difference on the % and the absolute count of ILC2 in the whole blood and there were also no differences according to the severity of interstitial lung disease or the disease duration (data not shown). Collectively, our data indicate that patients with SSc are characterised by a significant reduction in the proportion and number of circulating ILC2, which is correlated with the extent of skin fibrosis.

ILC2 are increased in human SSc skin and correlated with the extent of fibrosis

Since circulating ILCs were decreased, we further characterise ILCs infiltration in the skin. We first extracted cells from the skin and performed flow cytometry analysis. Representative staining depicting the gating strategy for ILCs and subpopulation categorisation is shown in figure 2A and online supplemental figure 2A. As suspected, we found that the percentage of total ILCs among CD45$^+$ cells was increased in the SSc skin compared with that in the HD skin (figure 2B). When evaluating the repartition of ILC subsets among the total skin ILCs, we observed that 69% were ILC2 in the HDs vs 77% in the patients with SSc, 27% were ILC1 in the HDs vs 19% in the patients with SSc (figure 2C), while ILC3 were barely detectable. When analysed among CD45$^+$ cells, a significant increase in ILC2 was observed in the SSc skin compared with the HD skin (figure 2D). The ILC1 frequency was similar in the patients with SSc and HDs (online supplemental figure 2AB), even if the proportion among ILCs was decreased in the patients with SSc compared with that in the HDs (figure 2C). The increased percentage of ILC2s among CD45$^+$ skin cells was correlated with the extent of skin fibrosis (figure 2E), whereas the ILC1 percentage did not show this correlation (online supplemental figure 2BC).

To validate this analysis and gain more insights on the precise localisation and quantification of ILC2 in the skin, we performed tissue immunofluorescence staining. Representative staining of ILC2 (Lin$^-$DAPI$^+$CRTH2$^+$) for an HD and an SSc patient is depicted in figure 2H,I. An analysis of the ILC2 distribution and quantification revealed that the number of ILC2 per surface area (mm^2) and the percentage of ILC2 (per total cell count) were increased in the patients with SSc compared with the HDs in the dermis (figure 2F

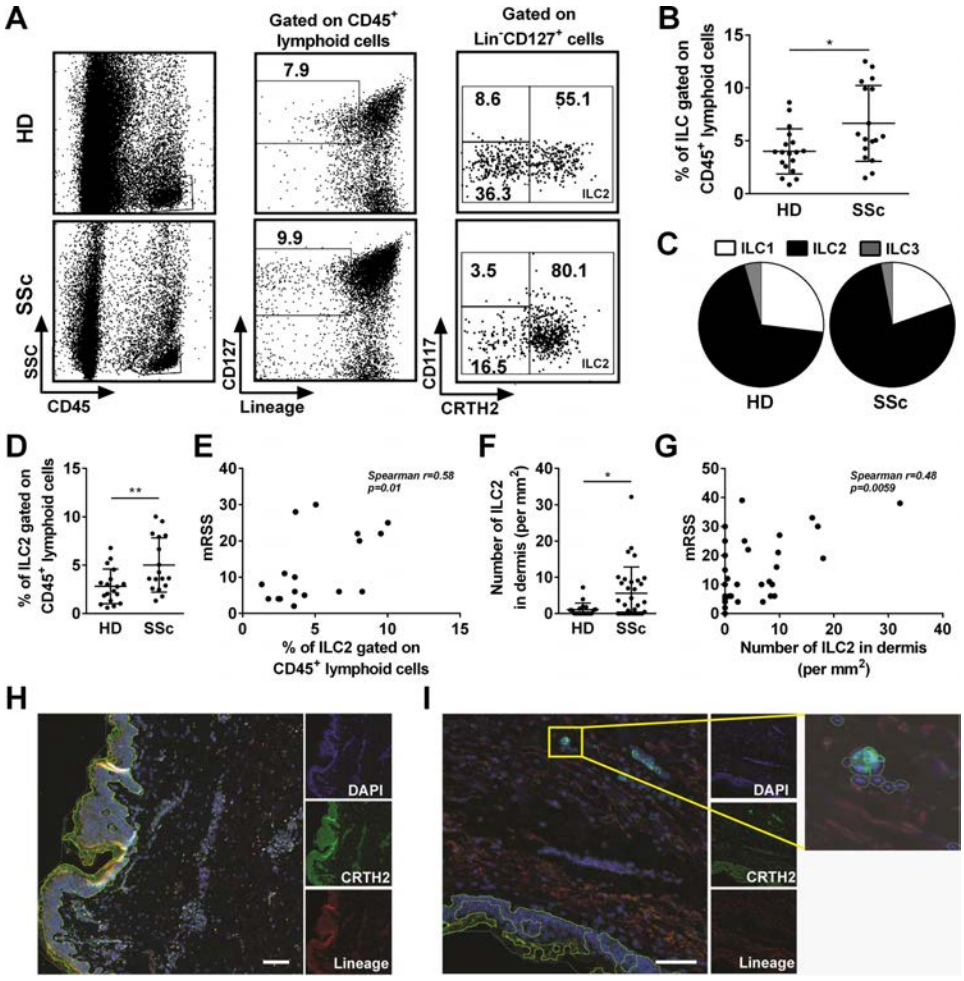

Figure 2 Characterisation of cutaneous innate lymphoid cells (ILC)-2 (ILC2) in patients with systemic sclerosis (SSc) and healthy donors (HDs). (A) Representative dot plot of cutaneous ILCs in the HD and SSc skin samples and (B) ILC frequency quantification. (C) Proportion of ILC subsets in the skin of HDs and patients with SSc. (D) Percentage of ILC2 among $CD45^+$ lymphoid cells in the HD and SSc skin samples. (E) Positive correlation between the percentage of ILC2 among $CD45^+$ cells and the extent of cutaneous fibrosis (modified Rodnan skin thickness score (mRSS)). (F) Number of ILC2 per mm^2 in the dermis of HD and SSc skin. (G) Correlation between the extent of cutaneous fibrosis (mRSS) and the number of ILC2 per mm^2 in the dermis. (H and I) Representative picture of an immunofluorescence assay using anti-CRTH2-based immunofluorescence (green) and antilineage (CD3, CD11b and FcεR1)-based immunofluorescence (red) performed to detect $CRTH2^+Lin^-$ ILC2 in the HD and SSc skin samples (scale bars=100 µm). Bar graphs show data as the mean±SEM (n=18–20 and 17–32 for HD and SSc, respectively). Comparisons between groups were calculated using Mann-Whitney U test. *P<0.05; **p<0.01.

and online supplemental figure 2CD). Of interest, the number of ILC2 per mm^2 in the dermis was positively correlated with the extent of skin fibrosis as assessed by mRSS at the time of biopsy (figure 2G).

Overall, these results show that ILC2 are increased in human SSc skin and their frequency in the dermis is associated with skin fibrosis.

KLRG1 expression on skin ILC2 is modified over the course of the disease

To determine the phenotype of cutaneous ILC2 in the SSc skin, we studied the expression of several markers: HLA DR, OX40L, CCR10, CCR6, CLA, TSLPR and KLRG1 (figure 3A,B). The percentage of ILC2 expressing HLA-DR, OX40L, CCR6, CCR10, CLA and TSLPR was similar in the skin of the patients with SSc and HDs. In sharp contrast, the percentage of KLRG1-positive cells was significantly lower in the SSc skin (figure 3A,B). Interestingly, the percentage of ILC2 $KLRG1^+$ tends to decrease with the extent of cutaneous fibrosis (figure 3C).

Altogether, these results indicated that surface KLRG1 expression on ILC2 decreases in the SSc skin and may correlate with the extent of skin fibrosis thus raising the question of the functional relevance of this observation.

TGFβ promotes in vitro KLRG1 modulation of ILC2 and impacts IL10 secretion

The relevance of KLRG1 in ILC2 modulation are uncertain and remains to be proven, particularly in human SSc. To identify the factors implicated in KLRG1 modulation, we sorted ILC2 from the peripheral blood of HDs (online supplemental figure 3A) and expanded this population in vitro in the presence of IL1β and IL2 as previously described.[20] At the end of the culture, ILC2 were consistently $CD127^+CRTH2^+$ (online supplemental figure 3B) and characterised by high expression of GATA3 (online supplemental figure 3C).[21] We then tested cytokines, specifically involved in human SSc, for their ability to modulate KLRG1 expression on expanded ILC2. IL33, IL4, TSLP and IL25 did not modify KLRG1 expression while

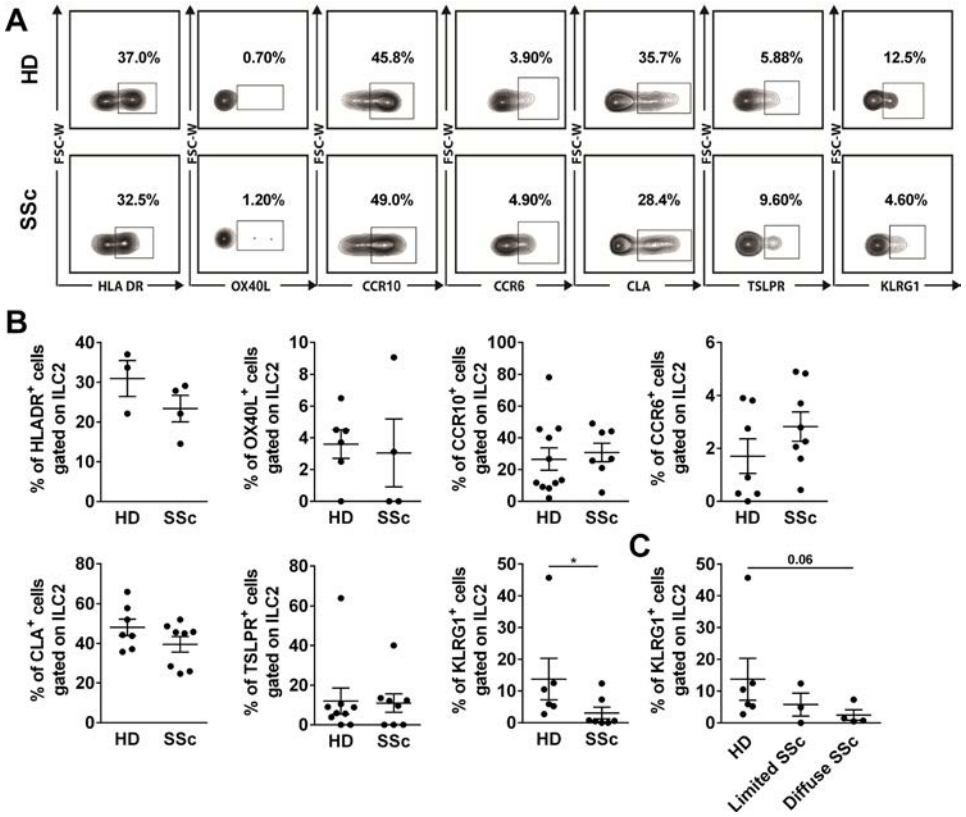

Figure 3 Decrease of killer cell lectin-like receptor G1 (KLRG1) expression on innate lymphoid cells (ILC)-2 (ILC2) in the skin of patients with systemic sclerosis (SSc). (A and B) Expression of HLA DR, OX40L, CCR10, CCR6, CLA, TSLPR and KLRG1 on skin ILC2 in the healthy donors (HDs) and patients with SSc. (C) Percentage of KLRG1$^+$ cells among ILC2s in patients with SSc with limited (Rodnan score <10) and diffuse (Rodnan score >10) in patients with SSc. Bar graphs show data as the mean±SEM (n=3–11 and 4–8 for HD and SSc, respectively). Comparisons between groups were calculated using Mann-Whitney U test. *P<0.05.

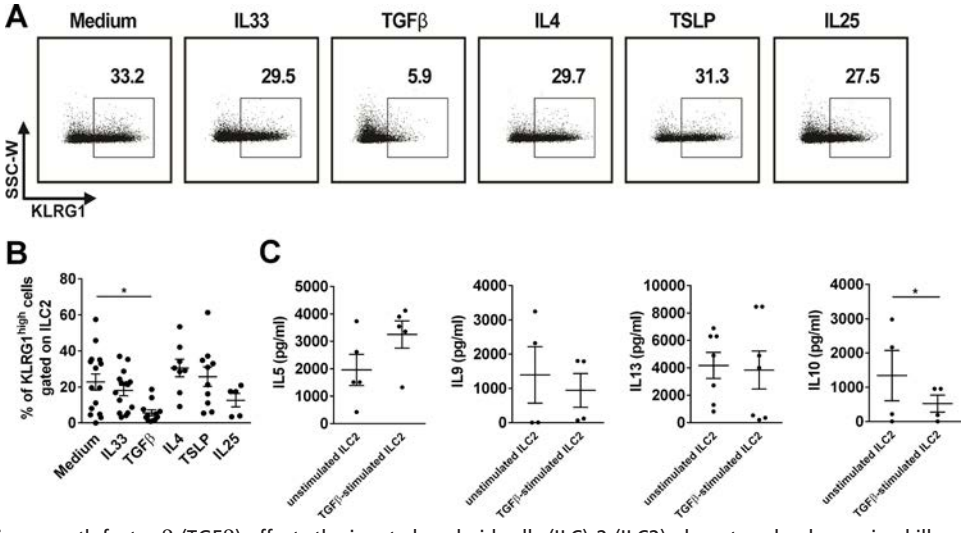

Figure 4 Transforming growth factor-β (TGFβ) affects the innate lymphoid cells (ILC)-2 (ILC2) phenotype by decreasing killer cell lectin-like receptor G1 (KLRG1) expression and interleukin (IL)10 production. (A) Representative dot blot of KLRG1 expression and (B) percentage of KLRG1$^+$ cells in unstimulated ILC2 (medium) and ILC2 stimulated with IL33, TGFβ, IL4, thymic stromal lymphopoietin (TSLP) and IL25 after 20 days of amplification. (C) Secretion of IL5, IL9, IL13 and IL10 assessed by a cytokine bead assay (CBA) in both types of ILC2 supernatants. Bar graphs show data as the mean±SEM (n=5–16 for KLRG1 expression and n=4–7 for CBA). Comparisons between groups were calculated using Kruskal-Wallis or Wilcoxon tests. *P<0.05.

TGFβ dramatically decreased the expression of KLRG1 on ILC2 (figure 4A,B).

We then evaluated the functional consequences of TGFβ exposure on ILC2 by analysing its impact on cytokine production. The levels of IL5, IL9 and IL13 were similar in ILC2 exposed or not to TGFβ (figure 4C). In contrast, the production of IL10 in TGFβ-primed ILC2 was significantly reduced compared with that of controls.

Overall, these data indicate that TGFβ downregulates KLRG1 expression on ILC2, whose phenotype is associated with a decreased capability to produce IL10.

IL10 decreased production by TGFβ-stimulated ILC2 leads to a profibrotic profile by fibroblasts

Our observations suggest that the KLRG1$^-$ ILC2 population could be involved in the fibrotic process. We therefore investigated the ability of TGFβ-stimulated ILC2 to modify the fibrotic response. To this end, supernatants (SN) from TGFβ-stimulated or unstimulated ILC2 were added to dermal fibroblasts. After 1 day of co-culture, the expression of type I collagen (*COL1A1*) and matrix metalloproteinase-1 (*MMP-1*) was evaluated. As expected, TGFβ alone increased *COL1A1* and decreased *MMP1* messenger RNA (mRNA) expression (figure 5A). TGFβ neutralisation completely blocked these changes. Interestingly, SN from TGFβ-stimulated ILC2 increased the *COL1A1* mRNA expression while the SN from unstimulated ILC2 did not affect the *COL1A1* mRNA levels. Importantly, TGFβ neutralisation in TGFβ-stimulated ILC2 did not affect these results, thereby ruling out the effect of the initial exogenously added TGFβ on the induction of *COL1A1* mRNA in fibroblasts. Furthermore, ILC2 SN dramatically increased the *MMP1* mRNA expression independently of the priming conditions (figure 5A). The profibrotic activity of TGFβ-stimulated ILC2 was emphasised by the *COL1A1*/*MMP1* ratio as a surrogate of collagen turnover, which was increased, suggesting an enhanced collagen deposition over degradation (figure 5B). While no difference of expression was observed for *COL1A2*, the SN of TGFβ-stimulated ILC2 also increased the mRNA of fibronectin (figure 5C).

Since the production of IL10 was specifically reduced in TGFβ-stimulated ILC2, we next evaluated the role of IL10 on their profibrotic activity. Of interest, when IL10 was neutralised in control ILC2 SN we observed enhanced *COL1A1* mRNA expression of dermal fibroblast (figure 5D). Conversely, the addition of IL10 to TGFβ-stimulated ILC2 SN led to a dramatic decrease in *COL1A1* mRNA expression. We then confirmed these results at the protein level (figure 5E). To further explore the fibroblast activation, we measured the proliferation and the differentiation into myofibroblasts. While no difference was observed regarding the proliferation (figure 5F), we found a significant increase expression of α-smooth muscle actin when fibroblasts were incubated with the SN of TGFβ-stimulated ILC2, independently of IL10 (figure 5G). As previously, we paid attention to incubate the SN of TGFβ-stimulated ILC2 with anti-TGFβ blocking antibody, excluding the possibility that residual TGFβ from ILC2 activation may have mediated myofibroblast differentiation.

Collectively, our data show that TGFβ favours the generation of KLRG1$^-$ ILC2 characterised by low IL10 production capacity, which simultaneously results in their enhanced profibrotic capacity.

Transcriptomic analysis reveals additional fibrotic potential of TGFβ-stimulated ILC2

To look further on the fibrotic potential of human ILC2, we evaluated the transcriptome of TGFβ-stimulated ILC2 compared with unstimulated ILC2 by RNAseq (online supplemental figure 4A). The heat map shows modification in gene expression of ILC2 triggered by TGFβ activation, with 2840 genes being differentially expressed (p-adjusted value <0.01). The enrichment analysis with R library Gprofiler2 (V.0.2.0), using Gene Ontology databases, indicated SMADs activation ('heteromeric SMAD protein complex'; GO:0071144; p adjusted value=4.01E^{-2} and 'SMAD protein complex'; GO:0071141; p adjusted value=4.45E^{-2}) and immune regulation (online supplemental table E3, E4, E5, E6 and E7). In the first 60 most upregulated genes (online supplemental table E8), we look at genes already described in fibrosis mechanisms. Interestingly, we observed an upregulation of *LTC4S* (leukotriene C4 synthase) (log2 fold change=2.71; p adjusted value=9.89E^{-22}). Regarding downregulated genes, we found a decrease of *IL10* (log2 fold

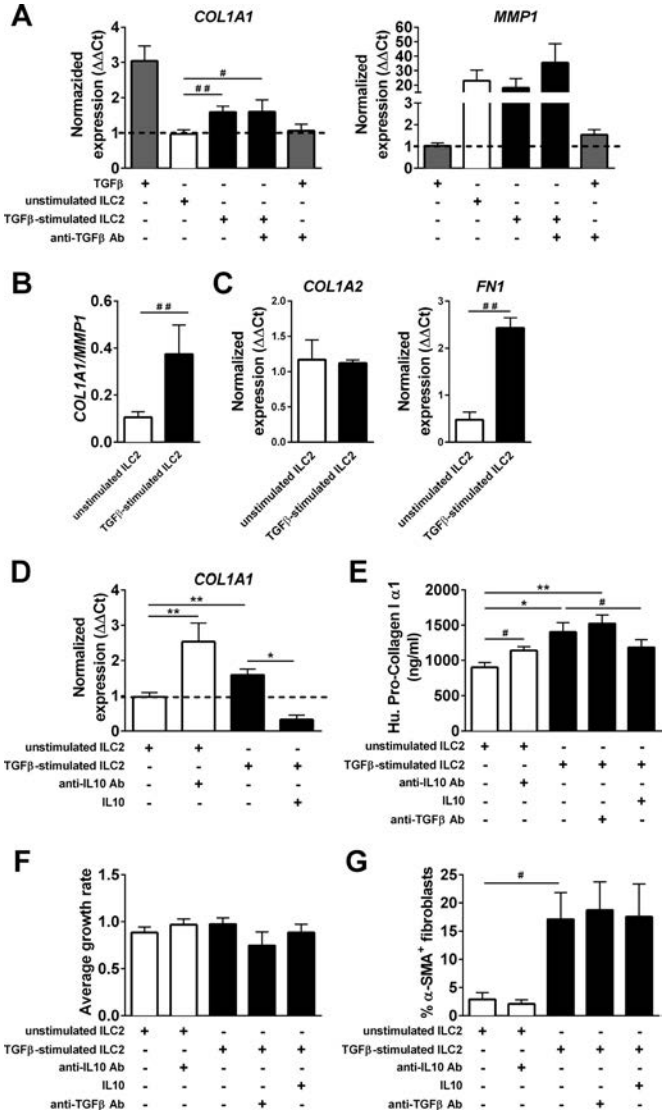

Figure 5 Transforming growth factor-β (TGFβ) exerts an indirect fibrotic role through innate lymphoid cells (ILC)-2 (ILC2) and interleukin (IL)10 secretion. (A) Q-RT-PCR analysis of type I collagen (*COL1A1*) and matrix metalloproteinase-1 (*MMP-1*) messenger RNA (mRNA) expression and (B) *COL1A1/MMP1* ratio, in fibroblasts from healthy donors (HDs), incubated with TGFβ or supernatant of unstimulated ILC2 or supernatant of TGFβ-stimulated ILC2 with or without blocking antibodies against TGFβ. (C) Q-RT-PCR analysis of *COL1A2* and *FN1* mRNA expression. (D) Q-RT-PCR analysis of *COL1A1* expression in fibroblasts incubated with supernatant of unstimulated ILC2 (in the presence or absence of anti-IL10 antibodies) and supernatant of TGFβ-stimulated ILC2 (with or without IL10). (E) Human pro-collagen I α1 concentration were analysed in the supernatants of fibroblasts from HDs after 48 hours of activation with ILC2 supernatants. (F) Average growth rate of fibroblasts were analysed after 48 hours of proliferation. (G) The percentage of α-smooth muscle actin positive fibroblasts were analysed after 72 hours of activation. Bar graphs show data as the mean±SEM (n=7–15). Comparisons between groups were calculated using the paired Wilcoxon (#) or the Kruskal-Wallis tests (*). *P<0.05; **p<0.01.

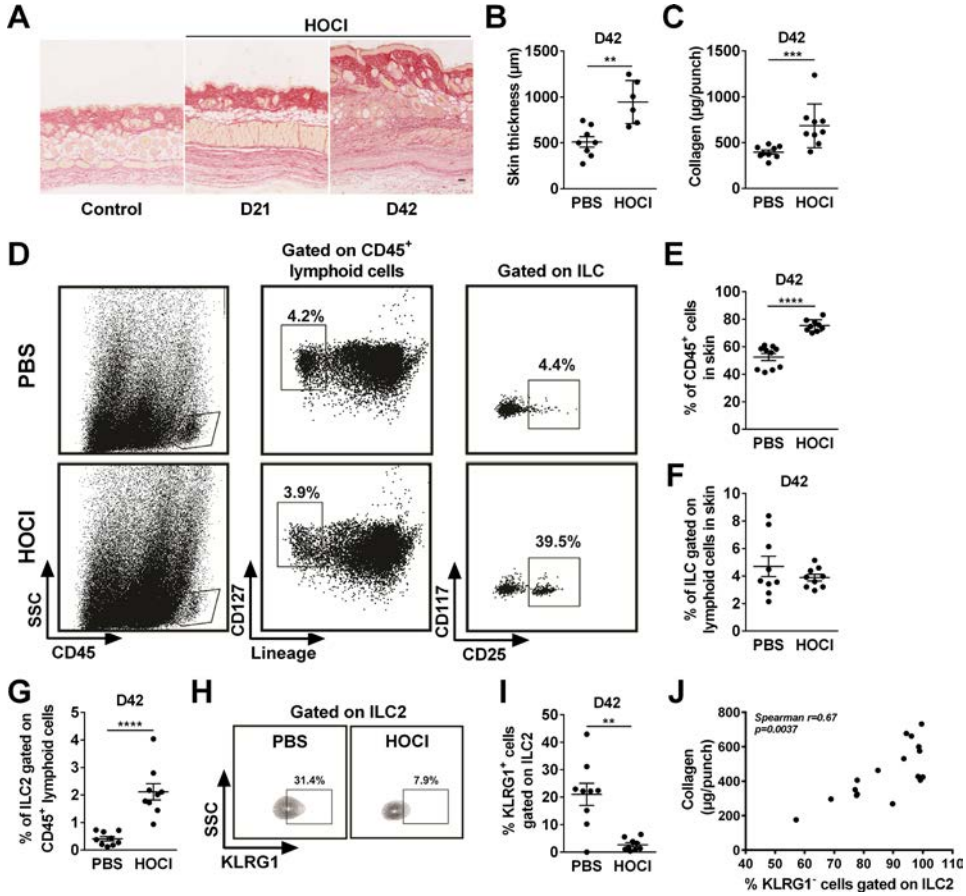

Figure 6 Characterisation of innate lymphoid cells (ILC)-2 (ILC2) in the skin of phosphate-buffered saline (PBS)-treated and hypochlorous acid (HOCl)-treated mice. (A) Picrosirius red staining of the control and systemic sclerosis (SSc) skin mice at day 42 (scale bar=10 µm). (B and C) Measure of skin thickness (µm) and collagen (µg) in the skin of PBS-treated and HOCl-treated mice at day 42. (D) Representative dot plot of cutaneous ILCs ($CD45^+$, Lin^-, $CD127^+$) and ILC2 ($CD45^+$, Lin^-, $CD127^+$, $CD25^+$) in both mice skin. (E) Percentage of $CD45^+$ cells in the skin of PBS-treated and HOCl-treated mice at day 42. (F) Percentage of ILC and (G) ILC2, among lymphoid cells in the skin of PBS-treated and HOCl-treated mice at day 42. (H and I) Percentage of $KLRG1^+$ cells among ILC2 in the skin of PBS-treated and HOCl-treated mice at day 42. (J) Positive correlation between the quantity of collagen (µg) and the percentage of $KLRG1^-$ ILC2. Bar graphs show data as the mean±SEM (n=10 per groups). Comparisons between groups were calculated using Mann-Whitney U test. **P<0.01; ***p<0.001; ****p<0.0001.

change=−3.71; p adjusted value=$1.36E^{-51}$), in agreement with the low IL10 expression at the protein level (figure 4C). Transcriptomic results for *IL10* and *LTC4S* were confirmed by Q-RT-PCR (online supplemental figure 4B).

$KLRG1^-$ ILC2 are enriched in the skin of hypochlorous acid-treated mice and their numbers correlate with the extent of skin fibrosis

To assess the in vivo relevance of our findings, we monitored ILC2 in the hypochlorous acid (HOCl)-induced mouse model of SSc, which recapitulates the main hallmarks of SSc.[22] At day 42, parallel to an increase in dermal thickness (figure 6A for a representative histology of the skin, figure 6B,C for skin thickness and collagen content, respectively), we observed a significant enrichment of the lymphoid cell infiltrate (figure 6D,E). Remarkably, while the frequency of ILCs was not changed (figure 6F), the frequency of ILC2 cells was more than 5 times greater in HOCl-treated skin mice compared with controls as detected by flow cytometry (figure 6G). In addition, the absolute value of cutaneous ILC2 was significantly higher in HOCl-treated mice compared with control mouse (online supplemental figure 5A). This ILC2 enrichment was specific, since the frequency and absolute count of total ILCs was not different in HOCl-treated and non-treated mice (figure 6F and online supplemental figure 5B). A slighter increase in ILC2 number and absolute count was already observed even before complete establishment of skin fibrosis (online supplemental figure 5C,D and E,F for skin thickness and collagen content, respectively). Furthermore, the frequency of KLRG1 in ILC2 was fourfold lower in HOCl-treated compared with control mice (figure 6H for a representative staining of ILC2 and figure 6I for quantification). Interestingly, we observed a strong positive correlation between the proportion of $KLRG1^-$ ILC2 and the extent of skin fibrosis (figure 6J).

The combined administration of IL10 and TGFβ inhibition is required to restore skin $KLRG1^+$ ILC2 and to prevent skin fibrosis

We have shown that the TGFβ-priming of human $KLRG1^-$ ILC2 enhances their profibrotic potential by specifically reducing their production of IL10, thus attenuating a negative feedback loop. We then addressed the question of the in vivo relevance of these findings by taking advantage of the HOCl mouse model of SSc. To inhibit TGFβ signalling, HOCl-treated mice received daily oral pirfenidone for 42 days, or PBS as control. In addition, they were injected or not with daily IL10. At day 42, the treatment with pirfenidone alone moderately reduced, without reaching statistical significance, the fibrotic response assessed as skin thickness and collagen content (figure 7A,B). The treatment with IL10 by itself had no effect on skin fibrosis. In contrast, when

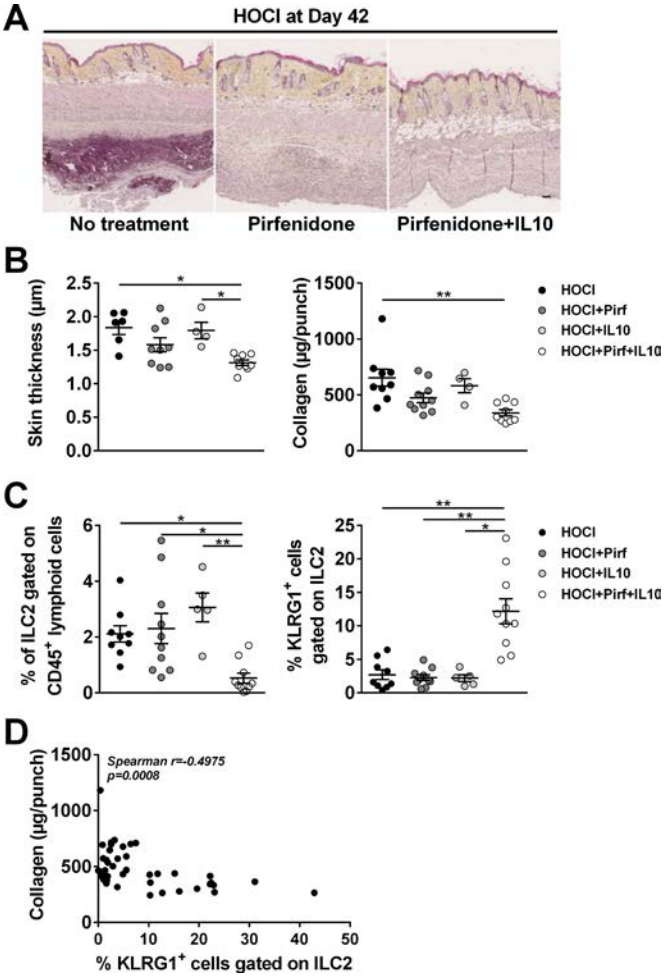

Figure 7 Interleukin (IL)10 and pirfenidone (Pirf) reduce fibrosis and affect the number of innate lymphoid cells (ILC)-2 (ILC2) in the skin of hypochlorous acid (HOCl)-treated mice at day 42. (A) H&E saffron staining of HOCl skin mice treated or untreated (scale bar=10 µm). (B) Measure of skin thickness (µm) and collagen (µg) in the skin of HOCl-untreated and HOCl-treated mice at day 42. (C) Percentage of ILC2 among lymphoid cells and percentage of KLRG1$^+$ cells among ILC2 in the skin of HOCl-untreated and HOCl-treated mice at day 42. (D) Negative correlation between collagen assay (µg) and the percentage of KLRG1$^+$ ILC2 in the skin. Bar graphs show data as the mean±SEM (n=10 per groups). Comparisons between groups were calculated using Kruskal-Wallis test. *P<0.05; **p<0.01.

pirfenidone and IL10 were administered jointly we observed a dramatic and statistically significant reduction in both the skin thickness and collagen content (figure 7B). Interestingly, the proportion of ILC2 was significantly lower and the frequency of their KLRG1$^+$ subset was significantly higher in HOCl mice that received the combined treatment with pirfenidone and IL10 when compared with all the other treatments (figure 7C). Not the least, the extent of collagen content in the skin was inversely correlated with the frequency of KLRG1$^+$ ILC2 (figure 7D). Altogether, these results demonstrate that the combined treatment with pirfenidone and IL10 strongly reduces skin fibrosis in vivo simultaneously reducing the number of ILC2 infiltrating the skin and enhancing their expression of KLRG1.

DISCUSSION

ILCs were recently identified as new important actors of the innate arm of the immune system. ILCs have been characterised and classified in recent years, and they have been directly implicated in many inflammatory conditions, including fibrosis, atopic dermatitis, asthma and inflammatory bowel disease.[23–26] SSc is a complex autoimmune connective tissue disease characterised by autoimmunity, widespread tissue fibrosis of the skin and internal organs and vasculopathic alterations. Its pathogenesis remains poorly understood, and a single treatment has not been approved over the last 50 years.

Our study directly implicates the impairment of ILC homeostasis as a potential contributor and new therapeutic strategy for SSc. Specifically, we observed that human skin biopsies from patients with SSc were characterised by a higher proportion of KLRG1$^-$ ILC2 positively correlated to the severity of the fibrotic process. Mechanistically, we demonstrated in vitro that TGFβ decreased KLRG1 expression on ILC2 and enhanced their profibrotic function through IL10 downregulation. Interestingly, in a mouse model of SSc recapitulating human findings, TGFβ inhibition associated with IL10 administration prevented the development of fibrosis while repopulating the skin via KLRG1$^+$ ILC2 in a synergistic manner. Altogether, those results revealed a previously undescribed mechanism in human SSc pathogenesis and paved the way for potential alternative therapeutic strategies based on TGFβ blocking associated with IL10 administration.

Few previous studies have investigated the role of ILCs in human SSc. One group described an increased number of CD4$^+$ group 1 ILCs,[27] while another group reported an increased proportion of ILC2 in the peripheral blood.[19] In contrast, our data showed a decrease in ILC2 numbers/proportions at the blood level and did not identify a difference for ILC1. We cannot exclude that immunomodulatory agents may have participated to the decrease of ILC2 in the blood. However, in the limit of the number of analysed patients, no impact on the increase amount of ILC2 in the skin of patients with SSc was observed. These contradictory observations also raised specific questions about the phenotypic definition of low-number circulating cells. Although key researchers in the field have reviewed this important question,[28] differences in gating strategies, antibodies or even methods may explain these discrepancies. In our gating strategy, one may argue that the gate for ILC is too close from the lineage positive cells. As shown in the online supplemental figure 1B, we pay attention to evaluate that the population edging the lineage positive cells also contained ILC1, ILC2 and ILC3 subsets. Regarding selected markers in our experiments, we used the CD5 in the lineage marker to remove CD4$^+$ T lymphocytes. A recent paper showed that CD5$^+$ ILCs are functionally immature and very close to progenitor cells.[29] Although CD5 could be expressed in a small proportion of ILC1, ILC2 and ILC3, these cells become functionally active cytokine-secreting ILCs when they downregulate CD5 and migrate to tissues. Therefore, using CD5 as we have done, appears to be a reasonable trade-off for eliminating CD4 T cells without losing mature cells. Finally, by using two complementary strategies, we observed an increase in the proportion of ILC2 in the SSc skin in accordance with a previously published report.[19]

Extensive characterisation of human skin-homing ILC2 from patients with SSc revealed subtle phenotypic changes associated with the fibrotic process. SSc skin is populated by KLRG1$^-$ ILC2, whereas skin-resident ILC2 express KLRG1 in normal skin from humans and mice. Interestingly, KLRG1 expression levels have been described as a marker to separate iILC2 (KLRG1$^+$) and nILC2 (KLRG1$^-$). iILC2 could be transient progenitors and develop into nILC2 or ILC3-like cells.[30] Although this dichotomy has mainly been described in the field of infection in mice, our results show that this observation is

true in humans and would be directly associated with the fibrotic process. Our results in SSc suggest KLRG1$^+$ ILC2 could migrate from the blood to the skin and switch into profibrotic KLRG1$^-$ ILC2. Functional studies in vitro revealed that TGFβ, which is known to be elevated in patients with SSc, was a key cytokine involved in KLRG1 downregulation, thus mirroring the effect of TGFb on KLRG1-expressing CD8$^+$ T cells.[31] Unexpectedly, we observed that KLRG1$^-$ ILC2 triggered profibrotic responses on dermal fibroblast, which was mainly due to an imbalance in production between profibrotic factors and antifibrotic factors. Moreover, we observed that the low capability to secrete IL10 was directly responsible for the profibrotic effect. The role of IL10 as an antifibrotic agent has been previously demonstrated. Indeed, two distinct models of fibrosis, one in chronic renal insufficiency and another in hepatic damage induced by biliary duct ligation, provide evidence for the impact of decreased levels of IL10 in the occurrence and maintenance of fibrosis.[32 33] Moreover, the genetic delivery of IL10 significantly attenuated TGFβ production in the lungs of mice subjected to bleomycin-induced pulmonary fibrosis. This effect was still observed when IL10 was delivered at later time points when fibrosis was already established.[34] Therefore, our results established a direct link between TGFβ and low secretion of IL10 at the tissue level through ILC2.

To look further on the fibrotic potential of ILC2, we evaluated the transcriptome of TGFβ-stimulated ILC2 compared with unstimulated ILC2 and found an increase of the leukotriene C4 synthase expression, suggesting that activated ILC2 may release leukotriene C4 (LTC4). Since LTC4 is a potent inducer of collagen synthesis by dermal fibroblasts,[35] the ILC2-LTC4 axis in SSc skin fibrosis will require further investigations.

