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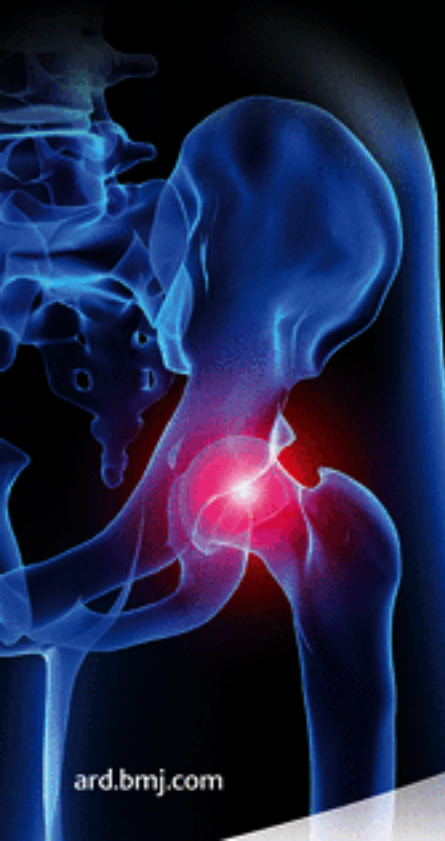

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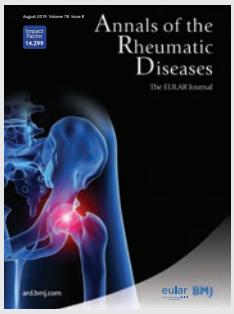

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Why did IL-23p19 inhibition fail in AS: a tale of tissues, trials or translation?

Stefan Siebert, Neal L Millar, Iain B McInnes

Clinical trials investigating biologic immune targeting therapeutics should deliver insight regardless of direction of the primary clinical outcome. Given the remarkable specificity of the 'molecular scalpels' now consequent upon the pharmacologic biologic revolution, it is imperative to learn lessons, particularly from those studies whose outcomes challenge pathogenetic wisdom. In this context, progress in understanding and treatment of the spondyloarthritides (SpA) and related extra-articular manifestations, especially psoriasis and inflammatory bowel disease (IBD), has been remarkable in the last decade. This group of phenotypically related, but still rather heterogeneous conditions share common genetic and pathogenetic features, leading to the notion that common clinical responses across the SpA spectrum should arise from specific immune-targeted interventions. This notion may shortly be disabused.

Whereas initial therapeutic advances in SpA comprised adoption of tumour necrosis factor (TNF) inhibitors from rheumatoid arthritis, major recent therapeutic breakthroughs followed identification of a substantial role for the IL-23/IL-17 pathway in pathogenesis. These studies integrated insights from a composite of genome-wide association studies (GWASs), postfunctional genomic studies, tissue analyses and a variety of preclinical models. Advances have been most marked in psoriasis with 'PASI100' response rates of around 50%–70% following IL-17A or IL-23p19 inhibition.[1–5] Subsequently, IL-12/23p40, IL-23p19 and IL-17A inhibitors demonstrated efficacy in psoriatic arthritis (PsA), although this has been somewhat less penetrant in terms of high-hurdle responses.[6–11] The IL-17A inhibitor, secukinumab, has recently also been shown to be efficacious in patients with active ankylosing spondylitis (AS).[12] Studies of IL-23 inhibition for the treatment of AS were commenced based on this suggestive preclinical and human data resource.

In *Annals of the Rheumatic Diseases*, Baeten and colleagues present a phase II clinical trial evaluating risankizumab, a humanised monoclonal antibody targeting the p19 subunit of IL-23, in patients with active AS.[13] The authors and editors should be congratulated for bringing these data into the public domain to advance our understanding of underlying disease pathogenesis. The trial evaluated three doses of risankizumab compared with placebo in biologic-naive patients with active AS. Risankizumab doses were selected based on data from a phase I study in psoriasis and were shown to have superior efficacy to the p40 IL-23 inhibitor ustekinumab in a subsequent phase II trial in psoriasis.[5] However, the current study of risankizumab in patients with active AS failed to meet the primary endpoint (ASAS40 at week 12) and demonstrated no convincing improvements in clinical or MRI outcomes compared with placebo, despite a dose-dependent reduction in C reactive protein with risankizumab.

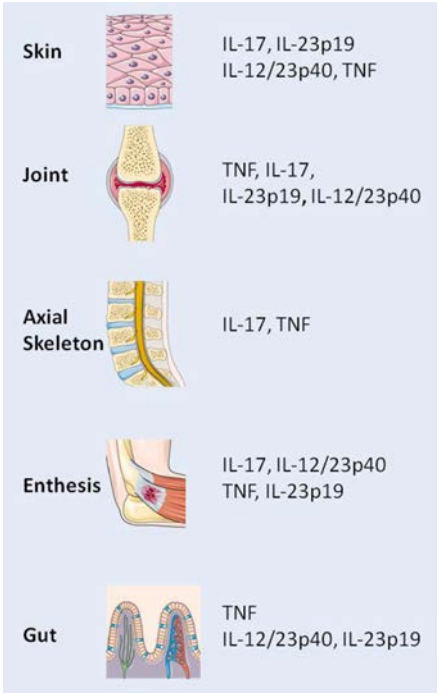

Figure 1 Proposed notional emerging tissue cytokine hierarchy based on current clinical trial data. There is now increasing evidence suggesting that different cytokines may enjoy distinct hierarchical roles in tissues across the spondyloarthritis spectrum. This figure highlights those pathways with demonstrable effects in each discrete tissues against those in which clinical responses were not observed. In the absence of formal head-to-head studies, these comparators should be taken as potential rather than proven. Future analyses are now required to ascribe formal within-tissue hierarchies.

While these results may initially appear surprising in light of the efficacy of IL-17A inhibition in AS, the study by Baeten *et al* should be more wisely considered in the wider context of increasingly tissue-discrete results for IL-23/IL-17 inhibition across the SpA spectrum (figure 1). In cutaneous psoriasis, the dominant role of the IL-23/IL-17 pathway is firmly established and has led to impressive results with an increasing array of inhibitors of these cytokines reaching the clinic. In PsA, while IL-17A and IL-23 (both p40 and p19) inhibition has demonstrated efficacy for synovial and entheseal disease, the results are more modest and have not met the high hurdles seen in cutaneous psoriasis. A study using paired biopsies of skin and synovium in patients with PsA reported a dominant IL-17 gene signature in lesional skin compared with a stronger TNF signature in synovium[14] perhaps suggesting that the clinical trial data may have pathogenetic correlates. In Crohn's disease, IL-23 inhibition with p40 (ustekinumab) and p19 (risankizumab) inhibitors has demonstrated efficacy in phase II/III studies.[15 16] In contrast, a phase II study of IL-17A inhibition with secukinumab did not meet its primary outcome and a phase II study of brodalumab, an IL-17RA inhibitor, was prematurely stopped, with numerical worsening of Crohn's disease in the treatment groups for both studies.[17 18] Therefore, while preclinical data suggested a role for both IL-23 and IL-17A in the pathogenesis of Crohn's disease, inhibition of these cytokines led to divergent results in clinical trials. Indeed, it has been suggested subsequently that IL-17A may have pathogenic and protective roles in the gut, with IL-23-independent IL-17A production required for regulation of intestinal epithelial permeability via the tight junction protein occludin.[19] More recently, the IL-17F pathway has also emerged as having distinct mucosal biologic features.[20] Interestingly, AS has a strong association with IBD, with 15% of patients developing overt IBD and up to 60% exhibiting evidence of underlying subclinical microscopic colitis, which has

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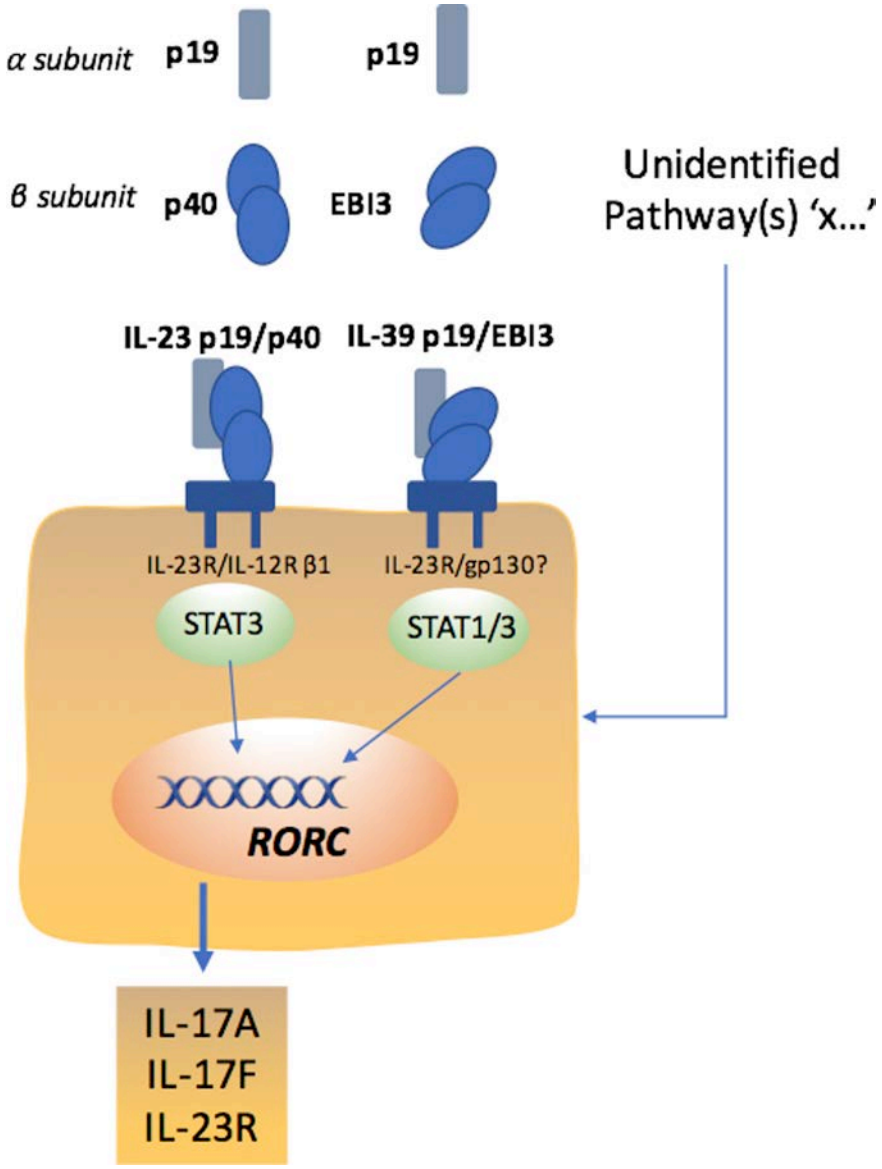

Figure 2 Potential cytokine pathways driving IL-17 responses in spondyloarthritis.

been proposed to contribute to the pathogenesis of AS.[21 22] In light of the data from the Crohn's disease trial programme, one might even have anticipated that IL-23 inhibition would be more effective than IL-17 inhibition in AS due to underlying IBD or subclinical colitis.

Taken together, clinical trial data have fundamentally challenged the notion that the pathogenic pathways driving disease in the tissues impacted in the SpA spectrum are truly common. We propose that each component tissue will comprise a specific immunologic pathology programme that reflects its evolutionary imperative for host defence, and as such therapeutic interventions must embrace such teleologic immunologic reality.

Could this be a trial design artefact? Several factors suggest that Baeten *et al* accurately describe the biological role of IL-23 in AS rather than eliciting issues concerning study design or outcome selection. Baseline characteristics of this study population do not differ significantly from those in previous studies of TNF or IL-17A inhibitors in active AS. While the primary efficacy outcome includes a significant subjective component, lack of efficacy was also observed for most secondary endpoints, including MRI imaging and biomarkers of bone remodelling. The authors eloquently consider issues relating to risankizumab dose and pharmacokinetics, suggesting that these are unlikely to account for the lack of observed efficacy. It does, however, remain possible that there is a fundamental problem with the tissue bioavailability of this molecule—however, more considered explanations are merited since the cutaneous benefits accrued on similar monoclonal approaches should not be readily ignored. The phase III trials in AS and non-radiographic axSpA of the IL-12/IL-23 inhibitor ustekinumab were recently terminated for not meeting key efficacy endpoints (ClinicalTrials.gov NCT02438787 and NCT02407223), despite a small open-label study suggesting efficacy in 20 patients with AS.[23] Taken together, these data suggest that, in contrast to IL-17A blockade, IL-23 inhibition is not an effective strategy for the treatment of AS, which raises the critical question—why?