Different SSc mice models have been used to evaluate different therapeutic strategies; however, none of them recapitulates the human observations *in integro*. The HOCl-treated mouse model has the advantage of summarising the three main characteristics of SSc: vasculopathy, deregulation of the immune response (including the production of autoantibodies) and cutaneous and pulmonary fibrosis. In our study, we only tested our hypothesis in one mouse model that constitutes a limitation. However, our data indicated that this model recapitulated the human skin findings, including the increased proportion of KLRG1$^-$ skin ILC2 proportions correlated to the fibrotic process. We were not able to increase lung fibrosis, precluding any further pulmonary investigation. Our work is thus limited to only one mouse model exploring cutaneous fibrosis. Many mouse models potentially useful to further assess the relevance of ILC in experimental animals could be considered, of which none fully recapitulates the features of human systemic sclerosis.[36] However, we believe that, within its limits, the HOCl mouse model we adopted, strongly support the results of our research principally and extensively made in humans. In detail, it met several important requirements for our demonstration: (i) development of skin fibrosis after an inflammatory period; (ii) accompanied by an increase of ILC2 in the skin; (iii) a positive correlation between skin fibrosis and number of ILC2 in the skin, as we have shown in humans. From a therapeutic perspective, most of the mouse models have implicated ILC2 through its deletion using *Rora*$^{sg/flox}$*Il7r*$^{Cre/+}$ mice[37] or *Rag1*$^{-/-}$ mice, for instance, coupled with the injection of depleting anti-Thy1 antibody.[38] Although these strategies offer a clear view of the global implication of ILC2 in a model, it is not a feasible strategy in humans and does not permit the characterisation of the implication of ILC2-skewed functionalities in a disease process, as it is the case in our setting. Therefore, we decided to adopt an alternative strategy that combines a well-accepted TGFβ inhibitor and IL10 supplementation, according to our in vitro data. Our results revealed a dramatic synergic effect on the fibrotic process while KLGR1$^+$ ILC2 were repopulating the skin. Although this therapeutic strategy does not formally directly prove the involvement of the KLRG1$^-$ population in the pathogenesis, it provides indirect evidence on the pathogenic loop implicating ILC2, IL10 and TGFβ. Moreover, our results could constitute the groundwork for proposing a clinical trial testing the combination of the TGFβ inhibitor and IL10 in patients with SSc. The US Food and Drug Administration recently approved targeting the TGFβ pathway for the treatment of idiopathic pulmonary fibrosis (IPF). In a phase III clinical trial, pirfenidone successfully reduced the progression of IPF and was associated with fewer deaths.[39] For unknown reasons, this effect in the patients with SSc seemed to be milder; thus, combining another strategy in this multifactorial disease could be an option. After the successful preclinical experiments, clinical studies using human recombinant IL10 are already in progress for the treatment of inflammatory bowel diseases with an acceptable safety profile.[40] Future studies using nintedanib instead of pirfenidone are also of interest since this molecule is used in interstitial lung disease in SSc.[41]

In conclusion, our study revealed that ILC2 may contribute to the fibrotic process observed in human SSc and the combination of the TGFβ inhibitor and IL10 could be a promising therapeutic strategy.

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Contributors PL and BA jointly designed the research, performed the experiments, collected, analysed and interpreted the data, wrote and revised the manuscript. MJ, PM, EL, DL, AG and VJ performed and analysed some experiments. EL, PL, JI, MJ and FB performed mice experiments. TS, CC, TP and PB provided intellectual input and edited the manuscript. PM, DL, PH, JS, JC, CR, PD, TS, EL and EF recruited the study participants and provided patients' samples and clinical data. CC-B and M-ET jointly designed and supervised the study and wrote the manuscript.

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Mechanosensitive TRPV4 is required for crystal-induced inflammation

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ABSTRACT

Crystal structures activate innate immune cells, especially macrophages and initiate inflammatory responses. We aimed to understand the role of the mechanosensitive TRPV4 channel in crystal-induced inflammation. Real-time RT-PCR, RNAscope in situ hybridisation, and *Trpv4*^{eGFP} mice were used to examine TRPV4 expression and whole-cell patch-clamp recording and live-cell Ca^{2+} imaging were used to study TRPV4 function in mouse synovial macrophages and human peripheral blood mononuclear cells (PBMCs). Both genetic deletion and pharmacological inhibition approaches were used to investigate the role of TRPV4 in NLRP3 inflammasome activation induced by diverse crystals in vitro and in mouse models of crystal-induced pain and inflammation in vivo. TRPV4 was functionally expressed by synovial macrophages and human PBMCs and TRPV4 expression was upregulated by stimulation with monosodium urate (MSU) crystals and in human PBMCs from patients with acute gout flares. MSU crystal-induced gouty arthritis were significantly reduced by either genetic ablation or pharmacological inhibition of TRPV4 function. Mechanistically, TRPV4 mediated the activation of NLRP3 inflammasome by diverse crystalline materials but not non-crystalline NLRP3 inflammasome activators, driving the production of inflammatory cytokine interleukin-1β which elicited TRPV4-dependent inflammatory responses in vivo. Moreover, chemical ablation of the TRPV1-expressing nociceptors significantly attenuated the MSU crystal-induced gouty arthritis. In conclusion, TRPV4 is a common mediator of inflammatory responses induced by diverse crystals through NLRP3 inflammasome activation in macrophages. TRPV4-expressing resident macrophages are critically involved in MSU crystal-induced gouty arthritis. A neuroimmune interaction between the TRPV1-expressing nociceptors and the TRPV4-expressing synovial macrophages contributes to the generation of acute gout flares.

Key messages

What is already known about this subject?

- Many exogenous or endogenously formed crystal structures with different compositions can act as damage-associated molecular patterns and in turn trigger NLRP3 inflammasome activation.
- Ion channels are critical signalling mediators in both innate and adaptive immune cells.

What does this study add?

- Macrophage-expressed TRPV4 is essential to the generation of crystal-induced joint pain and inflammation and inflammatory responses evoked by diverse crystalline structures.
- TRPV4 is critically involved in the activation of macrophage NLRP3 inflammasome and production of the inflammatory cytokine interleukin-1β induced by diverse crystalline materials but not non-crystalline NLRP3 inflammasome activators.
- A neuroimmune interaction between the TRPV1-expressing primary nociceptors and the TRPV4-expressing synovial macrophages is an important cellular mechanism behind acute gout flares.

How might this impact on clinical practice or future developments?

- Our findings show that macrophage-expressed TRPV4 is a common regulator of crystal-induced inflammatory responses and shed new insights into the development of new therapeutic interventions to treat inflammatory conditions associated with crystal disposition.

INTRODUCTION

Innate immune cells recognise a variety of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) to mount a rapid inflammatory response.[1 2] One way in which cells mediate this process is through the NLRP3 inflammasome, an intracellular sensor of both exogenous and endogenous stimuli such as microbes, misfolded proteins and adenosine triphosphate (ATP). Depending on the signal, this pathway activates a variety of cellular processes including inflammatory cytokine production and programmed cell death by pyroptosis to drive an innate antimicrobial response.[1] However, as with many host-protective responses, this process can also become highly pathologic.

Many exogenous or endogenously formed crystal structures with different compositions can act as DAMPs and in turn trigger NLRP3 inflammasome activation.[3 4] For instance, exogenous silica crystals and asbestosis fibres are well known to cause fibrosis and chronic airway disease. It is also recognised that innate immune cells such as macrophages (MΦs) internalise both endogenous and exogenous crystals

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and thereby activate the NLRP3 inflammasome, driving many inflammatory diseases such as gout, pseudogout, atherosclerosis and silicosis.[5 6] Currently, there are at least three hypotheses for MΦ recognition and NLRP3 inflammasome activation by crystals and nanoparticles: (1) The NLRP3 inflammasome senses the disturbance of cellular homeostasis rather than directly recognising various types of crystals[7]; (2) Distinct membrane-bound receptors serve as molecular sensors for distinct crystals to initiate NLRP3 inflammasome activation upon interaction[5] and (3) Phagocyte membrane engagement by crystals may directly activate the NLRP3 inflammasome in a receptor-independent manner.[8] However, the molecular events underlying the activation of the NLRP3 inflammasome induced by cell surface contact with crystals remain unclear.

Growing evidence shows that ion channels are critical signalling mediators in both innate and adaptive immune cells[9] and their expression and function on MΦs are tightly associated with NLRP3 inflammasome activation.[10] Transient receptor potential ion channels are important signalling components in both neurosensory and inflammatory pathways, giving way to their functions as polymodal sensors of environmental cues and as key mediators in sensory transduction.[11–15] Among them, the non-selective multifunctional cation channel TRPV4 is highly expressed in cartilage chondrocytes and activated by hypo-osmolality, heat and arachidonic acid metabolites.[16] TRPV4 has also been functionally linked to the skeletal dysplasia.[17 18] Although studies reported that TRPV4 is expressed by primary nociceptors and contributes to acute nociception caused by hypotonic stress and neuroinflammation,[19–21] emerging evidence also showed that TRPV4 expressed by non-neuronal cells including MΦs is involved in the development of acute/chronic itch and regulation of gastrointestinal motility.[22 23] However, it remains unknown whether and how TRPV4 channel is involved in crystal-induced inflammation in MΦs.

In this study, we show that MΦ-expressed TRPV4 is selectively involved in the inflammation and reflexive pain-related responses induced by both endogenous and exogenous crystals through activation of the NLRP3 inflammasome. Our results suggest that TRPV4 is a potential drug target for treating crystal-induced inflammatory disorders.

MATERIALS AND METHODS

Materials and methods are described in online supplemental file 1.

RESULTS

TRPV4 is required for monosodium urate (MSU) crystal-induced reflexive pain-related responses and inflammation in a mouse model of gout

To determine the role of TRPV4 in inflammatory joint disease, we first employed the well-established mouse model of gout.[24] As expected, wild-type (*wt*) mice treated with intra-articular (IA) injections of MSU crystals (0.8 mg per site) developed severe joint swelling and reflexive mechanical and thermal pain-related responses (figure 1A–C). Strikingly, intraperitoneal (i.p.) administration of GSK219, a potent and selective TRPV4 inhibitor,[23 25] either 3 days before injections of MSU crystals (figure 1A–E) or 5 hours after MSU crystal injections (online supplemental figure 1a–e) significantly reduced MSU crystal-induced reflexive pain-related responses as well as inflammation-related parameters including ankle oedema, leucocyte infiltration and myeloperoxidase (MPO) activity. The inhibitory effect of GSK219 was comparable to that of colchicine, a standard of care treatment for acute gout flares (figure 1A–E). Moreover, genetic ablation of TRPV4 function significantly alleviated MSU crystal-induced reflexive pain-related responses, and joint inflammation (ankle oedema, synovium lesion/inflammation, neutrophil infiltration and MPO activity) when compared with $Trpv4^{+/+}$ control mice (figure 1F–N), suggest that TRPV4 function is critically required for the generation of MSU crystal-induced gouty arthritis.

TRPV4 expression and function are increased in the setting of acute gout flares

Although TRPV4 is reported to be expressed by the primary nociceptors,[26 27] surprisingly, we did not detect TRPV4 expression and function in the dorsal root ganglion (DRG) cell bodies and nerve terminals in the skin using the $Trpv4^{eGFP}$ mice, RNAscope in situ hybridisation, and live-cell Ca^{2+} imaging on ex vivo DRG explants (online supplemental figure 2a–i). On the other hand, TRPV4-eGFP$^+$ cells were found in the synovial membrane and overlapped with well-known synovial MΦ markers such as F4/80, Cx3cr1 and CD68 (online supplemental figure 3a,b). To determine the functionality of TRPV4 in the synovial MΦs, we used live-cell Ca^{2+} imaging on sort-purified CD45$^+$ synovial MΦs using CD11b/MHCII (figure 2A–C). Application of a potent and selective TRPV4 activator GSK101 elicited a robust $[Ca^{2+}]_i$ response in the synovial MΦs isolated from the $Trpv4^{+/+}$ mice,[28 29] which was nearly abolished by GSK219 (figure 2A). GSK101-induced $[Ca^{2+}]_i$ response was absent in synovial MΦs from either the $Trpv4^{-/-}$ mice or the *Cre*$^+$ MΦ-specific *Trpv4 cKOs* ($Cx3cr1^{CreERT}$;$Trpv4^{f/f}$) in which TRPV4 expression is selectively ablated in MΦs after tamoxifen induction[22] (figure 2B,C). Moreover, GSK101 activated TRPV4-like whole-cell currents in the eGFP$^+$ synovial MΦs isolated from the $Trpv4^{eGFP}$ mice, which was inhibited by GSK219 (figure 2D). Flow cytometry further revealed that most of the TRPV4-eGFP$^+$/CD45$^+$ cells were CD11b$^+$/F4/80$^+$ (figure 2E). Strikingly, both TRPV4 mRNA transcripts (figure 2F) and the number of TRPV4-eGFP$^+$ cells were markedly increased in the joints 6 hours after IA injections of MSU crystals (0.8 mg/site) (figure 2G and online supplemental figure 3c). Consistent with the data from mouse studies, TRPV4 expression was significantly increased in PBMCs, the most relevant cell type of resident MΦs, from patients with acute gout flares when compared with that from healthy subjects (online supplemental figure 3d). In marked contrast, the expression of TRPV1, TRPA1, or TRPM8 was not significantly different between these two groups (online supplemental figure 3d). Moreover, PBMCs from gout patients displayed a stronger $[Ca^{2+}]_i$ response upon application of TRPV4 agonist GSK101 (100 nM) when compared with that from healthy control subjects (online supplemental figure 3e,f).

MSU crystal-induced inflammation and reflexive pain-related responses are attenuated in MΦ-specific *Trpv4 cKOs*

To test whether MΦ-expressed TRPV4 is required for MSU crystal-induced gouty arthritis, we used the MΦ-specific *Trpv4 cKOs* ($Cx3cr1^{CreERT}$; $Trpv4^{f/f}$).[22] Indeed, the *Cre*$^+$mice displayed significantly reduced reflexive pain-related responses, joint inflammation and synovium lesion when compared with the *Cre*$^-$ littermates in response to IA injections of MSU crystals (figure 3).

Since the synovial MΦs can arise from either tissue-resident or circulating monocyte-derived MΦs, we also tested the inducible MΦ-specific *Trpv4 cKOs* 4 weeks after tamoxifen administration when tissue-resident MΦs retain their identity but the monocyte-derived MΦs are replaced with *wt* MΦs produced by bone-marrow-derived progenitors as shown by fate mapping studies.[30]

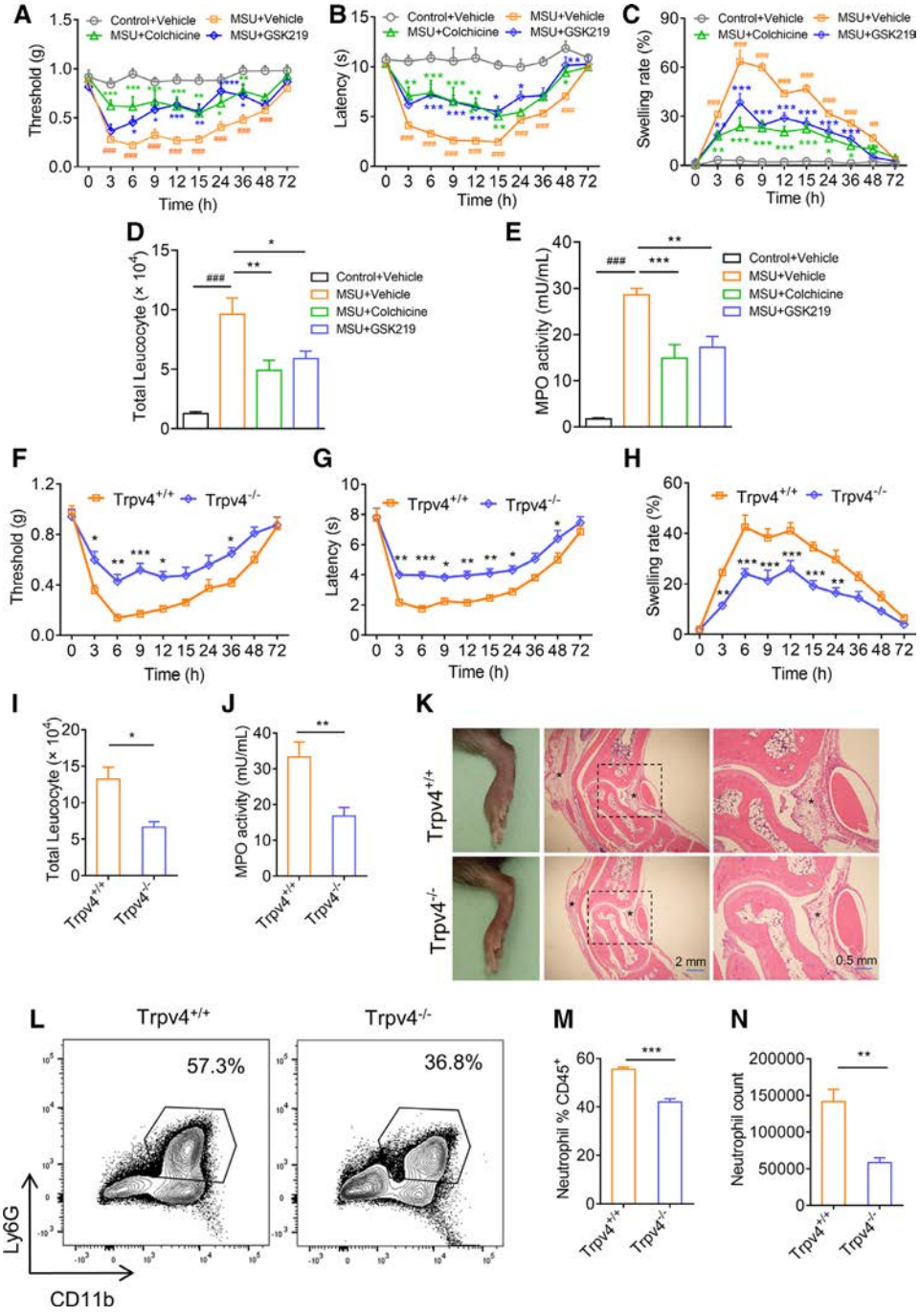

Figure 1 Pharmacological inhibition or genetic ablation of TRPV4 function reduces inflammation and reflexive pain-related responses in a mouse model of gout. (A–C) IA injections of MSU crystals (0.8 mg/site) in *wt* C57BL/6J mice produced reflexive mechanical (A) and heat (B) pain-related responses as well as joint swelling (C) in a time-dependent manner. Mice were treated with either GSK219 (5 mg/kg, i.p.) or vehicle 3 days before injections of MSU crystals. Colchicine (1 mg/kg, i.p.) was used as a positive control. n=6–8 for each group. (D, E) Increased leucocyte infiltration (D) and myeloperoxidase activity (E) were detected 6 hour after IA injections of MSU crystals in the presence of GSK219 (5 mg/kg, i.p.) or vehicle. Colchicine (1 mg/kg, i.p.) was used as a positive control. n=6 for the vehicle control and 7–8 for all other groups. (F–H) Time courses for reflexive mechanical (F) and heat (G) pain-related responses as well as joint swelling (H) induced by IA injections of MSU crystals in the *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice, n=7–8 for each group. (I, J) Both total leucocyte infiltration (I) and myeloperoxidase activity (J) were measured 6 hours after IA injections of MSU crystals, n=7–8 for each group. (K) Representative HE-staining showing oedema and inflammation of ankles in both *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice 6 hours after IA injections of MSU crystals. Magnifications, ×5 (middle) and ×20 (right). Asterisks indicate the infiltrating leukocytes. n=3 independent repeats with similar results for both groups. (L) Representative FACS plots of neutrophils in the articular cavity from the *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice subjected to IA injections of MSU crystals. (M, N) Summarised data on the right show the percentile of neutrophils within the CD45^{+} population (M) and the comparison of the neutrophil counts (N) between the *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice subjected to IA injections of MSU crystals. n=6 for all groups. Statistical significance was determined using Tukey post hoc tests (multiple comparisons, (A–C), one-way ANOVA with Tukey's post-test (D–E), two-way ANOVA followed by Bonferroni's post hoc test (F–H), and Student's t test (I, J, M, N). When compare to Control + Vehicle group, $^{\#\#}$P<0.001, $^{\#\#\#}$P<0.001. When compare to other groups, *P<0.05, **p<0.01, ***p<0.001. ANOVA, analysis of variance; FACS, fluorescence activated cell sorting; IA, intra-articular; i.p, intraperitoneal; MSU, monosodium urate; wt, wild-type.

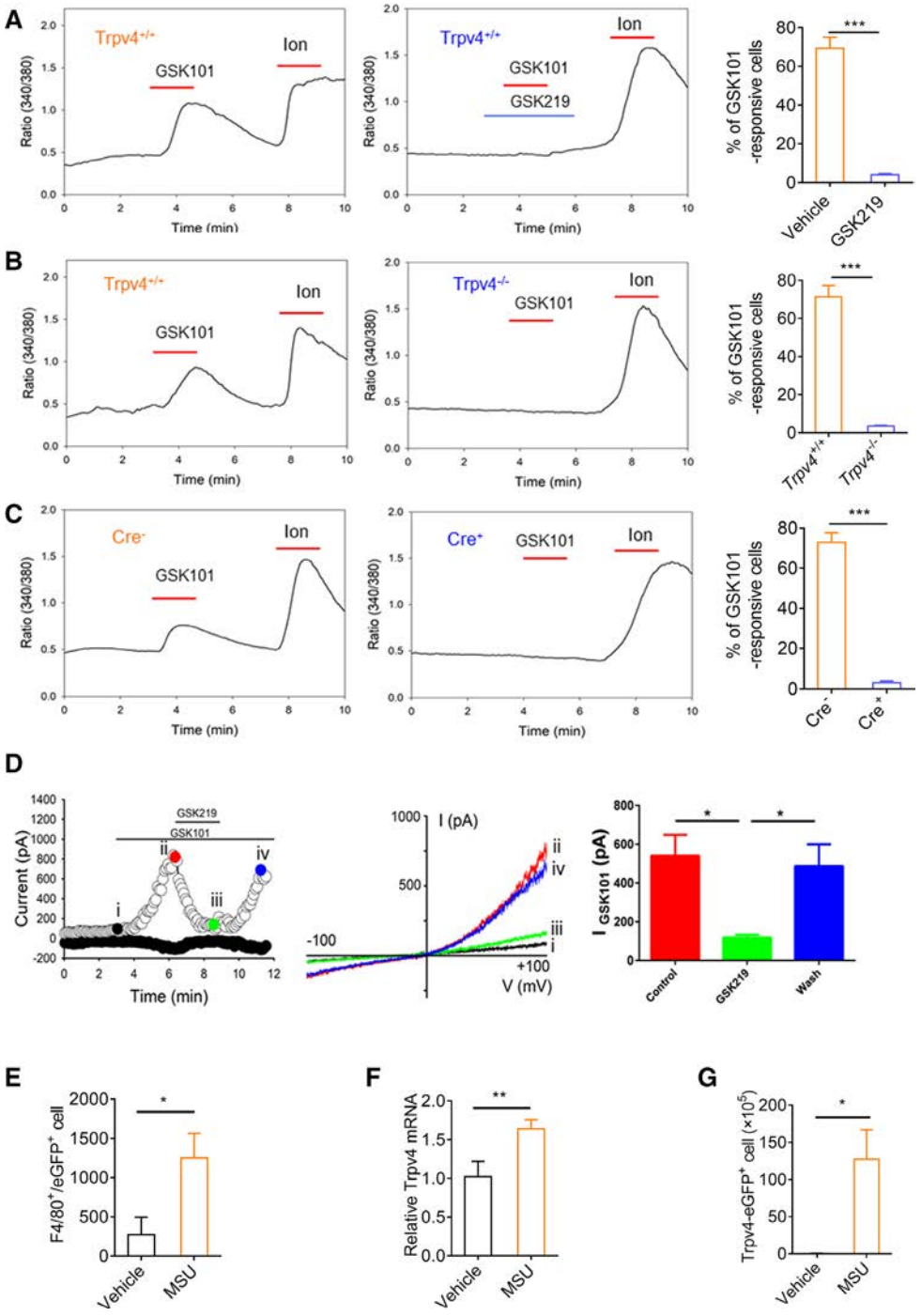

Figure 2 TRPV4 is functionally expressed by the synovial MΦs and TRPV4 expression and the number of TRPV4$^+$ MΦs are increased by MSU crystals. (A) Representative traces showing GSK101 (0.3 µM)-elicited $[Ca^{2+}]_i$ response in freshly dissociated synovial MΦ single-cell suspensions from the *Trpv4*$^{+/+}$ mice. Preapplied and coapplied GSK219 (1 µM) abolished the GSK101 action. Summarised data on the right show the reduction of the percentage of GSK101-responsive synovial MΦs by GSK219. (B) Representative traces showing that GSK101 induced a $[Ca^{2+}]_i$ response in the synovial MΦ single-cell suspensions from the *Trpv4*$^{+/+}$ but not *Trpv4*$^{-/-}$ mice. Summarised data on the right show that genetic ablation of TRPV4 function reduces the percentage of GSK101-responsive synovial MΦs. (C) Representative traces showing the GSK101-induced $[Ca^{2+}]_i$ response in the synovial MΦ single-cell suspensions from the *Cre*$^-$ but not *Cre*$^+$ *Cx3cr1*^{CreERT}*; Trpv4*$^{f/f}$ mice. Summarised data on the right show reduced the percentage of GSK101-responsive synovial MΦs isolated from the MΦ-specific TRPV4 *cKOs*. (D) Left: Representative time course of membrane currents evoked by GSK101 (0.3 µM) at +100 mV and −100 mV membrane potentials with and without coapplied GSK219 (1 µM). Horizontal bars denote the time courses for applications of GSK101 and GSK219. Middle: Representative current-voltage curves taken at time points i, ii, iii and iv (colour coded) from the time course on the left. A ramp protocol elicited by a voltage ramp from −100 mV to +100 mV was used. Quantification of the effect of GSK219 on GSK101-activated whole-cell membrane current recorded at +100 mV is shown on the right. (E) Flow cytometry shows that the number of TRPV4-eGFP$^+$ cells increased significantly 6 hours after IA injections of MSU crystals (0.8 mg/site). (F) TRPV4 mRNA expression in the synovial lining of the *Trpv4*^{eGFP} mice treated with vehicle or MSU crystals. (G) Quantification of the number of the F4/80$^+$/eGFP$^+$ synovial MΦs in response to treatment with MSU crystals or vehicle. Statistical significance was determined using Student's t-test (A–H), *p<0.05, **p<0.01, ***p<0.001. n=5–6 per group. IA, intra-articular; MSU, monosodium urate.

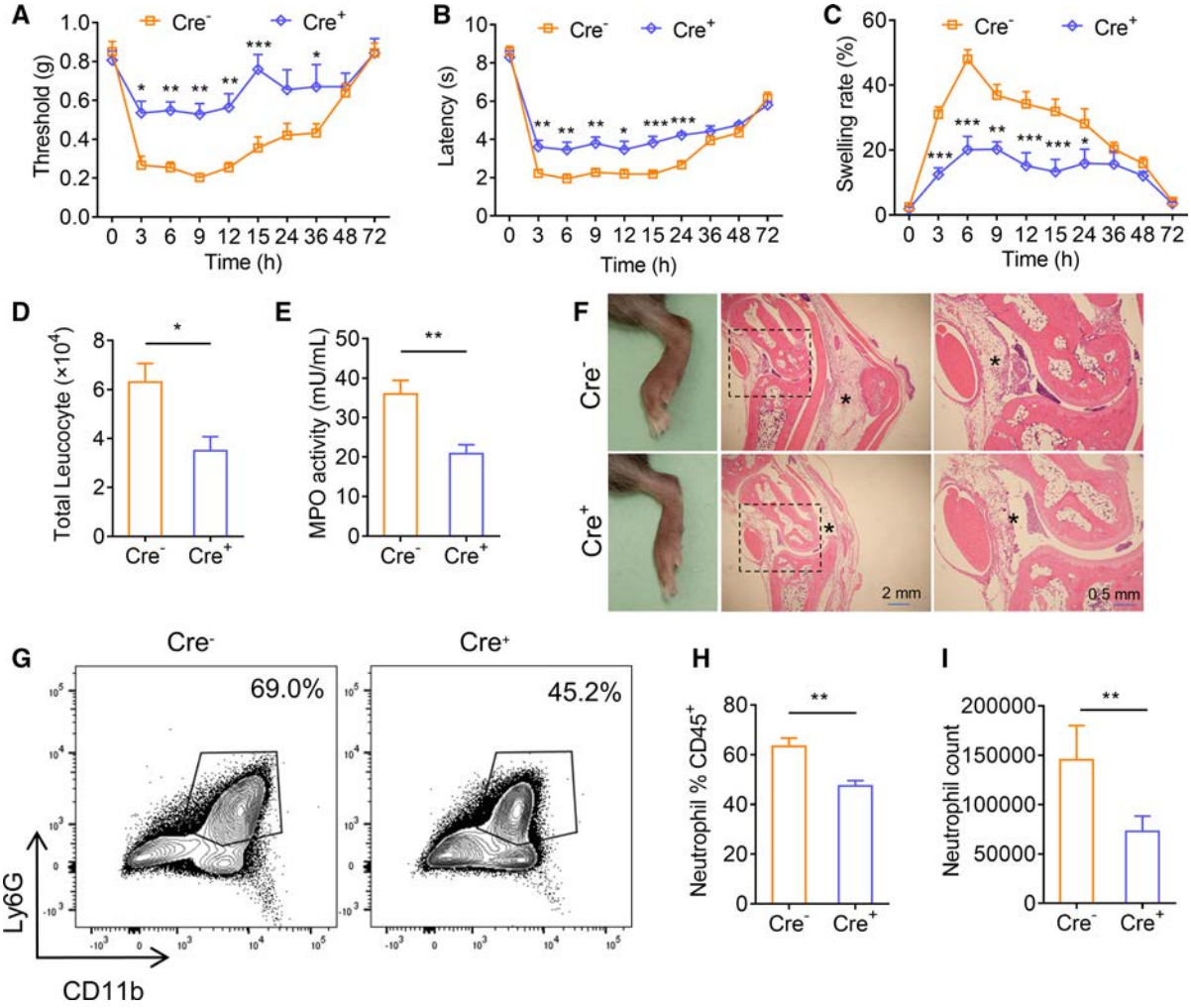

Figure 3 MΦ-specific *Trpv4 cKOs* display reduced joint swelling and reflexive pain-related responses in MSU crystal-induced gouty arthritis. (A–C) Time courses for reflexive mechanical (A) and heat (B) pain-related responses as well as joint swelling (C) induced by IA injections of MSU crystals (0.8 mg/site) in both *Cre*$^+$ and *Cre*$^-$ *Cx3cr1*^{CreERT}; *Trpv4*$^{f/f}$ mice (Experiments were performed 1 week after the last tamoxifen administration). n=6–8 for each group. (D, E) Changes of total leucocyte infiltration (D) and myeloperoxidase activity (E) in both *Cre*$^+$ and *Cre*$^-$ *Cx3cr1*^{CreERT}; *Trpv4*$^{f/f}$ mice 6 hours after IA injections of MSU crystals (0.8 mg/site), n=6–8 for each group. (F) Representative HE-staining showing oedema and inflammation of ankle sections 6 hour after IA injections of MSU crystals (0.8 mg/site) in the *Cre*$^+$ and *Cre*$^-$ *Cx3cr1*^{CreERT}; *Trpv4*$^{f/f}$ mice. Magnifications, ×5 (middle) and ×20 (right). Asterisks indicate the infiltrating leukocytes. n=3 independent experiments with similar results for both groups. (G) Representative FACS plots of synovial neutrophils from both *Cre*$^-$ and *Cre*$^+$ *Cx3cr1*^{CreERT}; *Trpv4*$^{f/f}$ mice subjected to IA injections of MSU crystals. (H, I) Summarised data show the comparison of the percentile of neutrophils within the CD45$^+$ population (H) and the neutrophil count (I) between the *Cre*$^-$ and *Cre*$^+$ *Cx3cr1*^{CreERT}; *Trpv4*$^{f/f}$ mice subjected to IA injections of MSU crystals. n=5 for each group. Statistical significance was determined using two-way ANOVA, followed by Bonferroni's post hoc test (A–C), and Student's t-test (D, E, H, I), *p<0.05, **p<0.01, ***p<0.001. ANOVA, analysis of variance; IA, intra-articular; MSU, monosodium urate.

Both reflexive pain-related responses and ankle swelling were still significantly reduced in the *Cre*$^+$ *Cx3cr1*^{CreERT}; *Trpv4*$^{f/f}$ mice when compared with their *Cre*$^-$ littermates (online supplemental figure 4). These results recapitulate the reduced reflexive pain-related responses and inflammation phenotypes displayed by the *Trpv4*$^{-/-}$ mice and further demonstrate that TRPV4 expression in the tissue-resident MΦs is the primary contributor to MSU crystal-induced gouty arthritis.

TRPV4 is also functionally expressed by neutrophils[31] and articular chondrocytes that are involved in age-related and post-traumatic osteoarthritis (OA).[32] Indeed, in addition to synovial MΦs, the number of eGFP$^+$ neutrophils was also markedly increased by MSU crystals as revealed by flow cytometry and immunofluorescence (online supplemental figure 5a–c). Notably, we and others have used neutrophil infiltration as an inflammation marker for MSU crystal-induced gouty inflammation.[33] To test if TRPV4 expression in these cell types also contributes to gouty inflammation and reflexive pain-related responses, we generated the neutrophil-specific (*S100a8*^{Cre}; *Trpv4*$^{f/f}$) and cartilage-specific (*Col2a1*$^{CreERT+}$; *Trpv4*$^{f/f}$) *Trpv4 cKOs*. Neither of these *Cre*$^+$ *cKOs* displayed changes in ankle swelling and reflexive pain-related responses after IA injections of MSU crystals compared with their respective *Cre*$^-$ groups (online supplemental figure 5d–i). Consistent with the absence of TRPV4 expression and function in primary nociceptors, genetic ablation of TRPV4 function in the TRPV1 lineage neurons (*Trpv1*^{Cre}; *Trpv4*$^{f/f}$) had no effect on MSU crystal-induced gouty arthritis (online supplemental figure 5j–l). Together, these results suggest that neutrophil-expressed, articular chondrocyte-expressed or sensory nociceptor-expressed TRPV4 is dispensable for the development of MSU crystal-induced gouty arthritis.

TRPV4 function is critically involved in MSU crystal-induced NLRP3 inflammasome activation and interleukin-1β production

Activation of NLRP3 inflammasome is a hallmark of MΦ-mediated inflammatory responses, which requires at least two signalling events.[7 34] The first event involves nuclear factor-κB (NF-κB)-dependent upregulation of NLRP3 along with pro-interleukin (IL)-1β, which is triggered by PAMPs including Toll-like receptors, DAMPs or cytokines. Of note, NF-κB signalling also promotes the production and release of tumour necrosis factor-α (TNF-α), which can be independent of NLRP3 inflammasome activation. The second event involves the assembly of a complex of multiple proteins including NLRP3, ASC (the adaptor molecule apoptosis-associated speck-like protein), and pro-caspase-1, resulting in the activation of caspase-1. Subsequently, active caspase-1 processes pro-IL-1β to mature IL-1β, which is then released from dying MΦs.

Strikingly, application of the TRPV4 activator GSK101 was sufficient to induce the production of IL-1β in a concentration-dependent manner in the *Trpv4*$^{+/+}$ bone marrow derived macrophages (BMDMs) primed with lipopolysaccharide (LPS) but not in LPS-unprimed group, which could be blunted by either pharmacological inhibition or genetic ablation of TRPV4 function (figure 4A,B and online supplemental figure 6a), although GSK101 did not affect pro-IL-1β and pro-caspase-1 in the lysates (Input) of LPS-primed BMDMs isolated from the *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice (online supplemental figure 6a). Similar to GSK101, application of MSU crystals also markedly increased the IL-1β production from LPS-primed *wt* BMDMs (figure 4C and online supplemental figure 6a) while the levels of both pro-IL-1β and pro-caspase-1 were comparable in lysates (Input) of LPS-primed and MSU crystal-treated BMDMs isolated from either *Trpv4*$^{+/+}$ or *Trpv4*$^{-/-}$ mice (online supplemental figure 6a). GSK219 suppressed MSU crystal-induced IL-1β production from LPS-primed BMDMs in a concentration-dependent manner (figure 4C). MSU crystal-induced IL-1β production was also abolished in the LPS-primed BMDMs isolated from either the *Trpv4*$^{-/-}$ mice or *Cre*$^{+}$ MΦ-specific *Trpv4 cKOs* when compared with their respective control groups (figure 4D,E and online supplemental figure 6a). Moreover, LPS-primed BMDMs from mice deficient in NLRP3 or caspase-1 were unable to release cleaved IL-1β in response to either MSU crystals (figure 4F) or GSK101 (figure 4G). These results suggest that TRPV4 is involved in the second event of NLRP3 inflammasome activation in LPS-primed BMDMs and NLRP3-caspase-1 signalling is required for TRPV4-mediated IL-1β production induced by both MSU crystals and GSK101. Consistent with results from mouse BMDMs, MSU crystals also induced robust inflammasome activation in cultured primary human MΦs (THP-1 cells) and human PBMCs isolated from healthy subjects, which was significantly reduced by GSK219 (figure 4H,I, and online supplemental figure 6b,c), suggesting that TRPV4 is a key mediator of MSU crystal-induced NLRP3 inflammasome activation in innate immune cells from both rodents and humans.

Interestingly, neither GSK101 nor GSK219 showed any significant effects on the release of TNF-α from the LPS-primed *wt* BMDMs (online supplemental figure 7a,b). Moreover, TNF-α production was not affected in the LPS-primed BMDMs isolated from either the *Trpv4*$^{-/-}$ mice or the *Cre*$^{+}$ MΦ-specific *Trpv4 cKOs* when compared with their respective controls (online supplemental figure 7c,d), suggesting that TRPV4 is unlikely involved in the first event of NLRP3 inflammasome activation and TRPV4 function is not required for TNF-α production in our MSU crystal-induced gout model.

Distinct mechanisms are involved in TRPV4-mediated NLRP3 inflammasome activation induced by GSK101 and MSU Crystals

Although the precise mechanism of MSU crystal-induced NLRP3 activation is not completely understood, the involvement of several key events including lysosomal rupture, activation of reactive oxygen species (ROS), and intracellular Ca^{2+} signaling has been reported.[35] As TRPV4 regulates numerous cellular functions through intracellular Ca^{2+} signalling, we first tested BAPTA-AM, a cell-permeant Ca^{2+} chelator, on GSK101- and MSU crystal-induced IL-1β production from the LPS-primed *wt* BMDMs. Surprisingly, BAPTA-AM effectively blocked GSK101-induced IL-1β release while it only suppressed about half of MSU crystal-induced IL-1β release (figure 5A,B). In contrast, cytochalasin D, an actin polymerisation inhibitor that blocks >90% of phagocytosis, nearly abolished MSU crystal-induced IL-1β production without a significant effect on the GSK101-induced IL-1β production (figure 5A,B). These results suggest that the effect of GSK101-induced IL-1β production is largely dependent on TRPV4-mediated intracellular Ca^{2+} signalling while MSU crystals can induce IL-1β production through phagocytosis without completely relying on TRPV4-dependent intracellular Ca^{2+} signalling.

Previous studies also showed that crystals can use receptor-independent membrane-based immune sensing to initiate the activation of NLRP3 inflammasome, which is blunted by a non-selective cation channel blocker ruthenium red, also a TRPV4 channel blocker.[36 37] We thus speculated that MΦ-expressed TRPV4 might be involved in the cell surface contact with various crystals. Indeed, both pharmacological blockade and genetic ablation of TRPV4 function significantly reduced MΦ phagocytosis (figure 5C,D and online supplemental figure 8a,b). Phagocytosis of crystal structures is known to produce large amounts of ROS.[38] Interesting, application of MSU crystals but not GSK101 promoted ROS production in the LPS-primed BMDMs in a TRPV4-dependent manner (figure 5E). Consistent with these results, IL-1β production induced by MSU crystals but not GSK101 was markedly reduced by two ROS scavengers: ethyl ester of glutathione (GSH-EE) and N-acetylcysteine (figure 5A,B).

Increased phagocytosis triggered by crystal structures also leads to lysosomal damage resulting in the cellular release of lysosomal contents such as cathepsin B (CTSB) that can be sensed by NLRP3 inflammasome.[39 40] Both MSU crystals and GSK101 significantly increased CTSB release from LPS-primed BMDMs, which was suppressed by either genetic or pharmacological inhibition of TRPV4 function (figure 5F,G). Further, IL-1β production induced by both GSK101 and MSU crystals was blunted by CA-074Me, a selective inhibitor of CTSB (figure 5A,B). Collectively, these findings suggest that TRPV4 is critically involved in the phagocytosis of MSU crystals by MΦs, which leads to the production of ROS and lysosomal leakage and ultimately NLRP3 inflammasome activation. On the other hand, the GSK101-induced NLRP3 inflammasome activation is accomplished by lysosomal leakage following TRPV4-dependent intracellular Ca^{2+} signalling (figure 5H).

TRPV4 is involved in NLRP3 inflammasome activation produced by crystalline but not non-crystalline inflammasome activators

Besides MSU crystals, other medically relevant crystals, such as calcium pyrophosphate dihydrate (CPPD) which causes pseudogout, SiO_2, and alum adjuvant, as well as classic non-crystalline NLRP3 inflammasome activators such as ATP and pore-forming toxins nigericin and gramicidin also activate

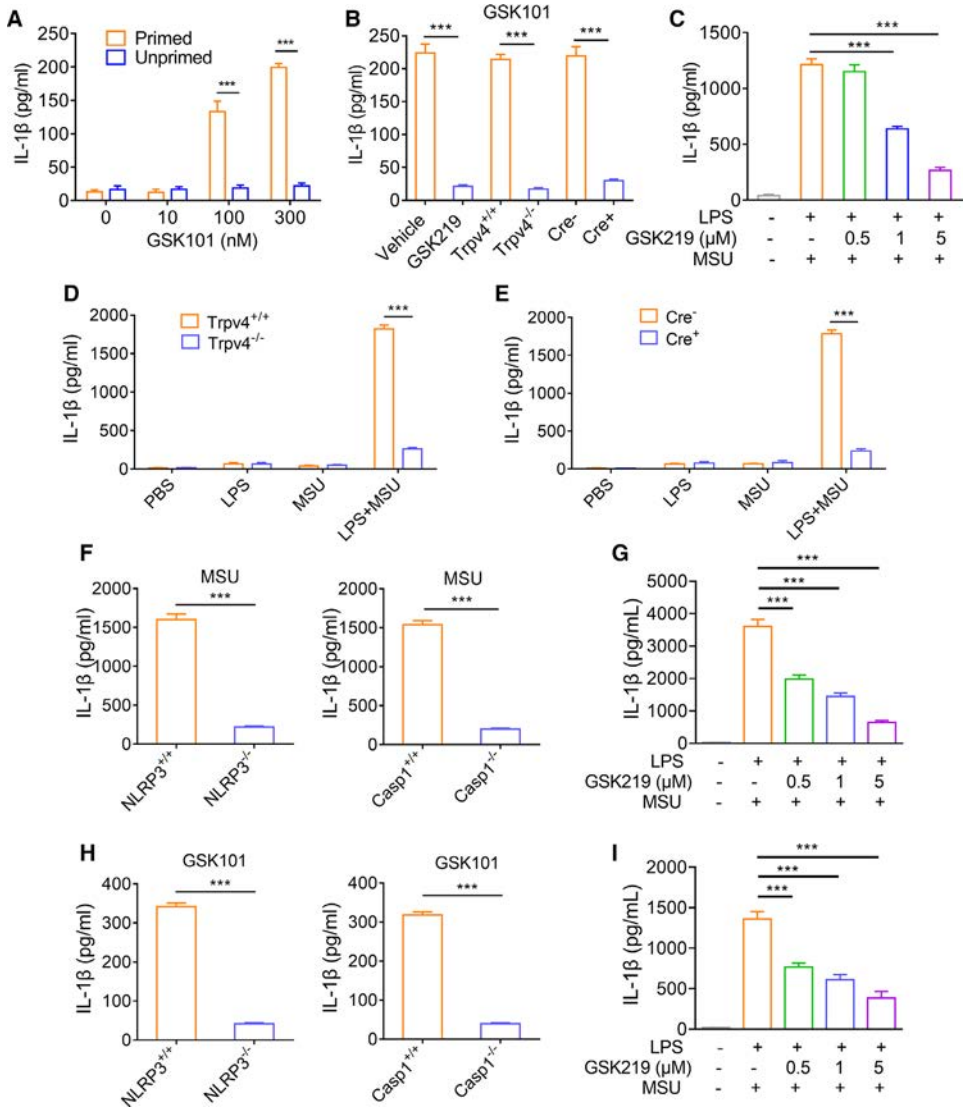

Figure 4 TRPV4 function is critical to MSU crystal-induced NLRP3 inflammasome activation and IL-1β production. (A) GSK101 promoted IL-1β production in LPS-primed but not in LPS-unprimed BMDMs in a concentration-dependent manner. (B) MSU crystal-induced IL-1β production from LPS-primed BMDMs was severely suppressed by TRPV4 antagonism and genetic ablation of TRPV4 function in both global *Trpv4 KO* mice and MΦ-specific *Trpv4 cKOs* compared with their respective controls. (C) MSU crystal-induced IL-1β production from LPS-primed BMDMs was inhibited by GSK219 in a concentration-dependent manner. (D) MSU crystal-induced IL-1β production was markedly reduced in LPS-primed BMDMs isolated from the $Trpv4^{-/-}$ mice when compared with the $Trpv4^{+/+}$ mice. (E) MSU crystal-induced IL-1β production was markedly reduced in LPS-primed BMDMs isolated from the Cre^{+} $Cx3cr1^{CreERT}$; $Trpv4^{f/f}$ mice when compared with their Cre^{-} littermates. Note PBS, LPS, and MSU crystals alone did not increase IL-1β production in (D, E). (F) MSU crystal-induced IL-1β production in LPS-primed BMDMs from NLRP3-deficient mice and Casp-1-deficient mice and their respective control mice. (G) ELISA analysis of IL-1β in supernatants from PMA-differentiated THP-1 cells treated with various concentrations of GSK219 and then stimulated with MSU crystals (200 μg/mL). (H) GSK101-induced IL-1β production in LPS-primed BMDMs from NLRP3-deficient mice and Casp-1-deficient mice and their respective control mice. (I) ELISA analysis of IL-1β in supernatants from human PBMCs pretreated with various concentrations of GSK219 and then stimulated with MSU crystals (200 μg/mL). Statistical significance was determined using two-way ANOVA, followed by Bonferroni's post hoc test (A), Tukey post hoc tests (multiple comparisons, (C, H, I), and Student's t-test (B, D–G). ***P<0.001. n=6 per group. ANOVA, analysis of variance; IL-1β, interleukin 1β; LPS, lipopolysaccharide; MSU, monosodium urate; PBS, phosphate buffered saline; PMA, phorbol-12-myristate-13-acetate; THP, human myeloid leukemia mononuclear cells.

NLRP3 inflammasome and increase IL-1β production. We, thus, tested if TRPV4 is involved in the signalling pathways shared by these activators. Surprisingly, GSK219 pretreatment or genetic ablation of TRPV4 function from LPS-primed MΦs markedly reduced IL-1β production induced by CPPD, alum, or silica crystals (figure 6a), whereas IL-1β production induced by ATP, nigericin or gramicidin was unaffected (online supplemental figure 9a–c), suggesting that TRPV4 function is essential for the activation of NLRP3 inflammasome by commonly used crystalline NLRP3 activators but not the noncrystalline activators ATP and pore-forming toxins in vitro.

To further investigate the role of TRPV4 in crystal-induced inflammation in vivo, we transorally instilled silica crystals into the $Trpv4^{-/-}$ mice or Cre^{+} $Cx3cr1^{CreERT}$; $Trpv4^{f/f}$ *Trpv4 cKOs* and their respective controls. Flow cytometry detected a robust neutrophil infiltration in bronchoalveolar lavage fluids from both the $Trpv4^{+/+}$ mice and Cre^{-} $Cx3cr1^{CreERT}$; $Trpv4^{f/f}$ *Trpv4 cKOs*, which was significantly reduced in the $Trpv4^{-/-}$ mice and

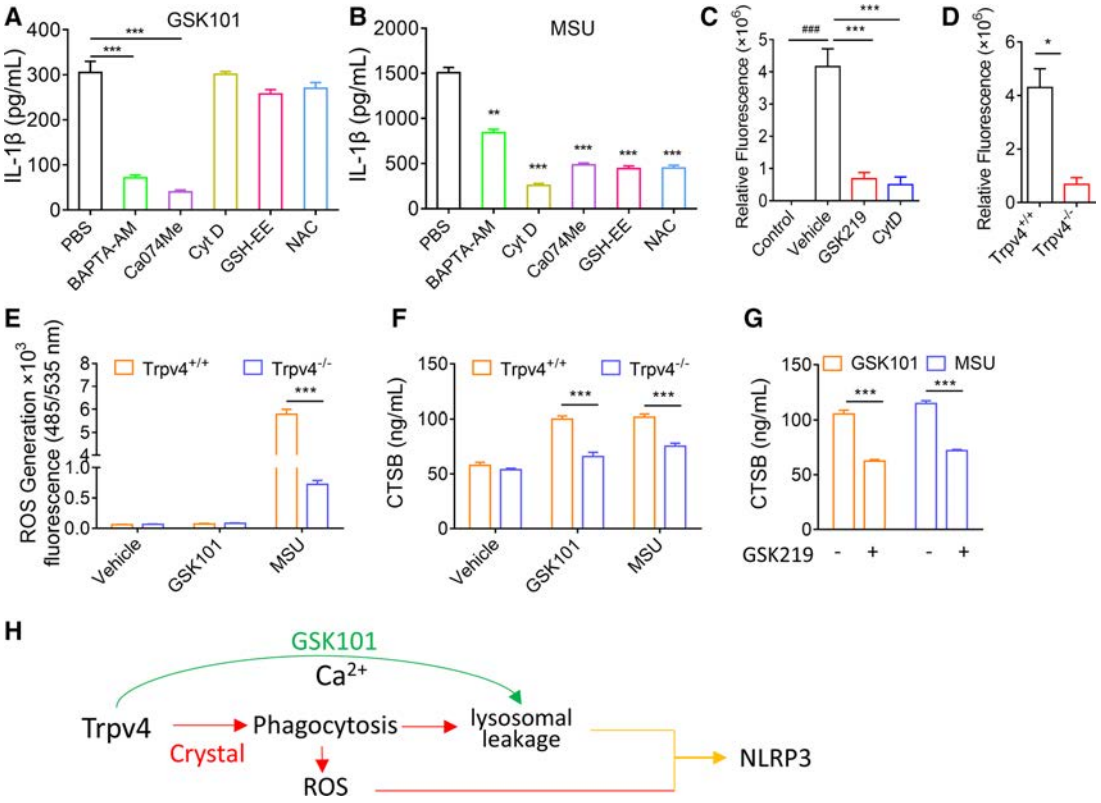

Figure 5 GSK101 and MSU crystals use distinct mechanisms to drive TRPV4-dependent NLRP3 inflammasome activation. (A, B) GSK101- or MSU crystal-induced IL-1β production from LPS-primed BMDMs in the presence of various chemical inhibitors: Bapta-AM (a cell permeant Ca^{2+} chelator), cytochalasin D (Cyt D, a potent phagocytosis inhibitor), CA-074Me (a selective inhibitor of CTSB), or ROS scavengers GSH-EE (ethyl ester of glutathione) and NAC (N-acetylcysteine). PBS is the vehicle control for all chemicals. (C) Effect of GSK219, Cyt D on phagocytosis of pHrodo Red *Escherichia coli* BioParticles in LPS-primed BMDMs using a fluorescent microplate reader. (D) Phagocytic activity of BMDMs from the *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice was measured using a fluorescence plate reader. (E) GSK101-induced and MSU crystal-induced ROS production in LPS-primed BMDMs from the *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice. (F) GSK101- and MSU crystal-induced increase in CTSB levels in LPS-primed BMDMs isolated from the *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice. (G) Effect of GSK219 on CTSB production induced by GSK101 or MSU crystals in LPS-primed *wt* BMDMs. (H) Schematic diagram of the working hypothesis. Statistical significance was determined using Student's t-test (A–G). When compare to Control group, $^{\#\#}$P<0.001. When compare to other groups, *P<0.05, **p<0.01, ***p<0.001. n=4–5 for each group. IL-1β, interleukin; MSU, monosodium urate; ROS, reactive oxygen species; wt, wild-type.

Cre^{+} *Cx3cr1*^{CreERT}*; Trpv4*$^{f/f}$ *Trpv4 cKOs*, respectively (figure 6B). Similar effect was also observed for MSU, CPPD and aluminium hydroxide crystals in a mouse model of peritonitis in which the recruitment of neutrophils induced by i.p. injections of diverse crystals was markedly reduced by genetic ablation of TRPV4 function (figure 6C–E), confirming that TRPV4 is required for crystal-induced inflammation in vivo.

Neuroimmune interaction between the TRPV1-expressing sensory nociceptors and TRPV4-expressing MΦs contributes to MSU crystal-induced gouty arthritis

Prior studies have demonstrated that cytokines released from activated immune cells contribute to MSU crystal-induced pain and inflammation through sensitising TRPV1-expressing nociceptive sensory neurons, suggesting that the TRPV1-expressing primary nociceptors are the downstream mediator of MSU crystal-induced sensory hypersensitivity.[41 42] Moreover, besides pain-related responses, MSU crystal-induced inflammation was also inhibited in mice deficient in nociceptor-expressed TRPA1 or TRPV1 channels,[41–47] which prompted us to hypothesise that activation of the TRPV1-expressing primary nociceptors during MSU crystal-induced acute gout flares can further enhance joint inflammation by promoting the function of the TRPV4-expressing MΦs, forming a positive feedback loop. To test this hypothesis, we selectively ablated the TRPV1-expressing primary nociceptors with a super potent TRPV1 agonist resiniferatoxin (RTX).[48] As expected, RTX treatment effectively reduced the reflexive mechanical (online supplemental figure 10a) and thermal (online supplemental figure 10b) pain-related responses. Strikingly, joint swelling (online supplemental figure 10c), IL-1β expression (online supplemental figure 10d), the number of neutrophils and MΦs (online supplemental figure 10e,f), the MPO activity (online supplemental figure 10g), and the TRPV4 mRNA expression in synovial resident MΦs (online supplemental figure 10h) in the MSU crystal-induced gout model were all markedly attenuated, supporting our hypothesis that the MSU crystal-induced MΦ-dependent inflammation requires the presence of the TRPV1-expressing primary nociceptors and a neuroimmune interaction between the TRPV1-expressing primary nociceptors and the TRPV4-expressing synovial MΦs is essential for the generation of MSU crystal-induced acute gout flares.

DISCUSSION

In this study, we showed that MΦ-expressed TRPV4 is selectively involved in the inflammatory responses induced by diverse crystals. The conclusion was supported by multiple experimental measures. First, we showed that TRPV4 is functionally expressed by MΦs and TRPV4 expression is upregulated in both MSU crystal-stimulated mouse synovial MΦs and PBMCs from human patients with acute

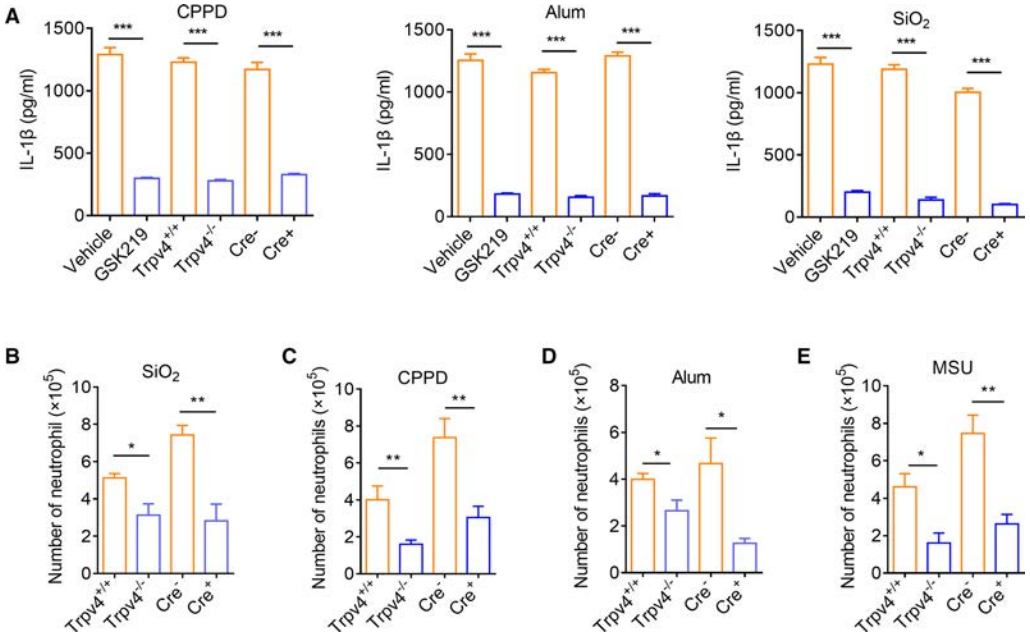

Figure 6 TRPV4 mediates both in vitro and in vivo inflammatory responses produced by crystalline but not non-crystalline NLRP3 inflammasome activators. (A) Pharmacological inhibition (GSK219, 1 μM) and genetic ablation of TRPV4 function using both global and MΦ-specific *cKOs* severely reduced IL-1β release from LPS-primed BMDMs stimulated with CPPD (100 ng/mL), alum (200 ng/mL) or SiO_2 (100 ng/mL). (B) Orotracheal instilled silica crystals increased neutrophil infiltration in the bronchoalveolar lavage fluid in the *Trpv4*$^{+/+}$ and *Cre*$^{-}$ *Cx3cr1*^{CreERT}*; Trpv4*$^{f/f}$ mice, which was significantly reduced in the *Trpv4*$^{-/-}$ and *Cre*$^{+}$ *Cx3cr1*^{CreERT}*; Trpv4*$^{f/f}$ mice, respectively. (C–E) Intraperitoneal injections of MSU crystals (C), CPPD (D), or alum (E) crystals markedly promoted neutrophil infiltration in the *Trpv4*$^{+/+}$ and *Cre*$^{-}$ *Cx3cr1*^{CreERT}*; Trpv4*$^{f/f}$ mice, which was significantly reduced in the *Trpv4*$^{-/-}$ and *Cre*$^{+}$ *Cx3cr1*^{CreERT}*; Trpv4*$^{f/f}$ mice, respectively. Statistical significance was determined using Student's t-test (A–E). *P<0.05, **p<0.01, ***p<0.001. n=5–6 for each group. IL-1β, interleukin 1 β; MSU, monosodium urate.

gout flares. Second, we provided evidence that TRPV4 function is required for the activation of the NLRP3 inflammasome and subsequent IL-1β production induced by diverse crystals including MSU, CPPD and SiO_2 but not non-crystalline NLRP3 inflammasome activators ATP, nigericin or gramicidin. Third, the reflexive pain-related responses and inflammation induced by IA injections of MSU crystals were markedly attenuated by both pharmacological inhibition and genetic ablation of TRPV4 function. Last, the TRPV1-expressing nociceptors are required for MSU crystal-induced gouty arthritis through increasing TRPV4 expression, the number of synovial resident MΦs and the release of MΦ-derived cytokines. which supports the hypothesis that a neuroimmune axis of the TRPV4-expressing resident MΦs-TRPV1-expressing primary nociceptors plays a critical role in the generation of crystal-induced gouty arthritis in mice. Of note, our findings might explain why the crystals are in patients for years without causing overt gout flares. It is well known that acute gout flares are tightly associated with the levels of uric acid in patients. For example, the accumulation of high level of uric acid caused by a diet rich in red meat, seafood and beverages sweetened with fructose[49] might activate TRPV4-expressing synovial resident MΦs, resulting in release of inflammatory cytokines which subsequently provoke action potential firing in nociceptors, thereby transmitting pain to the brain and driving the neurogenic inflammation to release neurotransmitters such as substance P and calcitonin gene related peptide (CGRP) in the affected joints and promote joint inflammation through regulating macrophage functions,[50 51] forming a positive feedback loop and driving the production of acute gout flares.

Our findings also challenge the long-held assumption that TRPV4 is expressed by primary nociceptors mediating neurogenic inflammation and nociception[52–56] because we did not find any evidence supporting a functional expression of TRPV4 in the primary nociceptors. First, we did not detect TRPV4 expression in DRG neurons using RNAscope in situ hybridisation which otherwise revealed a robust expression of TRPV1 in many DRG neurons. Second, GSK101 did not elicit TRPV4-dependent Ca^{2+} response in DRG neurons where capsaicin induced a robust Ca^{2+} response. Third, the MSU crystal-induced gouty arthritis was not significantly affected in sensory neuron-specific TRPV4 *cKOs* using the *Trpv1*^{Cre} line which covers ~80% of the nociceptors.[57] These results demonstrate that TRPV4 unlikely engages in crystal-induced joint pain and inflammation through a direct neurogenic action, like that mediated by nociceptor-expressed TRPV1 and TRPA1 channels.[43 58] It should be noted that nerve injury and inflammation can induce macrophage expansion in the DRG,[59 60] leading to heightened pain responses. TRPV4 expression in these MΦs but not sensory neurons might contribute to the generation of pain-related responses. Future studies are required to test this possibility.

TRPV4 is functionally expressed in articular chondrocytes in multiple species,[61–63] and promotes distinct mechanoelectrical transduction pathways to regulate the metabolic response of chondrocytes to dynamic loading.[64 65] Interestingly, although chondrocyte-expressed TRPV4 was reported to contribute to age-related OA,[32] knockout of TRPV4 channels was shown to promote the development of OA in male mice.[66] Moreover, IA injections of a TRPV4 agonist stimulated chondrocyte anabolic changes and decreased the length of the damaged area in a surgically induced rat model of OA, suggesting that TRPV4 activation protects rats from the development of OA.[67] Surprisingly, our results using cartilage-specific *Trpv4 cKOs* showed that the chondrocyte-expressed TRPV4 does not contribute to MSU crystal-induced gouty arthritis. Therefore, chondrocyte-expressed TRPV4 plays distinct roles in the settings of different types of joint inflammation.

The classic NLRP3 inflammasome activators including ATP, nigericin, gramicidin and diverse crystals (MSU, CPPD, alum and SiO_2) use distinct mechanisms to activate the NLRP3 inflammasome.[68] For instance, ATP acts on the P2×7 receptors while nigericin and gramicidin are pore forming toxins, although membrane permeability is engaged in both pathways.[69–71] On the other hand, crystal signalling involves phagolysosome and lysosome rupture.[7] Our data showed that TRPV4 is involved in the NLRP3 inflammasome activation induced by diverse crystals but not ATP and pore-forming toxins. Therefore, TRPV4 likely engages in the crystal-induced signalling in MΦs which is separate from these classics signalling pathways. Although the precise mechanism how TRPV4 channel is activated in the gout model remains unclear, our findings suggest that mechanosensitive TRPV4 channel might be critically involved in the cell surface contact by various crystals and be directly activated by swelling-related cell volume change (online supplemental figure 11) associated with the phagocytosis of MSU crystals, which subsequently causes lysosomal leakage and drives ROS production, leading to NLRP3 inflammasome activation in the joint MΦs.[39 72 73] On the other hand, TRPV4-mediated NRLP3 inflammasome activation induced by GSK101 relies primarily on intracellular Ca^{2+} signaling and subsequent lysosomal leakage although the relationship between the TRPV4-mediated intracellular Ca^{2+} signaling and lysosomal leakage requires further investigation. Considering TRPV4 is activated by many forms of physical and chemical stimuli,[74 75] it might play a critical role in inflammation through converging many signalling pathways driving NLRP3 inflammasome activation.

In summary, we demonstrated that the TRPV4-expressing resident MΦs are the key mediator of MSU crystal-induced gouty arthritis in mice. Mechanistically, the mechanosensitive TRPV4 channel can be selectively activated by crystal-induced cell volume change, which leads to NLRP3 inflammasome activation and subsequent production and release of inflammatory cytokines that drive the joint inflammation and pain-related responses. We also showed that this process requires the presence of the TRPV1-expressing nociceptors and demonstrated that a neuroimmune interaction between the TRPV1-expressing primary nociceptors and the TRPV4-expressing synovial MΦs is a critical cellular mechanism underlying acute gout flares. Identification of the function of TRPV4 in crystal-induced inflammation should facilitate the development of new therapeutic interventions to treat inflammatory conditions associated with crystal disposition.

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TRANSLATIONAL SCIENCE

14-3-3 epsilon is an intracellular component of TNFR2 receptor complex and its activation protects against osteoarthritis

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ABSTRACT

Objectives Osteoarthritis (OA) is the most common joint disease; however, the indeterminate nature of mechanisms by which OA develops has restrained advancement of therapeutic targets. TNF signalling has been implicated in the pathogenesis of OA. TNFR1 primarily mediates inflammation, whereas emerging evidences demonstrate that TNFR2 plays an anti-inflammatory and protective role in several diseases and conditions. This study aims to decipher TNFR2 signalling in chondrocytes and OA.

Methods Biochemical copurification and proteomics screen were performed to isolate the intracellular cofactors of TNFR2 complex. Bulk and single cell RNA-seq were employed to determine 14-3-3 epsilon (14-3-3ε) expression in human normal and OA cartilage. Transcription factor activity screen was used to isolate the transcription factors downstream of TNFR2/14-3-3ε. Various cell-based assays and genetically modified mice with naturally occurring and surgically induced OA were performed to examine the importance of this pathway in chondrocytes and OA.

Results Signalling molecule 14-3-3ε was identified as an intracellular component of TNFR2 complexes in chondrocytes in response to progranulin (PGRN), a growth factor known to protect against OA primarily through activating TNFR2. 14-3-3ε was downregulated in OA and its deficiency deteriorated OA. 14-3-3ε was required for PGRN regulation of chondrocyte metabolism. In addition, both global and chondrocyte-specific deletion of 14-3-3ε largely abolished PGRN's therapeutic effects against OA. Furthermore, PGRN/TNFR2/14-3-3ε signalled through activating extracellular signal-regulated kinase (ERK)-dependent Elk-1 while suppressing nuclear factor kappa B (NF-κB) in chondrocytes.

Conclusions This study identifies 14-3-3ε as an inducible component of TNFR2 receptor complex in response to PGRN in chondrocytes and presents a previously unrecognised TNFR2 pathway in the pathogenesis of OA.

Key messages

What is already known about this subject?

- TNFR2 was reported to inhibit inflammation and prevent bone loss in inflammatory arthritis. Whether and how TNFR2 signalling is involved in chondrocyte metabolism and OA remain largely unknown.

What does this study add?

- This study identifies the intracellular signalling molecule 14-3-3ε as a novel component of the TNFR2 receptor complex and uncovers a new strategy for activating this key pathway of anti-inflammation in OA and other related diseases. This study also identifies Elk1 as a previously unrecognised transcription factor which is required for TNFR2 anabolic signalling in chondrocytes.
- This study establishes a novel TNFR2 signalling paradigm to orchestrate chondrocyte anabolism and combat the inflammation/catabolism via PGRN/TNFR2/14-3-3ε/Elk-1 anabolic and PGRN/TNFR/14-3-3ε/NF-κB anticatabolic cascade, respectively, thereby protecting against OA.
- This study advances our understanding of TNFR2 signalling pathway in chondrocytes and OA. In addition, the results of this study will also have broader application to the understanding of cartilage haemostasis and musculoskeletal degenerative diseases in general.

How might this impact on clinical practice or future developments?

- The chondroprotective effects of PGRN on OA support the concept that targeted activation of TNFR2 signalling by PGRN, particularly its derivative Atsttrin, would be an effective therapeutic candidate for treating OA.

INTRODUCTION

Osteoarthritis (OA) is the most common cause of chronic disability and its prevalence is continuously increasing.[1] Despite the high prevalence and morbidity of OA, effective disease modifying treatments capable of intervening this degradative cascade are not currently available, and the molecular mechanisms involved in OA's initiation and progression remain poorly understood.[2] Although it is unclear whether the primary cause of OA is cartilage damage, OA chondrocytes undergo a series of complex changes in the disease progression,

impacting proliferation, catabolism and ultimately death.[3] Chondrocytes themselves are major protagonists in this regulatory cascade—not just the target of external biomechanical and biochemical stimuli but are themselves the source of proteases, cytokines and inflammatory mediators that promote the deterioration of articular cartilage.[4 5]

Accumulating evidences indicate that OA is a low-grade chronic inflammatory disease[6 7] and inflammation is thought to play a critical role in the pathogenesis of OA. TNFα signalling has received great attention due to its position at the apex of the proinflammatory cytokine cascade and its dominance in the pathogenesis of various diseases, including arthritis.[8] TNFα is one of the major proinflammatory cytokines detected in synovial fluid and is a widely studied regulator of catabolic processes in chondrocytes.[9] TNFα signals through two specific TNF receptors, TNFR1 and TNFR2. TNFR1 is ubiquitously expressed by nearly all cell types and appears to be the dominant receptor responsible for mediating TNFα's inflammatory activity and has been extensively studied.[9 10] Conversely, TNFR2 exhibits a restricted expression, and knowledge concerning TNFR2 signalling remains largely unclear. Our global genetic screen led to the identification of TNFR2 as the high-affinity binding receptor of progranulin (PGRN),[11] a multifaceted growth factor known to regulate chondrocyte homeostasis and its deficiency causes susceptibility to OA.[12–15] In contrast to TNFα, which demonstrates higher affinity for TNFR1 than TNFR2, PGRN exhibits 600-fold higher binding affinity to TNFR2 than TNFα.[11]

Emerging evidences indicate that distinct from TNFR1, TNFR2 signalling plays anti-inflammatory and protective roles in several diseases and conditions, including neurodegenerative and cardiac diseases.[16–18] TNFR2 was also reported to inhibit inflammation and prevent bone loss in inflammatory arthritis.[19–21] Although we previously reported that PGRN's protection against OA mainly depended on TNFR2,[12] whether and how TNFR2 signalling is involved in chondrocyte metabolism and OA remain largely unknown. In this study, we took advantage of the knowledge gained through previous studies from several laboratories including ours and performed several unbiased screens, including biochemical copurification and proteomics screens, bulk RNA-seq analysis, single cell transcriptomic analysis, transcription factor activity screen, combined with various genetically modified chondrocytes and mouse models, which led to the identification of the signalling molecule 14-3-3 epsilon (14-3-3ε) and transcription factor Elk-1 as essential components of TNFR2 signalling to mediate PGRN's chondroprotective and therapeutic activities against OA.

RESULTS

14-3-3ε is an intracellular component of TNFR2 receptor complex in response to PGRN in chondrocytes

Our previous findings that PGRN binds to TNFR2 with high affinity[11] and protects chondrocytes against OA[12] prompted us to identify additional components of the TNFR2 receptor complex in response to PGRN treatment. For this purpose, the intracellular domain (ICD) of TNFR2 was cloned into the PGEX-3X vector to express a fusion of GST to TNFR2ICD. As illustrated in figure 1A, GST (serving as a control) or GST-TNFR2ICD was affinity-purified on glutathione-agarose beads and used as a bait to trap proteins from PGRN-treated human chondrocytes. These samples were then analysed by mass spectrometry (MS) and MS/MS spectra were searched against the Uniprot database. After subtracting the hits that were also trapped by the GST column, eight proteins were found to specifically bind to TNFR2 (figure 1A). Identification of TRAF1 and TRAF2, two known TNFR2-binding proteins, among the eight hits validated the technique. The protein ranking first was 14-3-3ε, a critical intracellular signalling mediator that belongs to 14-3-3 family.[22–24]

To characterise the role of 14-3-3ε in chondrocytes, we generated inducible chondrocyte specific 14-3-3ε deficient mice (hereafter referred to as 14-3-3ε^{Agc1}) by crossing 14-3-3ε$^{f/f}$ mice[25] with Agc1-Cre^{ERT2} mice[26] in which Cre-mediated recombination is induced by tamoxifen (online supplemental figure 1). 14-3-3ε^{Agc1} mice were born in a Mendelian ratio and tamoxifen treatment of 14-3-3ε^{Agc1} mice resulted in no overt phenotype. Protein and gene analysis indicated the tamoxifen treatment could specifically delete 14-3-3ε in cartilage in 14-3-3ε^{Agc1} mice (figure 1B, online supplemental figure 1). Coimmunoprecipitation using both anti-14-3-3ε and anti-TNFR2 antibodies with the lysate of chondrocytes isolated from tamoxifen-treated 14-3-3ε$^{f/f}$ and 14-3-3ε^{Agc1} mice was performed to validate the interaction between TNFR2 and 14-3-3ε. The results revealed that TNFR2 was specifically detectable in the immunoprecipitated complex from 14-3-3ε$^{f/f}$ but not 14-3-3ε^{Agc1} chondrocytes in response to PGRN treatment, or PGRN plus TNFα treatment, but not to TNFα treatment alone (figure 1B). These results, together with our biochemical co-purification/MS results, indicate that 14-3-3ε was specifically recruited to TNFR2 following PGRN treatment in chondrocytes.

14-3-3ε is down-regulated in OA cartilage

In a separate effort to isolate OA-associated genes, we performed bulk RNA-seq analysis using cartilage isolated from normal patients without arthritis and patients with OA. Total RNA was isolated from three samples of non-arthritic cartilage and four samples of OA cartilage (Kellgren-Lawrence Grade 3 or 4). Genes (900 total, 600 upregulated, 300 downregulated) were differentially expressed in OA versus normal (fold change >2, false discovery rate (FDR)<0.00001, adjusted $p<0.05$) (figure 1C, online supplemental figure 2a). Gene set enrichment analysis (GSEA) indicated altered gene expression pattern in OA cartilage compared with non-arthritic cartilage. Specifically, pathways known to be implicated in OA pathogenesis, including inflammatory response, interferon alpha and gamma response, apoptosis and oxidative phosphorylation, were upregulated in OA cartilage compared with non-arthritic cartilage (online supplemental figure 2b–f). Analysis of the genes differentially regulated between OA and non-arthritic cartilage, with a special interest in PGRN and 14-3-3ε, revealed that GRN (gene encoding PGRN) expression is significantly upregulated in OA cartilage (figure 1D), which was in line with previous reports,[13] and intriguingly, YWHAE (gene encoding 14-3-3ε) exhibited a trend of reduced expression in OA cartilage (figure 1E).