The preclinical evidence supporting IL-23p19 blockade in AS was robust per current standards of 'a priori' proof-of-concept (POC). As noted above, GWASs clearly implicate the IL-23R pathway in disease risk and progression. The use of minicircle DNA technology to express IL-23 in the hepatocytes of B10.RIII mice resulted in a destructive polyarthritis that was found to be independent of $CD4^+$ T cells[24] while a further animal study[25] using similar technology revealed that systemic expression of IL-23 in normal mice was sufficient to induce the major features of SpA (enthesitis, sacroiliitis and aortic root inflammation), putatively through activation of a novel population of innate $CD3^+$, $CD4^-CD8^-$ and retinoic acid receptor–related orphan nuclear receptor γt (RORγt)–positive T cells located in the entheses of these mice. While the precise source of IL-23 was not identified, a further study suggested that these cells were tissue-resident $Vγ6^+γδ$ T cells[26] promoting bone growth through IL-17[27] and were therefore a putative pathogenic cell population linking IL-23-induced inflammation to bone growth in the enthesis. Follow-on human studies revealed the number of γδ T cells that produce IL-17 and express IL-23R was elevated in peripheral blood in people with AS.[28] Interestingly, only one study[29] has confirmed the presence of $CD4^-CD8^-$ T cells in human entheseal digests. Further examination of T-cell subsets indicated that a high proportion of these cells were likely γ/δ T cells, but no functional analysis of these human subsets was undertaken. Human tissue analysis[30] revealed a significantly higher incidence of $IL\text{-}23^+$ cells in patients with AS with the majority of IL-17-producing cells comprising myeloperoxidase-positive and CD15-positive neutrophils rather than $CD3^+$ T cells, suggesting that IL-17-producing cells other than Th17 cells are relevant in local inflammation in this population. There is evidence to support IL-23-independent induction of IL-17 from γδT cells and innate lymphoid cells.[31] Thus, given this

disparate cell expression profile between mouse and human studies, it is plausible (but not yet proven) that IL-23-independent sources of IL-17 (eg, via ILCs, $CD15^+$ neutrophils) are of pathogenetic importance in driving disease chronicity in AS. Furthermore, given the presumed central role of RORγt as a 'master' transcription factor of the type 17 response in entheseal disease and recent evidence that it acts in an IL-23 independent fashion,[19] we contend that there could be a molecular argument that p19 inhibition alone will prove insufficient to effectively target the type 17 immune response evident in the axial component of the SpA spectrum.

While the IL-12 family cytokines have pleiotropic functions with parallel unique characteristics, much attention initially focused on the p19 subunit combination with the p40 subunit forming IL-23(p19/40). Subsequent signalling was considered pivotal through the IL-23R/IL-12Rβ1 receptors with downstream STAT3 proposed in autoimmune diseases, including AS.[32] Although four bona fide members have thus far been described, promiscuous chain-pairing between alpha (IL-23p19, IL-27p28, IL-12/IL-35p35) and beta (IL-12/IL-23p40, IL-27/IL-35Ebi3) subunits predicts six possible heterodimeric IL-12 family cytokines.[33] Indeed, emerging evidence has highlighted that the p19 and EBi3 form, a novel p19/Ebi3 heterodimer termed IL-39, mediates inflammation in lupus-like MRL/lpr mice[34] and importantly anti-mouse IL-39 polyclonal antibodies ameliorate autoimmune symptoms in lupus-like mice.[35] A similar association between p19 and EBI3 was suggested in damaged keratinocytes, possibly contributing to wound healing by dampening inflammatory responses[36] linking stromal and immune responses of the p19 subunit. Thus, given increasing evidence that local damage may provide a trigger for SpA,[37] the intriguing heterodimerisation of the p19 subunit towards IL-39 provides a plausible, potential alternate mechanism driving pathology . More studies around the role of p19 in this context, and indeed how p19 inhibiting antibodies modify such biology, will be important in axSpA. Moreover, it is becoming apparent that T_H17 cells are not homogeneous, with a large body of work indicating an inherent instability of T_H17 cell populations.[38] Data concerning the role of IL-23 in the generation of non-T_H17 Treg cells are conflicting. Some studies suggest that IL-23 promotes the accumulation of Treg cells in the gut,[39] which are probably non-T_H17 Treg cells.[40] Conversely, IL-23 promotes the stability of pathogenic T_H17 cells through the transcription factor PR domain zinc finger protein 1 (PRDM1).[41] These findings suggest that increased numbers of T_H17 cells in patients with AS might not result from preferential differentiation of naive T cells with particular reference to early IL-23p19 expression, but rather through a reduced plasticity of mature T_H17 cells. Thus more information as to the biology of IL-23 in the joint and GI mucosa is required. Having previously been relatively underinvestigated, the involvement of IL-17-producing $CD8^+$ T cells (T_C cells) and innate lymphoid cells in autoimmune inflammation has now been documented in both humans and mice.[42] In particular, recent data suggest enrichment of articular T_C cells across multiple SpA subtypes[43] and identify a phenotypic signature for IL-17^+CD8^+ T cells, consisting of type 17 and tissue-associated markers[44] implicating such cells as important contributors to the pathogenesis of axSpA. How such cells relate to IL-23 biology is now also requires further investigation.

Taken together, established AS may have 'transitioned pathogenetically' to a mature type 17 phenotype, which is unresponsive to IL-23p19 blockade and other upstream treatment strategies (eg, IL-6 inhibition[45]) that might otherwise modulate type 17 cell differentiation. As a consequence, at a molecular level, the IL-23p19 subunit appears not the only regulatory agent for targeting the type 17 response in AS, and, by corollary, neutralising this upstream molecule seems to be less effective than specifically targeting IL-17A in AS (figure 2). The identity of additional drivers to the IL-17 response and indeed other effector pathologic pathways should now be sought.

The apparent failure of IL-23p19 inhibition in AS serves further as salutary reminder of the complexity of chronic polygenic human inflammatory diseases but, paradoxically, helps advance our understanding of these diseases by redefining our understanding of the importance of a pathway within the pathogenetic hierarchy. Preclinical modelling, even supported by state-of-the-art genetic and postgenomic functional studies, remain imperfect in their predictive use. With the expansion of therapeutic novel modes of action, the next decade will offer unparalleled opportunities to build 'new knowledge on old' as clinical trial datasets accrue; as such, we can build pathogenetic understanding based on truly human disease models. Thus, the importance of confirming apparently persuasivepreclinical results in humans remains key, while there remain lessons to be learnt about applying animal data to humans. Considering tissue-specific and time-specific cytokine responses and hierarchies when developing novel therapies would seem wise.

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Consensus-based recommendations for the management of juvenile localised scleroderma

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ABSTRACT

In 2012, a European initiative called Single Hub and Access point for paediatric Rheumatology in Europe (SHARE) was launched to optimise and disseminate diagnostic and management regimens in Europe for children and young adults with rheumatic diseases. Juvenile localised scleroderma (JLS) is a rare disease within the group of paediatric rheumatic diseases (PRD) and can lead to significant morbidity. Evidence-based guidelines are sparse and management is mostly based on physicians' experience. This study aims to provide recommendations for assessment and treatment of JLS. Recommendations were developed by an evidence-informed consensus process using the European League Against Rheumatism standard operating procedures. A committee was formed, mainly from Europe, and consisted of 15 experienced paediatric rheumatologists and two young fellows. Recommendations derived from a validated systematic literature review were evaluated by an online survey and subsequently discussed at two consensus meetings using a nominal group technique. Recommendations were accepted if ≥80% agreement was reached. In total, 1 overarching principle, 10 recommendations on assessment and 6 recommendations on therapy were accepted with ≥80% agreement among experts. Topics covered include assessment of skin and extracutaneous involvement and suggested treatment pathways. The SHARE initiative aims to identify best practices for treatment of patients suffering from PRDs. Within this remit, recommendations for the assessment and treatment of JLS have been formulated by an evidence-informed consensus process to produce a standard of care for patients with JLS throughout Europe.

INTRODUCTION

In 2012, a European project called Single Hub and Access point for paediatric Rheumatology in Europe (SHARE) was launched to optimise and disseminate diagnostic and management regimens in Europe for children and young adults with rheumatic diseases.[1] As currently no international or European consensus exists with regard to the assessment and treatment of juvenile rheumatic diseases, defining clear guidelines is one of the most important aims of the SHARE initiative. In this paper, we focus on juvenile localised scleroderma (JLS) consensus-based recommendations.

METHODS

An international committee of 15 experts in paediatric rheumatology was established to develop consensus-based recommendations for JLS.[2] European League Against Rheumatism (EULAR) standard operating procedures for developing best practice were used.[3] Ten experts were part of the SHARE consortium; five other experts were asked to take part to the project due to their consolidate clinical experience in the management of JLS.

Systematic literature search

The electronic databases PubMed/MEDLINE, Embase and Cochrane were searched in August 2013 and subsequently in January 2015. All synonyms of JLS were searched in MeSH/Emtree terms, title and abstract. Reference tracking was performed in all included studies (full search strategy in online supplementary figure S1). Fellows (RC, FS) and experts (FZ, IF) selected the relevant papers for validity assessment (inclusion and exclusion criteria shown in online supplementary figure S1): 53 out of 1550 papers were eventually selected. All full-text scored papers are listed in the online supplementary list 1.

Validity assessment

Every relevant paper dealing with 'diagnosis', 'assessment' and 'therapy' studies has been independently assessed for methodological quality by two experts, who extracted data using a predefined scoring system.[4] Disagreements were resolved by the opinion of a third expert. Adapted classification tables for assessment and therapeutic studies were used to determine the level of evidence and strength of each recommendation.[5]

Recommendation development

As part of the EULAR standard operating procedure,[3] experts assessed validity and level of evidence and described the main results and conclusions of each paper. This information was examined by two experts (FZ, IF) and used to formulate 18 provisional recommendations. These drafted recommendations were at first presented to the expert committee in an online survey (100% response rate) and subsequently revised accordingly to responses. The derived recommendations were then presented to the expert committee and discussed using a nominal group technique in two face-to-face

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Table 1 Recommendations regarding diagnosis and assessment

		L	S	Agreement (%)
	Overarching principle			
	All children with suspected localised scleroderma should be referred to a specialised paediatric rheumatology centre.	4	D	100
1	LoSSI, which is part of LoSCAT, is a good clinical instrument to assess activity and severity in JLS lesions and is highly recommended in clinical practice.	3	C	90
2	LoSDI, which is part of LoSCAT, is a good clinical instrument to assess damage in JLS and is highly recommended in clinical practice.	3	C	90
3	Infrared thermography can be used to assess activity of the lesions in JLS, but skin atrophy can give false-positive results.	4	D	90
4	A specialised US imaging, using standardised assessment and colour Doppler, may be a useful tool for assessing disease activity, extent of JLS and response to treatment.	4	D	100
5	All patients with JLS at diagnosis and during follow-up should be carefully evaluated with a complete joint examination, including the temporomandibular joint.	2a	C	100
6	MRI can be considered a useful tool to assess musculoskeletal involvement in JLS, especially when the lesion crosses the joint.	3	C	100
7	It is highly recommended that all patients with JLS involving face and head, with or without signs of neurological involvement, have an MRI of the head at the time of the diagnosis.	3	C	90
8	All patients with JLS involving face and head should undergo an orthodontic and maxillofacial evaluation at diagnosis and during follow-up.	2b	B	90
9	Ophthalmological assessment, including screening for uveitis, is recommended at diagnosis for every patient with JLS, especially in those with skin lesions on the face and scalp.	2a	C	100
10	Ophthalmological follow-up, including screening for uveitis, should be considered for every patient with JLS, especially in those with skin lesions on the face and scalp.	3	C	100

JLS, juvenile localised scleroderma; L, level of evidence; LoSCAT, Localized Scleroderma Cutaneous Assessment Tool; LoSDI, Localized Scleroderma Skin Damage Index; LoSSI, Localized Scleroderma Skin Severity Index; S, strength of recommendation; US, ultrasound.

meetings on March 2014 in Genova (Italy) and on March 2015 in Barcelona (Spain). At both meeting, a non-voting expert (SJV) facilitated the process. Recommendations were accepted when ≥80% of the experts agreed.

RESULTS

The literature search yielded 1550 papers; after the application of inclusion/exclusion criteria, title/abstract and full-text screening, 53 papers (26 for assessment and 27 for treatment) were selected and sent to the expert committee for validity assessment. Following a consensus-based methodology, the scleroderma working group of SHARE formulated 22 recommendations for the management of JLS. In total, 1 overarching principle, 10 recommendations on assessment and 6 on therapy were accepted with ≥80% agreement among the experts. Topics include assessment of skin, extracutaneous involvement and treatment suggestions at disease onset and in refractory disease.

We briefly describe the recommendations with corresponding supporting literature. Tables 1 and 2 summarise the recommendations for JLS, their levels of evidence, recommendation strength and percentage of agreement between experts. Of note, two recommendations derive from randomised controlled trials (level of evidence 1b, strength of evidence A), while three derive from expert opinions (level of evidence 4, strength of evidence D).

Overarching principle

JLS includes a group of disorders whose manifestations are confined to the skin and subdermal tissues and, with rare exceptions, do not affect internal organs. The most widely used classification includes five subtypes: circumscribed morphoea, linear scleroderma, generalised morphoea, pansclerotic morphoea, and the mixed subtype where a combination of two or more of the previous subtypes is present.[2] It is a rare condition in children as the incidence is 3.4 cases per million children per year, the vast majority represented by the linear subtype.[6] The female to male ratio of JLS is 2.4:1, the mean age at onset is approximately 7.3 years,[7] although the disease can start as early as at birth.[8] The severity of the disease varies widely from isolated plaques to generalised morphoea, and to extensive linear lesions involving limbs, trunk and/or the face and head.[9]

Given the rarity of the disease, the expert group agreed that patients with suspected JLS should be referred to a specialised paediatric rheumatology centre for clinical assessment and treatment (table 1).

Assessment of skin lesions

The assessment of disease activity is crucial in patients with JLS. At the time of diagnosis and during follow-up, it is fundamental to determine whether a lesion is active in order to establish an appropriate treatment regimen. Indeed, quantifying the activity of specific lesions is important in order to evaluate the response

Table 2 Recommendations regarding treatment

	L	S	Agreement (%)
Systemic corticosteroids may be useful in the active inflammatory phase of JLS. At the same time as starting systemic corticosteroids, MTX or an alternative DMARD should be started.	2b	C	100
All patients with active, potentially disfiguring or disabling forms of JLS should be treated with oral or subcutaneous methotrexate at 15 mg/m²/week.	1b	A	100
If acceptable clinical improvement is achieved, methotrexate should be maintained for at least 12 months before tapering.	3	C	100
Mycophenolate mofetil may be used to treat severe JLS or MTX-refractory or MTX-intolerant patients.	2a	B	100
Medium-dose UVA1 phototherapy may be used to improve skin softness in isolated (circumscribed) morphoea lesions.	1b	A	100
Topical imiquimod may be used to decrease skin thickening of circumscribed morphoea.	3	C	100

DMARD, disease-modifying antirheumatic drug; JLS, juvenile localised scleroderma; L, level of evidence; MTX, methotrexate; S, strength of recommendation; UVA1, ultraviolet A1.

to therapy. As for disease activity and severity, the experts agreed on using both multiparametric scoring systems and instrumental techniques (table 1).

LoSCAT (Localized Scleroderma Cutaneous Assessment Tool) is a scoring system that includes a Skin Severity Index (LoSSI) and a Skin Damage Index (LoSDI).[10 11] LoSSI is a validated clinical instrument that allows to assess activity and severity of JLS lesions. Indeed, it correlates well with disease activity evaluated by clinicians.[12] LoSSI includes four domains (body surface area involvement, degree of erythema, skin thickness and appearance of new lesion or old lesion extension), each one graded from 0 to 3, in 18 anatomic sites.[10] LoSDI assesses damage by a similar scoring system. It includes three domains: skin atrophy, subcutaneous tissue loss and hypo-hyperpigmentation.[11] Although this method does not evaluate the real size of the lesions, it can be performed by physicians in daily practice without the need for special equipment.

Infrared thermography (IT) is a non-invasive technique that detects infrared radiation and provides an image of the temperature distribution across the body surface.[13] IT has been shown to be of value in the detection of active lesions with high sensitivity (92%) but moderate specificity (68%).[13] False-positive results are related to the fact that old lesions lead to marked atrophy of skin, subcutaneous fat and muscle, with increased heat conduction from deeper tissues.

High-frequency ultrasound can detect several abnormalities such as increased blood flow related to inflammation as well as increased echogenicity due to fibrosis and loss of subcutaneous fat.[14 15] The main limits of this tool are its operator dependency and the lack of standardisation.

Assessment of extracutaneous involvement

Although cutaneous and subcutaneous involvement is prominent, almost 20% of patients with JLS present extracutaneous manifestations[16] which are more frequent in patients with linear scleroderma and consist essentially of arthritis, neurological findings or other autoimmune conditions. Based on published data and clinical experience, the experts approved six recommendations regarding the assessment and monitoring of the extracutaneous manifestations of JLS.

Articular involvement is the most frequent extracutaneous feature being present in up to 19% of patients.[16] It can manifest with limited range of joint motion from contractures and/or arthritis.

Articular involvement is more common in children with the linear subtype, but it can be present in any subtype of JLS.[16] Therefore, all patients with JLS should be evaluated with a comprehensive joint examination at diagnosis and during follow-up. Joint symptoms are more common in patients with linear scleroderma and the affected joint does not always correlate with the site of the cutaneous lesion. Children with JLS who develop arthritis often have positive rheumatoid factor, and sometimes an elevated erythrocyte sedimentation rate.[7] A few studies, conducted mainly in adults,[17 18] reported a positive correlation between MRI and clinical findings of arthritis, especially during treatment. In addition to the literature evidence, the expert panel reported a positive experience in using this non-invasive tool to assess musculoskeletal involvement in JLS.

Central nervous system (CNS) involvement, although rare, has been reported in children with JLS, especially in those with linear scleroderma of the face and scalp.[19] The most frequent signs and symptoms are seizures and headache, although behavioural changes and learning disabilities have been also described.[16 20] Abnormalities on MRI, such as calcifications, white matter changes and vascular malformations or vasculitis, have also been reported.[21] Considering that most of these changes have been reported in the linear scleroderma of the face/head, it is mandatory to perform an MRI of the head in every patient with facial/scalp lesions. The lesions may occur distant to the skin lesions and do not apparently represent a skin down to deep tissue full thickness pathology. These patients should also be screened for ocular abnormalities[16] as literature shows a correlation between ophthalmological and neurological involvement in patients with linear forms.[22] Among the ocular manifestations, anterior uveitis is the most frequent one although there can be direct involvement of the eye, eyelid, eyelashes and orbit with the JLS lesions. Being usually asymptomatic, an ophthalmological screening is recommended at the time of diagnosis and during follow-up.

Indeed, since linear scleroderma of the face is significantly associated with odontostomatologic abnormalities,[23] an orthodontic and maxillofacial evaluation at diagnosis and during follow-up is recommended. Joint approaches to treatment may be needed, including with plastic surgery input, when there are severe wasting of facial fat compartments or in the linear scalp lesions. A comprehensive review on the most recent advances on monitoring and treatment of JLS has been recently published.[24]

Treatment

Over the years, many treatments have been tried for JLS[24] frequently without significant evidence base. Management decisions should be based on the particular subtype of disease, the site of lesions and on the degree of activity.

Most recent reported data show effectiveness of systemic corticosteroids in association with methotrexate (MTX) in patients with active JLS, particularly in progressive linear scleroderma and generalised or pansclerotic morphoea. Experience with steroids for treatment of active disease in children is reported in many papers, mainly in combination with MTX.[25 26] Literature evidence suggests that systemic corticosteroids are effective and well tolerated in the active phase of the disease and this was confirmed by the expert panel.[27] Data from the literature mainly suggest two administration regimens: oral prednisone at a dosage of 1–2 mg/kg/day for a period of 2–3 months with subsequent gradual tapering,[28] or pulsed high-dose intravenous methylprednisolone (30 mg/kg) with various administration schedules.[25 26] As far as the preferred administration route and dosage is concerned, no agreement has been achieved by the expert committee, therefore both alternatives are accepted. In the future, comparative trials of the two regimes could be considered.

As for the disease-modifying antirheumatic drugs that should be started in combination with corticosteroids, experts recommend MTX as first-step treatment. The only randomised double-blind placebo-controlled trial published to date clearly shows the safety and efficacy of oral MTX in the treatment of JLS, initially in combination with corticosteroids.[28] A weekly regimen of 15 mg/m^2 MTX as single oral or subcutaneous dose is recommended. During the first 3 months of therapy, a course of corticosteroids, namely prednisone, should be used as adjunctive 'bridge therapy'.[28] Prolonged remission off medication is more likely to occur in patients treated for more than 12 months after achieving clinical remission on medication.[29 30] Therefore, once an acceptable clinical improvement is achieved, MTX should be maintained for at least 12 months before tapering, although longer term treatments are also frequently used.

As for safety, several reports show that low-dose MTX is safe and well tolerated in the paediatric population,[25–30] with a low rate of non-severe side effects including nausea, headache and transient hepatotoxicity.[26 28 30]

If MTX is ineffective or the disease relapses after a period of clinical remission (ie, cutaneous disease progression or severe extracutaneous manifestations) or in the case of MTX-intolerant patients, mycophenolate mofetil (MMF) at a dose of 500–1000 mg/m^2 may be used, despite that lack of good evidence in the literature.[31] A retrospective study on efficacy of MMF, mostly in combination with MTX, in severe refractory JLS has shown clinical improvement in all patients and a good safety profile.[31] More trials on the safety and efficacy of MMF in a larger paediatric population with localised scleroderma are needed.

Circumscribed morphoea is generally of cosmetic concern only and should be treated with topical treatment. Some studies report efficacy of imiquimod (IMQ) in decreasing the skin thickening of isolated plaques of circumscribed morphoea.[32 33] IMQ is a novel immunomodulator which is effective in the treatment of keloids, genital warts and basal cell skin cancers. One of its modes of action is to upregulate a variety of cytokines including interferon α and γ. These interferons are capable of inhibiting collagen production by fibroblasts, likely by downregulating the production of transforming growth factor beta.[34 35] Although published literature includes mainly adult data in low numbers of patients,[33] IMQ appears to be safe in the paediatric population and despite limited evidence, the expert panel suggested its use in selected non-progressive or extended forms of JLS, although a formal trial is also recommended.

Phototherapy with ultraviolet (UV) light represents another possible therapeutic choice for JLS[36 37] although data on its use in children are scarce. Medium-dose UVA1 therapy seems to be effective in improving skin softness and reducing skin thickness with a good safety profile in adults with localised scleroderma.[36–38] Limitations for the use of phototherapy in children are the need for prolonged maintenance therapy, leading to a high cumulative dosage of irradiation, and the increased risk of potential long-term effects such as skin ageing and carcinogenesis.[39]

Although there are, to date, no published trials of biologics or combination treatments, surveys of clinical practice demonstrate that tacrolimus, cyclophosphamide and a number of biologics (including tumour necrosis factor or interleukin-6 inhibitors) are being used in some patients for resistant or CNS disease.[40–43] There is also no high-level evidence regarding when to stop MTX or other immunosuppressive treatments. The expert panel suggested considering the withdrawal of MTX (or alternative disease-modifying drug) once the patient is in remission and off steroids for at least 1 year.

Based on consensus recommendations, a flow chart was proposed for JLS treatment (figure 1).