To unravel the relative abundance and distribution of PGRN, TNFR2 and 14-3-3ε mRNA transcripts in different chondrocyte subpopulations, we performed single-cell RNA-seq (scRNA-seq) in chondrocytes isolated from human OA and normal cartilage. Similar to a recent study,[27] unbiased clustering based on known cell specific markers identified seven distinct cell clusters, including fibrocartilage chondrocytes, homeostatic chondrocytes, prehypertrophic chondrocytes (pre-HTCs), hypertrophic chondrocytes (HTCs), proliferative chondrocytes, effector chondrocytes and regulatory chondrocytes (figure 1F). As expected, almost all chondrocyte clusters expressed cartilage oligomeric matrix protein (COMP), a known cartilage marker (figure 1G).[28] Both *GRN* and *YWHAE* appeared to be abundant across all cell clusters, whereas *TNFRSF1B* (gene encoding

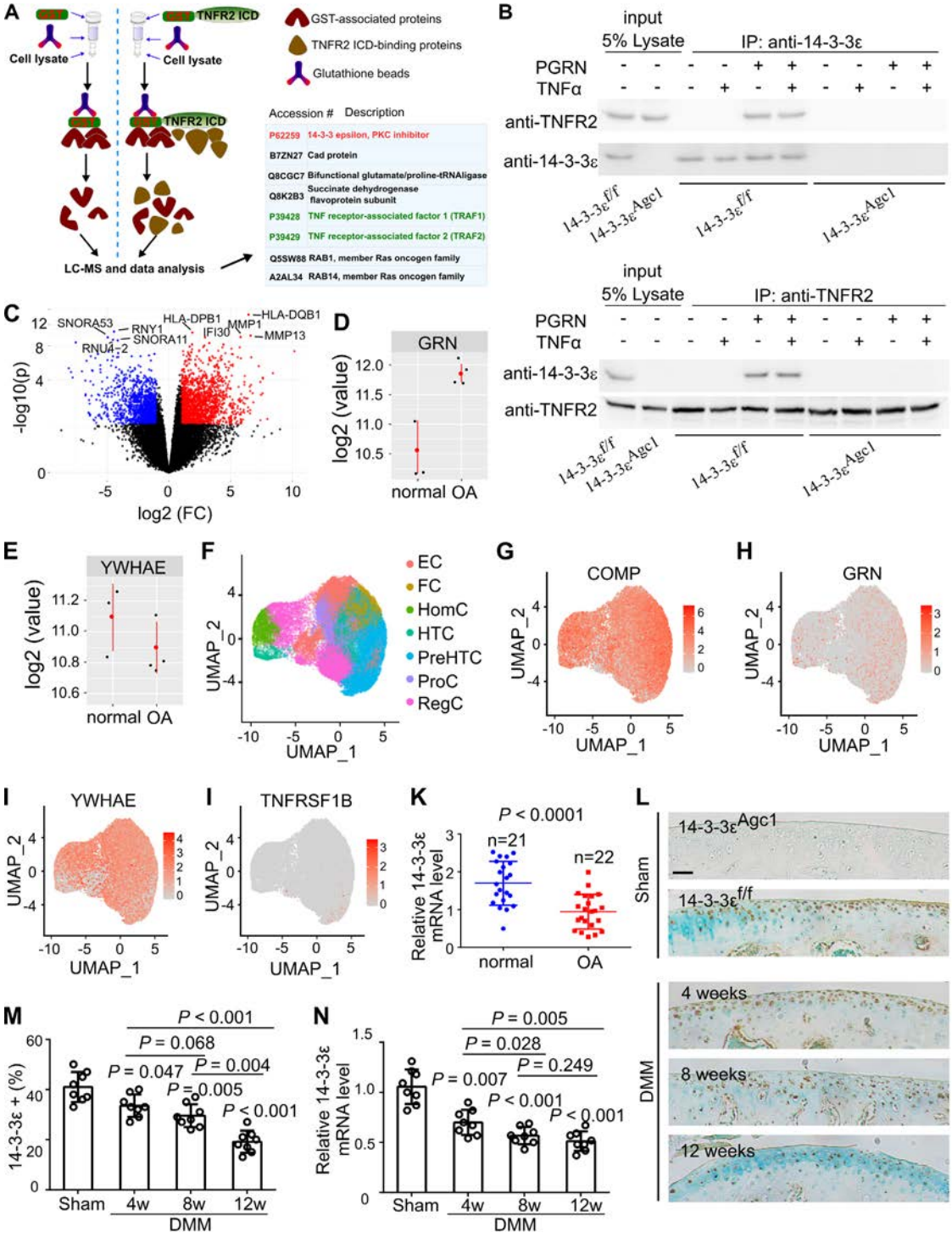

Figure 1 14-3-3ε is an intracellular component of TNFR2 complex in chondrocyte and downregulated in OA cartilage. (A) Schematic of the experimental design to identify potential molecules recruited to TNFR2 ICD in response to PGRN stimulation. Summary of the hits that were specifically recruited to activated TNFR2 complexes in human C28I2 chondrocytes. (B) Chondrocytes isolated from wildtype (WT) or 14-3-3ε^{Agc1} mice were treated with 10 ng/mL TNFα or/and 200 ng/mL PGRN for 30 min and then immunoprecipitated with 14-3-3ε or TNFR2 antibodies, and detection of TNFR2 and 14-3-3ε by immunoblotting. Results shown are representative of three biological replicates. (C) Volcano plots for gene expression of human OA (n=4) versus normal (n=3) cartilage. Genes in red (upregulated in OA) and blue (downregulated in OA) have Benjamini-Hochberg adjusted $p < 0.05$. (D, E) Relative mRNA expressions of GRN (gene encoding PGRN) (D) and YWHAE (gene encoding 14-3-3ε) (E) in human OA versus normal cartilage by RNA-seq. (F) Unbiased clustering of scRNA-seq data from human non-arthritic (n=3) and OA (n=4) revealed seven distinct cell clusters. (G–J) Expression of COMP (G), GRN (H), YWHAE (I) and TNFRSF1B (gene encoding TNFR2) (J) across the cell clusters. Each dot represents a single cell and colours correspond to the expression level of a gene in each cell. (K) qRT-PCR analysis of 14-3-3ε in human OA (n=22) and normal (n=21) cartilage. (L,M) Immunohistochemical staining of 14-3-3ε and quantification of 14-3-3ε positive cells in joint section collected from WT mice subjected to sham or DMM surgery (n=8 mice per group). Scale bar, 50 µm. (N) Relative 14-3-3ε mRNA level in cartilage isolated from sham or DMM operated mice (n=8 mice per group). Data are mean±SD. ICD, intracellular domain; DMM, destabilisation of the medial meniscus; OA, osteoarthritis; PGRN, progranulin.

TNFR2) exhibited a much more restricted expression pattern, mainly in pre-HTCs (figure 1H–J, online supplemental figure 3). Thus, TNFR2 appeared to be the rate-limiting component in PGRN/TNFR2/14-3-3ε complex implicated in regulation of chondrocyte metabolism. Furthermore, independent validation by quantitative PCR revealed significantly decreased 14-3-3ε expression in human OA cartilage compared with non-arthritic cartilage (figure 1K). In line with the decrease at mRNA level, 14-3-3ε protein level was also reduced in human arthritic cartilage as compared with non-arthritic controls (online supplemental figure 4).

We next examined the expression of 14-3-3ε in the course of OA using the surgically induced destabilisation of the medial meniscus (DMM) OA model in mice and found that similar to observations with human OA cartilage, the levels of 14-3-3ε protein and mRNA were also reduced in the course of OA (figure 1L–N).

Aged 14-3-3ε deficient mice exhibit severer OA-like phenotype

Considering that 14-3-3ε was isolated as inducible component of TNFR receptor complex in response to PGRN treatment and its levels were downregulated in OA, we therefore explored the potential contribution of 14-3-3ε to the development of naturally occurring OA in ageing mice. For this purpose, we generated inducible global 14-3-3ε knockout mice (hereafter referred to as 14-3-3ε-/-) by breeding 14-3-3ε$^{f/f}$ mice with Rosa26-Cre^{ERT2} mice in which Cre-mediated recombination is induced by tamoxifen (online supplemental figure 5a–c). PCR was implemented to confirm 14-3-3ε deletion efficiency in various tissues following tamoxifen administration in adult mice (online supplemental figure 5d). Thereafter, spontaneous changes in the histological features of the articular cartilage were analysed in 14-3-3ε-/- and 14-3-3ε$^{f/f}$ littermates at ages 3 and 18 months.

As expected, there was no 14-3-3ε expression in the cartilage of 14-3-3ε-/- mice at either age (figure 2A, online supplemental figure 6a). Histological evaluations of H&E, Safranin O and Movat pentachrome staining revealed that cartilage of 3-month-old 14-3-3ε-/- mice is indistinguishable from that of 14-3-3ε$^{f/f}$ littermates (online supplemental figure 6a); 14-3-3ε$^{f/f}$ and 14-3-3ε-/- cartilage show comparable cartilage features, including proteoglycan content, cartilage thickness and subchondral bone plate thickness at 3 months of age (online supplemental figure 6b–e). In general, histological staining of 18-month-old 14-3-3ε$^{f/f}$ mice displayed characteristic OA changes in joints, including proteoglycan loss, thinning of articular cartilage, thickening of the subchondral bone and osteophyte formation (figure 2A–D).[29 30] Moreover, at 18 months of age, 14-3-3ε-/- cartilage exhibited a more severe OA phenotype, illustrated by a significantly greater degree of proteoglycan loss and reduction of articular cartilage thickness relative to 14-3-3ε$^{f/f}$ littermates (figure 2A–D). Consistent with histological analysis, micro-CT analysis of undecalcified joint samples indicated that ageing 14-3-3ε-/- mice have more osteophyte formation and severer subchondral bone sclerosis than 14-3-3ε$^{f/f}$ littermates (figure 2E–G). Compared with 14-3-3ε$^{f/f}$ cartilage, 14-3-3ε-/- cartilage appeared to have significantly increased levels of aggrecan neoepitope, COMP fragments, ColX and MMP13, indicators of cartilage degradation and degeneration (figure 2H). Consistent with the observations of immunohistochemistry staining, the transcript levels of catabolic markers, matrix metalloproteinase (*Mmp13*) and a disintegrin and metalloproteinases with thrombospondin type 5 motif (*Adamts5*), and inflammatory response markers, cyclooxygenase-2 (*Cox-2*) and inducible nitric oxide synthase (*Nos2*)[31] were also significantly elevated in 14-3-3ε-/- cartilage relative to 14-3-3ε$^{f/f}$ cartilage (figure 2I–L). Collectively, 14-3-3ε deficiency mice exhibited exaggerated age-associated, naturally occurring OA phenotype, thereby suggesting that genetic deletion of 14-3-3ε might contribute to age-related OA-like phenotype. It is also noted that the expressions of 14-3-3ε were markedly lower in cartilage from 18-month-old 14-3-3ε$^{f/f}$ mice than those in 3-month-old 14-3-3ε$^{f/f}$ mice (figure 2A, online supplemental figure 6a), suggesting that 14-3-3ε may also be associated with an ageing phenomenon in addition to OA.

14-3-3ε is required for PGRN regulation of chondrocyte metabolism

Following isolation of 14-3-3ε as an effector recruited to the TNFR2 complex by PGRN, we sought to determine whether 14-3-3ε is involved in the regulation of chondrocyte metabolism and whether it is also important for PGRN/TNFR2 mediated regulation of chondrocyte metabolism. First, we generated 14-3-3ε knockout C28I2 human chondrocytes by employing CRISPR-Cas9 technique (figure 3A,B). Deletion of 14-3-3ε markedly inhibited the expressions of anabolic markers type II collagen (*Col2a1*), aggrecan (*Acan*) and *COMP* (figure 3C) and significantly enhanced TNFα-induced expressions of Adamts5 and Mmp13 (figure 3D). More importantly, PGRN/TNFR mediated stimulation of chondrocyte anabolism and inhibition of TNFα-induced catabolic/inflammatory response, including *Cox2 and Nos2*, were abolished in 14-3-3ε knockout human chondrocytes (figure 3C,D). Similar results were also observed in 14-3-3ε-/- mouse primary chondrocytes as compared with chondrocytes isolated from 14-3-3ε$^{f/f}$ littermates (online supplemental figure 7a–c). Furthermore, PGRN's regulatory effects on chondrocyte metabolism were also blunted in 14-3-3ε-/- chondrocytes as compared with 14-3-3ε$^{f/f}$ chondrocytes (online supplemental figure 7a–c).

We previously developed a PGRN-derived engineered protein called Atsttrin, composed of three TNFR2-binding fragments of PGRN, which exhibited therapeutic effects in both inflammatory arthritis and OA.[11 32] Similar to PGRN, Atsttrin enhanced anabolism and inhibited TNFα induced inflammatory catabolism in control C28I2 cells while these effects were compromised in 14-3-3ε knockout C28I2 cells (online supplemental figure 8a,b).

To further characterise the necessity of 14-3-3ε in PGRN/TNFR2 regulation of chondrocyte metabolism, Flag-tagged 14-3-3ε was re-expressed in 14-3-3ε knockout C28I2 human chondrocytes to determine whether re-expression of 14-3-3ε could functionally rescue the 14-3-3ε deficiency phenotype (figure 3E). Re-expression of 14-3-3ε in 14-3-3ε knockout C28I2 cells reversed the phenotype induced by 14-3-3ε deficiency; more importantly, it could also restore PGRN mediated regulation of chondrocytes in terms of enhanced anabolism and suppressed inflammatory cytokine-induced catabolism and inflammation (figure 3F,G). Collectively, these results indicated that 14-3-3ε exerts chondroprotective effects as an essential mediator of PGRN/TNFR2 signalling in regulating chondrocyte metabolism.

14-3-3ε is required for PGRN's therapeutic effects against OA in vivo

Following isolation of 14-3-3ε as an essential molecule mediating PGRN's effects on chondrocyte metabolism, we assessed whether 14-3-3ε was also critical for PGRN's protective and therapeutic effects in OA. To this end, we established the surgically

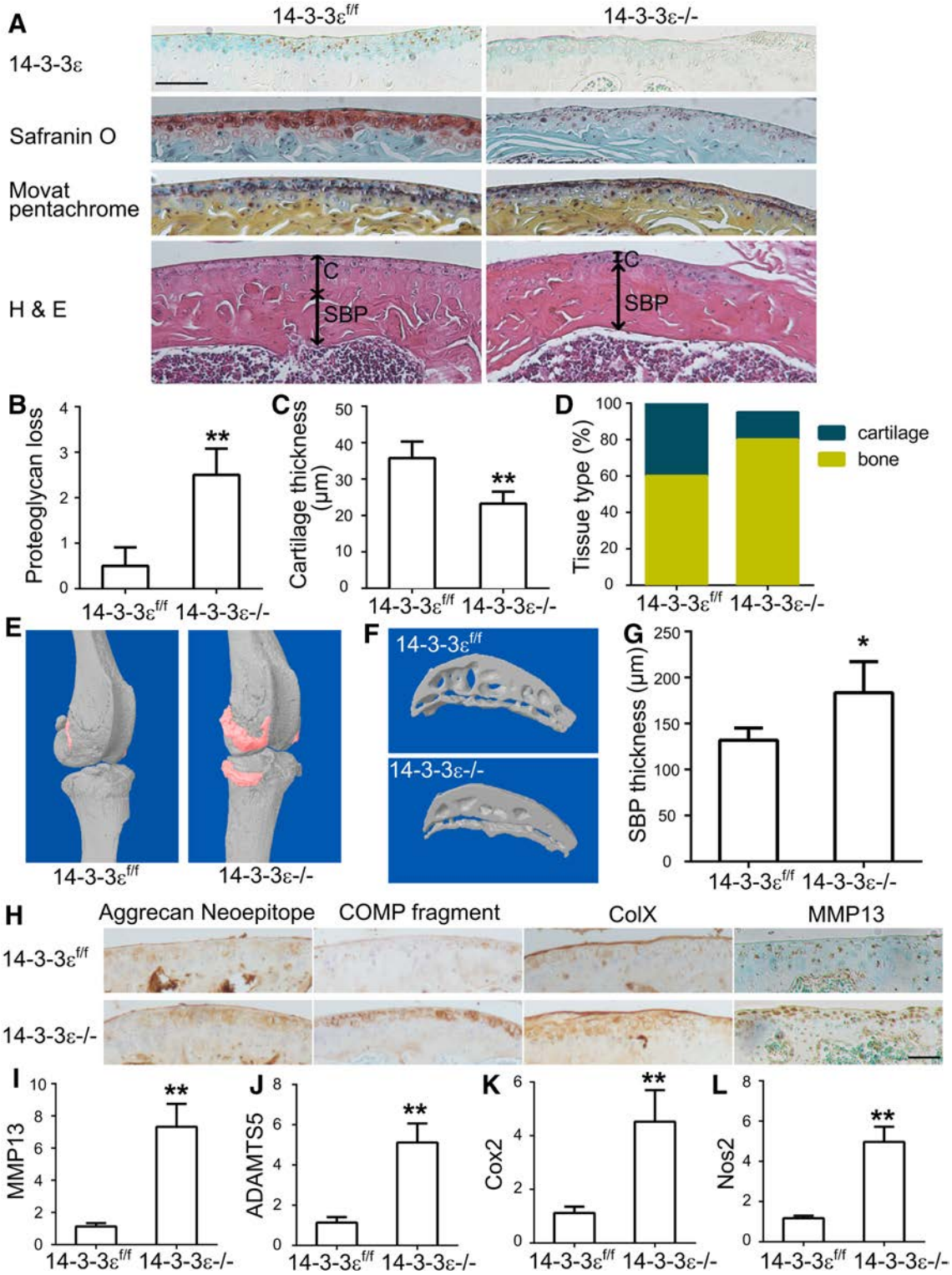

Figure 2 14-3-3ε deletion exaggerates naturally occurring phenotype with age. (A) Immunohistochemical staining for 14-3-3ε, Safranin O, Movat pentachrome and H&E staining in knee joint section collected from 14-3-3ε$^{f/f}$ (n=3) and 14-3-3ε-/- (n=4) mice at age 18 months. Scale bar, 100 µm. Representative image is shown. (B,C) Scoring of proteoglycan loss and cartilage thickness in 14-3-3ε$^{f/f}$ and 14-3-3ε-/- mice at age 18 months, respectively. (D) Quantitation of the composition of the articular cartilage in 14-3-3ε$^{f/f}$ and 14-3-3ε-/- mice at 18 months based on Movat pentachrome staining (yellow: bone; blue: cartilage). (E) Micro-CT scan and three-dimensional reconstruction of the knee joint from 18-month-old 14-3-3ε$^{f/f}$ and 14-3-3ε-/- mice, and the region marked in red is osteophyte. (F,G) Three-dimensional micro-CT images and quantification of thickness for the medial compartment of the tibial subchondral bone of 18-month-old 14-3-3ε$^{f/f}$ and 14-3-3ε-/- mice. (H) Representative image of immunohistochemical staining for Aggrecan neoepitope, COMP fragment, ColX and MMP13 in WT and 14-3-3ε-/- knee section at age 18 months. Scale bar, 50 µm. (I–L) Mmp13, Adamts5, Cox2 and Nos2 mRNA levels in cartilage from WT (n=3) and 14-3-3ε-/- (n=4) at age 18 months. Data are mean±SD; *p<0.05 or **p<0.01.

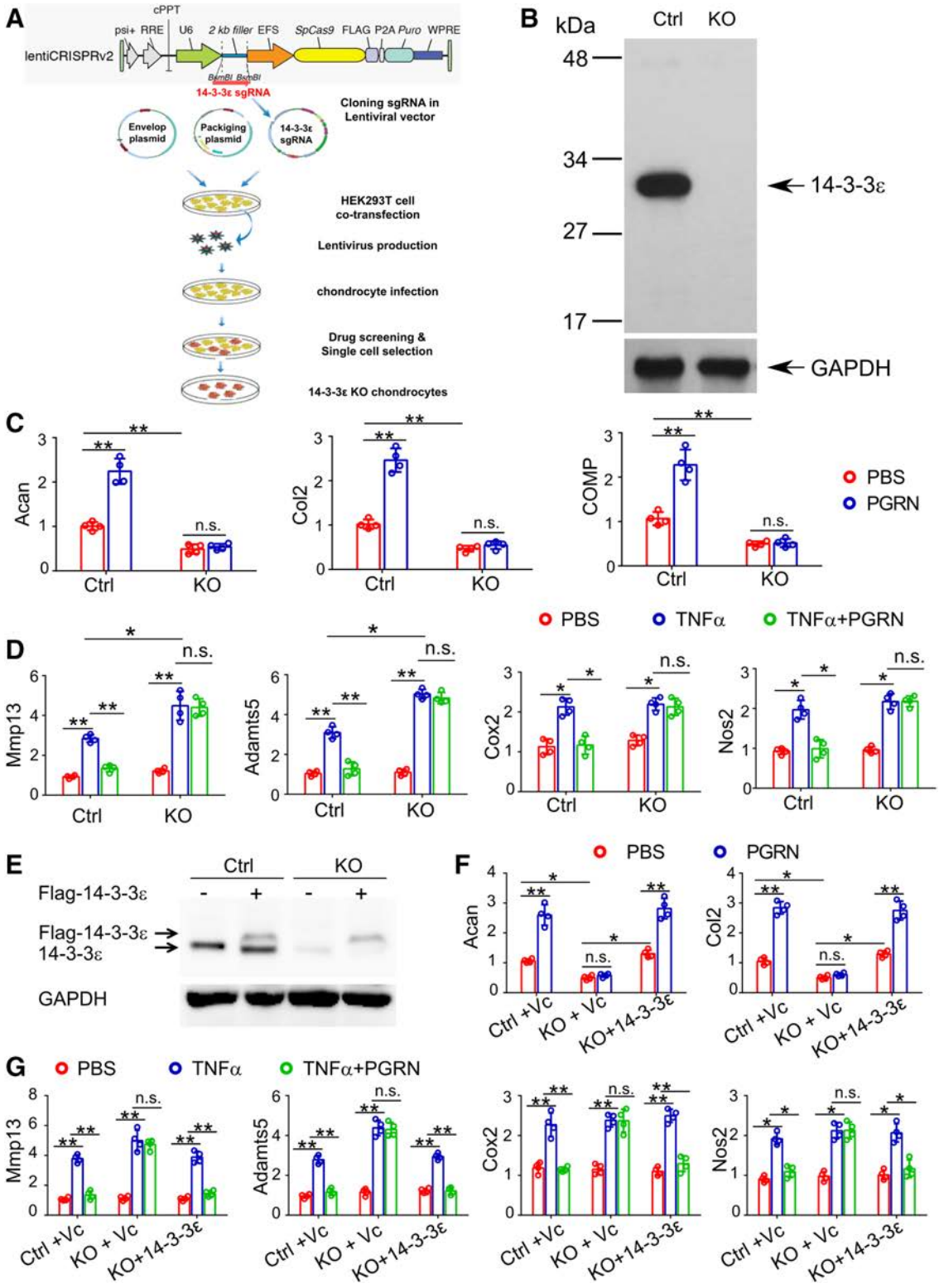

Figure 3 14-3-3ε is required for PGRN regulation of chondrocyte metabolism. (A) Schematic for generating 14-3-3ε-/- human C28I2 chondrocytes using CRISPR/Cas9 technology. (B) Western blotting to confirm the loss of 14-3-3ε in 14-3-3ε knockout C28I2 cells. Cell lysates were examined by immunoblotting with 14-3-3ε antibody. (C) mRNA levels of *Col2*, Acan and COMP in control and 14-3-3ε knockout C28I2 cells treated with or without 200 ng/mL PGRN for 24 hours, assayed by qRT-PCR analysis. (D) mRNA levels of Mmp13, Adamts5, Cox2 and Nos2 in control and 14-3-3ε knockout C28I2 cells treated with 10 ng/mL TNFα in the absence or presence of 200 ng/mL PGRN for 24 hours, assayed by qRT-PCR analysis. (E) Expression of Flag-14-3-3ε in control and 14-3-3ε knockout C28I2 cells, assayed by western blot. (F) mRNA levels of *Col2* and *Acan* in PGRN (200 ng/mL) treated control or 14-3-3ε knockout C28I2 cells with or without re-expression of 14-3-3ε, assayed by qRT-PCR analysis. (G) Control and 14-3-3ε knockout C28I2 cells with or without re-expression of 14-3-3ε were treated with 10 ng/mL TNFα in the absence or presence of 200 ng/mL PGRN for 24 hours. mRNA levels of Mmp13, Adamts5, Cox2 and Nos2 were measured by qRT-PCR. Data are mean±SD; n=4 biological replicates; *p<0.05 or **p<0.01. PGRN, progranulin.

induced DMM model in 14-3-3ε$^{f/f}$ and 14-3-3ε-/- mice, followed by intra-articular injection of PGRN three times per week for a total of 8 weeks starting from 4 weeks after surgery and OA phenotypes were analysed with a variety of techniques, including morphometric analysis, immunohistochemistry staining, ELISA and pain analysis (figure 4A).

Compared with 14-3-3ε$^{f/f}$ mice, 14-3-3ε-/- mice exhibited statistically severer cartilage erosion following DMM surgery (figure 4B). In addition, DMM-operated 14-3-3ε-/- mice exhibited slightly, yet statistically significant, higher Osteoarthritis Research Society International (OARSI) scores and thickening of the subchondral bone plate, two critical characteristics of OA, relative to 14-3-3ε$^{f/f}$ littermates (figure 4B,C). PGRN treatment ameliorated surgically induced OA pathogenesis in 14-3-3ε$^{f/f}$ mice as evidenced by significant reduction of articular cartilage destruction, along with substantial inhibition of osteophyte formation and thickening of subchondral bone plate (figure 4B,C). In addition, PGRN-mediated protection against OA in 14-3-3ε$^{f/f}$ mice, including reduction of articular cartilage destruction, osteophyte formation and thickening of subchondral bone plate, was almost abolished in 14-3-3ε -/- mice with DMM (figure 4B,C).

It is appreciated that concurrency of upregulation of matrix-degrading enzymes and accelerated matrix degradation promotes OA,[33] accordingly we assessed whether the expression of relevant effector molecules contributed to PGRN's regulation of OA and its dependence on 14-3-3ε. Immunohistochemistry staining demonstrated that 14-3-3ε deficiency correlated with upregulated MMP13, aggrecan neoepitope and COMP fragment. In addition, 14-3-3ε deficiency enhanced the expression of ColX, a marker for HTCs (figure 4D, online supplemental figure 9a). Conversely, PGRN treatment following DMM markedly reduced the levels of MMP13, aggrecan neoepitope, COMP fragment and ColX in 14-3-3ε$^{f/f}$ mice, and these PGRN-mediated effects were markedly attenuated by 14-3-3ε deletion (figure 4D, online supplemental figure 9a). 14-3-3ε deletion also engendered significant elevation of COMP protein fragments in sera, largely unresponsive to PGRN treatment, while serum levels of COMP fragments were meaningfully reduced in PGRN treated relative to phosphate-buffered saline (PBS) treated 14-3-3ε$^{f/f}$ mice (figure 4E). Additionally, DMM-induced OA pain was significantly reduced in PGRN treated 14-3-3ε$^{f/f}$ mice, but not 14-3-3ε-/- mice, although 14-3-3ε deletion does not further enhance DMM induced pain as reflected by statistical equivalence of pain scores from PBS treated mice irrespective of genotype (figure 4F). Collectively, these results reinforce 14-3-3ε's standing as a critical mediator of the PGRN/TNFR2 pathway in regulation of cartilage homeostasis and protection against OA pathogenesis.

We also examined whether PGRN and14-3-3ε are involved in macrophage polarisation. Deletion of PGRN and 14-3-3ε altered transcriptome of bone marrow derived macrophage which was stimulated with proinflammatory lipopolysaccharides (LPS)/IFNγ (polarised to M1) or anti-inflammatory IL-4 (polarised to M2) (online supplemental figure 10a,b). GSEA analysis demonstrated that both 14-3-3ε deficiency and PGRN deficiency significantly upregulated inflammatory response in macrophages compared with WT macrophages (online supplemental figure 10c–f). As OA is considered a chronic inflammatory disease, we then asked whether macrophage polarisation regulated by PGRN and 14-3-3ε also contributed to OA pathogenesis. Immunohistochemistry staining of F4/80, a marker of general macrophage, showed undistinguishable macrophage infiltration among the mice with different genetic backgrounds treated with or without PGRN (online supplemental figure 11a,b). Further phenotypic characterisation of macrophage in synovium revealed a significant decrease of M1 macrophage (iNos positive) and increase of M2 macrophage (CD206 positive) in the synovium of WT mice treated with PGRN compared with PBS. 14-3-3ε deletion skewed the macrophage towards M1 phenotype compared with WT, and, remarkably, PGRN induced reduction of M1 and enhancement of M2 in WT mice was largely abolished in 14-3-3ε-/- mice (online supplemental figure 11a,c,d). No obvious difference was observed in populations of synovial CD4 +T cells and mast cells, and the immune cells were also reported to be involved in OA,[34 35] between 14-3-3ε$^{f/f}$ and 14-3-3ε-/- mice with DMM with or without PGRN treatment, as detected by immunohistochemistry staining (online supplemental figure 12a–c). Although no obvious difference for synovial CD4+ T cells was observed, whether 14-3-3ε is important for PGRN regulation of T cell subpopulations, including regulatory T cells,[36] warrants further investigations. Collectively, these results suggested that macrophage phenotypic polarisation modulated by PGRN and 14-3-3ε may also contribute to the regulations of OA by anti-inflammatory PGRN/TNFR2/14-3-3ε signalling complex.

Deletion of 14-3-3ε in chondrocytes exaggerates surgically induced OA and counteracts PGRN regulation of cartilage homeostasis

Having determined that global 14-3-3ε deficiency exaggerated OA and blunted PGRN-mediated protection against OA, we next investigated the role of chondrocyte-specific 14-3-3ε in the pathogenesis of surgically induced OA. We thus established the DMM model in 14-3-3ε^{Agc1} mice and their littermate controls and Safranin O staining revealed that cartilage degeneration was substantially progressed in both 14-3-3ε^{Agc1} and 14-3-3ε$^{f/f}$ mice following DMM surgery. Deficiency of 14-3-3ε in chondrocytes exaggerated cartilage destruction, with higher OARSI score in 14-3-3ε^{Agc1} mice as compared with 14-3-3ε$^{f/f}$ controls at 4, 8 and 12 weeks after DMM surgery (online supplemental figure 13a,b). Accordingly, deficiency of 14-3-3ε associated with elevated serum levels of COMP fragments, a biomarker correlated with severity of cartilage degradation,[37] following DMM surgery as compared with 14-3-3ε$^{f/f}$ controls (online supplemental figure 13c).

To evaluate whether chondrocyte-specific 14-3-3ε was also important for PGRN regulation of cartilage homeostasis, we also established DMM model in 14-3-3ε^{Agc1} mice and their littermate controls and compared PGRN's therapeutic effects between genotypes at 12 weeks following surgery (figure 5A). Similar to 14-3-3ε-/- mice, genetic ablation of 14-3-3ε in chondrocytes elicited a slightly severer OA phenotype, including severe cartilage erosion, increased osteophyte development and thickening of subchondral bone plate as compared with 14-3-3ε$^{f/f}$ controls at 12 weeks post-DMM (figure 5B,C). PGRN treatment substantially attenuated the OA phenotype by inhibiting articular cartilage destruction, osteophyte development and thickening of subchondral bone plate in 14-3-3ε$^{f/f}$ mice while cartilage-specific 14-3-3ε deficiency dampened PGRN's protective effect against OA pathologies with reduced efficacy in preserving cartilage integrity and no ameliorative impact on osteophyte maturity and thickness of subchondral bone plate relative to that observed in 14-3-3ε$^{f/f}$ mice (figure 5B,C). Collectively, these results indicated that chondrocyte-expressed 14-3-3ε was required for maintaining cartilage homeostasis. Complimentary immunohistochemistry staining demonstrated marked reduction of MMP13, aggrecan neoepitope, COMP fragment and ColX observed in

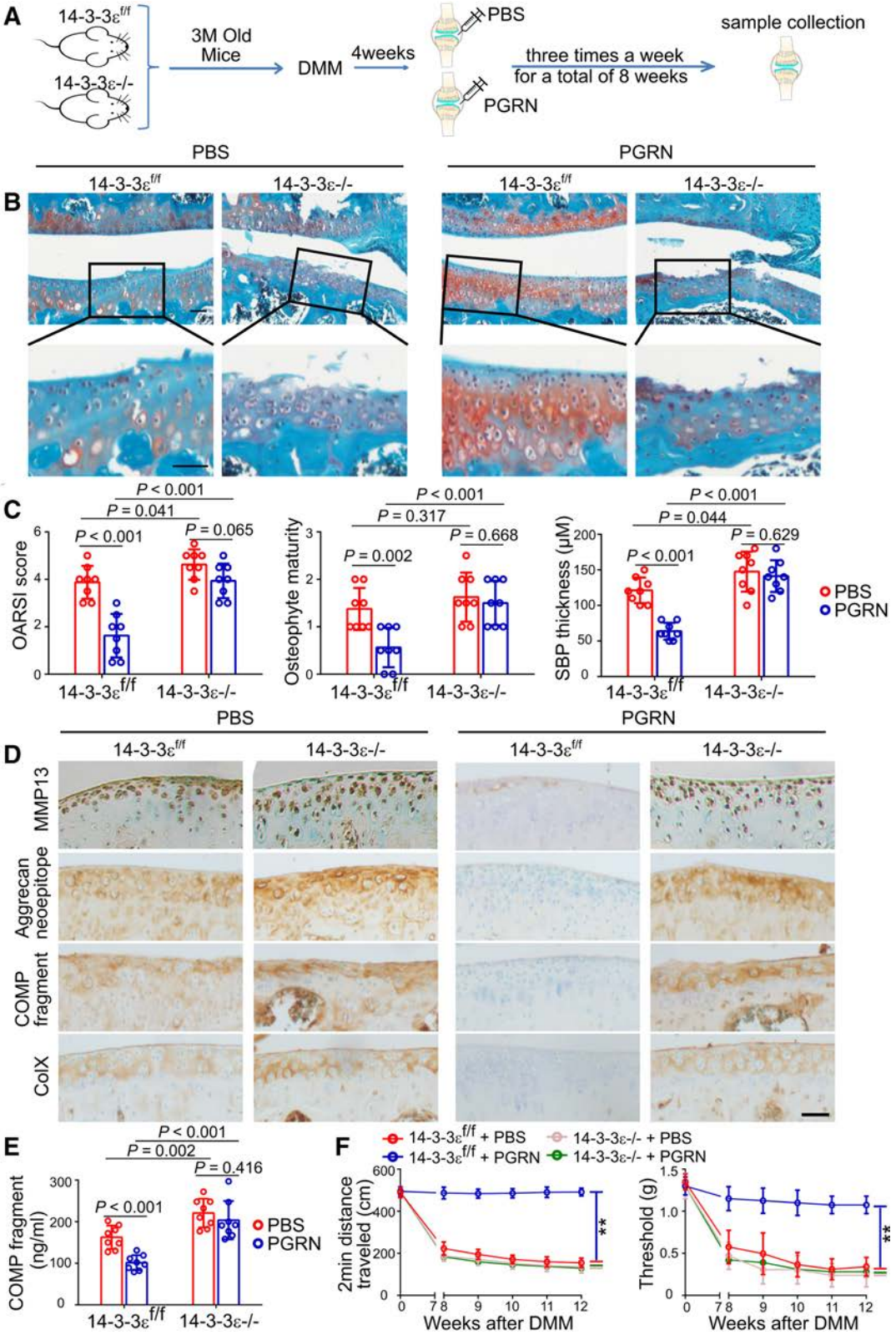

Figure 4 Global deletion of 14-3-3ε regulates OA pathogenesis and largely abrogates PGRN's therapeutic effects against OA. (A) Schematic of the experimental outline. 14-3-3ε$^{f/f}$ and Rosa26-ERT2;14-3-3ε$^{f/f}$ (ie, 14-3-3ε-/-) mice are injected with tamoxifen at 10 weeks old, and DMM operation is performed on 3-month-old mice. n=8 mice per group. (B) Representative images of Safranin O/Fast green stained sections of knee joints from 14-3-3ε$^{f/f}$ and 14-3-3ε-/- mice treated with or without PGRN for 8 weeks. Scale bar, 50 µm. (C) Quantitative analysis of OARSI score, osteophyte development and SBP thickness in different group of mice. (D) Representative images of immunohistochemical staining for MMP13, Aggrecan neoepitope, COMP fragment and ColX in knee joint sections of 14-3-3ε$^{f/f}$ and 14-3-3ε-/- mice treated with or without PGRN for 8 weeks. Scale bar, 50 µm. (E) Serum COMP fragment levels in 14-3-3ε$^{f/f}$ and 14-3-3ε-/- mice treated with or without PGRN for 8 weeks. (F) 2 min travel distance and von Frey pain assay in DMM-operated WT and 14-3-3ε-/- mice treated with or without PGRN at the indicated time after surgery. Data are mean±SD; **p<0.01. DMM, destabilisation of the medial meniscus; OA, osteoarthritis; PGRN, progranulin; SBP, subchondral bone plate.

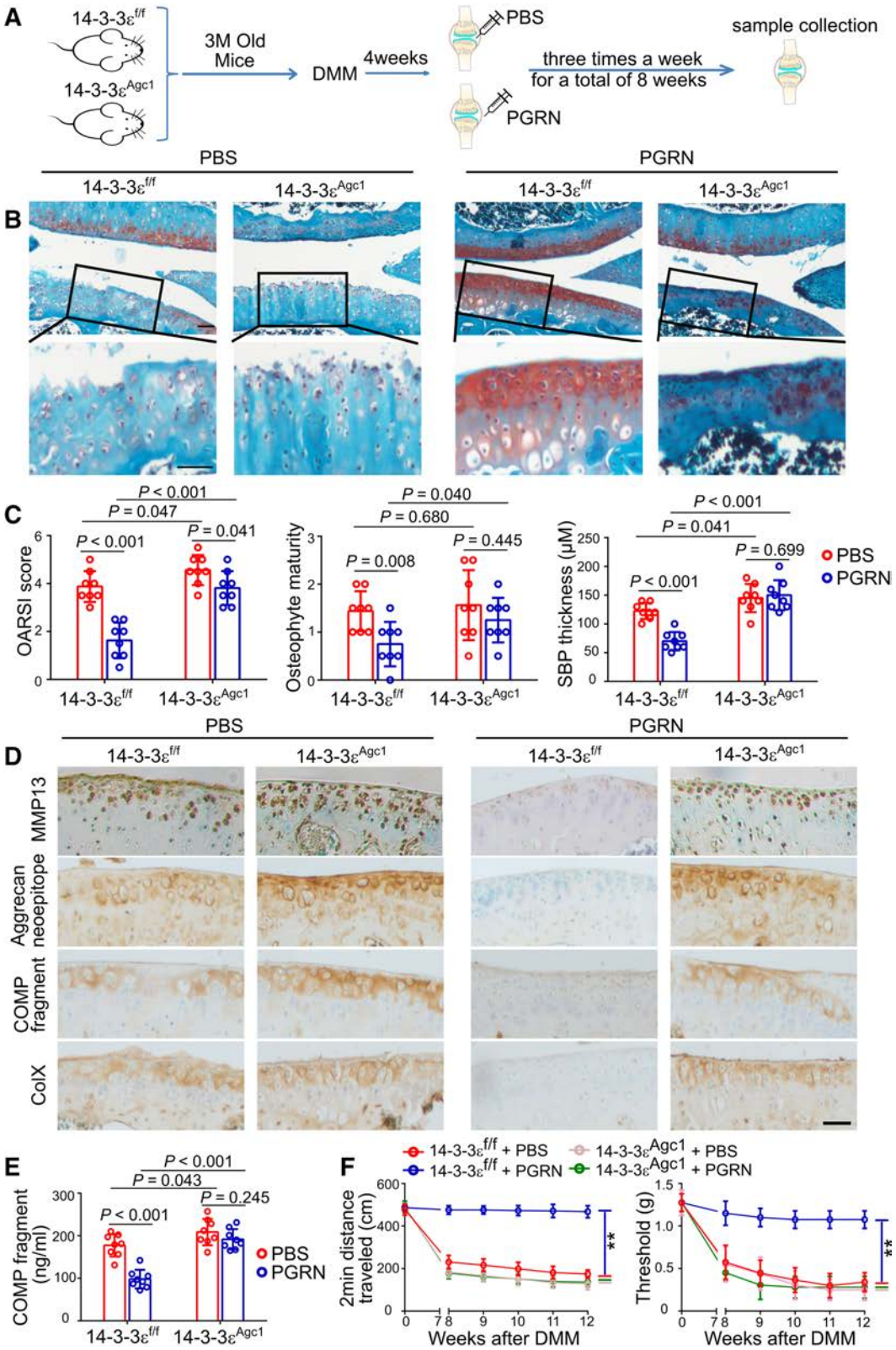

Figure 5 Chondrocyte specific deletion of 14-3-3ε attenuates PGRN mediated protection against experimental OA. (A) Schematic of the experimental outline. 14-3-3ε$^{f/f}$ and Agc1-ERT2; 14-3-3ε$^{f/f}$ (14-3-3ε^{Agc1}) mice are injected with tamoxifen at 10 weeks old, and DMM operation is performed on 3-month-old mice. n=8 mice per group. (B) Representative images of Safranin O/Fast green stained sections of knee joints from 14-3-3ε$^{f/f}$ and 14-3-3ε^{Agc1} mice treated with or without PGRN for 8 weeks. Scale bar, 50 µm. (C) Quantitative analysis of OARSI score, osteophyte development and SBP thickness in different groups of mice. (D) Representative images of immunohistochemical staining for MMP13, Aggrecan neoepitope, COMP fragment and ColX in knee joint sections of 14-3-3ε$^{f/f}$ and 14-3-3ε^{Agc1} mice treated with or without PGRN for 8 weeks. Scale bar, 50 µm. (E) Serum COMP fragment levels in 14-3-3ε$^{f/f}$ and 14-3-3ε^{Agc1} mice treated with or without PGRN for 8 weeks. (F) 2 min travel distance and von Frey pain assay in DMM-operated 14-3-3ε$^{f/f}$ and 14-3-3ε^{Agc1} mice treated with or without PGRN at the indicated time after surgery. Data are mean±SD; **p<0.01. DMM, destabilisation of the medial meniscus; OA, osteoarthritis; PGRN, progranulin; SBP, subchondral bone plate.