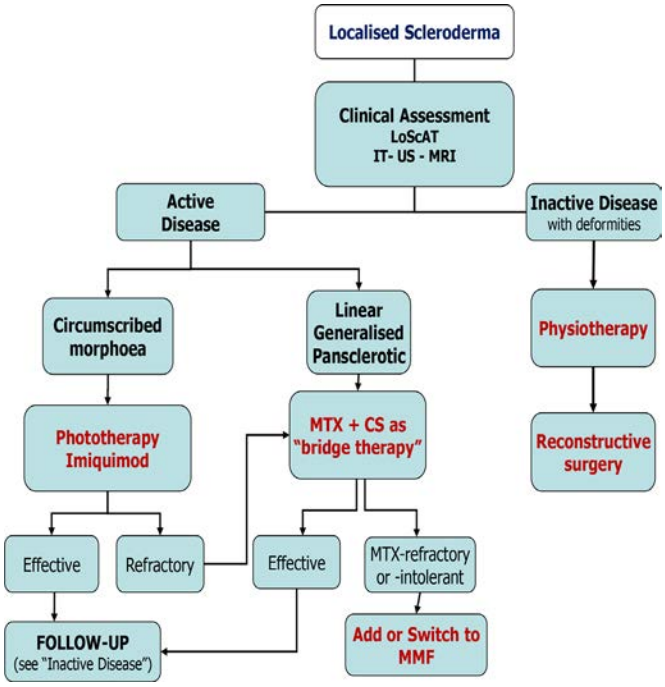

Figure 1 Flow chart for the treatment of newly diagnosed or refractory patients with juvenile localised scleroderma according to the clinical subtype. CS, corticosteroid; IT, infrared thermography; LoSCAT, Localized Scleroderma Cutaneous Assessment Tool; MMF, mycophenolate mofetil; MTX, methotrexate; US, ultrasound.

DISCUSSION

The scleroderma working group of SHARE formulated a total of 22 recommendations for the management of JLS, based on a systematic literature review and consensus procedure.

Topics include assessment of skin lesions and extracutaneous involvement, and the use of topical and systemic treatment options.

In total, 1 overarching principle, 10 recommendations on assessment and 6 on therapy were accepted with ≥80% agreement among the experts.

Close monitoring of patients' disease status and well-being by an experienced multidisciplinary and interdisciplinary team with expertise in localised scleroderma is essential for a good clinical outcome.

As in patients with idiopathic inflammatory myopathies or other rare connective tissue diseases all experts agree on the importance of managing JLS in specialised centres.[44] As with all significant rare disorders, concentrating care in a few centres gives rise to a larger physician experience. In addition, European and international sharing of patients in studies provides evidence to improve standards of care. An important message from both the literature and the experience of experts is the requirement of a global evaluation of patients with JLS, focusing attention on the skin lesions and on possible extracutaneous involvement, which even though are rare can also be severe and potentially disabling. Validated scores for disease activity and damage are proposed in order to perform a structured assessment of outcome over time and to closely check their effect on the growth in children.

Recent evidence highlights the importance of treating skin disease aggressively as it is associated with high morbidity both physically and psychologically. Long-term follow-up studies are warranted to clarify complication risks and predictors of poor outcome. Given the disease rarity, international collaboration is crucial to recruit sufficient patients for future clinical trials with both current and innovative drugs.

To conclude, this SHARE initiative is based on expert opinion informed by the best available evidence and provides recommendations for the diagnosis and treatment of patients with JLS, along with other paediatric rheumatic diseases, with a view to improving their outcome in Europe. We anticipate that these guidelines will likely be adopted by physicians caring for patients with JLS outside Europe.

It will now be important to broaden discussion and test the reliability of these recommendations to the wider scientific community and to the patients.

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Classification criteria for autoinflammatory recurrent fevers

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ABSTRACT

Background Different diagnostic and classification criteria are available for hereditary recurrent fevers (HRF)—familial Mediterranean fever (FMF), tumour necrosis factor receptor-associated periodic fever syndrome (TRAPS), mevalonate kinase deficiency (MKD) and cryopyrin-associated periodic syndromes (CAPS)—and for the non-hereditary, periodic fever, aphthosis, pharyngitis and adenitis (PFAPA). We aimed to develop and validate new evidence-based classification criteria for HRF/PFAPA.

Methods Step 1: selection of clinical, laboratory and genetic candidate variables; step 2: classification of 360 random patients from the Eurofever Registry by a panel of 25 clinicians and 8 geneticists blinded to patients' diagnosis (consensus ≥80%); step 3: statistical analysis for the selection of the best candidate classification criteria; step 4: nominal group technique consensus conference with 33 panellists for the discussion and selection of the final classification criteria; step 5: cross-sectional validation of the novel criteria.

Results The panellists achieved consensus to classify 281 of 360 (78%) patients (32 CAPS, 36 FMF, 56 MKD, 37 PFAPA, 39 TRAPS, 81 undefined recurrent fever). Consensus was reached for two sets of criteria for each HRF, one including genetic and clinical variables, the other with clinical variables only, plus new criteria for PFAPA. The four HRF criteria demonstrated sensitivity of 0.94–1 and specificity of 0.95–1; for PFAPA, criteria sensitivity and specificity were 0.97 and 0.93, respectively. Validation of these criteria in an independent data set of 1018 patients shows a high accuracy (from 0.81 to 0.98).

Conclusion Eurofever proposes a novel set of validated classification criteria for HRF and PFAPA with high sensitivity and specificity.

Key messages

What is already known about this subject?
- Hereditary recurrent fever (HRF) syndromes are genetic disorders secondary to mutations in genes involved in the innate immune response.
- A number of classification or diagnostic criteria have been developed in the past.
- Overall, these criteria lack accuracy and do not consider the results of genetic analyses, now an essential tool for the accurate diagnosis and classification of HRF.

What does this study add?
- We developed and validate new evidence-based classification criteria for HRF and periodic fever, aphthosis, pharyngitis and adenitis, combining international expert consensus, statistical evaluation of real patients from a large data set of patients in the Eurofever Registry.
- The new classification criteria combine for the first time clinical manifestations with genotype.

How might this impact on clinical practice or future developments?
- The use of these classification criteria is recommended for inclusion of patients in translational and clinical studies, but they cannot be used as diagnostic criteria.

INTRODUCTION

In the last 20 years the discovery of the inflammasome and the related genes of the now called systemic autoinflammatory diseases (SAIDs) has led to a completely new line of research. SAIDs are caused by exaggerated activation of the innate immune system, in the absence of high-titre autoantibodies or antigen-specific T-cells.[1 1] Recurrent (or periodic) fevers are characterised by inflammatory flares separated by intervals of general overall well-being. Some conditions are caused by a genetic defect and are collectively referred to as hereditary recurrent fever (HRF). Familial Mediterranean fever (FMF) is caused by mutations of *MEFV*[2 3];

mevalonate kinase deficiency (MKD), by mutations of the mevalonate kinase gene (*MVK*)[4 5]; tumour necrosis factor (TNF) receptor-associated periodic fever syndrome (TRAPS), by mutations of type I TNF receptor (*TNFSRF1A*)[6]; and cryopyrin-associated periodic syndromes (CAPS), by mutations of *NLRP3*.[7 8] More common forms of recurrent fever syndromes include periodic fever, aphthosis, pharyngitis and adenitis (PFAPA) syndrome, which is a multifactorial disorder.[9] So far, several clinical diagnostic and classification criteria have been proposed for HRF[10–15] and PFAPA.[9 16] Overall, these criteria lack accuracy and do not consider the results of genetic analyses, now an essential tool for the accurate diagnosis and classification of HRF.

This distinction between classification and diagnostic criteria is not always clear in clinical practice, and the two terms are often (wrongly) used interchangeably.[17] Classification criteria facilitate accurate identification of diseases for clinical or epidemiological studies, in this context reliably differentiating one autoinflammatory disease from another, but are not designed to diagnose that autoinflammatory disease; hence, classification criteria make the assumption that important disease mimics (eg, chronic infection or malignancy) have already been excluded. In contrast, diagnostic criteria are designed to positively rule in a specific diagnosis in an individual patient, while excluding all conditions with different overlapping disease manifestations based on derivation and validation in cohorts that include important disease mimics. As such, classification criteria cannot be used as diagnostic criteria.[18 19] The purpose of this study was to develop and validate new evidence-based classification criteria for HRF and PFAPA, combining international expert consensus and statistical evaluation of real patients from a large data set of patients in the Eurofever Registry.

METHODS

A multistep process using consensus formation techniques (Delphi and nominal group technique (NGT)) and statistical evaluations on real patients was used to develop and test the classification criteria[17] (online supplementary figure 1 and supplementary material), based on a methodological framework used successfully in previous studies in rheumatology.[20–25]

Step 1: selection of clinical, laboratory and genetic candidate variables

A panel of 162 international adult and paediatric experienced clinicians completed successive Delphi questionnaires in order to propose and then select and rank the variables (clinical manifestations, genetic analyses, laboratory examinations) from 1 (less important) to 10 (most important), for classification of each HRF[26] and PFAPA.[27]

Step 2: classification of patients from the Eurofever Registry

After selection (online supplementary figure 2), a random sample of 360 patients, 60 patients for each disease (FMF, TRAPS, MKD, CAPS, PFAPA and undefined recurrent fevers (uRF)) were selected from the Eurofever Registry.[28] The inclusion criteria for the enrolment in the registry have been previously described[28] (see online supplementary material).

Twenty-five international experienced clinicians/researchers and eight geneticists (total of 33 panellists) in the field of SAID blinded on patients' original diagnosis were invited to participate in a multiround, secured web process to classify each of the 360 patients into one of six mutually exclusive diagnoses.[29] Clinicians and geneticists worked separately in the first steps (clinicians blinded to genetic results and geneticists blinded to clinical data) and then together to reach consensus ≥80% on all classifiable patients.

Step 3: statistical analysis for the selection of the best candidate classification criteria

The statistical analysis plan (full details in the online supplementary material) foresaw the following steps:

- Evaluation through a univariate logistic regression of the relationship between each individual top variable identified in step 1 and each disease as derived from the panel's classification.
- Computer generation of more than 30 000 new candidate sets of classification criteria through linear combinations of genetic and clinical variables with improper linear modelling. Additionally, 11 sets of criteria were derived from the literature[9–16] or proposed by members of the panel based on their expertise.
- Identification of the top-performing criteria through ranking according to the Akaike information criterion (AIC), with best model having the lowest AIC.

Step 4: NGT Consensus Conference for the selection of the final classification criteria

The Consensus Conference was held in Genoa, Italy, on 18–21 March 2017. Clinicians and geneticists, who participated in the step 2 web consensus classification exercise, attended a meeting. The overall goal of the meeting was to decide on the final set of criteria, using a combination of statistical and consensus (≥80%) formation techniques with the consensus panel classification as reference standard.

Step 5: cross-sectional validation of the final classification criteria

The performance of the final set of classification criteria to discriminate patients with the different HRF and PFAPA was tested, using the original treating physician patients' diagnosis as reference standard for the cross-sectional validation, postconsensus, in a separate set of 1018 patients selected after random computer generation from the Eurofever Registry, which contains all variables included in the final set of classification criteria.

RESULTS

The demographic, clinical, genetic and laboratory features of the 360 patients randomly selected from the Eurofever Registry are provided in table 1 and online supplementary table 1.

A total of 100 different genotypes were reported in the 360 patients included in the classification process as reported in online supplementary table 2.

Nine patients with CAPS and two with TRAPS had no mutations detected using Sanger sequencing; thus, at the time of enrolment, somatic mosaicism could not be formally excluded in them. Low penetrant or incidental (non-confirmatory) genetic findings were also reported in 7 patients with PFAPA and 14 with uRF (online supplementary table 3).

Classification of patients from the Eurofever Registry

In the first two rounds, evaluation of clinical data by clinicians (blinded to genetic results) resulted in consensus of ≥80% for a total of 216 of 360 (60%) patients (figure 1); consensus was reached in 51 patients with MKD, 43 with TRAPS, 29 with FMF, 29 with CAPS, 26 with PFAPA and 38 with uRF. Similarly evaluation of demographic and genetic data by geneticists (blinded

Table 1 Demographic features of the 360 patients included in the study

	FMF n=60	CAPS n=60	MKD n=60	TRAPS n=60	PFAPA n=60	uRF n=60
Male	30 (50%)	32 (53%)	26 (43%)	35 (58%)	28 (47%)	28 (47%)
Paediatric/Adults	54/6	33/27	45/15	29/31	59/1	39/21
Age, years, median (range)	10.5 (7.0–15.5)	16.0 (8.9–31.6)	16.2 (9.1–23.0)	21.9 (10.5–41.1)	6.6 (3.8–9.5)	13.5 (8.2–26.4)
Age at disease onset, median (range)	3.4 (1.2–6.4)	3.0 (0.5–11.2)	0.4 (0.2–0.9)	3.4 (0.8–10.6)	1.5 (0.7–3.0)	5.9 (2.0–19.1)
Disease duration, median (range)	5.6 (2.7–10.2)	9.0 (4.6–19.1)	14.2 (7.9–20.8)	13.3 (6.8–23.2)	3.9 (2.3–6.8)	4.8 (3.0–8.2)
Episode duration, median (range)	3.0 (2.0–4.0)	2.0 (0.8–5.0)	5.0 (4.0–7.0)	8.0 (5.0–18.0)	4.0 (3.0–5.0)	4.0 (3.0–7.0)
Number episodes/year, median (range)	12.0 (10.0–20.0)	12.0 (6.0–25.0)	12.0 (10.0–16.0)	6.0 (4.0–12.0)	12.0 (12.0–18.0)	12.0 (5.0–13.0)

CAPS, cryopyrin-associated periodic syndromes; FMF, familial Mediterranean fever; MKD, mevalonate kinase deficiency; PFAPA, periodic fever, aphthosis, pharyngitis and adenitis; TRAPS, tumour necrosis factor receptor-associated periodic fever syndrome; uRF, undefined recurrent fevers.

to clinical data) in two separate rounds reached consensus on 319 of 360 (89%) with 278 (77%) patients with 80% consensus after the first round. At the end of the two initial rounds, 128 (36%) patients were concordant between the independent evaluation of both the clinicians and the geneticists. At the end of the fourth round, consensus was achieved in 281 of 360 (78%) as follows: 56 (95%) MKD, 39 (76%) TRAPS, 37 (70%) PFAPA, 36 (71%) FMF, 32 (63%) CAPS and 81 (85%) uRF (figure 1, online supplementary table 4). K (concordance coefficient) agreement between the panel reference standard classification and the original patient diagnosis by the treating physician was 0.99 for MKD, 0.87 for TRAPS, 0.86 for CAPS, 0.84 for FMF and 0.68 for PFAPA.

Statistical analysis for the selection of the best classification criteria

The top variables arising from step 1 (see the Methods section) were included in a univariate logistic regression analysis using the 281 patients for which consensus was achieved by the panel as outcome. Clinical variables positively and negatively associated with each disease are reported in online supplementary table 5 with the related OR and 95% CI. The strategy for the classification of the genotypes is described in online supplementary table 6.

A total of 198 over >30 000 possible new sets of classification criteria (available on request; 50 for CAPS, 45 for FMF, 44 for TRAPS, 32 for MKD and 22 for PFAPA) were retained, based on

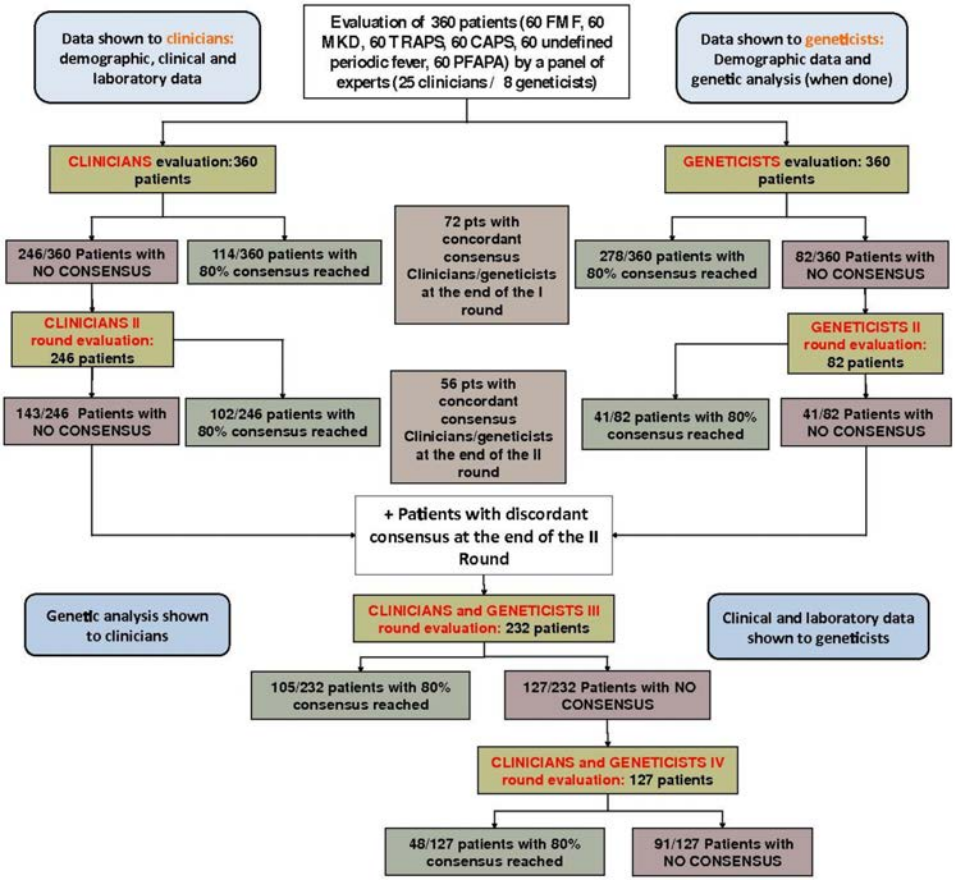

Figure 1 Flow chart of the consensus nominal group technique for classification of patients from the Eurofever Registry. CAPS, cryopyrin-associated periodic syndromes; FMF, familial Mediterranean fever; MKD, mevalonate kinase deficiency; PFAPA, periodic fever, aphthosis, pharyngitis and adenitis; pts, patients; TRAPS, tumour necrosis factor receptor-associated periodic fever syndrome.

Table 2 New Eurofever/PRINTO classification criteria for hereditary recurrent fevers and their performance in the 281 patients with consensus

CAPS	FMF	TRAPS	MKD
Presence of a *confirmatory NLRP3 genotype** and *at least one* among the following: ► Urticarial rash. ► Red eye (conjunctivitis, episcleritis, uveitis). ► Neurosensorial hearing loss. OR Presence of *not confirmatory NLRP3 genotype*† and *at least two* among the following: ► Urticarial rash. ► Red eye (conjunctivitis, episcleritis, uveitis). ► Neurosensorial hearing loss.	Presence of *confirmatory MEFV genotype** and *at least* one among the following: ► Duration of episodes 1–3 days. ► Arthritis. ► Chest pain. ► Abdominal pain. OR Presence of *not confirmatory MEFV* genotype‡ and *at least two* among the following: ► Duration of episodes 1–3 days. ► Arthritis. ► Chest pain. ► Abdominal pain.	Presence of confirmatory *TNFRSF1A* genotype* and at least one among the following: ► Duration of episodes ≥7 days. ► Myalgia. ► Migratory rash. ► Periorbital oedema. ► Relatives affected. OR Presence of a not confirmatory *TNFRSF1A* genotype† and *at least two* among the following: ► Duration of episodes ≥7 days. ► Myalgia. ► Migratory rash. ► Periorbital oedema. ► Relatives affected.	Presence of a *confirmatory MVK genotype** and *at least one* among the following: ► Gastrointestinal symptoms. ► Cervical lymphadenitis. ► Aphthous stomatitis.
Sensitivity: 1	Sensitivity: 0.94	Sensitivity: 0.95	Sensitivity: 0.98
Specificity: 1	Specificity: 0.95	Specificity: 0.99	Specificity: 1
Accuracy: 1	Accuracy: 0.98	Accuracy: 0.99	Accuracy: 1

A patient with (1) evidence of elevation of acute phase reactants (ESR or CRP or SAA) in correspondence to the clinical flares and (2) careful consideration of possible confounding diseases (neoplasms, infections, autoimmune conditions, other inborn errors of immunity) and a reasonable period of recurrent disease activity (at least 6 months) is classified as having hereditary recurrent fever if the criteria are met.
*Pathogenic or likely pathogenic variants (heterozygous in AD diseases, homozygous or in trans (or biallelic) compound heterozygous in AR diseases).
†Variant of uncertain significance (VUS). Benign and likely benign variants should be excluded.
‡In trans compound heterozygous for one pathogenic *MEFV* variants and one VUS, or biallelic VUS, or heterozygous for one pathogenic *MEFV* variant. See online supplementary table 7 for glossary.
AD, autosomal dominant; AR, autosomal recessive; CAPS, cryopyrin-associated periodic syndromes; CRP, C-reactive protein; ESR, erythocytes sedimentation rate; FMF, familial Mediterranean fever; MKD, mevalonate kinase deficiency; *MVK*, mevalonate kinase; PRINTO, pediatric rheumatology international trial organization; SAA, serum amyloid A; TRAPS, tumour necrosis factor receptor-associated periodic fever syndrome.

their AIC, for further evaluation at the Consensus Conference, together with 11 criteria from the literature (online supplementary figure 4).

NGT Consensus Conference for the selection of the final classification criteria

The performances of all the criteria chosen by the consensus in the 281 patients who reached a consensus are reported in tables 2 and 3 (see also glossary in online supplementary table 7).

The first disease discussed was FMF. After multiple voting sessions, all three tables of experts, which worked independently from each other, showed a complete convergent validity selecting the same top definition number 38 (online supplementary figure 4, session A), including genetic and clinical variables with a positive association (table 2). After general discussion, a second set of criteria based solely on clinical criteria was selected to be used as a possible tool for the indication for molecular analysis or as classification criteria in case genetic testing is not locally available

Table 3 Eurofever/PRINTO clinical classification criteria for PFAPA and hereditary recurrent fevers and their performance in the 281 for whom consensus was achieved

PFAPA	CAPS	FMF	TRAPS	MKD
At least *seven out of* eight: Presence ► Pharyngotonsillitis. ► Duration of episodes, 3–6 days. ► Cervical lymphadenitis. ► Periodicity. Absence ► Diarrhoea. ► Chest pain. ► Skin rash. ► Arthritis.	Presence of *at least two of five**: ► Urticarial rash. ► Cold/Stress-triggered episodes. ► Sensorineural hearing loss. ► Chronic aseptic meningitis. ► Skeletal abnormalities (epiphysial overgrowth/frontal bossing).	At least *six out of nine*: Presence ► Eastern Mediterranean ethnicity. ► Duration of episodes, 1–3 days. ► Chest pain. ► Abdominal pain. ► Arthritis. Absence ► Aphthous stomatitis. ► Urticarial rash. ► Maculopapular rash. ► Painful lymph nodes.	Score ≥5 points: Presence ► Fever ≥7 days (2 points). ► Fever 5–6 days (1 point). ► Migratory rash (1 point). ► Periorbital oedema (1 point). ► Myalgia (1 point). ► Positive family history (1 point). Absence ► Aphthous stomatitis (1 point). ► Pharyngotonsillitis (1 point).	Presence of *at least three of six*: ► Age at onset <1 years. ► Gastrointestinal symptoms. ► Painful lymph nodes. ► Aphthous stomatitis. ► Triggers. ► Maculopapular rash.
Sensitivity: 0.97	Sensitivity: 0.80	Sensitivity: 0.91	Sensitivity: 0.87	Sensitivity: 0.91
Specificity: 0.93	Specificity: 0.91	Specificity: 0.92	Specificity: 0.92	Specificity: 0.82
Accuracy: 0.99	Accuracy: 0.85	Accuracy: 0.97	Accuracy: 0.96	Accuracy: 0.92

*Modified by Kuemmerle-Deschner *et al*.[14] See online supplementary table 6 for glossary. See table 2 for prerequisite criteria.
CAPS, cryopyrin-associated periodic syndromes; FMF, familial Mediterranean fever; MKD, mevalonate kinase deficiency; PFAPA, periodic fever, aphthosis, pharyngitis and adenitis; PRINTO, pediatric rheumatology international trial organization; TRAPS, tumour necrosis factor receptor-associated periodic fever syndrome.

(online supplementary figure 4, session B). To this aim, definition number 12, including clinical variables with both positive and negative association with the disease, was chosen (table 3).