PGRN treated 14-3-3ε$^{f/f}$ mice which was largely absent in 14-3-3ε^{Agc1} mice (figure 5D, online supplemental figure 9b). Likewise, PGRN-mediated reduction of COMP fragments in serum of 14-3-3ε$^{f/f}$ mice was abolished in 14-3-3ε^{Agc1} mice (figure 5E). PGRN-triggered substantial reduction in DMM-induced OA pain in 14-3-3ε$^{f/f}$ mice was also abolished in 14-3-3ε^{Agc1} mice (figure 5F). In sum, the loss of PGRN's therapeutic efficacy against OA observed following global 14-3-3ε knockout was closely recapitulated following chondrocyte specific deletion of 14-3-3ε, thereby confirming that chondrocyte-expressed 14-3-3ε primarily contributed to and is required to mediate PGRN/TNFR2's protection against OA.

We also examined PGRN regulation on macrophage plasticity in 14-3-3ε^{Agc1} mice. Compared with PBS treated WT and 14-3-3ε^{Agc1} mice, PGRN did not change the total macrophages presented in the synovium (online supplemental figure 14a,b). However, PGRN treatment regulated macrophage plasticity. Specifically, PGRN inhibited proinflammatory M1 macrophage and skewed macrophages towards anti-inflammatory M2 macrophage (online supplemental figure 14a,c,d) in both WT and chondrocyte-specific 14-3-3ε^{Agc1} mice but not in global 14-3-3ε-/- mice (online supplemental figure 11a-d), highlighting the notion that 14-3-3ε is a critical mediator of PGRN/TNFR2 signalling in both chondrocytes and macrophages.

PGRN/TNFR2/14-3-3ε regulates chondrocyte metabolism by activating Elk-1 transcription factor

To further elucidate the molecular mechanisms by which the PGRN/TNFR2/14-3-3ε receptor complex regulates chondrocyte metabolism and OA, we performed transcription factor array to identify the transcription factor(s) activated by PGRN/TNFR2/14-3-3ε receptor complex. Among the 45 transcription factors examined, Elk-1, NF-κB and Stat3 showed more than twofold changes in transcriptional activity following PGRN treatment in WT primary chondrocytes, whereas these regulatory changes were abrogated in both TNFR2-/- and 14-3-3ε-/- chondrocytes (figure 6A), highlighting these three transcription factors as potential mediators of PGRN's regulation of chondrocyte metabolism in TNFR2- and 14-3-3ε-dependent manners. Among the three isolated transcription factors, Elk-1 is the only one for which activity is enhanced, while NF-κB and Stat3 were inhibited, by PGRN through TNFR2 and 14-3-3ε. The transcription factor Elk-1 is known to act downstream of ERK, and ERK activation induces phosphorylation of Elk-1, leading to transcriptional activation of target genes.[38] In addition, ERK signalling is also known to be required for PGRN/TNFR2 regulation of chondrocyte anabolism,[12] and we thus focused on examining the functional dependence of PGRN/TNFR2/14-3-3ε induced anabolism on the Elk-1 transcription factor. Indeed, Elk-1 luciferase reporter gene was activated by PGRN in WT articular chondrocytes, but this PGRN-mediated activation of Elk1 was completely lost in TNFR2-/- and 14-3-3ε-/- articular chondrocytes (figure 6B). Moreover, PGRN induced the phosphorylation of ERK and Elk-1in WT articular chondrocytes, and these activations were also abrogated in both TNFR2-/- and 14-3-3ε-/- articular chondrocytes (figure 6C,D).

We next re-expressed 14-3-3ε in 14-3-3ε knockout human C28I2 chondrocytes. Re-expression of 14-3-3ε efficiently restored PGRN-induced activations of ERK and Elk-1 (figure 6E,F), further indicating that 14-3-3ε represented an essential component in the PGRN/TNFR2 signalling cascade. Both pharmacological inhibition of ERK and siRNA knockdown of Elk-1 in human C28I2 chondrocytes markedly inhibited PGRN-activated Elk-1 transcriptional activity (figure 6G, online supplemental figure 15a). Accordingly, pharmacological inhibition of ERK significantly inhibited activation of ERK and Elk-1 by PGRN (figure 6H,I). In addition, siRNA knockdown of Elk-1 markedly reduced Elk-1 expression level and Elk-1 activation (online supplemental figure 15b,c). Notably, PGRN induced expressions of anabolic markers, including Col2, Acan and COMP, were abolished by U0126 and siRNA knockdown of Elk-1 (figure 6J, online supplemental figure 15d). These results confirmed that Elk-1 transcriptional activity was required for PGRN/TNFR2/14-3-3ε regulation of chondrocyte anabolism.

In addition to activating chondrocyte anabolism, PGRN also exerts anticatabolic function in chondrocytes by downregulating matrix-degrading enzymes and inflammatory response markers.[12] Therefore, we used the same strategy to isolate the downstream transcription factor(s) implicated in mediating PGRN's anticatabolic activity. For this purpose, we treated chondrocytes with TNFα in the absence and presence of PGRN and performed transcription factor array. Among 45 transcription factors, PGRN inhibited TNFα-activated transcription factors NF-κB, STAT1 and STAT1/STAT2 in WT articular chondrocytes, and this inhibition was abolished in 14-3-3ε-/- chondrocytes (online supplemental figure 16a). TNFα displayed the most potent activation of the NF-κB transcription factor, and PGRN is known to inhibit TNFα-mediated activation of NF-κB in inflammatory arthritis.[11] We thus selected NF-κB for functional validation in PGRN-mediated anticatabolism and its dependence on 14-3-3ε. PGRN significantly inhibited TNFα-induced NF-κB phosphorylation and transcriptional activity, an effect that was lost in 14-3-3ε knockout human chondrocytes (online supplemental figure 16b–d). The selective Ikk-2 inhibitor SC-514[39] significantly inhibited TNFα-activated NF-κB phosphorylation, to a comparable extent as PGRN, in control human chondrocytes (online supplemental figure 16c). Despite SC-514's effective inhibition of TNFα-activated NF-κB phosphorylation and expression of *Mmp13* and *Adamts5*, PGRN lost these inhibitions in 14-3-3ε knockout human chondrocytes (online supplemental figure 16d–f). These results indicated that PGRN inhibited TNFα-activated NF-κB in a non-canonical 14-3-3ε-dependent anticatabolic pathway in chondrocytes (online supplemental figure 16g).

DISCUSSION

TNFR2 signalling plays a protective and anti-inflammatory role in joint destruction,[19 20] and activation of TNFR2 by PGRN has been shown to protect against OA.[12] In this study, combined use of biochemical copurification and mass spectrometry led to the isolation of 14-3-3ε, an important intracellular signalling molecule, as a novel component recruited to TNFR2 complex in response to PGRN stimulation in human chondrocytes. By using multiple techniques including RNA-seq, single-cell transcriptomics, in vitro validations in mouse and human chondrocytes, alongside in vivo and ex vivo assessments of both spontaneous, age-related and surgically induced OA in genetically modified mice, we gain critical insights supporting the conclusion that 14-3-3ε is an essential mediator for the activation of the protective TNFR2 signalling by PGRN in OA.

We previously reported that both PGRN and its derivative Atsttrin promote chondrocyte anabolism through activating ERK.[12 32] Herein, transcription factor array and in vitro validation isolated Elk-1 as the critical transcription factor in PGRN/TNFR2/14-3-3ε signalling and its ERK dependent activation is required for PGRN/TNFR2/14-3-3ε regulation of chondrocyte anabolism. Elk-1 is a transcription factor involved in various biological processes, such as cell growth, differentiation and

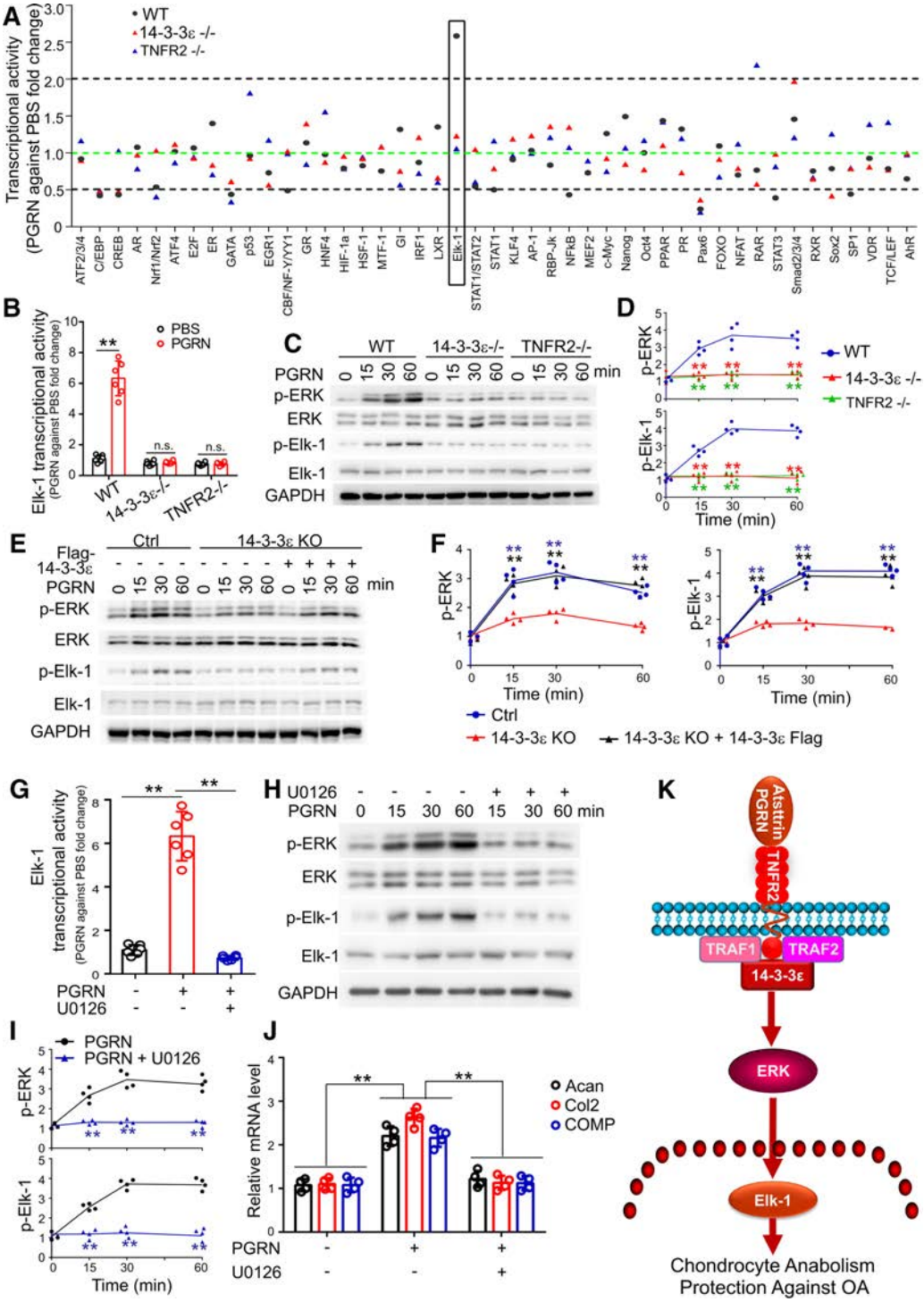

Figure 6 Transcription factor Elk-1 is indispensable for the regulation of chondrocyte anabolism by PGRN/TNFR2/14-3-3ε. (A) Transcriptional activities of 45 transcription factors are scanned using the transcription factor array. Primary articular chondrocytes isolated from WT, TNFR2-/- and 14-3-3ε mice are transfected with the construct included in the kit for 48 hours, followed by treatment with 200 ng/mL PGRN for another 24 hours. (B) Elk-1 transcription activity in primary articular chondrocytes isolated from WT, TNFR2-/- and 14-3-3ε-/- mice treated with PGRN for 24 hours. n=6 for each group. (C) Immunoblotting of indicated protein in WT, TNFR2-/- and 14-3-3ε-/- primary articular chondrocytes treated with PGRN for different time points, as indicated. n=4 for each group. (D) Densitometry analysis of immunoblotting results shown in (C). (E) Immunoblotting of indicated protein in control and 14-3-3ε knockout human C28I2 cells transfected with Flag-14-3-3ε construct prior to treatment with PGRN for indicated time. n=4 for each group. (F) Densitometry analysis of immunoblotting results shown in (E). (G) Elk-1 transcription activity analysis in human chondrocytes treated with 10 μM U0126 for 1 hour prior to treatment with 200 ng/mL PGRN for 24 hours. n=6 for each group. (H) Immunoblotting of indicated protein in human chondrocytes treated with 10 μM U0126 for 1 hour prior to treatment with 200 ng/mL PGRN for different time points, as indicated. n=4 for each group. (I) Densitometry analysis of immunoblotting results shown in (H). (J) mRNA levels of indicated molecules in human chondrocytes treated with 10 μM U0126 for 1 hour prior to treatment with 200 ng/mL PGRN for 24 hours. n=4 for each group. (K) A proposed model depicting the signalling pathway by which PGRN (its derivative Atsttrin as well) binds to TNFR2 and recruits 14-3-3ε to the receptor complex, leading to the activation of chondrocyte anabolism and protection against OA. Data are mean±SD; **p<0.01. PGRN, progranulin.

survival, wound healing and inflammation.[40] Activation of Elk-1 has been shown to attenuate oxidative and apoptotic response in human chondrocytes.[41] Both pharmacological and siRNA knockdown of Elk-1 abrogated PGRN induced anabolism (figure 6). Future studies will lead to better understating of Elk-1 directed gene expression via PGRN/TNFR2/14-3-3ε/ERK signalling pathway in the context of OA. Nonetheless, Elk-1 appears to be indispensable for the regulation of chondrocyte anabolism by the PGRN/TNFR2/14-3-3ε receptor complex (figure 6K).

Besides activating chondrocyte anabolism, PGRN/TNFR/14-3-3ε signalling could also inhibit chondrocyte catabolism. PGRN's promotion of anabolism and inhibition of catabolism rely on 14-3-3ε, and 14-3-3ε deficiency activates a catabolic cascade by upregulating matrix-degrading enzymes, Mmp13 and Adamts5.[42] NF-κB and Stat3 are found to be inhibited by PGRN in a TNFR2-dependent and 14-3-3ε-dependent manner in our transcription factor array. NF-κB and Stat3 activation by proinflammatory cytokines are shown to stimulate chondrocyte catabolism;[9 43 44] thus, inhibition of these two transcription factors by PGRN/TNFR2/14-3-3ε signalling contributes to PGRN's anticatabolic actions in chondrocytes. PGRN exhibits higher binding affinity to TNFR2 than does TNFα and comparable binding affinity to TNFR1 and TNFR2.[11] Interestingly, 14-3-3ε could also be recruited to TNFR1 on stimulation by PGRN (data not shown) although TNFR1 and TNFR2 mediate distinct signalling pathways,[45] suggesting that 14-3-3ε may act as a signalling switch of TNFRs in response to PGRN and TNFα stimulation. In addition, PGRN inhibited TNFα-activated NF-κB in a non-canonical 14-3-3ε-dependent manner in chondrocytes. Taken together, PGRN and its derivative Atsttrin exert their therapeutic and protective effects in OA through dual mechanisms: (a) primarily activating PGRN/TNFR2/14-3-3ε/Elk-1 anabolic pathway independent of TNFα (figure 6K) and (b) competing with TNFα to bind to TNFR1, thus simultaneously triggering PGRN/TNFR1/14-3-3ε/NF-κB anticatabolic signalling (online supplemental figure 16g).

Emerging evidences demonstrate that accumulations of immune cells, particularly activated macrophages in the synovium of joints, also affect OA progression.[46–50] Coincident with its role to mediate chondroprotective effects of PGRN/TNFR2 in chondrocytes, 14-3-3ε was also found to be required for PGRN regulation of macrophage polarisation in the course of OA, which may also explain the more prominent blockade of PGRN effects observed in global 14-3-3ε deficient mice than seen in chondrocyte-specific 14-3-3ε deficient mice. In brief, roles of PGRN/TNFR/14-3-3-ε in regulating chondrocyte metabolism and macrophage polarisation are all expected to contribute to the protective role of PGRN in the context of OA.

OA is a degenerative disease affecting the whole joints, including articular cartilage, subchondral bone and synovium.[51 52] In addition to deteriorating articular cartilage destruction, both global and chondrocyte-specific 14-3-3ε deletion caused more severe subchondral bone sclerosis, whereas activation of 14-3-3ε by PGRN through TNFR2 inhibited articular cartilage destruction, osteophyte formation and subchondral bone sclerosis. Although it is unclear how these events interact with each other and which event first occurs to initiate OA, the results provide genetic evidences that cartilage destruction, subchondral sclerosis and osteophyte development are highly correlated and targeted by PGRN/TNFR2/14-3-3ε signalling.

Pain is the common symptom of OA and a complex process involving structural changes in joint tissues, neuronal mechanisms and alterations of pain processing.[53] Our results demonstrated that PGRN treatment could alleviate OA pain in a 14-3-3ε-dependant manner, although much remains to be learnt about how PGRN/TNFR2/14-3-3ε signalling contributed to control OA pain. We previously reported that PGRN derived Atsttrin exhibited potent anti-inflammatory effects in several preclinical animal models of inflammatory arthritis, surpassing that of PGRN.[11] Moreover, results from several laboratories, including ours, demonstrate that Atsttrin signals through TNFRs and protects against OA in both mouse and rat OA models.[32 54] The current finding that Atsttrin's regulation of chondrocyte metabolism also relies on 14-3-3ε, further supports a strong case for testing this reagent in a clinical trial.

Both global and chondrocyte specific 14-3-3ε mice demonstrate that 14-3-3ε mediates chondroprotective and anti-inflammatory effects of PGRN in OA. Consistent with these findings, deletion of 14-3-3ε favours OA development in vivo in both naturally occurring with age and surgically induced OA. Intriguingly, extracellular 14-3-3ε secreted by osteoblasts/osteocytes was reported to induce the release of catabolic factors by chondrocytes.[55] This paradoxical controversy suggests that intracellular and extracellular 14-3-3ε might exert distinct effects on chondrocyte metabolism and may have different roles in the pathogenesis of OA.

In sum, this study reports discovery of intracellular 14-3-3ε as a crucial component of TNFR2 receptor complex in chondrocytes and OA, and establishes a novel TNFR2 signalling paradigm to orchestrate chondrocyte anabolism and combat the inflammatory/catabolism via PGRN/TNFR2/14-3-3ε/Elk-1 anabolic and PGRN/TNFR/14-3-3ε/NF-κB anticatabolic cascade, respectively, thereby protecting against OA. The chondroprotective effects of PGRN on OA support the concept that targeted activation of TNFR2 signalling by PGRN, particularly its derivative Atsttrin, would be an effective therapeutic candidate for treating OA.

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Contributors WF and CL designed the experimental plan and wrote the manuscript. WF executed most experiments. AH assisted with human OA sample collection and μCT data analysis. YC collected OA sample for single cell RNA-Seq and data analysis. WH performed RNA-seq data analysis. XD assisted with μCT data analysis. MC assisted with macrophage immunohistochemical staining. JM and YY assisted with protein immunoprecipitation. YD, WS and RL assisted with mouse studies. MA and JS provided cDNAs extracted from human normal and arthritic cartilage for qRT-PCR. ES, PL and RS provided human cartilage samples for bulk RNA-sea, scRNA-seq and for Immunoblotting. All authors were involved in editing the manuscript.

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Early reduction in circulating monocyte count predicts maintenance of remission in patients with rheumatoid arthritis treated with anti-TNF therapy

Maintenance of remission once achieved is becoming a critical goal for patients with rheumatoid arthritis (RA) as outcomes improve and advances in therapies continue.[1 2] Identification of biomarkers to facilitate tailoring of treatment is often linked to modest response criteria, but less frequently to the more stringent target of sustained remission. We have previously implicated monocytes as potential predictor of response to anti-tumour necrosis factor (TNF) through modulation of regulatory T cells, which may promote maintenance of remission through re-establishment of immune tolerance.[3] Circulating monocyte numbers are increased in RA but fall in patients who respond to TNF blockade.[4] Whether changes in monocyte numbers can also predict loss of remission, once achieved, to anti-TNF therapy is unknown. We therefore addressed whether the change in monocyte counts in the first year from initiation of anti-TNF therapy (baseline) would predict loss of remission (LOR) in patients who achieved sustained remission.

We extracted data (June 2020) from two independent cohorts of adult biologic-naïve patients with RA who attained sustained remission while treated with anti-TNF between January 2008 and December 2019 (online supplemental table 1). In this retrospective study, Disease Activity Score-28 (DAS28) with erythrocyte sedimentation rate ≤2.6 on at least two occasions (3–6 months apart) after initiation of anti-TNF therapy was used as the definition of remission, as this index was routinely calculated at the treating hospitals. A more stringent definition of remission based on the Clinical Disease Activity Index (CDAI ≤2.8)[5] was applied to a subset of patients (when this index could be derived from the data available). Circulating monocyte counts at initiation of anti-TNF (baseline) and three monthly for 12 months were obtained. Latent class mixed modelling was used to investigate trajectories of change in monocyte count over the first year of therapy (online supplemental methods). Kaplan-Meier estimator and propensity score-adjusted Cox regression were applied to

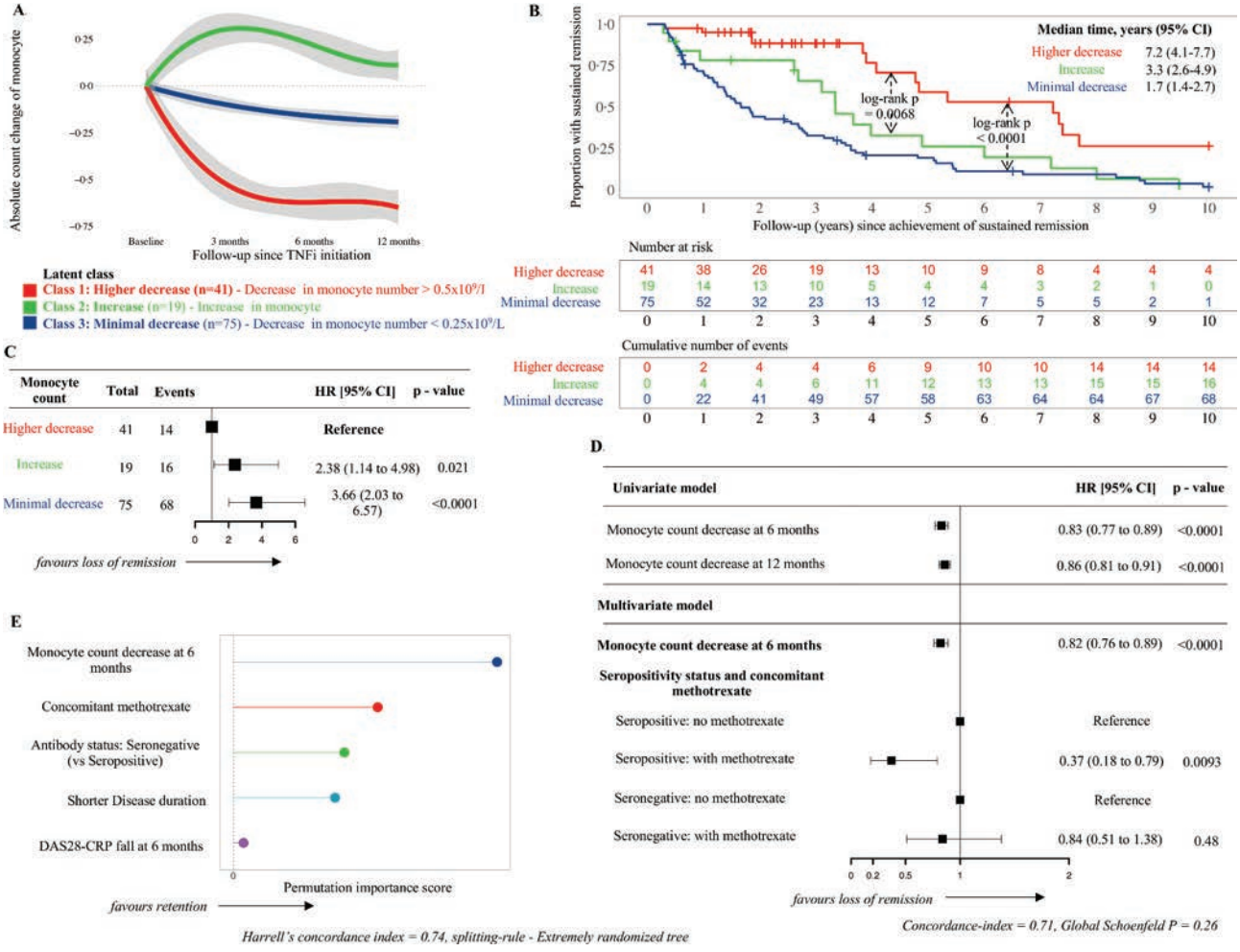

Figure 1 (A–E) Monocyte count change and other variables predicting loss of remission in anti-TNF-treated patients with rheumatoid arthritis. (A) Smoothed curves of the trajectories of a three-latent class model of peripheral blood monocyte count over the first year after initiation of anti-TNF. The thresholds of monocyte count change for each trajectory were derived from their mean change from baseline. (B) Kaplan-Meier curve of the monocyte latent class to predict loss of remission anti-TNF. (C) Cox regression to predict the loss of remission stratified by the latent class, adjusted by propensity score. (D) Univariate and multiple Cox regression model to predict loss of remission of anti-TNF using monocyte count change (by each 0.1×10^9/L unit decrease) at 6 and 12 months and adjusted by variables selected by partial least square regression. (E) Random forest model—to predict loss of remission of anti-TNF. The top five variables (ranked by permutation importance score) contributing to the loss of remission are shown. DAS28-CRP, Disease Activity Score-28 with C reactive protein; TNFi, tumour necrosis factor inhibitor.

predict LOR within the latent classes identified. Propensity scores were estimated for each patient using logistic regression adjusted for age, disease duration, sex, baseline DAS28 and C reactive protein (CRP), type of anti-TNF, antibody status, concomitant disease-modifying antirheumatic drug and prednisolone. Multivariate Cox regression and random forest were employed to determine the key factors affecting LOR (see online supplemental methods).

There were 92 and 43 patients who attained sustained remission in cohort 1 and 2, respectively, and follow-up data were available up to 10 years from achieving sustained remission. Mean monocyte count at baseline was 0.95×10^9/L with SD 0.29 (normal range: $0.2–1.0\times10^9$/L). Three latent classes were identified based on monocyte count changes in the first year from initiation of anti-TNF (figure 1A). The baseline characteristics of the three latent classes are shown in online supplemental table 2. Patients within class 1 associated with greatest reduction in monocyte count demonstrated longer time to LOR compared with those within class 2 (unadjusted log-rank p=0·0068) and class 3 (unadjusted log-rank p<0.0001) (figure 1B). There was no significant difference between class 2 and 3 with respect to time to loss of remission. Adjusted by propensity score, patients in class 2 and class 3 showed increased risk of LOR with HRs 2.38 (95% CI 1.14 to 4.98) and 3.66 (95% CI 2.03 to 6.57), respectively, compared with class 1 (figure 1C). These findings were confirmed when remission was defined using CDAI in a subset of patients (n=71) (online supplemental figure 1).

In the multivariate model, monocyte count reduction (for each drop by 0.1×10^9/L) at 6 months was associated with an incremental reduction of risk of LOR by 17% (95% CI 11% to 23%, p<0.0001). Concomitant methotrexate reduced the risk of LOR (HR: 0.37, 95%CI 0.18 to 0.78, p=0.0089), but only in seropositive patients, consistent with our previous data[6] (figure 1D). The best performing (by Harrell's concordance score) random forest model revealed five important variables to predict LOR (figure 1E). Decrease in monocyte count at 6 months was the strongest predictor of reduced risk of LOR, followed by use of methotrexate, seronegative status, shorter disease duration and fall of DAS28-CRP. We did not find any difference in monocyte count change as a predictor of LOR between adalimumab and etanercept, irrespective of concomitant use of methotrexate.

These data reveal that a substantial decrease in monocyte count in the first 6 months after initiation of anti-TNF is associated with a durable remission. Monocyte counts are routinely available in the clinic and our analysis provides a semiquantitative measure that could guide therapeutic decisions in patients treated with anti-TNF to ensure remission is maintained. Our data not only raise the possibility that tapering anti-TNF or methotrexate therapy[7] may be more appropriate for patients who have a substantial reduction in their monocyte count after commencing anti-TNF, but also highlight that stopping methotrexate completely is likely to risk a flare in disease, at least for seropositive patients. These findings warrant further prospective analysis of the relationship between monocytes and sustained remission in RA, and investigation into the underlying mechanisms.

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Nail involvement in psoriatic arthritis patients is an independent risk factor for carotid plaque

Psoriatic arthritis (PsA) is a chronic, inflammatory and immune-mediated disease that affects up to 30% of psoriasis (PsO) patients.[1] Nail involvement affects 80% of PsA patients and 30%–50% of

PsO patients. Nail PsO has been associated with worse quality of life, higher score on the PsO Area Severity Index, early disease onset, arthritis, depression and anxiety.[2] Patients with PsO and PsA have a higher risk of cardiovascular atherosclerotic morbidity and mortality than the general population. Nail PsO and cardiovascular disease have been seldom studied. Nail involvement in PsO patients has been associated to a higher prevalence of metabolic syndrome, higher risk of heart failure and higher cardiovascular risk overall.[3 4] If PsA patients with nail PsO also have a higher cardiovascular risk is unknown. We aimed to determine if nail involvement in PsA patients is associated with a higher prevalence of subclinical atherosclerosis by carotid ultrasound.

We performed a cross-sectional, observational and comparative study that included a total of 64 PsA patients consecutively recruited from a Preventive Cardiology-Rheumatology Clinic cohort of the University Hospital 'Dr. José E. González' in Monterrey, Mexico. Patients included in the cohort were 30–75 years old that fulfilled the 2006 Classification Criteria for Psoriatic Arthritis.

All PsA patients with nail involvement were included and patients without nail involvement were matched by age, gender and type 2 diabetes mellitus diagnosis. Patients with a previous cardiovascular atherosclerotic disease were excluded.

A B-mode carotid ultrasound was performed in all study subjects by a board-certified radiologist blinded to clinical information. Carotid plaque (CP) was defined as a carotid intima–media thickness (cIMT) ≥1.2 mm or a focal narrowing ≥0.5 mm of the surrounding lumen, and an increased cIMT was defined as a value ≥0.8 mm. Nail PsO Severity Index (NAPSI) was assessed in all patients. Distribution was evaluated with the Kolmogorov-Smirnov test. Comparisons were done with χ^2 test for qualitative variables and Student's t-test or Mann-Whitney's U test for quantitative variables. Correlation between NAPSI and cIMT (using 1.2 mm as the value of cIMT in patients with CP) was determined with Spearman's rank correlation coefficient (r). A $p<0.05$ was considered statistically significant.

A total of 64 patients were included (32 in each group). Clinical and demographic characteristics are shown in table 1. CP was significantly more prevalent in PsA patients with nail involvement (53.1% vs 25.0%, p=0.021). PsA patients with nail involvement also had higher cIMT values (0.85 mm vs 0.59 mm, p=0.026). Spearman's r showed a significant medium positive correlation between NAPSI and cIMT (r=0.314, p=0.012). A binary logistic regression, including traditional cardiovascular risk factors (hypertension, dyslipidaemia, obesity, active smoking, C reactive protein and erythrocyte sedimentation rate) demonstrated that nail involvement is an independent risk factor for the presence of CP with an OR 6.64 (95% CI: 1.71 to 25.74) (p=0.006).

Our results showed that nail involvement in PsA patients is independently associated to CP. This could be explained by the fact that nail involvement has been linked to severe skin manifestations and joint involvement, resulting from an increased inflammatory burden, that is, directly associated with the development of atherosclerosis.[5] In conclusion, PsA patients with nail involvement had a higher rate of CP and higher cIMT values than patients with PsA without it. Systematic evaluation of nail involvement in PsA patients may help identify high-risk individuals. More studies with a higher sample are necessary to confirm our findings and determine the precise role of carotid ultrasound evaluation in this population.

Table 1 Comparison of demographic characteristics and carotid ultrasound findings between PsA patients with nail involvement and matched PsA patients without it

	Nail involvement (n=32)	Without nail involvement (n=32)	P value
Age years, mean±SD	54.1±11.3	54.5±10.3	0.882
Female, n (%)	14 (43.8)	14 (43.8)	1.000
T2DM, n (%)	8 (25.0)	8 (25.0)	1.000
Hypertension, n (%)	11 (34.4)	16 (50.0)	0.206
Dyslipidaemia, n (%)	14 (43.8)	11 (34.4)	0.442
Obesity, n (%)	15 (46.9)	12 (37.5)	0.448
Active smoking, n (%)	6 (18.8)	8 (25.0)	0.545
Disease duration, years, median (IQR)	6 (4.0–10.0)	4 (2.0–6.7)	0.233
DAPSA, median (IQR)	20.3 (7.2–32.5)	14.1 (4.6–21.6)	0.187
NAPSI, median (IQR)	13 (5–20)	0	**<0.001**
PASI, median (IQR)	1.6 (0–4.2)	1.2 (0–2.15)	0.447
CRP mg/dl, median (IQR)	0.56 (0.31–1.36)	0.60 (0.35–1.03)	0.809
ESR mm, median (IQR)	16.5 (8.2–31.0)	15.5 (12–22.7)	0.957
Glucocorticoids, n (%)	7 (21.9)	3 (9.4)	0.168
Methotrexate, n (%)	21 (65.6)	23 (71.9)	0.590
bDMARD, n (%)	6 (18.8)	11 (34.4)	0.157
Carotid ultrasound findings			
Any carotid plaque, n (%)	17 (53.1)	8 (25.0)	**0.021**
Increased cIMT, n (%)	4 (12.5)	4 (12.5)	1.000
cIMT mm, median (IQR)	0.85 (0.59–1.18)	0.59 (0.50–0.87)	**0.026**

Bold values represent a *p<0.05*.
bDMARD, biological disease-modifying antirheumatic drugs; cIMT, carotid intima–media thickness; CRP, C reactive protein; DAPSA, disease activity in psoriatic arthritis; ESR, erythrocyte sedimentation rate; NAPSI, Nail Psoriasis Severity Index; PASI, Psoriasis Area Severity Index; T2DM, type 2 diabetes mellitus.

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Tofacitinib for the treatment of antineutrophil cytoplasm antibody-associated vasculitis: a pilot study

Antineutrophil cytoplasm antibody-associated vasculitis (AAV) is a group of necrotising vasculitis involving small vessels characterised by upper and lower respiratory tract, kidney, eye, ears–nose–throat, skin, gastrointestinal and neurological involvement. AAV includes granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA), and eosinophilic granulomatosis with

Table 1 Clinical characteristics and treatment of patients with AAV at enrolment

Number	Gender	Age	Diagnosis	AAV duration (year)	ANCA status (ULN of PR3: <20 RU/mL, ULN of MPO: <20 RU/mL)			Clinical manifestations	Previous treatment	Treatment before JAKi initiation	Treatment concomitant with JAKi	Systemic involvement	BVAS at enrolment	Follow-up (months)	Present dose of pred (mg/day)	Clinical evaluation
					At diagnosis	At enrolment	At last follow-up									
1	F	50	GPA	1	Negative	Negative	Negative	Protruding eyes, red eye scleritis, congested nose	Pred, CYC, CyA	Pred 10 mg +CYC	Pred 7.5 mg +JAKi	ENT (sinusitis and mass), eye (retro-orbital mass and scleritis), lung (nodules)	8	13	3.75	CR
2	M	54	GPA	1	cANCA(+), PR3 >200 RU/mL	cANCA(+), PR3: 72.7 RU/mL	cANCA(+), PR3: 61 RU/mL	Blurred vision and headache	Pred, CYC, HCQ, IVIG	Pred 25 mg+CYC +HCQ	Pred 25 mg +CYC+ HCQ+JAKi	ENT (sinusitis, nasal ulceration and otitis media), eye (scleritis), lung (nodules and cavitation)	4	10	2.5	CR
3	F	50	GPA	7	cANCA(+), PR3: 72.7 RU/mL	Negative	Negative	Protruding and swollen eyeball, headache	Pred, CYC, CyA, MMF	Pred40mg +MMF	Pred 40 mg +JAKi	ENT (sinusitis and otitis media), eye (retro-orbital mass), lung (fixed pulmonary infiltrates)	7	13	12.5	PR
4	F	35	GPA	0.25	pANCA(+), MPO 66 RU/mL	pANCA(+), MPO 66 RU/mL	pANCA(+), MPO 84.5 RU/mL	Lung infiltrate	/	/	Pred 15 mg +JAKi	Lung (fixed pulmonary infiltrates)	3	11	5	CR
5	M	31	GPA	8	cANCA(+) ，PR3 >200 RU/mL	cANCA(+), PR3 <20 RU/mL	Negative	Protruding and swollen eyeballs	Pred, CYC, HCQ, LEF, AZA, rituximab, CyA	Pred 15 mg +CyA	Pred 15 mg +CyA +JAKi	ENT (sinusitis, nasal crusting, mass), eye (retro-orbital mass)	5	8	15	CR
6	F	57	GPA	4	cANCA(+), PR3 75.5 RU/mL	cANCA(+), PR3 >200 RU/mL	cANCA(+), PR3: 84.6 RU/mL	Fever, fatigue, blurred vision, red eye, cough	Pred	Pred 30 mg	Pred 15 mg +JAKi	General, ENT (sinusitis), lung (nodules)	11	9	10	CR
7	F	38	MPA	5	pANCA(+), MPO>200 RU/mL	pANCA(+), MPO: 159 RU/mL	pANCA(+), MPO: 48.6 RU/mL	Numbness in limbs, proteinuria and hematuria	Pred, MMF, HCQ	Pred 10 mg +MMF	Pred 10 mg +MMF +JAKi	Kidney, nerve	18	9	5	CR
8	M	55	MPA	2.5	pANCA(+), MPO 86 RU/mL	Negative	Negative	Abdominal pain	Pred, CYC, HCQ, MMF	Pred 5 mg +HCQ +MMF	Pred 50 mg +HCQ +JAKi	Abdominal	6	9	12.5	CR
9	F	64	MPA	0.3	pANCA(+), MPO 96.3 RU/mL	pANCA(+), MPO 23.9 RU/mL	Negative	Cough, myalgia	Pred, CYC, HCQ	Pred 20 mg +HCQ +CYC	Pred 20 mg +HCQ +JAKi	General, lung (interstitial pneumonia)	1	6	5	CR
10	M	24	EGPA	1.25	Negative	Negative	Negative	Toe ulceration, pain	Pred, CYC, LEF, tacrolimus, rituximab	Pred 15 mg +rituximab	Pred 20 mg +MTX +JAKi	Cutaneous	4	7	7.5	CR

/, not applicable; AAV, antineutrophil cytoplasm antibody-associated vasculitis; ANCA, antineutrophil cytoplasm antibody; AZA, azathioprine; AZA, azathioprine; BVAS, Birmingham Vasculitis Activity Score; cANCA, cytoplasmic antineutrophil cytoplasm antibody; CR, complete remission; CyA, cyclosporine; CYC, cyclophosphamide; EGPA, eosinophilic granulomatosis with polyangiitis; ENT, ears–nose–throat; F, female; GPA, granulomatosis with polyangiitis; HCQ, hydroxychloroquine; IVIG, intravenous immunoglobulin; JAKi, Janus kinase inhibitor, tofacitinib; LEF, leflunomide; M, male; MMF, mycophenolate mofetil; MPO, myeloperoxidase; MTX, methotrexate; pANCA, perinuclear antineutrophil cytoplasm antibody; PR3, proteinase 3; PR, partial remission; Pred, prednisone; ULN, upper limit of normal.