The same approach was followed for the other HRFs (CAPS, TRAPS, MKD), leading to the selection of criteria with genetic and clinical variables (number 32 for CAPS, number 46 for TRAPS, number 37 for MKD) (table 2, online supplementary figures 6-8). As per the process to establish FMF criteria, a set of purely clinical criteria (ie, without genetic results) was also selected for each HRF, namely definitions number 20 and number 1 for MKD and TRAPS, respectively (table 3). For CAPS classification, the experts reached consensus on a modified version of recently published criteria.[14] The performance of the original Kummerle criteria (using two out of six criteria) in the context of the present study population displayed a good sensitivity (0.91), but a low specificity (0.80).[14] In contrast, when the variable 'musculoskeletal pain' was excluded, a higher specificity (0.94, with a sensitivity of 0.80) was achieved, if two out of five criteria are present (table 3). The most severe form of CAPS, chonic infantile neurological cutaneous articular (CINCA)/neonatal onset multisystemic inflammatory disorder (NOMID), displays a chronic rather than a recurrent disease course. Patients with CINCA were not included in the validation process described above. However, when the new genetic and clinical CAPS criteria were tested in a separate set of 70 patients with CAPS with chronic disease course enrolled in the Eurofever Registry, the sensitivity was 100% for the genetic and clinical criteria and 80% for the clinical criteria (not shown).

Clinical classification criteria for PFAPA were discussed between the 25 clinical panellists distributed in two tables (no geneticists). After discussion (online supplementary figure 8), definition number 13 (clinical variables with both positive and negative association) was chosen (table 3). During the Consensus Conference, the panel agreed on a few suggested mandatory criteria that should be fulfilled in all the patients before the application of the new classification criteria (table 3) with a consensus of 100% for point 1 and 96% for point 2.

Globally, convergent validity among the three tables of experts was obtained for the genetic and clinical definitions of FMF and CAPS, whereas for all the other definitions a partial convergent validity (agreement in two out of three tables) was reached, with the need for a final plenary voting session (online supplementary figures 4-8 and online supplementary table 8).

Cross-validation of the final classification criteria

The ability of the new classification criteria to discriminate among the different recurrent fevers and uFR was further tested in the validation data set of 1018 patients extracted from the Eurofever Registry (online supplementary table 9) using as reference standard for each patient the diagnosis given by the treating physician. In the last column of table 4, the genotype (score 0=negative/not done; score 1=not confirmatory; score 2=confirmatory) of patients not identified by the clinical criteria for HRF is reported. Notably, almost all the patients not classified by the clinical and genetic criteria displayed a negative or not confirmatory genotype (table 4). The performance of the new classification criteria (either clinical and genetic or clinical only) was generally superior (accuracy ranging from 0.81 to 0.98; table 4) to those already available in the literature (accuracy 0.56–0.94) (online supplementary table 10).

DISCUSSION

The present study provides new evidence-based classification criteria for the four 'classical' HRF (FMF, MKD, TRAPS, CAPS) and PFAPA, incorporating combined consensus expertise of clinicians and geneticists with statistical analyses in real patients from the Eurofever Registry. At variance with past work[15] these new classification criteria combine genetic and clinical variables to overcome the paradox of the absence of a role of the molecular analysis for the proper identification of patients affected by these (mainly) genetic conditions. As defined by the American College of Rheumatology, the proposed classification criteria have selected clinical and genetic findings able to identify the defined diseases and separate from other confounding autoinflammatory conditions.[18 19] Although these criteria may at times be helpful in clinical practice, they are explicitly not meant to be employed as diagnostic criteria. The advent of the so-called next-generation sequencing era resulted on one side to an increased availability of the molecular analysis at reduced costs but might often lead to difficulties in the proper interpretation of this large set of bioinformatic data. In fact, besides the identification of clearly pathogenic variants, in many circumstances (ie, low penetrance variants or variants of unknown significance, monoallelic variants in autosomal recessive diseases, presence of variants in more than one gene) the genetic results are not unequivocal and should be placed in the context of a pertinent clinical setting. In these latter cases, the classification of the patient is usually problematic, as clearly shown in the process of patients' validation in this study. For these reasons, the panel decided to introduce a distinction between a confirmatory (namely, surely or likely pathogenic variants) and not confirmatory (variants of unknown significance) genetic test. For the daily use of the new criteria, a parallel consensus classification effort by the genetic subcommittee of the INSAID project has established the pathogenicity of each currently known variant associated to HRF.[30] A differential approach for the interpretation of the biallelic variants was chosen for the two autosomal recessive diseases, namely MKD and FMF. MKD is caused by loss-of-function mutations in the *MVK* gene. The panel excluded the possibility of classifying a patient as an MKD in the absence of biallelic mutations of the *MVK* gene. Conversely, recent evidence has clarified that FMF is secondary to gain-of-function mutations of the *MEFV* gene, with a clear dose effect,[31 32] and therefore FMF could be classified with identification of either one or two pathogenic variants in exon 10 of *MEFV* in the presence of a consistent clinical phenotype. The same possibility was also considered for the two autosomal dominant diseases, CAPS and TRAPS, in the absence of confirmatory phenotype. In patients carrying variants of unknown pathogenic significance (such as R92Q and P46L for *TNFRSF1A*, or V198M for *NLRP3*),[33–36] only the presence of some very specific clinical variables would support the proper disease classification. In parallel with the elaboration of the definitive criteria that include genetic/clinical variables, the panel agreed on additional clinical criteria that should be used to (1) identify patients with recurrent fevers that would need to undergo genetic testing for molecular confirmation; (2) search for possible somatic mosaicism using NG in patients with a clear phenotype, but negative Sanger sequencing results; and (3) classify patients (eg, for epidemiological studies) even in those countries where routine genetic testing is not possible. For PFAPA, the contemporary evaluation of either positive (presence) and negative (absence) clinical variables yielded a much higher accuracy when compared with the classical modified Marshall's criteria.[16] Following the consensus meeting, the new sets of criteria were

Table 4 Performance of the new classification criteria to discriminate different recurrent fevers in the validation data set of patients extracted from the Eurofever Registry (N=1018)

	Positive/Negative	OR	Sensitivity	Specificity	Accuracy	AUC	Patients not fulfilling the criteria	Number of patients not fulfilling the clinical criteria but satisfying the clinical/genetic criteria
CAPS clinical + genetics	TP: 98/1013 TN: 877/1013 FP: 0/1013 FN: 38/1013	4490	0.72	1	0.96	0.86	27/38 pts, score 0 (71.05%) 10/38 pts, score 1 (26.32%) 1/38 pts, score 2 (2.63%)	
CAPS clinical	TP: 82/925 TN: 806/925 FP: 12/925 FN: 25/925	220.3	0.77	0.99	0.96	0.88	3/25 pts, score 0 (13.04%) 8/25 pts, score 1 (34.78%) 12/25 pts, score 2 (52.17 %)	Score 0: 0/3 Score 1: 1/8 Score 2: 11/12
FMF clinical + genetics	TP: 304/1010 TN: 664/1010 FP: 3/1010 FN: 39/1010	1725.3	0.89	1	0.96	0.94	12/39 pts, score 0 (30.77%) 26/39 pts, score 1 (66.67%) 1/39 pts, score 2 (2.56%)	
FMF clinical	TP: 283/940 TN: 568/940 FP: 39/940 FN: 50/940	82.4	0.85	0.94	0.91	0.89	3/50 pts, score 0 (6,12%) 26/50 pts, score 1 (53,06%) 20/50 pts, score 2 (40,82 %)	Score 0: 0/3 Score 1: 8/26 Score 2: 19/20
MKD clinical + genetics	TP: 45/1005 TN: 944/1005 FP: 0/1005 FN: 16/1005	5209.1	0.74	1	0.98	0.87	2/16 pts, score 0 (12.5%) 14/16 pts, score 1 (87.5%)	
MKD clinical	TP: 43/818 TN: 617/818 FP: 144/818 FN: 14/818	13.2	0.75	0.81	0.81	0.78	2/14 pts, score 1 (15.38%) 11/14 pts, score 2 (84.62%)	Score 1: 0/2 Score 2: 10/11
TRAPS clinical + genetics	TP: 73/1000 TN: 900/1000 FP: 1/1000 FN: 26/1000	2526.9	0.74	1	0.97	0.87	6/26 pts, score 0 (23.08%) 20/26 pts, score 1 (76.92%)	
TRAPS clinical	TP: 52/940 TN: 813/940 FP: 33/940 FN: 42/940	30.4	0.55	0.96	0.92	0.76	27/42 pts, score 1 (71.05%) 11/42 pts, score 2 (28.95 %)	Score 1: 8/27 Score 2: 11/11
PFAPA clinical	TP: 149/1001 TN: 752/1001 FP: 24/1001	61.4	0.66	0.97	0.9	0.82		

For explanation of the scores 0–1–2, see online supplementary table 6.
CAPS, cryopyrin-associated periodic syndromes; FMF, familial Mediterranean fever; FN, false negative; FP, false positive; MKD, mevalonate kinase deficiency; PFAPA, periodic fever, aphthosis, pharyngitis and adenitis; pts, patients.TN, true negative; TP, true positive; TRAPS, tumour necrosis factor receptor-associated periodic fever syndrome;

further validated in a large group of additional patients from the Eurofever Registry, showing a very high specificity when compared with previous literature criteria. As noticed, most of the diagnoses refuted by the new criteria had been in patients with either non-confirmatory or negative genetic tests results. It is therefore conceivable that the present new criteria will be more stringent in the classification of patients, by excluding a substantial proportion of patients carrying variants of unknown origin. The classification criteria we propose are accurate for the discrimination of one form of autoinflammation from another in the context of the six conditions considered herein, but very much have to be applied judiciously, after careful consideration of confounding diseases, as highlighted in table 2. These classification criteria are therefore intended for use for clinical, epidemiological or translational studies, but not for routine diagnostic purposes in individual patients.[37] That said, the purely clinical classification criteria might guide molecular testing approaches for individual cases, although this point requires future validation. One possible limitation of the present study is the lack of comparison groups including possible confounding conditions (chronic infections, neoplasms, immune deficiencies, autoimmune disease and metabolic diseases) presenting sometimes with a recurrent disease course. In daily practice confounding diseases with a true recurrent disease course are rather infrequent outside HRF and PFAPA, while the most challenging group of confounding conditions are the large emerging group of patients with uRF, many of whom may have a true monogenetic cause other than the four genetic diseases considered herein. For these reasons, the different HRFs have been used as controls for each individual condition with PFAPA and uRF as genetically negative controls. The panel of experts unanimously decided that the presence of elevation of acute phase reactants during disease flares (recorded at least in one occasion) should be considered as *mandatory* preliminary criterion for the use of the new classification criteria.[14] Some other relevant pathognomonic laboratory examinations, such as urinary mevalonic acid in MKD, were not available in the Eurofever Registry, probably reflecting the fact that it is not widely available for testing routinely. As such the panel recommended the importance for the diagnostic work-up, for example, with intracellular MVK enzyme activity and/or urinary mevalonic acid in MKD,[38] particularly for patients with convincing phenotypes but non-confirmatory genotype for MKD. Similarly, the response to some specific treatments (such as colchicine in FMF or anti-interleukin (IL)-1 in CAPS) or ethnic background (for FMF) could certainly be considered as additional elements to be considered in daily practice, especially for patients with non-confirmatory genotype, but are not good discriminators of the different forms of autoinflammatory disease considered herein. In conclusion, the present work allowed the proposal of novel evidence-based classification criteria for HRF and PFAPA with a high specificity. The use of these classification criteria is highly recommended for inclusion of patients in translational and clinical studies, including clinical trials, and should not be misused as diagnostic criteria.[17] The possible identification of new genetic entities in the heterogeneous group of undefined periodic fevers could require an update of the criteria in the future.

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Contributors MG, MH and NR coordinated the study, analysed the data and drafted the manuscript. SF, FV and CG analysed the data. FB and MPS performed statistical analysis. IA, JA, JIA, KB, EB-C, PAB, LC, IC, FDB, FD, ED, JF, RG-M, AG, VH, HH, TK, IK-P, JK-D, HJL, RML, AL, LO, DR, RR, YS, AS, NT, IT, YU, MvG, DK, DF and AM participated in the Delphi, patient evaluation and Consensus Conference. All authors evaluated and approved the manuscript.

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Patient consent for publication Not required.

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Provenance and peer review Not commissioned; externally peer reviewed.

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OPEN ACCESS

CLINICAL SCIENCE

Efficacy and safety of bimekizumab as add-on therapy for rheumatoid arthritis in patients with inadequate response to certolizumab pegol: a proof-of-concept study

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ABSTRACT

Objective Evaluate the efficacy and safety of dual neutralisation of interleukin (IL)-17A and IL-17F with bimekizumab, a monoclonal IgG1 antibody, in addition to certolizumab pegol (CZP) in patients with rheumatoid arthritis (RA) and inadequate response (IR) to certolizumab pegol.

Methods During this phase 2a, double-blind, proof-of-concept (PoC) study (NCT02430909), patients with moderate-to-severe RA received open-label CZP 400 mg at Weeks 0, 2 and 4, and 200 mg at Week 6. Patients with IR at Week 8 (Disease Activity Score 28-joint count C-reactive protein (DAS28(CRP))>3.2) were randomised 2:1 to CZP (200 mg every 2 weeks (Q2W)) plus bimekizumab (240 mg loading dose then 120 mg Q2W) or CZP plus placebo. The primary efficacy and safety variables were change in DAS28(CRP) between Weeks 8 and 20 and incidence of treatment-emergent adverse events (TEAEs).

Results Of 159 patients enrolled, 79 had IR at Week 8 and were randomised to CZP plus bimekizumab (n=52) or CZP plus placebo (n=27). At Week 20, there was a greater reduction in DAS28(CRP) in the CZP-IR plus bimekizumab group compared with the CZP-IR plus placebo group (99.4% posterior probability). The most frequent TEAEs were infections and infestations (CZP plus bimekizumab, 50.0% (26/52); CZP plus placebo, 22.2% (6/27)).

Conclusions PoC was confirmed based on the rapid decrease in disease activity achieved with 12 weeks of CZP plus bimekizumab. No unexpected or new safety signals were identified when neutralising IL-17A and IL-17F in patients with RA concomitantly treated with CZP, but the rate of TEAEs was higher with dual inhibition.

Key messages

What is already known about this subject?

- Significant increases in circulating T helper 17 cells and interleukin (IL)-17 production have been observed following inadequate response to tumour necrosis factor (TNF) inhibitors (anti-TNFs) in patients with rheumatoid arthritis.
- It has been hypothesised that this compensatory amplification of IL-17 biology may contribute to the impaired response to anti-TNF treatment in some patients; however, clinical data substantiating this hypothesis are conflicting.

What does this study add?

- We evaluated the efficacy and safety of dual neutralisation of IL-17A and IL-17F with bimekizumab, a monoclonal IgG1 antibody, in addition to certolizumab pegol in patients with rheumatoid arthritis and inadequate response to certolizumab pegol.
- Proof-of-concept was confirmed based on the rapid decrease in disease activity achieved with 12 weeks of certolizumab pegol and bimekizumab treatment, with no unexpected or new safety findings identified.

How might this impact on clinical practice or future developments?

- These findings support the potential to further explore concomitant neutralisation of multiple pathways in other patient populations where this treatment strategy may provide additional benefits.

INTRODUCTION

It is well documented that some patients with rheumatoid arthritis (RA), particularly those with poor prognostic factors, have an inadequate response (IR) to initial treatment with conventional synthetic disease-modifying antirheumatic drugs (csDMARDs), such as methotrexate (MTX). In these individuals, add-on treatment with tumour necrosis factor (TNF) inhibitors (anti-TNFs) is often considered.[1] As a class, anti-TNFs (adalimumab, certolizumab pegol, etanercept, golimumab and infliximab) comprise an effective treatment approach that has considerably improved the success of treatment for RA.[2 3] However, sustained disease remission is only achieved by <10% of patients, and there remains a group of patients who fail to respond, or do not achieve an adequate response, even with anti-TNFs.[4] Should patients fail to respond to one anti-TNF, they may be treated with another anti-TNF or a treatment with a different mode of action.[1]

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Significant increases in circulating T helper 17 (Th17) cells and interleukin (IL)-17 production have been observed following IR to anti-TNFs in patients with RA.[5–8] It has been hypothesised that this compensatory amplification of IL-17 biology may contribute to the impaired response to anti-TNF treatment in some patients; however, clinical data substantiating this hypothesis are conflicting. For example, phase 3 studies have shown that IL-17A blockade with secukinumab has minimal efficacy in patients with RA who have IR or intolerance to anti-TNFs,[9–11] suggesting inhibition of IL-17A alone is insufficient to neutralise the inflammatory response in RA. Conversely, a phase 2 study demonstrated a modest but statistically greater American College of Rheumatology 20% improvement criteria (ACR20) response with ixekizumab, another higher affinity anti-IL-17A, compared with placebo after 12 weeks' treatment in patients with RA with IR to anti-TNF therapy.[12]

In addition to IL-17A, Th17 cells secrete a number of pro-inflammatory cytokines including IL-22, IL-26, interferon (IFN)-γ, TNF, granulocyte-macrophage colony-stimulating factor, C-C motif chemokine ligand 20 and another member of the IL-17 family, IL-17F.[13] Both IL-17A and IL-17F have been shown to independently co-operate with other cytokines to mediate chronic inflammation[14]; they share ~50% structural homology and overlapping but non-redundant biological functions,[15–17] suggesting IL-17F may also play an important role in RA. Bimekizumab is a monoclonal immunoglobulin G1 antibody that potently and selectively neutralises the biological function of both IL-17A and IL-17F.[18] In a proof-of-concept (PoC) study in patients with psoriatic arthritis, bimekizumab demonstrated rapid, profound responses in joint and skin, with no unexpected safety findings.[18] In the phase 2b BE ABLE 1 study, rapid and substantial improvements were achieved with bimekizumab in patients with moderate-to-severe psoriasis.[19] These data support the rationale for targeting both IL-17A and IL-17F in immune-mediated inflammatory disease. For those patients with RA and IR to anti-TNFs, neutralisation of both IL-17A and IL-17F in addition to TNF inhibition may reduce disease activity compared with inhibition of TNF alone. However, the potential safety effects of inhibiting these three cytokines together are not known.

Here, we report the efficacy and safety results of a phase 2a, randomised, double-blind, placebo-controlled PoC study (NCT02430909) evaluating certolizumab pegol, a Fc-free, PEGylated anti-TNF that provides rapid and sustained improvements to many patients with RA,[20 21] in combination with bimekizumab in patients with moderate-to-severe RA who had an IR to certolizumab pegol.

METHODS

Study design and treatment

This was a multicentre phase 2a, randomised, double-blind, placebo-controlled PoC study (NCT02430909) to assess the efficacy and safety of certolizumab pegol plus bimekizumab in patients with moderate-to-severe RA and IR to certolizumab pegol. This study was conducted in accordance with the principles of the Declaration of Helsinki and the International Conference on Harmonisation Guidance for Good Clinical Practice. Independent institutional review board approvals were obtained, and all patients provided written informed consent in accordance with local requirements.

During an 8-week open-label, run-in period, patients received certolizumab pegol 400 mg at Weeks 0, 2 and 4, and then 200 mg at Week 6 (figure 1). Patients who responded to certolizumab pegol during the open-label run-in period remained on this treatment. Patients with IR to certolizumab pegol at Week 8, defined as Disease Activity Score 28-joint count C-reactive protein (DAS28(CRP))

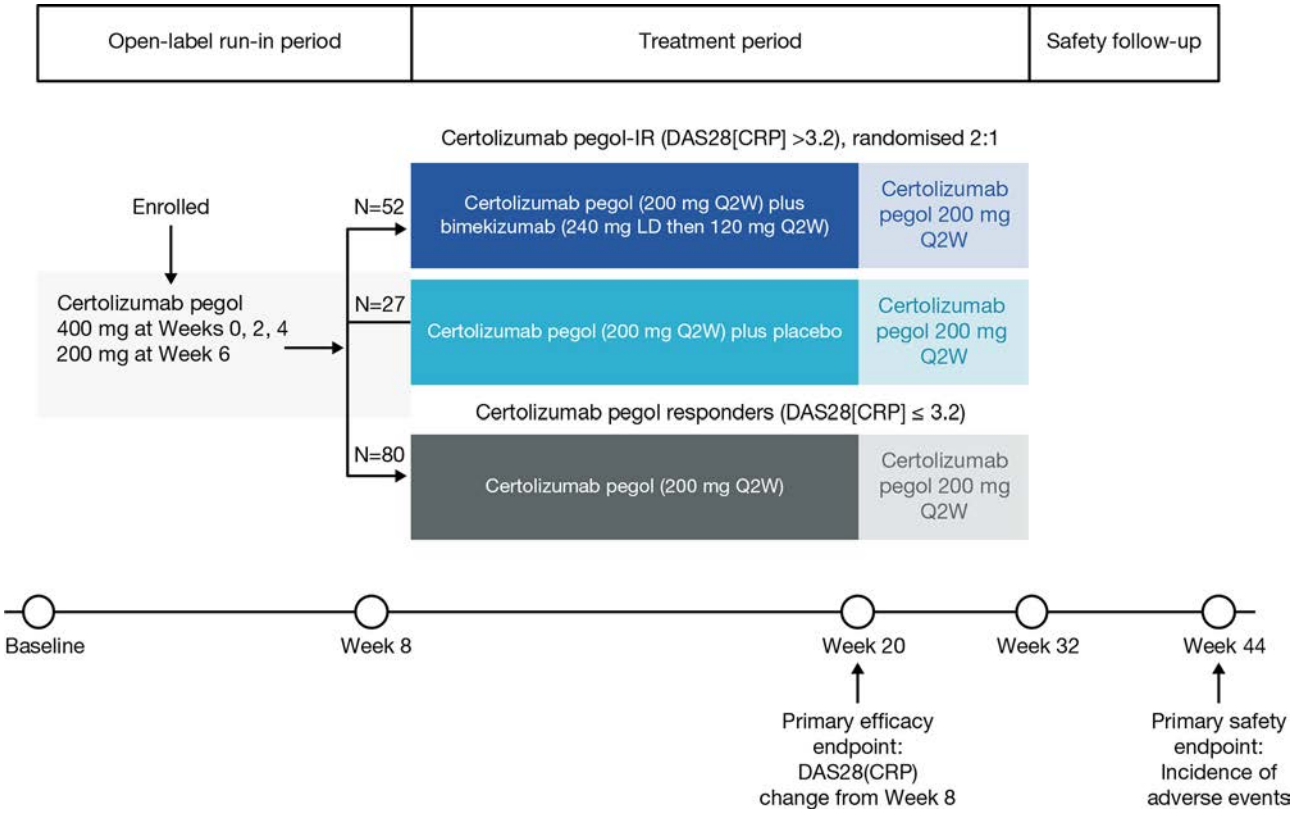

Figure 1 Study design. DAS28(CRP), Disease Activity Score 28-joint count (C-reactive protein); Q2W, once every 2 weeks.