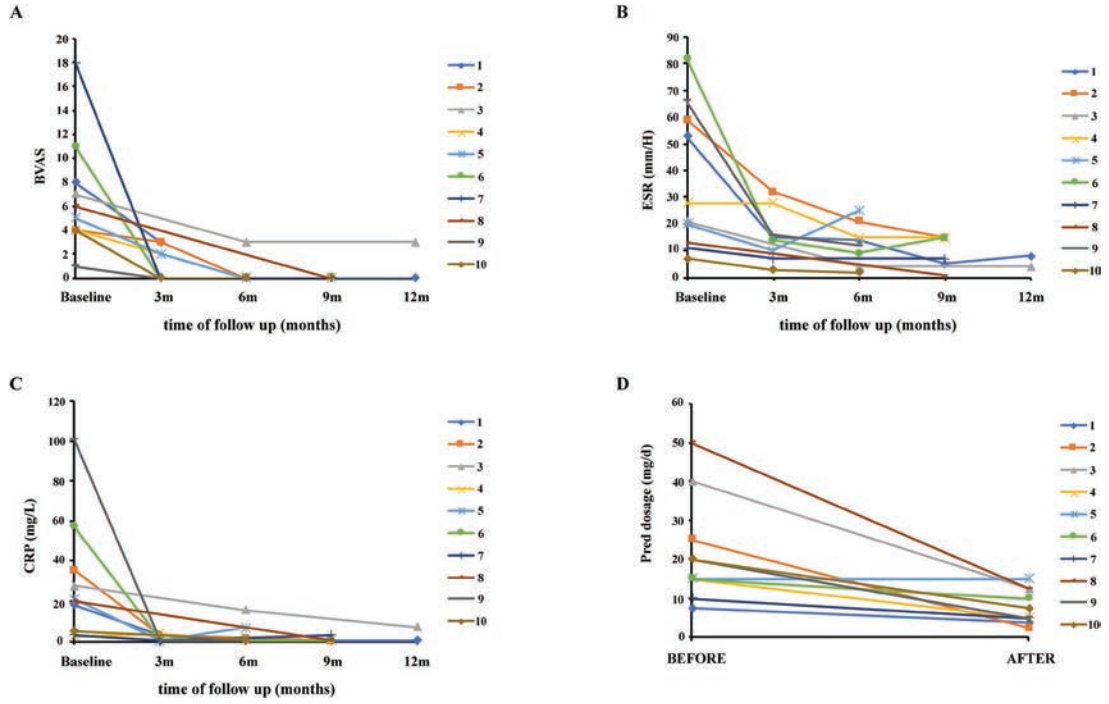

Figure 1 (A) Changes in the disease activity score (BVAS 2003) during patient follow-up. (B,C) Changes in the ESR and CRP levels during tofacitinib treatment. (D) Changes in the glucocorticoid level from baseline to the end of follow-up. BVAS, Birmingham Vasculitis Activity Score; CRP, C reactive protein; ESR, erythrocyte sedimentation rate; Pred, prednisone.

polyangiitis (EGPA).[1] To date, maintenance therapy to prevent disease relapse remains the main therapeutic challenge for patients with AAV.

Previous studies indicate that T cells and associated cytokine production (eg, interleukin (IL)-6, IL-10, IL-12, IL-23 and type 1 interferons) play an important role in the pathogenesis of AAV[2–4] via activation of the Janus kinase (JAK)/signal transducer and activator of transcription pathway.[5] Tofacitinib is a JAK1/3 inhibitor that functions by suppressing the activity of the JAK family of non-receptor tyrosine kinases (RTKs) and has been used successfully for the treatment of rheumatoid arthritis, psoriatic arthritis, Behçet's disease and systemic lupus erythematosus[6–9]; however, the use of tofacitinib for the treatment of AAV has not been reported. Of particular interest, imatinib mesylate, an RTK inhibitor, has been reported to be an effective treatment for patients with EGPA.[10] Therefore, we hypothesised that tofacitinib-mediated inhibition of JAK signalling may represent an effective therapy for active AAV.

In this study, we aimed to explore the efficacy and safety of tofacitinib 5 mg two times per day in 10 patients with AAV (6 with GPA, 3 with MPA and 1 with EGPA) with a confirmed diagnosis according to the modified classification criteria of the 1990 American College of Rheumatology[11 12] and nomenclature of the 2012 Chapel Hill Consensus Conference[1] with a Birmingham Vasculitis Activity Score (BVAS)[13] of ≥1. With the exception of one case of new-onset GPA, the other nine patients with AAV exhibited disease relapse and had previously received a combination of glucocorticoids (GCs) and multiple immunosuppressants. The follow-up time varied from 6 months to 13 months (average: 9.5 months) and was conducted by the same medical team. At each visit, the clinical manifestation, adverse events, level of C reactive protein (CRP), erythrocyte sedimentation rate (ESR) and BVAS were evaluated. At the same time, the amount of GCs was also recorded. The response to treatment was defined as follows[14]: (1) complete remission (CR), defined as the absence of disease activity (BVAS=0); (2) partial remission (PR), defined as at least 50% reduction of BVAS and no new manifestations; and (3) treatment resistance, defined as less than a 50% reduction or increased disease activity after 4–6 weeks of treatment.

The demographic data and clinical characteristics of the 10 patients with AAV are presented in table 1. Nine patients achieved CR (BVAS=0); one patient achieved PR (BVAS decreased from 7 to 3); and no patient relapsed during the follow-up (figure 1A). For the five patients with GPA with eye involvement, scleritis, blurred vision and swelling of the eyeball quickly resolved. In the patient with EGPA, the skin ulcers quickly healed and the eosinophils returned to normal levels following 1 month of tofacitinib administration. Although a significant improvement in the CRP and ESR levels was observed at the third month, the CRP results were more convincing (figure 1B,C). The average dosage of GCs also significantly decreased from 21.75 mg/day at baseline to 7.88 mg/day at the end of the follow-up (figure 1D). The dose of tofacitinib was reduced to 5 mg/day in two patients without the occurrence of a relapse. One patient developed mild symptoms of an upper respiratory infection, and the other developed low-grade fever and fatigue. The symptoms resolved quickly with continuous use of tofacitinib. No other adverse events were observed.

The excessive activation of effector T cells and the dysfunction of regulatory T cells play a vital pathogenic role in AAV.[3] Tofacitinib is a JAK inhibitor that can inhibit Th1 and Th17 cell differentiation by suppressing RORγt and T-bet expression.[15] Although this study was limited by the small number and heterogeneity of patients, improvements in clinical symptoms and inflammatory indicators were observed in patients with AAV following treatment with tofacitinib.

The findings of our study suggest that tofacitinib is well tolerated and effective for patients with non-organ-threatening AAV, sparing the dose of GCs. However, what we cannot ignore is that drugs of tyrosine kinase inhibitor including JAKi has the

potential risk of inducing vasculitis (eg, leucocytoclastic vasculitis, IgA vasculitis and EGPA) as previously reported.[16–18] Therefore, a prospective randomised controlled clinical trial is warranted to further confirm the efficacy and safety of tofacitinib treatment for patients with AAV.

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Immunotherapies and COVID-19 mortality: a multidisciplinary open data analysis based on FDA's Adverse Event Reporting System

During the COVID-19 pandemic, the risks and potential benefits of immunotherapies for the treatment of autoimmune disorders are still not well defined, and many cohort studies neither took the epidemiological dynamics of COVID-19 nor the potential capacities of the local healthcare systems in their outcome analysis into account. Due to a pronounced heterogeneity in the outcome reports of different participating countries, the large 'COVID-19 Global Rheumatology Alliance registry' addressed this issue using a 'cluster design' and shed light on factors associated with a more severe COVID-19 course in their study population.[1] We here present data of the US Food and Drug Administration (FDA) Adverse Event Reporting System (FAERS),[2] a postmarketing, self-reporting, open-access pharmacovigilance platform that contains international data of COVID-19 cases. Sources of FAERS are voluntary reports from healthcare professionals and consumers. We combine this data set with local measurements of the course of the pandemic (from Oxford University's 'Our World in Data'[3]) and the potential resiliency of the respective healthcare systems (from 'World Bank'; see online supplemental table 1 for full source information). Only patients with the diagnosis of an autoimmune disorder and a single immunotherapy (required group size: n≥100) at the time point of COVID-19 were analysed by multivariable regression analysis (online supplemental figure 1), limiting the generalisability of our data, for example, concerning combination therapy scenarios (online supplemental figure 1).

The mean age of patients in our cohort (n=2103) was 51.3 years (range 3–92 years; SD 14.9), female sex was more prevalent (1372/2103, 65.2%) and the majority of cases was reported in the USA/Canada (1285/2103, 61.1%). Inflammatory joint disease (846/2103, 40.2%), multiple sclerosis (474/2103, 22.5%) and inflammatory skin disease (435/2103, 20.7%) were the most prevalent diagnoses. Anti-tumour necrosis factor α (TNFα) therapies were the most frequently used medications for the underlying autoimmune disease (714/2103, 34%), followed by anti-CD20 therapies (388/2103, 18.4%). Additional cohort characteristics are shown in online supplemental table 2 and the monthly distribution of cases and cases by country in online supplemental figure 2.

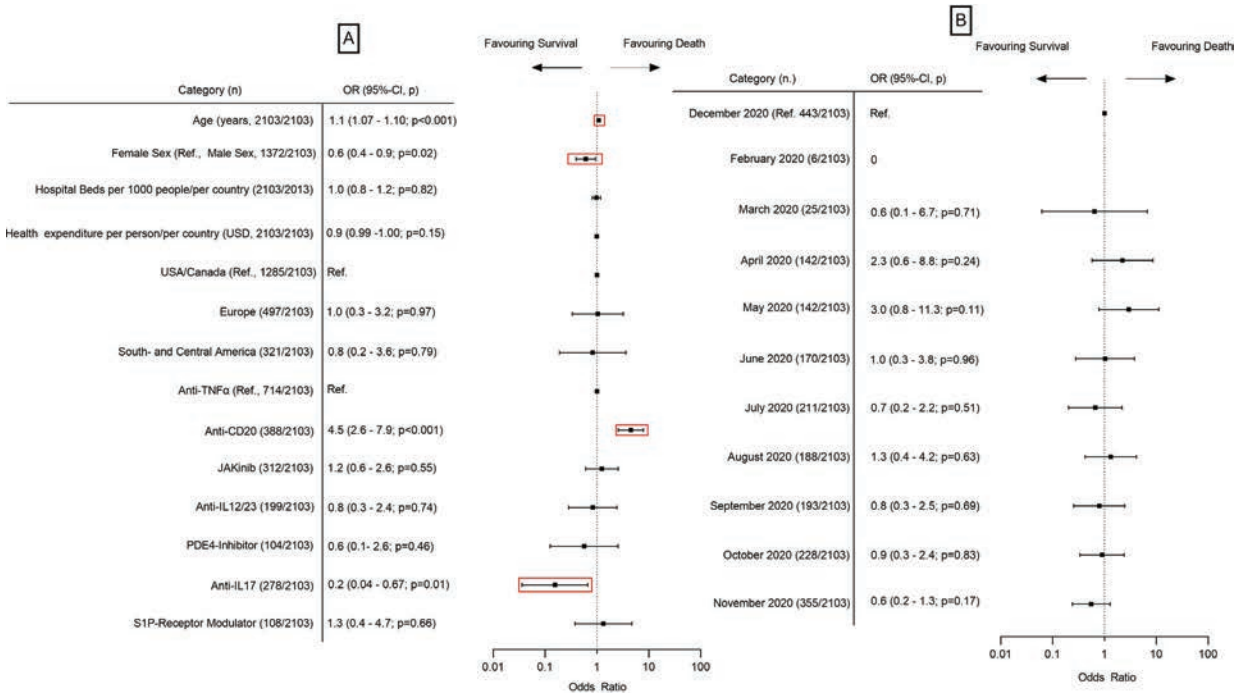

Figure 1 MLR showing the OR for 'death': 95% CIs and p values of the independent variables on a 10-log scale. (A) ORs for age, sex, hospital beds per 1000 persons/per country, health expenditure per person/per country (in US$), region and immunotherapy. (B) ORs for each month of 'initial FDA received date'. To control for the potential 'expectation bias' of FAERS, we included the variable 'ratio of total reports to FAERS to the number of reported deaths to FAERS for each drug in 2018–2020 excluding COVID-19 cases' (2.8; 0.1 to 84.2; p=0.56; not displayed). As reference groups (Region, Immunotherapy, Month), we chose the one with the highest number. Nagelkerke's R^2 is 0.24. For February 2020, no deaths have been reported to FAERS; therefore, the OR for February is 0. MLR was conducted with SPSS V.25 (IBM, USA, 2017). For more information regarding multivariable analysis adjusted for "month of event" instead of "initial FDA received date" see online supplemental figure 3 and for univariable analysis online supplemental figure 4. Not displayed: 'case fatality rate per month of initial FDA received date/country' (OR 0.5; 0.0 to 2479.3; p=0.86), 'new cases per population per month and country' (OR 51.5; 0.00 to 3.124E+37; p=0.93) and 'new deaths per population per month and country' (OR 0; p=0.54). CD-20, Cluster of Differentiation-20; FAERS, US Food and Drug Administration (FDA) Adverse Event Reporting System; IL-12/23, interleukin 12/23; IL-17, interleukin 17; JAKinib, Janus kinase inhibitor; MLR, multivariable logistic regression; PDE4-Inhibitor, phosphodiesterase-4 inhibitor; S1P-Receptor Modulator, sphingosine-1 phosphate receptor modulator; TNFα, tumour necrosis factor α.

In all, 26.3% of the reported patients were hospitalised (553/2103), and the overall reported mortality rate in our cohort was 5.1% (107/2103; for other outcomes, see online supplemental table 3). In the multivariable logistic regression analysis, age (OR per year 1.1; 95% CI 1.07 to 1.1; p<0.001) and female sex (OR 0.6; 95% CI 0.4 to 0.9; p=0.02) were significant predictors of mortality. Regarding immunotherapies, patients under anti-CD20 therapies had an increased mortality (OR 4.5; 95% CI 2.6 to 7.9; p<0.001), whereas those under anti-IL17 therapies had a reduced mortality (OR 0.2; 95% CI 0.04 to 0.67; p=0.01) compared with anti-TNFα therapies (reference group; figure 1).

In summary, using international open data sets and adjusting for local infectious diseases dynamics and the potential resilience of the national healthcare systems, our study demonstrates that anti-CD20 therapies are associated with a higher COVID-19 mortality risk in people with autoimmune disorders. This finding is in line with other cohort studies.[1 4] Regarding the potential protective capacities of anti-IL17 treatments, further studies are needed. This study also identified age and male sex as relevant predictors of COVID-19-associated mortality, which should therefore be taken into account in individual risk–benefit assessments. Our study has several limitations, for example, the fact that FAERS reports basic information on patients. We could not analyse disease-specific characteristics, comorbidities and risk factors, which have previously shown to influence mortality risks, thus representing a limitation of our analysis. Furthermore, adjustment for individual disease groups was not possible due to multicollinearity to immunotherapies. Biological therapies and recently approved oral immunotherapies are over-represented compared with classical immunotherapies, pointing towards a selection bias of FAERS. Furthermore, we cannot report the method of SARS-CoV-2 detection, as this information is not included in the FAERS data set.

Finally, we consider the use of combined open-access, pharmacoepidemiological data and a multidisciplinary approach, despite its limitations, as a valuable tool to address the various issues posed by the SARS-CoV-2 pandemic. Our findings might represent a complement to already published data and call for intensified investigations within larger cohort and translational studies.

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Methotrexate and glucocorticoids, but not anticytokine therapy, impair the immunogenicity of a single dose of the BNT162b2 mRNA COVID-19 vaccine in patients with chronic inflammatory arthritis

Strategies aimed at expediting immunisation campaigns against COVID-19 include providing single vaccine doses to individuals with previous exposure to SARS-CoV-2 and delaying second doses. While such approaches are effective at the population level, immunogenicity yielded by one dose of vaccines in immunocompromised patients may be alarmingly low.[1 2] Biological (b) and targeted synthetic (ts) disease-modifying antirheumatic drugs (DMARDs) interfere with the immune system at multiple levels and may variably reduce response to viral vaccines.[3] Limited data on small numbers of rheumatic patients with variable diagnoses and treatments hamper definitive conclusions on the possible impact of immune-mediated inflammatory diseases, immunomodulatory drugs or both on the efficacy of the new generation of mRNA vaccines.[4 5]

Here we present interim data analysis on the immunogenicity of the BNT162b2 COVID-19 vaccine in 140 patients with chronic inflammatory arthritis all treated with b/tsDMARDs at the Division of Rheumatology of the IRCCS Policlinico San Matteo University Hospital of Pavia, receiving the first dose of vaccine between 24 and 31 March 2021. Patients were advised to discontinue both the b/tsDMARD and concomitant methotrexate around vaccination. In particular, the following suggestions were made: (1) for all the bDMARDs and methotrexate, withholding of therapy in the 7 days before and after vaccination; and (2) for tsDMARDs, withholding of therapy from the day before until day 7 after vaccination. For glucocorticoids and conventional synthetic DMARDs other than methotrexate, no modifications were advised. Blood samples were obtained immediately before vaccination and at day 21 after the first dose. Serum samples were tested using chemiluminescent immunoassay (LIAISON SARS-CoV-2 S1/S2 IgG; DiaSorin) for the quantitative characterisation of SARS-CoV-2 anti-S1 and anti-S2 IgG antibodies, with values >15 AU/mL indicating a positive result. Demographic and clinical variables were retrieved from the last available rheumatological assessment (median (IQR) 14 (5–19) days before vaccination) (table 1). The b/tsDMARD was predominantly an anticytokine therapy (67.1%), followed

Table 1 Demographic and clinical characteristics of the study population, stratified for response to the first dose of the BNT162b2 mRNA COVID-19 vaccine

	All n=140 patients	Responders n=85	Non-responders n=55	P value
Age, mean (SD), years	55.7 (14.4)	50.9 (13.9)	63.3 (11.6)	**<0.001**
Females, n (%)	95 (67.9)	53 (62.4)	42 (76.4)	0.12
BMI, mean (SD)	25.89 (5.24)	25.37 (5.31)	26.69 (5.09)	0.19
Smoking, n (%)	23 (16.4)	14 (16.5)	9 (16.4)	0.83
Hypertension, n (%)	47 (33.6)	23 (27.1)	24 (43.6)	0.07
Obesity, n (%)	26 (18.6)	15 (17.6)	11 (20)	0.89
CCI, mean (SD)	0.57 (0.92)	0.47 (0.91)	0.73 (0.93)	0.11
≥1 comorbidity*, n (%)	50 (35.7)	23 (27.1)	27 (49.1)	**0.01**
Previous COVID-19*, n (%)	20 (14.3)	19 (22.4)	1 (1.8)	**0.002**
Diagnosis, n (%):				
RA	83 (59.3)	40 (47.1)	43 (78.2)	<0.001
PsA	29 (20.7)	20 (23.5)	9 (16.4)	0.42
SpA	28 (20)	25 (29.4)	3 (5.5)	0.001
Disease duration, mean (SD), years	13.7 (8.2)	12.9 (8.7)	14.9 (7.2)	0.15
Active disease†, n (%)	34 (24.3)	22 (25.9)	12 (21.8)	0.73
Glucocorticoids, n (%)	53 (37.9)	26 (30.6)	27 (49.1)	**0.04**
Prednisone dose, mean (SD), mg/day	3.7 (1.8)	3.7 (1.5)	3.7 (2.1)	0.94
csDMARDs, n (%)	80 (57.1)	38 (44.7)	42 (76.4)	<0.001
MTX	66 (47.1)	27 (31.8)	39 (70.9)	<0.001
SSZ	12 (8.6)	10 (11.8)	2 (3.6)	0.17
LFN	5 (3.6)	3 (3.5)	2 (3.6)	0.67
CYA	1 (0.7)	0 (0)	1 (1.8)	0.83
MTX dose, mean (SD), mg/week	14.7 (5.2)	14.6 (5.3)	14.7 (5.2)	0.93
Days of MTX withholding, mean (SD)	16.4 (3.5)	16.1 (3.4)	16.6 (3.6)	0.65
Adherence to MTX withholding‡, n (%)	33 (50)	13 (48.1)	20 (51.3)	0.99
b/tsDMARDs, n (%)	140 (100)	85 (100)	55 (100)	
TNFi	61 (43.6)	39 (45.9)	22 (40)	0.61
IL-6Ri	14 (10)	8 (9.4)	6 (10.9)	0.99
IL-17/IL-23i	19 (13.6)	17 (20)	2 (3.6)	0.01
CTLA4Ig	30 (21.4)	9 (10.6)	21 (38.2)	<0.001
JAKi	12 (8.6)	9 (10.6)	3 (5.5)	0.46
PDE4i	4 (2.9)	3 (3.5)	1 (1.8)	0.94
Days of b/tsDMARD withholding, mean (SD)	22 (13.2)	23.7 (14.7)	19.4 (10.2)	0.06
Adherence to b/tsDMARDs withholding‡, n (%)	96 (68.6)	62 (72.9)	34 (61.8)	0.23

Bold indicates statistically significant values (p<0.05).
*Based on patient-reported history of swab-confirmed SARS-CoV-2 infection and/or on prevaccine anti-S IgG levels >15 AU/mL (LIAISON SARS-CoV-2 S1/S2 IgG; DiaSorin).
†Above the threshold of low disease activity according to the appropriate composite index: DAS28 >3.2; DAPSA >14; ASDAS-PCR >2.1.
‡Recommendations for timing of immunomodulatory therapies in relation to vaccination: (1) for all the bDMARDs and MTX, withholding of therapy in the 7 days before and after vaccination; (2) for tsDMARDs, withholding of therapy from the day before until day 7 after vaccination; and (3) for glucocorticoids and csDMARDs other than MTX, no modifications.
§Among those listed in the Charlson Comorbidity Index.
ASDAS-CRP, Ankylosing Spondylitis Disease Activity Score calculated with C reactive protein; BMI, body mass index; b/ts,biological/targeted synthetic CCI, Charlson Comorbidity Index; cs, conventional synthetic; CTLA4Ig, cytotoxic T lymphocyte associated protein-4 immunoglobulin; CYA, cyclosporine; DAPSA, Disease Activity in Psoriatic Arthritis; DAS28, Disease Activity Score on 28 joints; DMARD, disease-modifying antirheumatic drug; IL-17/IL-23i, interleukin-17/interleukin-23 inhibitor; IL-6Ri, interleukin-6 receptor inhibitor; JAKi, Janus kinase inhibitor; LFN, leflunomide; MTX, methotrexate; PDE4i, phosphodiesterase-4 inhibitor; PsA, psoriatic arthritis; RA, rheumatoid arthritis; SpA, spondyloarthritis; SSZ, sulphasalazine; TNFi, tumour necrosis factor inhibitor.

by CTLA4Ig (21.4%) and Janus kinase inhibitors (8.6%). Treatment also included low-dose glucocorticoids (mean (SD) prednisone dose 3.8 (1.9) mg/day; ≤5 mg/day in 98.6% of the cases) in 38.5% of the patients and conventional synthetic DMARDs (mostly methotrexate) in 56.6%. Arthritis was on average well controlled, with 74.8% of the patients being in low disease activity.

Fifty-five patients (39.3%) were non-responders based on anti-S IgG levels at day 21. As shown in table 1, non-responders were more frequently on methotrexate and/or glucocorticoids; among the different b/tsDMARDs, CTLA4Ig was more common and interleukin-17/23 inhibitors were less common in non-responders. Results were confirmed when patients with a known history of swab-confirmed COVID-19 (n=9) or prevaccine antibody levels indicative of previous SARS-CoV-2 infection (n=11) were excluded (online supplemental table S1). Collectively, seroconversion decreased from 85.4% among patients not receiving neither methotrexate nor glucocorticoids to 33.3% among those on both therapies (figure 1A); such significant trend was confirmed after exclusion of patients with previous COVID-19 (figure 1B). At multivariable analysis, methotrexate and glucocorticoids independently predicted failure to achieve immunogenicity with adjusted ORs (95% CI) of 7.46 (2.88 to 19.33) and 2.69 (1.04 to 5.89), even with the inclusion of patients with previous stimulation by SARS-CoV-2 (online supplemental figure 1S 1C, table S2). In contrast, the effect of CTLA4Ig was restricted to patients with no history of COVID-19 (figure 1C and D and online supplemental table S2). The lower rates of seroconversion observed in patients with RA compared with other arthritis were largely dependent on covariates such as age and type of immunomodulatory treatment. Of note, neither adherence to the recommendations on methotrexate withholding (followed by 50% of the patients) nor the interval of b/tsDMARD discontinuation significantly impacted on the results (online supplemental table S2). The negative impact of methotrexate and glucocorticoids was confirmed in the larger subgroup of patients on tumour necrosis factor inhibitors (online supplemental table S3). Importantly, both drugs also impaired the magnitude of the antibody response among patients who seroconverted (figure 1E and F). The negative association of methotrexate and glucocorticoids with antibody levels was dose dependent (online supplemental table S4). Although the vast majority of the patients was receiving prednisone doses ≤5 mg/day, differences were already seen between the group

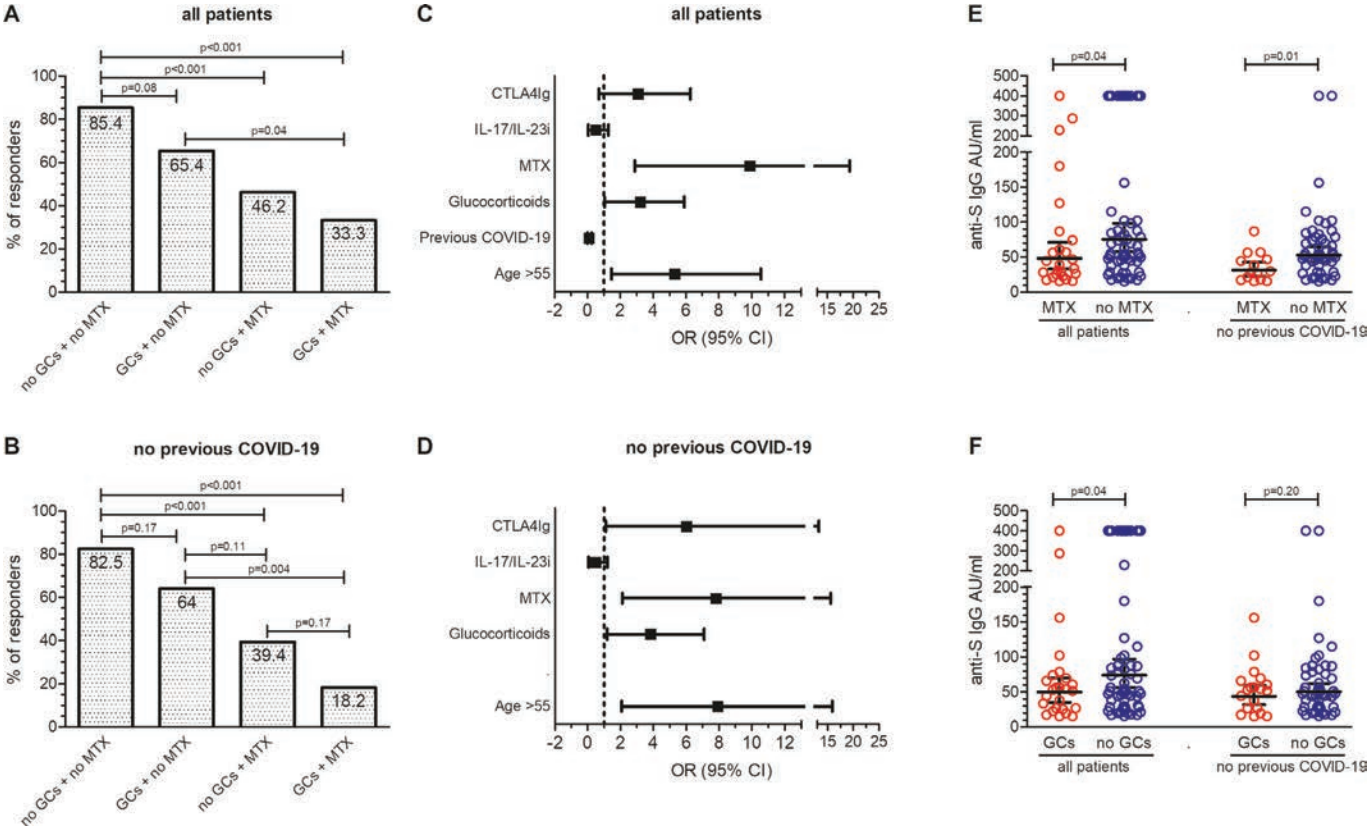

Figure 1 Impaired immunogenicity of a single dose of the BNT162b2 mRNA COVID-19 vaccine associated with methotrexate and glucocorticoids. (A and B) Rates of response to the first dose of mRNA COVID-19 vaccine in patients on treatment with biological or targeted synthetic disease-modifying antirheumatic drugs ((A) overall population, n=140; (B) patients with previous exposure to SARS-CoV-2 infection excluded, n=120) stratified for concomitant therapy with glucocorticoids (GCs), methotrexate (MTX) or both. (C and D) Forest plots illustrating factors associated with non-response to the first dose of mRNA COVID-19 vaccine in the overall population (C) and after exclusion of patients with previous exposure to SARS-CoV-2 infection (D). (E and F) Comparisons of anti-S IgG antibody levels in patients achieving response to the first dose of mRNA COVID-19 vaccine (levels above the cut-off of 15 AU/mL at day 21) stratified for concomitant therapy with MTX (E) and GCs (F). Data are shown as geometric mean values with 95% CIs.

treated with >2.5 mg/day and the group treated with ≤2.5 mg/day (geometric mean (95% CI) anti-S IgG levels 69.81 (150.95) vs 39.85 (83.82) AU/mL, p=0.07).

Deeper characterisation of memory B cell and T cell responses after each of the two doses of mRNA vaccines is needed to assist the optimal vaccination strategy in rheumatic patients on immunosuppressive treatments. Furthermore, the impact of specific medications, such as those interfering with interferon-driven responses, needs to be more extensively evaluated in larger patient cohorts. Equally important, strategies of methotrexate withholding, alone or in combination with b/tsDMARDs, should be established through randomised clinical trials. Notwithstanding these limitations, the high rate of response (>80%) following a single dose of mRNA COVID-19 vaccine among patients not receiving neither methotrexate nor glucocorticoids found here approaches the immunogenicity reported in registration trials of BNT162b2[6] and confirms the low impact of most anticytokine therapies on vaccination.[3] However, impaired humoral responses associated with methotrexate and glucocorticoids, even at low doses, impose caution before endorsing delayed second dose boosts in rheumatic patients.

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serum samples and revised the manuscript critically. DL contributed to the analysis of serum samples, to the interpretation of the results and revised the manuscript critically. FB contributed to the interpretation of data and revised the manuscript critically for important intellectual content. AM contributed to the analysis and interpretation of data and revised the manuscript critically for important intellectual content. CM conceived the work and revised the manuscript critically for important intellectual content. All the authors provided final approval of the version to be published.

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Unexpected impact of COVID-19 lockdown on spinal mobility and health perception in spondyloarthritis

Physical therapy (PT) forms the cornerstone of non-pharmacological treatment in axial spondyloarthritis (SpA).[1] The effect of temporary cessation of PT on SpA outcomes is unknown. Therefore, we evaluated the impact of a lockdown during the COVID-19 pandemic on physical activity patterns, spinal mobility and health perception in SpA.

Patients of the BeGIANT cohort, a Belgian multicentre prospective observational registry of newly diagnosed patients with SpA, completed an online questionnaire during the first lockdown in Belgium (March/April 2020), followed by a standardised clinical examination immediately thereafter. The online questionnaire (online supplemental file 1), developed jointly with patient advocacy groups, assessed changes in PT, exercise and sport regimens. We also probed the impact of a lockdown on health perception, including completion of the 36-Item Short Form Survey (SF-36) Questionnaire, Bath Ankylosing Spondylitis Disease Activity Index and Bath Ankylosing Spondylitis Functional Index. Patients from this lockdown cohort were clinically assessed by measuring Bath Ankylosing Spondylitis Metrology Index (BASMI) and chest expansion immediately after the lockdown (May 2020). Data were compared with the last available measurements as part of the standardised follow-up.

The online questionnaire was completed by 185 patients during the third week of the lockdown, of whom 65 patients (35 men, (mean±SD) age 40.8±11.6 years, symptom duration of 10.5±8.0 years) visited our outpatient clinic immediately post-lockdown. All 65 patients had stable disease and therapy prior to the lockdown. Among 33 (51%) patients following PT, 30 discontinued due to inaccessibility as a consequence of the lockdown.

Overall, no significant differences in spinal mobility measures were observed before and after lockdown. However, chest expansion was markedly lower immediately after the lockdown (before: 5.9±0.33 cm; after: 4.3±0.26 cm, $p \leq 0.0001$, figure 1A), and not limited to patients who temporarily ceased PT (online supplemental table 1). Intriguingly, chest expansion was significantly related ($\rho = 0.270$, $p = 0.037$) to 'the role of limitations due to emotional problems' subscale of the SF-36. All BASMI outcomes stayed stable over time whereas chest expansion in the lockdown cohort normalised to pre-lockdown values on the next regular clinical visit after the lockdown (pre: 5.9±0.33 cm; post: 6.1±0.34 cm, $p = 1.0$). To determine the clinical relevance of our findings, we used long-term follow-up data from 202 other BeGIANT patients (online supplemental table 2), during a similar time frame before the COVID-19 pandemic. As shown in figure 1B, chest expansion remains remarkably stable over time, underscoring the unusual nature of the observed reduction in chest expansion coinciding with a COVID-19 lockdown. As for the patient's general health perception during the lockdown, one-third reported this to be worse, while two-thirds reported no difference. Importantly, none of the patients displayed COVID-19 suggestive symptoms nor a positive test result during the evaluated period.

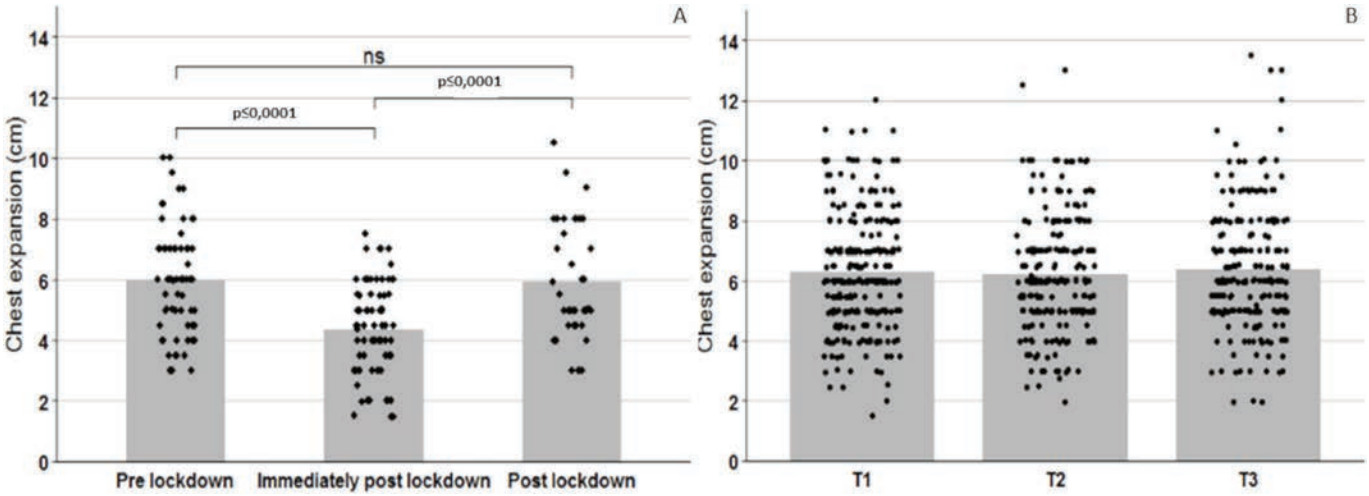

Figure 1 (A) Chest expansion in 65 patients with SpA was measured pre-lockdown (left panel), immediately after lockdown (middle panel) and 6 months after the pre-lockdown measurement (right panel). Significance (α=0.05) was determined using an ANOVA test for repeated measures. The grey boxes represent the mean, ***=p≤0.0001. (B) Standardised prospective follow-up of chest expansion in a nationwide cohort of patients with SpA (BeGIANT) over a period of 2 years, under stable established pharmacological therapy (n=202). Significance (α=0.05) was determined using an ANOVA test for repeated measures. Chest expansion at follow-up at month 12 (T1) at month 18 (T2) and at month 24 (T3). The grey boxes represent the mean. ANOVA, analysis of variance; ns, not significant; SpA, spondyloarthritis.

Because all clinical assessments from the lockdown onwards were performed in patients wearing a mask, changes in chest expansion were not influenced by wearing a mask.

While it was anticipated that temporary cessation of PT due to the lockdown would strongly impact the overall mobility of patients with SpA, this could not be demonstrated in our study. Likely explanations include a relatively short interruption of PT and the finding that most patients remained equally active by performing exercises and/or sports at home. A striking observation was the reduced chest expansion, independently from prior PT, normalising again after the lockdown. Although this observation was significantly associated with emotional distress, the study design did not enable to demonstrate a causal relationship. However, it is documented that emotional/psychological stress can influence the pulmonary function or evoke difficulties with breathing.[2–4] Psychological distress during the COVID-19 pandemic was recently demonstrated in patients with rheumatic disease, yet the impact on mobility remains unclear.[5 6] Thus, chest expansion might be vulnerable to emotional distress and should be interpreted with caution under such conditions.

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European League against Rheumatism/ American College of Rheumatology classification criteria for systemic lupus erythematosus: the laboratory immunologist's point of view

We read with great interest the new European League against Rheumatism/American College of Rheumatology (EULAR/ACR) classification criteria for systemic lupus erythematosus (SLE) by Aringer *et al*,[1] that recommend a positive antinuclear antibody (ANA) test as an entry criterion for SLE classification. According to these criteria, ANA testing has to be performed by a *highly sensitive* screening method before initiating the cascade work algorithm. This is a strategic move compared with the previous criteria by the Systemic Lupus International Collaborating Clinics (SLICC)[2] in which ANA was considered just one of the immunological items together with the more specific antibodies against Sm and dsDNA. With this new concept, ANA testing has been separated from its corresponding level tests, strengthening the idea that ANA is necessary to define the clinical lupus phenotype.

However, as laboratory immunologists we have some concerns about the ANA definition both in the previous and new criteria. The SLICC criteria which considered 'an ANA above laboratory reference range' as a criterion, left a great deal to arbitrariness about which method to use, and when the indirect immunofluorescence (IIF) method on HEp-2 cells is used, to the discriminating screening titer. Referring instead to the new classification criteria, we would like to note that the provision 'Testing by immunofluorescence on HEp-2 cells or a solid phase ANA screening immunoassay with at least equivalent performance' is somehow inaccurate, in that equivalence might be understood in this context in terms of sensitivity at the expense of specificity, which is a hallmark for classification purposes.[3] While we acknowledge that specificity can be achieved by completing the classification algorithm and that the ACR/EULAR decision has been made in view of ongoing work on the standardisation of autoimmune diagnostics and its potential future advances, we maintain that this is a risky definition that might be misinterpreted, thereby encouraging the use of alternative methods, even when they lack (or provide suboptimal) clinical specificity.

Hopefully, the American College of Rheumatology Antinuclear Antibody Task Force will take a position towards the individual alternative methods.[4–6] The great majority of studies performed in recent years[7–9] have mainly analysed the performance of new solid-phase screening assay, the so-called connective tissue disease screen test, which is currently the main 'competitor' to the ANA-IIF, having shown itself to be the closest in terms of sensitivity and specificity.[10 11] However, since a lot of different methods are available today to the clinical lab, they can be inappropriately used as an alternative to the ANA-IIF test, without a solid evidence of being really equivalent. Therefore, efforts in standardising diagnostic tests and their brisk evolution and perfection make it fundamental nowadays not only to suggest the test name among the criteria, but to define and take a strong position on the method to use, and better yet, on what method not to use.

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Response to: 'European League against Rheumatism/American College of Rheumatology classification criteria for systemic lupus erythematosus: the laboratory immunologist's point of view' by Infantino *et al*

In their letter, Drs Infantino, Manfredi and Bizzaro express concerns regarding the low specificity of antinuclear antibodies (ANA) for systemic lupus erythematosus (SLE) classification.[1] In particular, they propose that the entry criterion definition of positive ANA in the new European League Against Rheumatism (EULAR)/American College of Rheumatology (ACR) classification criteria as 'Antinuclear antibodies (ANA) at a titre of ≥1:80 on HEp-2 cells or an equivalent positive test at least once. Testing by immunofluorescence on HEp-2 cells or a solid phase ANA screening immunoassay with at least equivalent performance is highly recommended'[2 3] could be associated with low specificity for SLE.

While specificity is important for classification criteria, as Dr Infantino and colleagues correctly stress,[1] it is important to take both the overall test characteristics of ANA and its position as an *entry criterion* into account. The systematic literature review and metaregression of published ANA data on patients with SLE performed as part of the EULAR/ACR SLE classification criteria project[4] showed a relevant loss in *sensitivity* at a titre of 1:160 and above (table 1). At the titre of 1:80 selected for the EULAR/ACR 2019 classification criteria for SLE, specificity of the ANA test by itself is around 75% (table 1), far lower than the final specificity of 93.4% that the new set of EULAR/ACR criteria reached in the validation cohort.[2 3] This is because an entry criterion on its own has limited influence on increasing specificity. It is just the first step before the application of many other criteria that ultimately improve the specificity of SLE classification.

ANA have an inherent inability to differentiate between SLE and other connective tissue diseases, so that high specificity is not realistic. Precisely therefore the position of this test was changed to that of an entry criterion.[5] This was also more in line with the use of ANA as a highly sensitive screening parameter for connective tissue diseases. Given the role of the ANA test as an entry criterion, it was more important to provide a solution for centres without access to HEp-2 immunofluorescence than to try further improve specificity by more specific ANA tests. This said, we fully agree with Dr Infantino and colleagues that high quality ANA testing is extremely important and support efforts to standardise these tests.

For the EULAR/ACR criteria, issues concerning ANA test *sensitivity* using some HEp-2-cell substrates, raised by Pisetsky and colleagues,[6] are likely to have more impact on the EULAR/ACR criteria than ANA specificity issues considered by Infantino *et al*.[1] For diagnostic purposes, however, where a positive ANA result will often lead to several additional tests, ANA specificity plays an important role. We therefore agree that high quality ANA testing is crucial and that steps are necessary towards reaching this goal.

Table 1 ANA sensitivity and specificity per ANA titre as per metaregression of published data on 13 080 patients with SLE[4] 2017, American College of Rheumatology

Cut-off	Sensitivity		Specificity	
ANA titre	%	95% CI	%	95% CI
1:40	98.4	97.6 to 99.0	66.9	57.8 to 74.9
1:80	97.8	96.8 to 98.5	74.7	66.7 to 81.3
1:160	95.8	94.1 to 97.1	86.2	80.4 to 90.5
1:320	86.0	77.0 to 91.9	96.6	93.9 to 98.1

ANA, antinuclear antibodies; SLE, systemic lupus erythematosus.

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New 2019 SLE EULAR/ACR classification criteria are valid for identifying patients with SLE among patients admitted for pericardial effusion

The new 2019 SLE European League Against Rheumatism/American College of Rheumatology (EULAR/ACR) classification criteria for systemic lupus erythematosus (SLE) have been recently published.[1] Seritis is a prominent—often inaugural—feature of active SLE. Low titers of antinuclear antibodies (ANA) have been frequently reported in patients with idiopathic pericarditis.[2 3] Of note, ANA positivity at a titer ≥1/80 is now mandatory as an entry criterion in the 2019 SLE EULAR/ACR classification criteria. Although classification criteria have theoretically no individual diagnostic purpose, we aimed at testing this new criteria set in unselected patients with pericardial effusion.

In a retrospective study performed in the Department of Internal Medicine, University Paris Diderot, a French competence centre for rare systemic autoimmune diseases (AIDs), all consecutive adult patients hospitalised from January 2009 to January 2019 for pericardial effusion were reviewed. Clinical and biological data collected at time of the diagnosis of pericardial effusion were analysed.

Over a 10-year period, 137 patients were admitted for pericardial effusion. Search for ANA was systematically performed at diagnosis in all but 8 (n=129) and measured at a titer ≥1:80 on Hep-2 cells in 49 patients (38%) that were eventually separated in three groups:

1. Seventeen (34.7%) patients with a final diagnosis of SLE based on senior clinician judgement.
2. Six (12.2%) patients with a final diagnosis of AID other than SLE including primary Sjögren's syndrome (n=2), undifferentiated connective-tissue disease (n=2) and systemic sclerosis (n=2).
3. Twenty-six (53.1%) patients with a diagnosis of idiopathic pericarditis after exclusion of malignancy, tuberculosis and systemic inflammatory diseases with a median 12.3 (1.6–29.8) months follow-up

The characteristics of the patients are listed in table 1. Three sets of lupus criteria (SLE ACR-1997,[4] SLE SLICC[5] and 2019 SLE EULAR/ACR criteria) were applied in all ANA-positive patients. The 2019 SLE EULAR/ ACR criteria were met in 100% of patients with SLE, 33.3% of patients with non-SLE AID and 11.5% of patients with idiopathic pericarditis. Thus, this new set of criteria for SLE offered a higher sensitivity (100%) but a lower specificity (84.38%) as compared with the former criteria, for the diagnosis of SLE in patients with pericardial effusion (online supplementary table S1). Interestingly, the 2019 SLE EULAR/ACR classification score was higher in SLE patients (median: 30 (11–45)) as compared with non-SLE AID (median: 8 (6–12), p=0.0006) and idiopathic pericarditis patients (median: 6 (5–12), p<0.00001). Moreover, the 2019 classification set score strongly correlated with the Systemic Lupus Erythematosus Disease Activity Index activity score[6] as shown online supplementary figure S1 (R^2 =0.8105, p<0.00001). Setting the 2019 SLE EULAR/ACR classification threshold score >12 (out of a theoretical maximum of 51) instead of ≥10 increased the specificity of 2019 SLE EULAR/ ACR criteria from 84.38% to 100%. Overall, in patients with pericardial effusion and positive ANA, the diagnosis of SLE could be ruled out when 2019 SLE EULAR/ACR criteria score was <10 and confirmed when the score was >12.

Table 1 Patients clinical and biological characteristics

	SLE n=17	Non-SLE AID n=6	Idiopathic n=26	P value*
General features				
Median age at diagnosis, years (range)	32.4 (18.4–46.7)	47.2 (30.5–61.1)	51.4 (24.5–79.9)	0.028
Female, n (%)	17 (100)	6 (100)	11 (42.3)	0.0001
SLEDAI, median (range)	15 (6–38)	–	–	–
Clinical features, n (%)				
Fever	5 (29.4)	1 (16.7)	7 (26.9)	1.000
Pericardial effusion†	17 (100)	6 (100)	26 (100)	1.000
Acute pericarditis‡	8 (47.1)	5 (83.3)	21 (80.8)	0.044
Cardiac tamponade§	0 (0)	1 (16.7)	15 (57.7)	0.0001
Pleural effusion	10 (58.8)	5 (83.3)	19 (73.1)	0.507
Non-scarring alopecia	0 (0)	0 (0)	0 (0)	1.000
Oral ulcers	2 (11.8)	0 (0)	0 (0)	0.151
Subacute cutaneous lupus	1 (5.9)	0 (0)	0 (0)	0.395
Acute cutaneous lupus	2 (11.8)	0 (0)	0 (0)	0.151
Delirium	1 (5.9)	0 (0)	0 (0)	0.395
Psychosis	0 (0)	0 (0)	0 (0)	1.000
Seizure	2 (11.8)	0 (0)	0 (0)	0.151
Joint involvement	11 (64.7)	2 (33.3)	0 (0)	<0.00001
Biological features, n (%)				
Leucopenia	6 (35.3)	0 (0)	0 (0)	0.002
Thrombocytopenia	5 (29.4)	0 (0)	0 (0)	0.006
Autoimmune haemolysis	2 (11.8)	0 (0)	0 (0)	0.151
Proteinuria >0.5 g/24	11 (64.7)	0 (0)	1 (3.8)	0.006
Histological features¶, n (%)				
Class II or V lupus nephritis	3 (17.6)	0 (0)	0 (0)	0.055
Class III or IV lupus nephritis	6 (35.3)	0 (0)	0 (0)	0.002
Immunological features, n (%)				
ANA** ≥1/80	17 (100)	60(100)	26 (100)	1.000
ANA≥1/160	17 (100)	60(100)	13 (50)	0.0004
ANA≥1/320	17 (100)	60(100)	6 (23.1)	<0.00001
ANA≥1/640	16 (94.1)	5 (83.3)	4 (15.4)	<0.00001
ANA≥1/1280	12 (70.6)	4 (66.7)	1 (3.8)	<0.00001
Anti-dsDNA antibodies	13 (76.5)	0 (0)	2 (7.7)	<0.00001
Anti-Sm antibodies	3 (17.6)	0 (0)	0 (0)	0.055
aPL antibodies††	9 (52.9)	0	5 (19.2)	0.044
Low C3	12 (70.6)	0 (0)	0 (0)	<0.00001
Low C4	12 (70.6)	1 (16.7)	0 (0)	<0.00001
Sets of criteria, n (%)				
SLE ACR-1997	16 (94.1)	0 (0)	0 (0)	<0.00001
SLE SLICC	16 (94.1)	0 (0)	0 (0)	<0.00001
2019 SLE EULAR/ACR	17 (100)	2 (33.3)	3 (11.5)	<0.00001
Score, median (range)	30 (11–45)	8 (6–12)	6 (5–12)	<0.00001

*As compared between SLE and idiopathic and determined using Mann-Whitney test for continuous variables and Fisher's test for categorical variables.
†Pericardial effusion defined as ultrasound evidence for pericardial effusion.
‡Acute pericarditis defined as pericardial chest pain associated with pericardial effusion on ultrasound.
§Cardiac tamponade defined as a life-threatening compression of the heart requiring surgical drainage.
¶Histological features on renal biopsy according to ISN/RPS 2003 classification.
**Antinuclear antibodies (ANA) titer measurement on Hep-2 cells.
††aPL antibodies included anticardiolipin antibodies (IgG or IgM) at medium or high titer (>40 GPL or MPL units) and/or positive anti-β2GP1 antibodies (IgG or IgM) and/or positive lupus anticoagulant.
ACR, American College of Rheumatology; AID, autoimmune disease;ANA, antinuclear antibodies on Hep-2 cells; aPL, antiphospholipid; C3 and C4, serum complement components C3 and C4; dsDNA, double-stranded DNA;SLE, systemic lupus erythematosus;SLEDAI, Systemic Lupus Erythematosus Disease Activity Index; SLICC, Systemic Lupus International Collaborating Clinics; Sm, Smith.

Comparative analysis showed that SLE patients were younger, more frequently female and had higher titers of ANA while cardiac tamponade was more frequent in idiopathic pericarditis. Interestingly, non-SLE AID features appeared to belong to a spectrum between SLE and idiopathic pericarditis.

In conclusion, this study shows that the new 2019 SLE EULAR/ACR criteria for SLE are helpful in clinical practice for the diagnosis of SLE in patients admitted for pericardial effusion.

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Response to: 'New 2019 SLE EULAR/ACR classification criteria are valid for identifying patients with SLE among patients admitted for pericardial effusion' by Sacre *et al*

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In their letter, Dr Sacre and colleagues[1] describe an interesting retrospective study on 129 patients with pericardial effusion, of whom 17 were diagnosed with systemic lupus erythematosus (SLE). The authors arrive at a reassuring sensitivity of 100% for the new European League Against Rheumatism/American College of Rheumatology (EULAR/ACR) 2019 classification criteria.[2 3] However, specificity was clearly lower at 84%, below the specificity of the ACR and the Systemic Lupus International Collaborating Clinics (SLICC) criteria. The latter is a somewhat unexpected result. Based on the experience of the last months, the most common reasons for suboptimal specificity in applying the EULAR/ACR 2019 criteria are incorrect attribution to SLE and reliance on non-specific serological tests. Given the data presented by Dr Sacre and colleagues,[1] both may also have played a role in misclassifying 2 of 6 patients with other autoimmune disease and 3 of 26 patients with idiopathic pericarditis as having SLE.

In addition to serositis, EULAR/ACR classification criteria items in non-SLE patients were limited to fever, joint involvement and low C4 in patients with other autoimmune diseases.[1] By attribution rule,[2–4] these items should not be counted for SLE if due to another autoimmune disease. Similarly, patients without autoimmune disease had fever and proteinuria besides serositis. Again, these should not be counted if in fact attributed to an infection.

Moreover, 2 of 26 patients without an autoimmune disease were reported to have antibodies against double-stranded DNA (dsDNA). This should depend on an assay with at least 95% specificity against relevant disease controls,[2 3] usually a Crithidia test or radioimmune assay. Such test would be unlikely to become positive in idiopathic pericarditis. Anti-dsDNA tests of lower specificity, on the other hand, should not be counted.

While we thank Dr Sacre and colleagues[1] for their interesting data and while the sensitivity results are reassuring, we would like to remind authors of the importance of following the attribution rule of the EULAR/ACR criteria, that the criteria items should only be counted if there is no more likely alternative explanation.[2–4] Since this single rule has replaced the exclusion criteria for individual items, not following the attribution rule will inevitably lead to underestimating specificity when the new SLE classification criteria are applied.

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Development and initial validation of diagnostic gene signatures for systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a complex and heterogeneous rheumatic disease with variable clinical features. The correct diagnosis of SLE is still challenging, partially due to the complexity and heterogeneity of SLE pathogenesis. A new classification criteria for SLE with excellent sensitivity and specificity has been recently proposed by the European League Against Rheumatism and the American College of Rheumatology.[1] Apart from autoantibodies such as anti-double-stranded DNA antibodies, novel molecular biomarkers may help to improve the performance of SLE classification criteria, but they are not included in the new classification criteria, which is largely attributed to the limited availability in the clinical setting or insufficient evidence.[1] Transcriptome studies using either microarray or RNA sequencing mainly aim to investigate the aberrant RNA expression levels of genes on a genome-wide scale, and have been widely used in the clinical research of rheumatic diseases. Studies using transcriptome analysis also have provided deeper insights into the pathogenic mechanism of SLE. Differently expressed genes (DEGs) derived from microarray-based peripheral blood transcriptome data can also be used as diagnostic or prognostic biomarkers for autoimmune diseases, but their use in SLE is still not well established.[2 3] In this study, we aimed to develop a genetic signature for SLE diagnosis through bioinformatic analyses of whole blood transcriptome data.

To overcome the limited clinical utility caused by the low consistency and the risk of noise discovery in microarray-based studies, robust rank aggregation (RRA) analysis was used to integrate data from multiple transcriptome datasets. RRA is a useful integration approach in pooling data from heterogeneous datasets and can help to identify the mostly aberrantly expressed genes between patients with SLE and controls across multiple datasets, which may lead to a SLE diagnostic gene signature with both high reproducibility and high stability.[4] Fourteen whole blood transcriptome datasets with at least 20 patients with SLE and 10 controls were integrated with RRA, which included GSE110685, GSE112087, GSE99967, GSE110169, GSE88884, GSE65391, GSE72509, GSE45291, GSE49454, GSE61635,

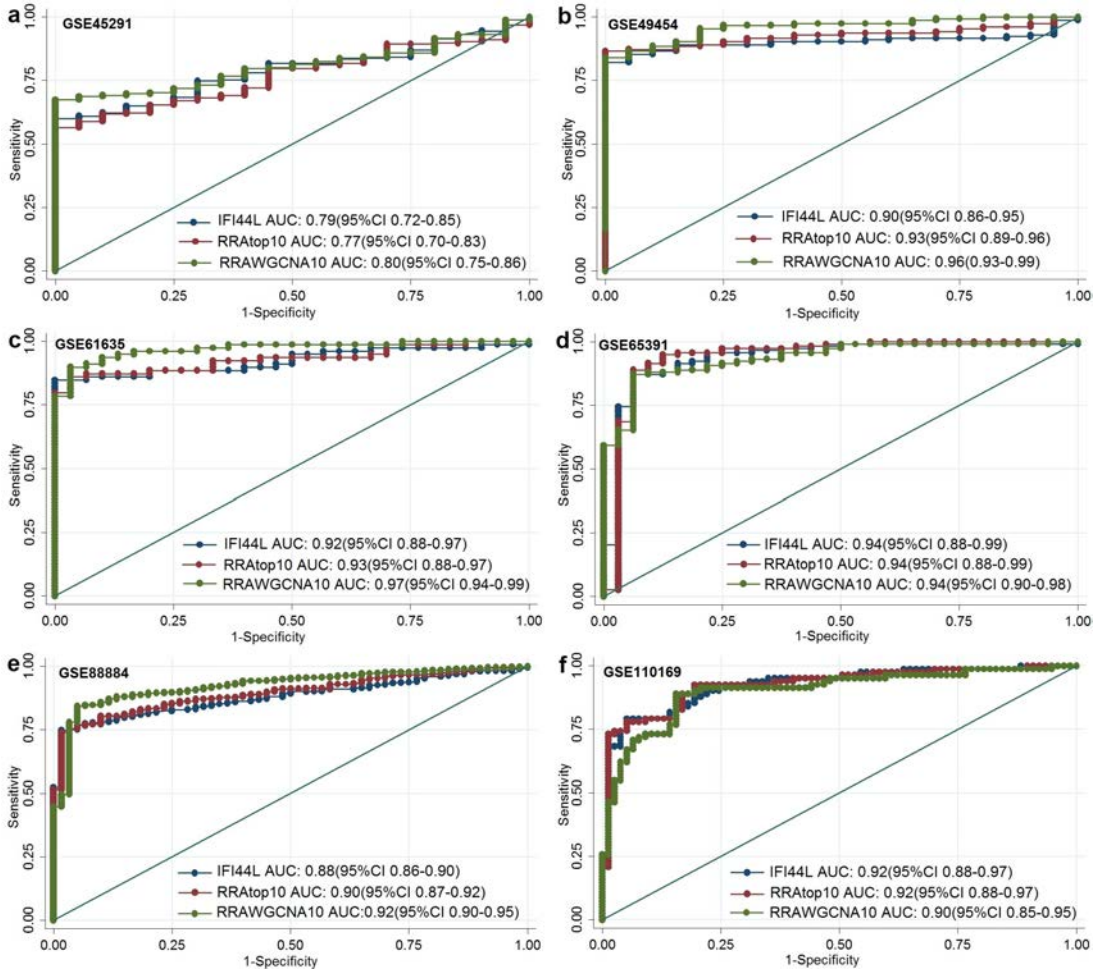

Figure 1 Assessment of the diagnostic performance of gene signatures in systemic lupus erythematosus (SLE) in six microarray datasets. (A) Diagnostic performance of RRAtop10, RRAWGCNA10 and IFI44L alone in GSE45291 (292 patients with SLE and 20 controls). (B) Diagnostic performance of RRAtop10, RRAWGCNA10 and IFI44L alone in GSE49454 (157 patients with SLE and 20 controls). (C) Diagnostic performance of RRAtop10, RRAWGCNA10 and IFI44L alone in GSE61635 (79 patients with SLE and 30 controls). (D) Diagnostic performance of RRAtop10, RRAWGCNA10 and IFI44L alone in GSE65391 (118 patients with SLE and 32 controls). (E) Diagnostic performance of RRAtop10, RRAWGCNA10 and IFI44L alone in GSE88884 (1760 patients with SLE and 60 controls). (F) Diagnostic performance of RRAtop10, RRAWGCNA10 and IFI44L alone in GSE110169 (82 patients with SLE and 77 controls). Receiver operating characteristic curve analyses were performed using the enrichment score of RRAtop10 and RRAWGCNA10 in each individual calculated through Gene Set Variation Analysis. AUC, area under the curve.

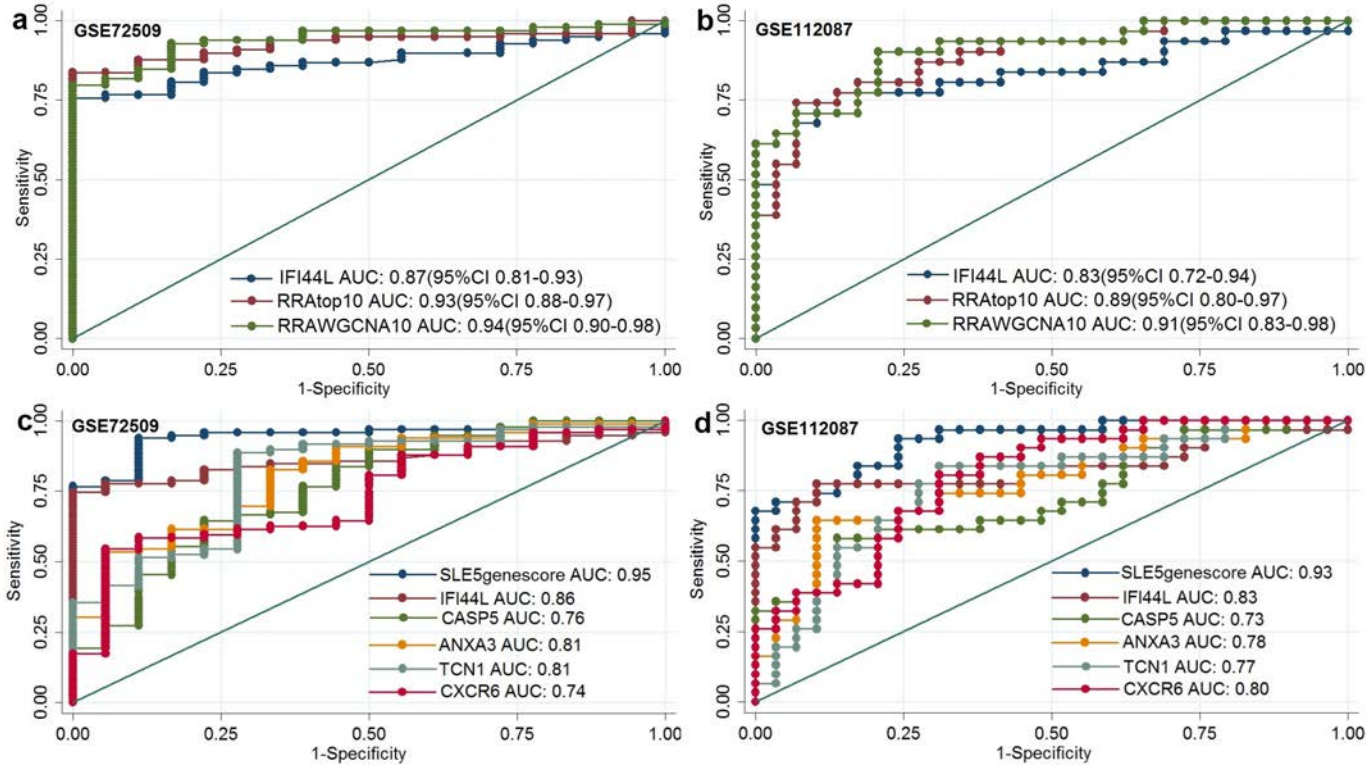

Figure 2 Assessment of the diagnostic performance of gene signatures in systemic lupus erythematosus (SLE) in two datasets using RNA sequencing. (A) Diagnostic performance of RRAtop10, RRAWGCNA10 and IFI44L alone in GSE72509 (99 patients with SLE and 18 controls). (B) Diagnostic performance of RRAtop10, RRAWGCNA10 and IFI44L alone in GSE112087 (31 patients with SLE and 29 controls). (C) Comparison of SLE5genescore with single gene in diagnosing SLE in the discovery dataset GSE72509 (99 patients with SLE and 18 controls). (D) Comparison of SLE5genescore with single gene in diagnosing SLE in the validation dataset GSE112087 (31 patients with SLE and 29 controls). Receiver operating characteristic curve analyses were performed using the enrichment score of RRAtop10 and RRAWGCNA10 in each individual calculated through Gene Set Variation Analysis, and SLE5genescore was calculated through the log2-transformed TPM expression values of five genes including IFI44L, CASP5, ANXA3, TCN1 and CXCR6. AUC, area under the curve.

GSE50635, GSE39088, GSE20864 and GSE17755. RRA outcomes suggested that most of those top 100 DEGs were from type I interferon–related pathways such as IFI44L, IFI27 and IFIT1. The top 10 upregulated genes included IFI44L, IFI27, RSAD2, IFIT1, HERC5, IFIT3, IFI44, OASL, CMPK2 and USP18 and were all from type I interferon–related pathways, which were preliminarily selected as one SLE diagnostic genetic signature referred to as RRAtop10 in this study.

Because gene signatures developed by combining genetic biomarkers from multiple functional modules may have the ability of leading to a more accurate diagnosis than gene signature derived from one single co-expression module,[3] the co-expression pattern of those top 100 upregulated DEGs in GSE88884 (1760 patients with SLE and 60 controls) was further analysed using weighted gene coexpression network analysis (WGCNA).[5] Based on the gene co-expression modules calculated above, a more complex gene signature was developed by selecting at most 10 genes from each upregulated co-expression module. This complex gene signature (referred to as RRAWGCNA10) consisted of 34 key genes from six independent co-expression modules and included IFI44L, IFI27, RSAD2, IFIT1, HERC5, IFIT3, IFI44, OASL, CMPK2, USP18, LHFPL2, RRM2, CEACAM6, CEACAM8, DEFA4, HP, LCN2, MMP8, OLFM4, OLR1, RNASE2, TCN1, ANKRD22, CASP5, CEACAM1, CLEC4D, DHRS9, DYNLT1, FCGR1B, TLR5, TNFAIP6, TNFSF13B, ANXA3 and SLC26A8. Among those 34 genes, the first 10 genes were identical to those genes in RRAtop10 and were all from the same co-expression module. Based on the enrichment score of these genetic signatures in each individual calculated through Gene Set Variation Analysis, the diagnostic role of genetic signatures was assessed by receiver operating characteristic curve analysis.[6] To evaluate the performance of those diagnostic gene signatures, the area under the curve (AUC) was calculated in six validation datasets using microarray (GSE45291, GSE49454, GSE61635, GSE65391, GSE88884 and GSE110169) and two validation datasets using RNA sequencing (GSE72509 and GSE112087). The performance of those two diagnostic gene signatures was compared with IFI44L, which was the top upregulated gene in the RRA analysis.

As shown in figures 1 and 2, as the most aberrantly expressed gene in the whole blood of patients with SLE, IFI44L alone could provide some assistance in diagnosing SLE with AUCs arranging from 0.79 and 0.94 among those six microarray datasets (figure 1A–F) and two RNA-sequencing datasets (figure 2A,B). Compared with IFI44L alone, RRAtop10 had a better performance in diagnosing SLE in only two datasets including GSE88884 (p=0.02; figure 1E) and GSE72509 (p=0.02; figure 2A), but not in the other six datasets such as GSE45291 (p=0.35; figure 1A) and GSE112087 (p=0.10; figure 2B). Compared with IFI44L alone, RRAWGCNA10 had a better performance in diagnosing SLE in four datasets including GSE49454 (p=0.01; figure 1B), GSE61635 (p=0.03; figure 1C), GSE88884 (p=0.0001; figure 1E) and GSE72509 (p=0.03; figure 2A), but not in the other four datasets such as GSE45291 (p=0.80; figure 1A) and GSE112087 (p=0.18; figure 2b). In addition, RRAWGCNA10 also had better

performance than RRAtop10 in diagnosing SLE in two datasets including GSE61635 (p=0.04; figure 1C) and GSE88884 (p=0.005; figure 1E), and had comparable performance with RRAtop10 in the other datasets. The outcomes above supported that gene signatures derived from whole blood transcriptional profiles could provide some assistance to the diagnosis of SLE, and gene signatures consisting of genes from multiple co-expression modules may have better diagnostic performance.

To improve the clinical application potential of SLE gene signatures, a simpler gene signature was developed using multivariate logistic regression analysis. Those genes analysed in the logistic regression analysis were key genes from main co-expression modules related to SLE in the WGCNA above, and only one candidate gene was selected from each module. Among those two RNA-sequencing datasets, GSE72509 (99 patients with SLE and 19 controls) was selected as the discovery dataset and GSE112087 (31 patients with SLE and 29 controls) was selected as the validation dataset. Owing to the obvious difference in data form between RNA-sequencing data and microarray data, datasets using microarray were not analysed in this part. Through logistic regression analysis in GSE72509, a five-gene diagnostic score for SLE (SLE5genescore) was developed through the log2-transformed transcripts per million (TPM) expression values of five genes including IFI44L, CASP5, ANXA3, TCN1 and CXCR6. The five-gene diagnostic score was calculated using the following formula: $0.76\times$IFI44L$+0.18\times$CASP5 $+0.87\times$ANXA3$+0.74\times$TCN1$-0.53\times$CXCR6. As shown in figure 2, SLE5genescore had an obvious better performance than single gene in diagnosing SLE in both the discovery dataset (GSE72509, AUC=0.95 (95% CI 0.90 to 0.99), p<0.05; figure 2C) and the validation dataset (GSE112087, AUC=0.93 (95% CI 0.87 to 0.99), p<0.05; figure 2D).

In summary, this study developed useful gene signatures for SLE diagnosis through bioinformatic analyses of whole blood transcriptomic data, which could effectively differentiate SLE in different datasets and may provide some assistance in the classification of SLE. Nevertheless, more studies are needed to further validate their performance in diagnosing SLE in different clinical settings.

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Response to: 'Development and initial validation of diagnostic gene signatures for systemic lupus erythematosus' by Wang *et al*

In their letter, Dr Wang and colleagues[1] correctly remark that no novel molecular biomarkers were included in the European League Against Rheumatism/American College of Rheumatology (EULAR/ACR) 2019 classification criteria for systemic lupus erythematosus (SLE), although a variety of such markers were considered in the process, including the type I interferon signature.[2 3] As cited by Wang *et al*,[1] sufficient evidence and worldwide availability were of importance for inclusion into the list of classification criteria items.

Their approach presently is on the opposite side of the field, where early hypotheses are generated. Dr Wang and colleagues[1] used robust rank aggregation for analysing multiple transcriptome data sets. Essentially all hits in this approach were interferon-regulated genes. Accordingly, the authors worked at enriching for genes of other modules, and over several steps arrived at a five-gene score, which was superior to any single gene in distinguishing SLE from healthy individuals.

This approach is interesting, and may in the end lead to markers relevant for diagnosis, as discussed by Dr Wang *et al*,[1] but eventually also for classification. However, testing against various other autoimmune diseases, such as in the EULAR/ACR SLE classification project,[2 3] has not yet been shown. This in our view is relevant given, for example, the presence of an interferon signature also in other autoimmune diseases.[4–6] Once this is successfully done, it will be interesting to see whether the combination of established criteria and such novel markers further improve classification.

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NCF1-339 polymorphism and systemic lupus erythematosus

We read the publication on 'NCF1-339 polymorphism is associated with altered formation of neutrophil extracellular traps, high serum interferon activity and antiphospholipid syndrome (APS) in systemic lupus erythematosus (SLE)' with great interest.[1] Linge *et al* concluded that "we revealed a striking connection between the ROS deficient NCF1-339 genotypes and the presence of phospholipid antibodies and APS".[1] The NCF1-339 rs201802880 polymorphism is a single mutation that might cause the molecular change. Due to the mutation, the molecular weight change and the alteration of the phenotypic expression is the result. In the present report, Linge *et al* studied on only one genetic polymorphism and did not assess the possible effects of other genetic polymorphisms that might have clinical association with SLE. The examples of those genetic polymorphisms are paraoxonase-1 gene and PAL-1 polymorphisms.[2 3] Further studies to access the possible confounding factors of other genetic polymorphisms are required.

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Response to: 'NCF1-339 polymorphism and systemic lupus erythematosus' by Joob and Wiwanitkit

In the comment by Joob and Wiwanitkit,[1] questions are raised regarding the role of the NCF1-339 rs201802880 polymorphism in systemic lupus erythematosus (SLE) in our recent publication.[2] One concern relates to the possibility of a molecular weight change due to the polymorphism and if this could alter phenotypic expression. Studies of functional effects of the polymorphism in question have been demonstrated both in this report as well as in earlier work by our group.[3 4] NCF1 ($p47^{phox}$) plays an vital role in the assembly and stability of the extensively studied nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2 complex[5] and the nucleotide shift from C to T at NCF1-339 alters the amino acid from arginine to histidine at a PX domain of the NCF1 protein.[6] This domain has been shown to be of crucial importance in the membrane binding and mutations in the NCF1-339 position reduces reactive oxygen species (ROS) response,[4 7] providing a mechanistic explanation for the observed ROS-decreasing effect of the polymorphism. We have not considered comparing molecular weight of polymorphic NCF1 and non-polymorphic molecules. Placing a polymorphism in a functional context provided by current and previous findings is far more relevant in our opinion. We find it unlikely that a potential molecular weight difference would add any significant information relevant to the biological/pathological context presented.

A second concern was raised about the potential role of other polymorphisms and possible functional consequences. Joob and Wiwanitkit are particularly interested in the putative effects of polymorphisms in the paraoxonase-1 gene and PAL-1. Our aim with this study was to determine the functional effect of NCF1-339 polymorphisms on neutrophil extracellular traps, type I interferon activity and to find potential associations with clinical phenotypes in SLE. The striking association between NCF1-339 polymorphisms and the antiphospholipid syndrome does not exclude associations with other polymorphisms but this was beyond the scope of this investigation.

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Regulatory T cell frequencies in patients with rheumatoid arthritis are increased by conventional and biological DMARDs but not by JAK inhibitors

Regulatory T (Treg) cells play an important role in controlling immune responses. Their frequency is decreased in many autoimmune diseases, including rheumatoid arthritis (RA). We read with great interest the article by Rosenzwajg *et al* which presents the results of a clinical trial with low-dose interleukin-2 (ld-IL-2).[1] The authors report that Treg cell frequencies were significantly increased following ld-IL-2 administration in 46 patients with autoimmune diseases. Among them, four patients had RA and received a background therapy with low-dose prednisolone (PRED) or methotrexate (MTX). The study demonstrates that ld-IL-2 administration is a successful strategy to overcome Treg cell deficiency and to increase the ratio between Treg cells and effector T cells in patients with RA and other autoimmune diseases.

Here we want to draw attention to the fact that Treg cell frequencies in patients with RA can be increased by some, but not by all, antirheumatic drugs and that the background treatment can therefore affect the results of clinical trials with IL-2. Using flow cytometry, we analysed the ex vivo frequency of $CD25^{high}CD127^{low}FoxP3^{high}$ $CD4^+$ Treg cells in the peripheral blood of 112 patients with RA and 19 healthy individuals (figure 1A). To confirm the suppressive capacity of the Treg cells, we assessed their ability to suppress effector T cells using a classical Treg suppression assay (figure 1B, C). Our results and previous findings from other groups demonstrate that MTX (≥15 mg/week) and various biological disease-modifying anti-rheumatic drugs (DMARDs) efficiently upregulate Treg cell frequencies to an almost normal level (figure 1D).[2–5] We observed a significant increase in Treg cell frequencies in the peripheral blood of patients treated with MTX, adalimumab, etanercept, golimumab and tocilizumab. Concomitant medication with MTX did not further increase the percentage of Treg cells in patients treated with the biologicals. The RA patients in the study by Rosenzwajg *et al* were either treated with low-dose PRED (<15 mg/day) or with MTX (≤20 mg/week). Interestingly, the fold change from baseline in Treg cells reported by Rosenzwajg *et al* was higher in the low-dose-PRED group compared with the group with MTX background therapy. This could probably be due to higher

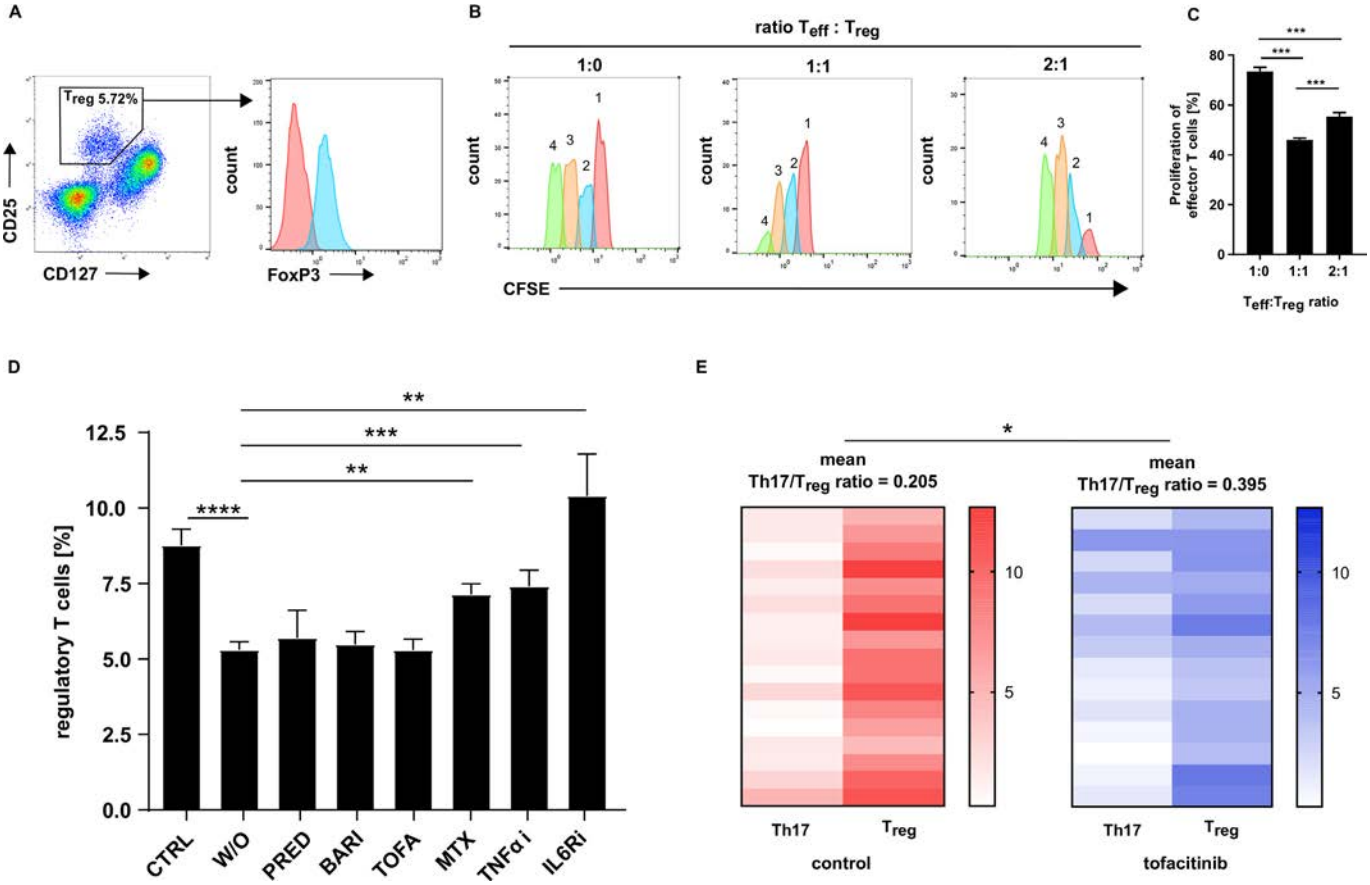

Figure 1 Frequency of regulatory T (Treg) cells in the peripheral blood of patients with rheumatoid arthritis. (A) CD4 +T cells were analysed ex vivo by flow cytometry. A representative example of the gaiting strategy is shown. (B,C) A representative example of a classical Treg suppression assay is shown. The assay was performed as described previously.[7] (D) Percentage of $CD25^{high}CD127^{low}FoxP3^{high}$ Treg cells in patients treated with low-dose prednisolone (<15 mg/day) (PRED, n=10), baricitinib (BARI, n=29), tofacitinib (TOFA, n=19), methotrexate (≥15 mg/week) (MTX, n=24), tumor necrosis factor-α inhibitors (TNF-αi, n=10: adalimumab (n=4), etanercept (n=4) and golimumab (n=2)) or interleukin (IL)-6R inhibitors (IL-6Ri, tocilizumab, n=10). Healthy individuals (CTRL, n=19) and untreated patients (W/O, n=10: first diagnosis n=8 and untreated for ≥6 weeks n=2) served as controls. (E) Th17/Treg cell ratio in healthy controls (red heat map) and patients treated with tofacitinib (blue heat map); *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; data are presented as mean±SEM; significant differences were determined using the Kruskal-Wallis test and the unpaired Mann-Whitney test. CFSE, carboxyfluorescein succinimidyl ester.

baseline Treg cell levels in the MTX group. In striking contrast to MTX and biological DMARDs, we observed no increase in Treg cell frequencies in patients treated with the Janus kinase (JAK)1/2 inhibitor baricitinib or the JAK1/3 inhibitor tofacitinib (figure 1D). Additional treatment with MTX had no influence on the results.

The peripheral blood of patients with RA is characterised by a higher percentage of Th17 cells and the balance between Th17 cells and Treg cells is shifted.[6] In RA patients, the Th17/Treg cell balance is not recovered by JAK inhibitors, although the percentage of Th17 cells is significantly suppressed (figure 1E). Even though JAK inhibition is an efficient treatment in RA, an increase in Treg cells could be beneficial for the patients. The influence of combined treatment with ld-IL-2 and JAK inhibitors on Treg cells has not been investigated yet. However, it is likely to be very low as JAK1 and JAK3 are located downstream of the IL-2 receptor. Taken together, our data confirm that MTX and biological DMARDs increase Treg cell frequencies. Moreover, they reveal that the percentage of Treg cells is not modified by JAK inhibitors. We suggest that the ability of background therapy to increase Treg cell frequencies is a critical factor that should be taken into account when planning future clinical studies on ld-IL-2 treatment.

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Contributors AM, PSW, and DMK made substantial contributions to the study concept and design. AM, PSW, KK, JS, JvT and THH made substantial contributions to the acquisition of the data. AM, PSW, KK, JS, JvT, THH and DMK drafted the article or revised it critically for important intellectual content. All authors reviewed the draft and approved the submission of the manuscript.

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Response to: 'Regulatory T cell frequencies in patients with rheumatoid arthritis are increased by conventional and biological DMARDs but not by JAK inhibitors' by Meyer *et al*

Commenting on Meyer *et al*[1] our recent publication[2] 'Immunological and clinical effects of low-dose interleukin-2 (IL-2) across 11 autoimmune diseases in a single, open clinical trial', Dr Meyer and colleagues point to the issue of concomitant background therapies that could affect the effects of low-dose IL-2. They reported a significant increase of Treg cell frequencies in peripheral blood of patients with rheumatoid arthritis treated with methotrexate, adalimumab, etanercept, golimumab and tocilizumab, which they discuss could affect the increase expected after an IL-2 treatment.

Only four rheumatoid arthritis patients were included in our study, all being treated with low-dose corticosteroids and/or low doses of immunosuppressants. Their baseline Treg evaluation was similar to that of patients with other diseases treated with similar background therapies (figure 1A). In addition, in the whole cohort of patients, we did not observe significant differences in baseline Treg values for patients receiving (1) 'non-specific immunological therapy' (including medications like nonsteroidal anti-inflammatory drugs for joint pain, or ursodeoxycholic acid for sclerosing cholangitis); (2) low-dose corticosteroids and/or low doses of immunosuppressants; compared with patients receiving (3) anti-tumour necrosis factor-α (TNFα) or (4) 'combination of biological disease modifying antirheumatic drug and low doses of immunosuppressants' (figure 1B). Furthermore, as reported in our study, there were no significant differences in the Treg response to low-dose IL-2 according to the background treatment.[2]

We believe that baseline levels of Tregs in peripheral blood cannot be a criteria for low-dose IL-2 treatment decision nor a robust enough biomarker of the clinical response. First, IL-2 treatment should not be limited to patients (or diseases) with qualitative or quantitative Treg deficiency as the mere presence of an autoimmune disease signals a Treg insufficiency, that is, that Tregs are unable to control the effector immune response as they should[3]; second, Treg peripheral blood levels may not accurately reflect Treg counts at sites where they are needed for therapeutic efficacy, that is, tissues or draining lymph nodes; third, IL-2 treatment not only expands Tregs, but also improves their fitness[2 4] and reshapes their T cell receptor repertoire, both parameters likely playing a role in IL-2 therapeutic activity.

IL-2 has pleiotropic[3] dose-dependent[4] biological activities and patients show some heterogeneity in response to it. Future studies will have to look for biomarkers of IL-2 activity through multiple omics studies, so as to optimise precision medicine-driven modalities.

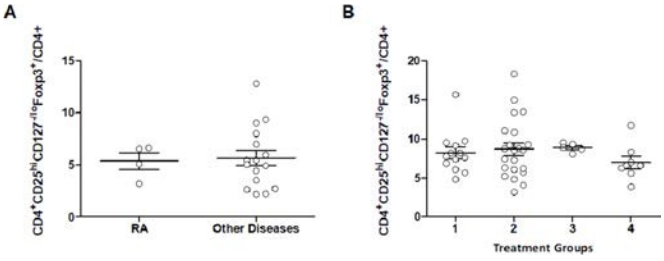

Figure 1 Percentage of Tregs at baseline in patients included in the TRANSREG study.[2] (A) Treg percentages in rheumatoid arthritis patients compared with all other patients receiving low-dose corticosteroids and/or low doses of immunosuppressants. (B) Comparison of baseline Treg percentages in patients according to their background treatment: group 1: non-specific immunological therapy; group 2: low-dose corticosteroids and/or low doses of immunosuppressants; group 3: anti-TNFa; group 4: combination of biological disease-modifying antirheumatic drug and low doses of immunosuppressants. RA, rheumatoid arthritis.

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Contributors MR and RL analysed data; DK drafted the reply.

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Competing interests MR, RL, and DK are inventors for patent applications related to the therapeutic use of ld-IL2, which belongs to their academic institutions and has been licensed to ILTOO Pharma. MR and DKhold shares in ILTOO Pharma. No other potential conflicts of interest relevant to this article were reported

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Correspondence to 'Slope sign': a feature of large vessel vasculitis?

We have read with great interest the observation of Dasgupta *et al*.[1] We would like to propose an extended definition of slope sign in giant cell arteritis (GCA) formerly named slide sign,[2 3] and present our method of assessing it by using the anteromedial ultrasound examination method of the large supraaortic vessels.[4]

Slope sign is a pathologically increased intima-media thickness (IMT)[5] that spreads over a long arterial segment and slides down to a normal brachial artery where a normal intima-media structure (double line) is observed. This definition contains a description of pathological findings in the axillary artery—a typical location for vasculitis in GCA, contrasting with normal findings in brachial artery—which is usually not involved by vasculitis.[6] Slope sign may not be limited to the place of arterial bifurcation of the subscapular artery, which is a typical location of atherosclerotic plaque. A transition zone should be observed between the involved axillary artery and the uninvolved brachial artery to generate the slope sign. Consequently, visualisation of this zone in a single image helps to conclude on the presence of pathological IMT compared with the nearby normal vessel (figure 1). In addition to thickness, wall structure should be assessed: in a normal brachial artery intima-media appears as a double line, which disappears due to the inflammation in the axillary artery.[7] Thus, the slope sign is best-observed at a long longitudinal view that avoids skipping areas or inadequate imaging from a series of short scans.

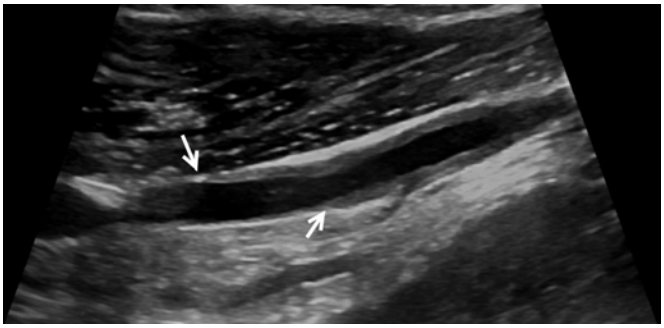

Figure 1 Tapering IMT leading to normalisation of vasculitic changes at the brachial artery at the point indicated by arrows.

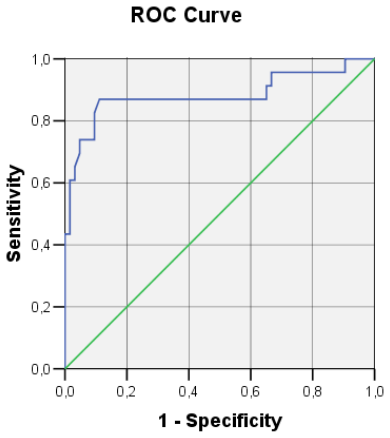

Figure 2 Performance of axillary to brachial IMT ratio for the diagnosis of axillary arteritis. ROC, receiver operating characteristic.

Table 1 Cut-off values for slope sign (axillary to brachial IMT ratio) and increased IMT in the axillary artery (GCA patients vs controls)

	Area under the curve	Optimal cut-off	Sensitivity (%)	Specificity (%)
Slope sign (axillary to brachial IMT ratio)	0.883	2.05	87.0	88.9
Increased IMT (axillary artery)	0.969	0.81 mm	87.0	93.7

Maximal IMT value from bilateral ultrasound measurements was chosen. Minimal difference between sensitivity and 1–specificity was chosen for optimal IMT cut-off values for vasculitis.
GCA, giant cell arteritis; IMT, intima-media thickness.

The statement that the slope sign 'may help to differentiate vasculitis from arteriosclerosis and other causes of arterial wall thickening'[1] requires some attention. Lack of slope sign is well recognisable in the cases of general arterial wall thickening, for example, calciphylaxis or amyloidosis.[8] However, the visualisation of a short transition zone may not be enough to differentiate the edge of vasculitis from non-calcified atherosclerotic plaque. Atherosclerosis is common and sometimes involves the axillary artery at the level of bifurcation of the subscapular artery (a region of turbulent flow predisposing to atherosclerosis). Yet, this is also a typical location of the slope sign in vasculitis. Therefore, we recommend examining the long course of axillary artery (both proximal and distal part) by using the anteromedial method (continuous ultrasonographic examination of the large supraaortic vessels), as vasculitis usually spreads along the whole arterial region of the axillary artery, up to the subclavian artery in contrast with atherosclerosis. The length of the slope in vasculitis is usually long, while atherosclerosis presents with a short slope.

Validation of the slope sign in 214 consecutive patients referred to fast track GCA clinic in Szczecin between 2011 and 2015 was performed. Out of 81 patients diagnosed with GCA axillary vasculitis was found in 23. In 50 patients, isolated PMR was diagnosed. In 83 patients, another diagnosis was confirmed and they served as controls. Lack of healthy controls is consistent with a real-life scenario but might had impact on results. In all patients with axillary vasculitis, slope sign was present. We calculated the slope sign reference range defined as axillary to brachial IMT ratio (figure 2). Statistical analyses were performed with STATA software (version 12.0; StataCorp). The area under the curve for the slope sign ratio was smaller compared with increased axillary IMT consistent with vasculitis (table 1).

Consequently, we think that the definition of the ultrasonographic slope sign should be descriptive and we agree with Dasgupta *et al* that slope sign is a helpful feature for large vessel GCA diagnosis.

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Contributors MM: idea of slide/slope sign, material collection, presentation of the calculations and preparation of the manuscript. APD: idea of writing a commentary, images collection, idea of anteromedial ultrasound examination method and

preparation of the manuscript. MB: preparation of the manuscript. ABH: images collection and preparation of the manuscript.

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Response to: 'Correspondence to 'Slope sign': a feature of large vessel vasculitis?' by Milchert *et al*

We are grateful for the interest in our article and agree with Milchert *et al*[1] that the slope sign deserves a precise definition. Intimal medial thickness (IMT) of the axillary artery is the current way of assessing large vessel giant cell arteritis (LV-GCA) by ultrasound (US), with a cut-off value of greater than 1.0 mm suggesting LV-GCA.[2] Other causes of arterial wall thickening can mimic vasculitis such as atherosclerosis and it is often challenging to differentiate between atherosclerosis and vasculitis related thickening of the axillary artery.[3] 'Slope sign' is a visual definition of a smooth, homogenous, gradual transition from an abnormal axillary arterial segment with increased IMT to an arterial segment with normal IMT. This should help differentiate between vasculitic and atherosclerotic wall thickening.[4] We suggest that sonographers should seek to demonstrate the slope sign in all cases of suspected LV-GCA through the use of panoramic views, if necessary, of the axillary artery from the humeral neck to the origin of the circumflex branch.

We agree with Milchert *et al*[1] that, in LV-GCA the slope is usually long. However, we disagree that atherosclerosis can demonstrate a sharp or short slope. Our observations are that abrupt, atherosclerotic thickening involves a focal area of arterial wall and therefore cannot be mistaken for a slope. We also have observed that the echotexture of the vasculitic IMT is hypoechoic which transitions smoothly to a normal echotexture. The retrospective analysis by Milchert *et al*[5] validates our finding. However, we do not think the axillary-brachial IMT ratio is a surrogate for the slope sign since it does not assess the gradual transition which is a critical part of the definition.

We agree that this sign requires validation and assessment as a marker of disease extent in prospective studies. Our ongoing multicentre HAS GCA study (National Institute for Health Research number 264294), exploring quantitative US halo score as a prognostic and monitoring tool in GCA, has the assessment of slope sign as one of its objectives.

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Clarification regarding the statement of the association between the recombinant zoster vaccine (RZV) and gout flares

We have read with great interest the article addressing the risk of gout flares after vaccination in a case crossover study by Yokose *et al*.[1] The authors suggest that vaccines may be associated with an increased odds of gout flares potentially via activation of the NLRP3 inflammasome. The study was conducted between 2003 and 2010 prior to the availability of the recombinant zoster vaccine (RZV); however, the authors make reference to two phase III clinical trials for RZV which, they state, demonstrated a higher risk of gout flares in the vaccine group. Even though RZV was not assessed in the study by Yokose *et al*, we would like to clarify this statement about RZV and gout flares.

The two large pivotal phase III clinical trials, ZOSTER-006/ZOE-50 (NCT01165177) and −022/ZOE-70 (NCT01165229), that involved a total of 29 305 subjects ≥50 years of age who received at least one dose of RZV (n=14 645) or placebo (n=14 660) were designed to assess the efficacy and safety of the RZV vaccine, but not to statistically assess a potential risk of gout among RZV recipients.[2–4]

We confirm that the analysis of the unsolicited adverse events (AE) reported during 30 days after each vaccination showed a numerical imbalance in the reporting rate of gout. Indeed, there were 27 (0.18% (95% CI 0.12 to 0.27)) versus 8 (0.05% (95% CI 0.02 to 0.11)) (unadjusted risk rario=3.38 (95% CI 1.49 to 8.60)) subjects in the RZV and placebo groups, respectively, who experienced an AE of gout or gouty arthritis.[5] However, these are not necessarily reported 'flares' as it includes both newly diagnosed (or incident) gouts and recurrent gouts (potential flares). Any AE was collected regardless of whether it was newly diagnosed or recurrent. As part of the safety monitoring, the risk of a newly diagnosed gout was analysed separately from the risk of a recurrent gout in a descriptive analysis. Differentiation between incident and recurrent gouts was done by retrospective review of the documented patient's medical history. It is worth noting that GSK may not have had access to full patient medical history information, including that classically used for diagnosis ascertainment, such as baseline serum uric acid levels or presence of monosodium urate crystals in synovial fluid.

It is important to note that the total number of cases of incident and recurrent gout reported was low (35 reported cases in the pooled analysis). Majority of the gout events in the RZV group were non-serious and mild-to-moderate in severity. Of the total of patients reporting an episode of gout after vaccination, 19 in the RZV group and 3 in the placebo group reported an episode of gout for the first time, while 8 subjects in the RZV group versus 5 subjects in the placebo group were reportedly known to have pre-existing (chronic) gout at baseline and experienced a recurrent episode of gout (gout flare) after vaccination.[6] From the data, it appears that newly diagnosed episodes of gout were more frequent than acute gout flares, regardless of gout stage (ie, intercritical period or currently having gouty arthritis or chronic tophaceous gout). However, available data cannot be used to draw conclusions in this regard, since majority were non-serious reports for which there is inconsistent clinical data completeness for assessment, for example, in terms of dietary habits, baseline serum uric acid levels, medical history, clinical narrative and diagnostic tests. In addition, multiple factors contribute to pathophysiology of gout and the individuals who reported episodes of gout after vaccination also had various confounding factors and medical conditions that are well-known risk factors for gout (eg, diabetes, hypertension, chronic kidney disease, hypercholesterolaemia, therapy with digoxine, β-blockers, diuretics, etc), and which may have explained the occurrence of the reported gout attack. Given that the two large pivotal phase III clinical trials were not designed to statistically assess a potential risk of gout among RZV recipients, one cannot exclude that the observed imbalance might be a chance finding, considering that the prevalence of gout in RZV target population (adults 50 years of age or older) is common.[5]

In view of all these caveats, the numerical imbalance observed should be interpreted with caution, and further data are needed to investigate a possible putative association between RZV vaccination and increased risk of gout, if any. The RZV postmarketing safety surveillance includes currently under development targeted safety studies designed to investigate the risk of incident gout.

Regarding the hypothesis of a potential mechanistic link between gout and NLRP3 inflammasome activation, it should be noted that, although activation of this pathway has been reported in vitro, it is controversial whether it has a role in the adjuvant effect of alum in vivo.[7] Similarly, QS-21 is able to trigger activation of NLRP3 in vitro and caspase-1 cleavage in the lymph node draining the injection site,[8] but studies in NLRP3-deficient mice showed that this pathway has no impact on the adjuvant effect of QS-21 or QS-21-containing adjuvants in vivo.[9 10] It is important to note that, for the vaccine to be the trigger of gout symptoms, it would have to directly affect the joint environment where the inflammation occurs. Given that the immune-stimulatory effect is local to the site of injection,[11] a direct trigger of gout by adjuvanted vaccines through caspase-1 activation is an unlikely hypothesis. Uric acid may be produced locally as a result of vaccine-induced inflammatory response through the release of DNA by dying innate cells (such as neutrophils) after they have been recruited at the site of injection. This has been shown to play a role in the adjuvant effect of alum.[12] It is not known, however, whether this effect occurs in humans and whether it would be significant enough to cause an increase in uric acid level in blood. Therefore, assessing changes in circulating uric acid blood level postvaccination could help verify this hypothesis, keeping in mind that an increase in serum uric acid levels (asymptomatic hyperuricaemia) may not necessarily translate into monosodium urate crystal formation in the joints and acute gout.

While the article by Yokose *et al*[1] proposed a potential association between vaccines and gout flares, the authors also highlighted methodological and statistical limitations of the study (eg, self-reporting, representativeness of the population, lack of information on the type of vaccine administered) that should be considered when drawing conclusions based on the Yokose results. A prospective study designed to measure multiple variables, such as age and sex, medical history of gout and medications, exact time of immunizations, vaccine used, reactogenicity and so on would help assess possible risks of gout in the context of vaccination. Any finding of an association should then be complemented with mode of action and mechanistic studies designed to understand potential putative mechanisms.

Finally, we concur with the authors on the importance of what vaccination brings to public health and the continuous need to evaluate the benefit/risk balance of vaccines.

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Response to: 'Clarification regarding the statement of the association between the recombinant zoster vaccine (RZV) and gout flares' by Didierlaurent *et al*

We read with great interest the correspondence from Didierlaurent *et al*[1] regarding our recent report on the association between vaccination and risk of gout flares using a case-crossover design.[2] In particular, we appreciate the clarification that the authors provided regarding the unsolicited adverse events reported during the 30 days after each vaccination, including episodes of gout, with incident gout cases surpassing reports of recurrent gout flares. While this is a notable difference from our online case-crossover study which included only patients with known gout and assessed for recurrent gout flares, this raises the intriguing possibility of the vaccine 'unmasking' gout in susceptible individuals, whether mediated by the effect of the vaccine adjuvant on the inflammasome pathway or another mechanism. It is well-recognised that patients with incident gout have a history of chronic hyperuricemia that leads to the asymptomatic deposition of monosodium urate (MSU) crystals in and around joints long before the first clinically apparent flare of gout.[3] For example, studies of patients with asymptomatic hyperuricaemia, a prerequisite condition for the eventual development of gout, have demonstrated that approximately 25% of patients have evidence of asymptomatic MSU deposits when assessed with advanced imaging techniques such as ultrasound or dual-energy CT.[4] Thus, while the results of our study may not be directly applicable to these patients who reported incident gout after recombinant zoster vaccine vaccination, the available data to date collectively call for future studies including patients with and without existing gout.

We agree with the authors that the hypothesised mechanisms underlying the potential association between vaccination and gout flares involving the activation of the inflammasome are derived from *in vitro* studies[5 6] and has not been definitively demonstrated *in vivo*. The authors also raise the intriguing possibility of the risk of gout flares being mediated by serum urate change, similar to other known triggers for gout flares such as diuretics[7 8] and alcohol use,[9–11] as a result of the release of DNA material by dying innate cells after they have been recruited at the site of injection.[12] Serial measurements of serum urate before and after vaccination can be a readily implementable first step to further elucidate this possibility.

Finally, we reiterate that the benefits of vaccinations far outweigh the possible small risks of gout flares.

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Open-label randomised pragmatic trial (CONTACT) comparing naproxen and low-dose colchicine for the treatment of gout flares in primary care

We read with great interest the article by Roddy *et al*[1], published in the Annals of Rheumatic Disease, an open-label study that examined 399 patients presented at primary care centres with exacerbation of gout. The patients were allocated to two treatment arms, one group received naproxen and the second group treated with colchicine, and the primary outcome measured changes in the pain level from baseline over the first 7 days after presentation. Diarrhoea and headaches were more commonly reported in the colchicine group and given there was no difference between the two groups in the primary outcome, the authors recommended naproxen as the first line of treatment in acute gout arthritis, if no contraindication exists.

Although the authors pointed out that naproxen might be associated with decreased risk of cardiovascular events compared with the other non-steroidal anti-inflammatory drugs (NSAIDs) commonly used in gout, a recent metanalysis conducted by Bally *et al*[2] demonstrated that all NSAIDs, including naproxen, have been linked to a higher risk of myocardial infarction. Therefore, given the evidence of serious adverse effects, we do not concur with the recommendation naproxen should be considered as the only first step treatment option. Furthermore, patients with a history of hypertension and/or diabetes mellitus were included in the study, and colchicine would be a reasonable first therapeutic option from a safety profile in this subgroup of patients.

Regarding the side effect profile, diarrhoea was evident in 45.9% of patients in the colchicine group versus 20% in the naproxen group, a significantly higher frequency compared with the 23% seen in the low-dose colchicine arm in the Acute Gout Flare Receiving Colchicine Evaluation (AGREE) trial.[3] We wonder why the authors did not use a lower dose regimen from day 2 to 4, for example, the prophylactic dose regime of 0.5–1 mg/day, a widely use dose with an acceptable safety profile supported by the European League Against Rheumatism guidelines.[4]

Lastly, given the individual patient's characteristics, comorbidities, preferences and shared decision making, we believe that NSAIDs and colchicine have an essential role in the management of gout flares, and both regimens can be used as first-line treatment.

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Response to: 'Open-label randomised pragmatic trial (CONTACT) comparing naproxen and low-dose colchicine for the treatment of gout flares in primary care' by Parperis *et al*

We concluded that naproxen should be considered as first-line treatment for gout flares in primary care based on there being no difference between naproxen and colchicine in pain intensity (the primary outcome), more analgesic use and self-reported side-effects in the colchicine group, and evidence that naproxen was cost-effective.[1] We note the Bayesian meta-analysis by Bally *et al* in which all non-steroidal anti-inflammatory drugs (NSAIDs), including naproxen, were associated with increased risk of myocardial infarction.[2] However, this review was limited by including only studies undertaken in healthcare databases risking bias due to residual confounding and, as the review's authors acknowledge, measuring drug dispensing or prescribing and not actual drug intake. A meta-analysis of individual patient data from 280 randomised trials of NSAID versus placebo found that naproxen did not significantly increase major vascular events or vascular deaths, in contrast to other NSAIDs.[3]

We acknowledge that the colchicine dose in our pragmatic trial differed from that subsequently recommended in the European League Against Rheumatism (EULAR) recommendations.[4] Pragmatic trials evaluate interventions as prescribed, managed and used in routine clinical practice.[5] We used the UK recommended colchicine dose,[6] consistent with the British Society for Rheumatology gout management guideline.[7] Furthermore, the EULAR recommendations advocate a loading dose of colchicine of 1 mg followed by 0.5 mg 1 hour later in patients presenting within 12 hours of flare onset,[4] as per the Acute Gout Flare Receiving Colchicine Evaluation (AGREE) trial,[8] without making a dose recommendation for patients with longer flare durations. Over two-thirds of our participants initiated medication over 24 hours after flare onset, hence the appropriateness of this dose regimen for our trial population is uncertain.

Our findings support informed decision-making based on an assessment of the balance of benefits and harms. Our conclusion was not that naproxen should be considered as the *only* first treatment option for gout flares, as stated by Parperis,[9] but importantly contained the caveat that naproxen should be considered as first-line treatment in primary care *in the absence of contraindications*. While colchicine would be a reasonable first therapeutic option in patients with cardiovascular risk factors, this is consistent with our conclusion that the choice of treatment should be influenced by the presence or absence of comorbidities.

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Association between osteoporosis and statins therapy: the story continues

It was with great interest that we read the study conducted by Leutner *et al*[1] that investigated the relationship between the use of statins and osteoporosis. As identified in the correspondence by Shih-Wei Lai, there is a long-standing interest in the relationship between statins and bone, with numerous observational studies identifying a protective effect; however, results are inconsistent.[2–4] In their study, Leutner *et al* identified that the use of statins was associated with an impressive 3.62-fold increased risk of being diagnosed with osteoporosis, and this association was observed in both men (OR 3.35) and women (OR 3.90. However, we note that if the crude ORs are calculated on the data matched for age and sex (derived from table 1 of the manuscript), the association between statin use and osteoporosis disappears with ORs of 1. Matching is one method to avoid the influence of confounding.[5] Due to the large difference between the reported ORs and the matched numbers, we are unsure how age and sex were included in the authors logistic regression model.

The authors further identify a very impressive dose-response relationship, which is identified in figure 2 of the manuscript. While we agree with the conclusion that it is highly important for future studies to consider the individual statins and dosage when examining the risk of osteoporosis, we would like to make an addition to consider statin potency. In the conclusion, the authors propose that the mediating effect of statins on bone is the inhibition of sex hormones via HMG-CoA reductase inhibition. However, we note that the inhibition of the synthesis of cholesterol influencing sex-hormones, based on HMG-Co-A-reductase inhibition, would also be affected by varying potencies of different statins.[6–8] Thus, we would not expect the results to be similar at identical average daily doses. Rather, simvastatin >40 mg would be comparable to 20 mg atorvastatin and 10 mg rosuvastatin. When comparing the results in the current study at these doses, the dose-dependent relationship becomes less clear. As a result, we postulate that within the drug, dose-response is likely a result of confounding by indication, whereby sicker (eg, frailer) patients who are prescribed higher statin doses are also more likely to be at risk for having a diagnosis of osteoporosis.[9] This confounding is intensified when non-users are the referent group.

While the authors draw a strong conclusion regarding the relationship between statins and osteoporosis, we believe that the impressive results are likely a product of unmeasured confounding. Moreover, due to the nature of a cross-sectional design, the authors cannot demonstrate a temporal relationship between statin use and osteoporosis diagnosis, nor were they able to assess time on therapy to assess a biologically plausible relationship.[10] To conclude that there is a dose-dependent relationship between statins and osteoporosis, these methodological issues need to be addressed. Thus, we believe that the results reported in this study should be interpreted with great caution.

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Response to: 'Association between osteoporosis and statin therapy: the story continues' by Burden and Weiler

We have read with great interest the correspondence of Burden and Weiler[1] referring to our previously published manuscripts.[2 3] First, they sharply observe that the crude OR between the statin group and the matched cohort shows no effect and therefore inquire as to how exactly we adjust for age. In our original study, we grouped patients according to their medications, computed mean age and sex within these medication groups as covariates, and performed a weighted multivariate regression on the risk of osteoporosis within the different medication groups (one 'observation' corresponds to one medication group, weighted proportionally to the patients in that group). This grouping by medication was performed for computational convenience due to our large cohort. We confirmed that our results do not qualitatively change if instead of grouping the patients we evaluate a regression model in which each observation is one patient. In response to the correspondence by Burden and Weiler, we have now additionally performed a multivariate regression as reported in the original paper, except that we used the 'matched cohort' as the control group rather than all patients without statins. This change in the reference group did not qualitatively affect the results either. Using the matched cohort, we observed an OR of 0.83 (95% CI 0.76to 0.91) for 0–10 mg simvastatin, 0.98 (CI 0.94 to 1.03) for 10–20 mg, 1.26 (CI 1.19 to 1.32) for 20–40 mg, 1.92 (CI 1.71 to 2.16) for 40–60 mg and 3.82 (CI 2.88 to 5.07) for 60–80 mg. These results render it highly unlikely that age was a potential confounding factor. Note that the distribution of patients over these dosage groups is uneven, with 53% of patients having dosages between 0 and 20 mg, 39% dosages of 20–40 mg and 8% with >40 mg. Therefore, if we pool all patients in one group, the risk-decreasing or neutral association in the low-dosage patients 'cancels out' the risk increase of higher dosages, resulting in the observed balanced crude OR.

Statins are among the most prescribed medications worldwide. The main mechanism of statins is inhibition of hydroxymethylglutaryl-CoA (HMG-CoA) reductase, which in further consequence reduces the synthesis of cholesterol.[4] Cholesterol is the basic substance for the synthesis of vital hormones such as cortisol or sex hormones. Our cross-sectional study was the first to investigate different types of statins and their dosages in detail, although earlier studies showed that statins could reduce sex hormone levels.[5–9] Thus we have hypothesised that the higher potencies and dosages of statins and their stronger cholesterol-lowering effect could inhibit the synthesis of vital hormones such as sex hormones more strongly and, therefore, be related to associated diseases such as osteoporosis. Our results show that dosages of 0–10 mg of pravastatin were related to a 32% decreased risk of being diagnosed with osteoporosis when compared with controls—similar results could be observed for the low-potency statin lovastatin. Interestingly, there was no significant overrepresentation of diagnosed osteoporosis in higher dosages of pravastatin or lovastatin. The first significant overrepresentation of osteoporosis in statin-treated patients could be observed in higher dosages of 40–60 mg of simvastatin and thus there was a 64% higher risk of being diagnosed with osteoporosis. In their correspondence, Burden and Weiler make the point that—following our hypothesis—effects observed for simvastatin with dosages >40 mg should be comparable to 20 mg atorvastatin and 10 mg rosuvastatin. Indeed, for atorvastatin 10–20 mg (20–40 mg) we found an OR of 1.35 (1.78). Both ORs are within the confidence intervals of simvastatin 40–60 mg (1.64, CI 1.31 to 2.07); hence these results are comparable. For even higher dosages, results should be interpreted with care because of the decreasing sample sizes and the increasing CIs. Results for rosuvastatin are at present not conclusive with respect to the impact of statin potency. For 20–40 mg we observed an OR of 2.04 with a relatively large CI of 1.31 to 3.18. For even higher dosages, sample sizes were not sufficient to obtain results. Findings for rosuvastatin should therefore be interpreted with care, particularly as—in contrast to simvastatin and atorvastatin—it is not metabolised by CYP3A4,[10 11] which is also mainly involved in the metabolisation of sex hormones such as oestrogen.[12–14] Because of the cross-sectional study design, at present we cannot establish potential time-ordering between the beginning of statin treatment and onset of osteoporosis. It therefore goes without saying that we are only able to report correlations and not causative interactions. We were able to exclude potential confounding factors such as age (as described above), sex and certain comorbid diseases (eg, rheumatoid arthritis, ischaemic heart disease, stroke, diabetes, nicotine dependency or overweight and obesity), in order to rule out confounding by indication stemming from these diagnoses. These robustness tests did not change our results qualitatively. Nevertheless, large prospective studies are certainly needed in order to validate our recently proposed mechanism of the possible inhibiting effect of statins on sex hormones.

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