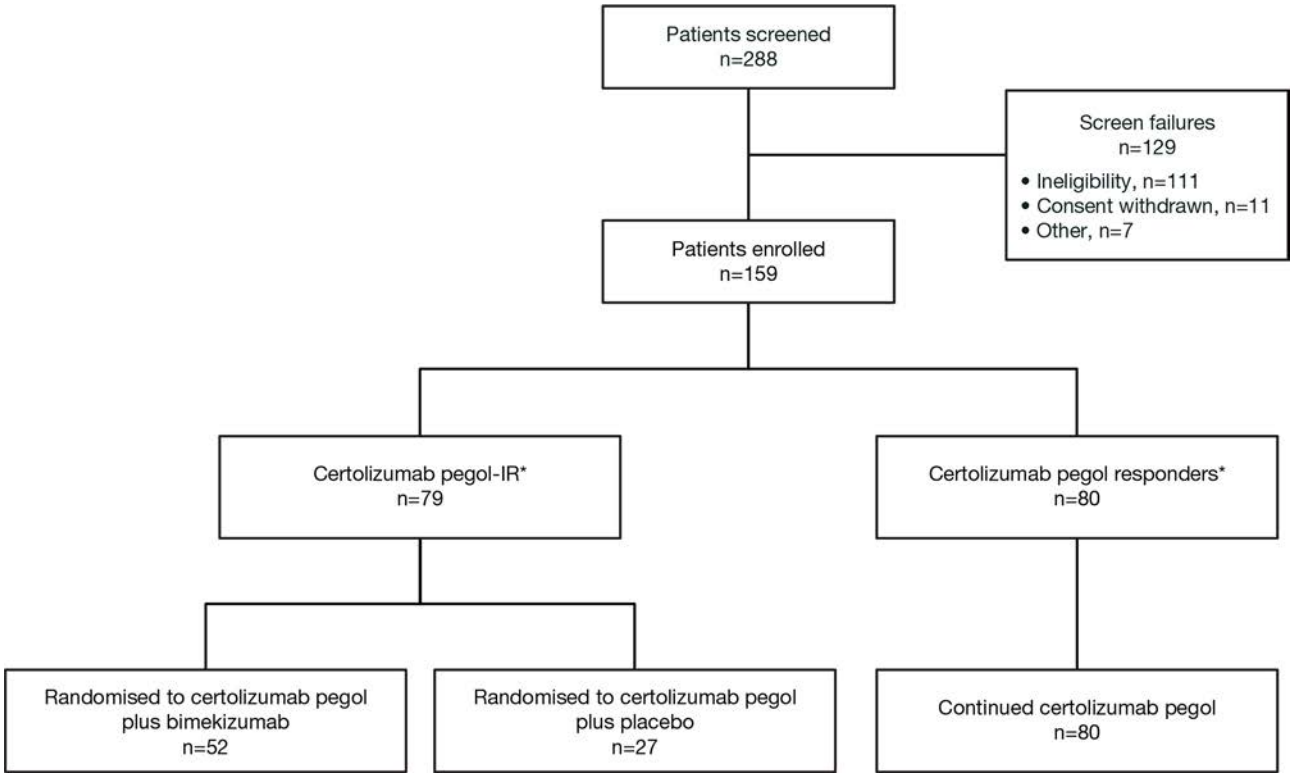

Figure 2 Patient disposition. *Inadequate response was defined as Disease Activity Score 28-joint count (C-reactive protein) >3.2. IR, inadequate response.

>3.2, were randomised 2:1 to certolizumab pegol (200 mg every 2 weeks (Q2W)) plus bimekizumab (240 mg loading dose then 120 mg Q2W) or certolizumab pegol (200 mg Q2W) plus placebo. At Week 20, the add-on therapy (bimekizumab or placebo) was withdrawn; certolizumab pegol continued until Week 32, after which their treatment was determined by their clinician outside of the study protocol. There was a final follow-up visit at Week 44, 12 weeks after the end of study treatment.

Patients

Patients were aged 18–69 years with a diagnosis of adult-onset moderate-to-severe RA of ≥6 months' duration as defined by ACR/European League Against Rheumatism 2010 classification criteria.[22] Additional inclusion criteria were body mass index 18–35 kg/m^2, with a body weight of ≥50 kg (men) or 45 kg (women); ≥6 tender joints (out of 68), ≥6 swollen joints (out of 66) and ≥10 mg/L CRP; and IR to ≥1 csDMARD. Patients with IR to csDMARDs could continue on stable doses of any permitted csDMARD; patients with a history of IR, but not currently receiving csDMARDS, were also eligible.

Key exclusion criteria were previous exposure to anti-TNFs, IL-17 inhibitors or bimekizumab; receipt of any investigational drug or experimental procedure within 90 days prior to baseline; and receipt of prohibited medications (online supplementary table S1). Patients with an active/high risk of infection, active or latent tuberculosis, known central nervous system demyelinating disorder or neoplastic disease within 5 years of study entry (with the exception of definitively treated basal or squamous carcinoma of the skin or carcinoma in situ) were excluded.

Assessments

The primary efficacy endpoint was change in DAS28(CRP) between Weeks 8 and 20. Secondary efficacy endpoints were DAS28(CRP) remission (DAS28(CRP)<2.6) at Week 20, percentage of improvement in ACR criteria (ACRn), ACR20, ACR50 and ACR70 response between Weeks 8 and 20. DAS28(CRP) remission by visit was an exploratory endpoint. The primary safety endpoint was the incidence of adverse events (AEs) at follow-up (Week 44); results of clinical laboratory tests was an additional safety endpoint. Treatment-emergent AEs (TEAEs) that occurred during treatment with either bimekizumab or placebo were defined as any AE that started or worsened on or after the first dose of bimekizumab or placebo, up to 140 days after the last dose.

Statistical methods

The primary and secondary efficacy endpoints were analysed using a Bayesian approach. At the design and analysis stage, an informative prior[23] (equivalent to approximately 13 patients) was assumed for the primary endpoint; this allowed for information borrowing from a previous study[21] to augment the control data from the certolizumab pegol-IR plus placebo group (online supplementary methods). A Bayesian analysis of covariance was conducted with treatment as factor and Week 8 DAS28(CRP) as covariate. In addition, several sensitivity analyses were conducted for the primary efficacy endpoint (online supplementary methods). The change from Week 8 in the individual components of DAS28(CRP) at Week 20 was summarised for each treatment group using descriptive statistics. Additional analyses included Boolean, DAS28(CRP)[3] and Clinical Disease Activity Index (CDAI) remission (online supplementary methods). A Bayesian analysis using a logistic model with vague prior distributions was conducted for the secondary efficacy variables (DAS28(CRP) remission, ACRn, ACR20, ACR50 and ACR70 response). These endpoints were plotted over time by

Table 1 Demographics and baseline disease characteristics

	Certolizumab pegol–IR plus bimekizumab (n=52)	Certolizumab pegol–IR plus placebo (n=27)	Certolizumab pegol responders (n=80)
Demographics, n (%)			
Age, median (range), years	53 (26–69)	57 (30–67)	58 (21–69)
Gender			
Male	7 (13.5)	4 (14.8)	13 (16.3)
Female	45 (86.5)	23 (85.2)	67 (83.8)
Race			
Caucasian	52 (100)	27 (100)	80 (100)
Duration of RA			
<2 years	12 (23.1)	8 (29.6)	17 (21.3)
≥2 years	40 (76.9)	19 (70.4)	63 (78.8)
History of extra-articular features	7 (13.5)	1 (3.7)	7 (8.8)
Anti-CCP positive	39 (75)	19 (70.4)	66 (82.5)
Rheumatoid factor positive	39 (75)	22 (81.5)	64 (80.0)
Prior csDMARDs*	49 (94.2)	27 (100)	79 (98.8)
Methotrexate	43 (82.7)	22 (81.5)	76 (95)
Methotrexate sodium	6 (11.5)	4 (14.8)	5 (6.3)
Concomitant csDMARDs†	42 (80.8)	26 (96.3)	71 (88.8)
Methotrexate	29 (55.8)	20 (74.1)	63 (78.8)
Methotrexate sodium	6 (11.5)	3 (11.1)	3 (3.8)
Disease characteristics, mean (SD)			
SJC‡	13.6 (6.4)	16.2 (7.9)	11.1 (5.4)
TJC‡	20.6 (10.5)	26.2 (12.3)	20.3 (9.8)
PtAAP	72.9 (15.5)	73.1 (17.8)	63.2 (21.4)
PtGADA	74 (14.2)	77.7 (17)	64.3 (20.9)
HAQ-DI	1.7 (0.6)	1.9 (0.4)	1.6 (0.6)
DAS28(CRP)	6.1 (0.7)	6.2 (0.8)	5.7 (0.8)

All patients received certolizumab pegol during the 8-week open-label run-in period.
*Prior medications include any medications that started prior to the start date of study medication.
†Concomitant medications are medications taken at least 1 day in common with the study medication, ie, whose start date is prior to the date of last study medication administration plus 14 days, and whose stop date is either missing, or on or after the date of first study medication administration.
‡SJC and TJC were based on 66 and 68 counts, respectively.
Anti-CCP, anti-cyclic citrullinated peptide; csDMARD, conventional synthetic disease-modifying antirheumatic drug; DAS28(CRP), Disease Activity Score 28-joint count (C-reactive protein); HAQ-DI, Health Assessment Questionnaire–Disability Index; PtAAP, Patient's Assessment of Arthritis Pain; PtGADA, Patient's Global Assessment of Disease Activity; RA, rheumatoid arthritis; SCJ, swollen joint count; TJC, tender/painful joint count.

treatment group including the 95% confidence interval (CI) using a Wilson approximation.

Sample size calculations were based on a Bayesian analysis of the primary endpoint (online supplementary methods). A sample size of 60 patients across both treatment groups was deemed sufficient to determine the primary endpoint success criterion of the ≥97.5% probability that the change in DAS28(CRP) from Week 8 was greater for the certolizumab pegol-IR plus bimekizumab group than the certolizumab pegol-IR plus placebo group. The study had an 89% probability of detecting a difference of 0.7 in DAS28(CRP) change from Week 8 between the treatment groups at Week 20.

All analyses were performed using SAS V.9.3 or later, R V.2.10.1 or later, or WinBUGS V.1.4.

RESULTS

Patients

Of 159 patients enrolled, 79 had IR to certolizumab pegol at Week 8 and were randomised to certolizumab pegol plus bimekizumab (n=52) or certolizumab pegol plus placebo (n=27) (figure 2). Patients who achieved low disease activity (DAS28(CRP)≤3.2; n=80) at Week 8 continued to receive open-label certolizumab pegol.

At baseline, demographics and disease characteristics were similar between the certolizumab pegol-IR plus bimekizumab group and the certolizumab pegol-IR plus placebo group (table 1). Disease characteristics at randomisation (Week 8; online supplementary table S2) were also similar between groups, although Patient's Assessment of Arthritis Pain and Patient's Global Assessment of Disease Activity (PtGADA) were numerically higher in the certolizumab pegol-IR plus bimekizumab group (53.4% and 53.7%, respectively) compared with the certolizumab pegol-IR plus placebo group (46% and 45.6%, respectively). Most patients received concomitant csDMARDs (80.8% in the certolizumab pegol-IR plus bimekizumab group and 96.3% in the certolizumab pegol-IR plus placebo group) (table 1). Demographics and characteristics for the certolizumab pegol responders group are also detailed in table 1.

Efficacy

PoC was confirmed based on the primary efficacy endpoint, with a greater reduction in DAS28(CRP) in the certolizumab pegol-IR plus bimekizumab group compared with the certolizumab pegol-IR plus placebo group from Week 8 to Week 20 (99.4% posterior probability by Bayesian analysis using an informative prior distribution). The estimated posterior group mean DAS28(CRP) change from Week 8 to Week 20 for the certolizumab pegol-IR plus bimekizumab group and the certolizumab pegol-IR plus placebo group were -1.41 (95% credible interval (CrI) -1.72, 1.09) and -0.82 (95% CrI -1.15, 0.49), respectively, with an estimated posterior mean treatment difference of 0.58 (95% CrI 0.13, 1.05). The results of sensitivity analyses of the primary efficacy variable were consistent with and supportive of the primary analysis (online supplementary results). The observed mean (SD) change from Week 8 to Week 20 in DAS28(CRP) was -1.40 (1.32) in the certolizumab pegol-IR plus bimekizumab group and -1.04 (0.90) in the certolizumab pegol-IR plus placebo group.

At Week 20, a greater percentage of patients in the certolizumab pegol-IR plus bimekizumab group achieved DAS28(CRP) remission compared with the certolizumab pegol-IR plus placebo group (97.6% posterior probability); the estimated posterior mean treatment difference in DAS28(CRP) remission was 17.8% (95% CrI 0.3, 33.8). The percentage of patients achieving DAS28(CRP) remission in the certolizumab pegol-IR plus bimekizumab group and the certolizumab-IR plus placebo group was similar during Weeks 2 to 8, and numerically higher during Weeks 10 to 26. By Week 32, after a further 12 weeks of certolizumab pegol treatment without the add-on therapy (bimekizumab or placebo), the percentage of patients achieving DAS28(CRP) remission was similar between the certolizumab pegol-IR plus bimekizumab group and certolizumab pegol-IR plus placebo group (figure 3A and online supplementary table S5).

Analysis of the DAS28(CRP) components showed numerically larger reductions in mean swollen joint count (SJC) and tender joint count (TJC) in the certolizumab pegol-IR plus placebo group from Week 8 to Weeks 20 and 32 compared with the

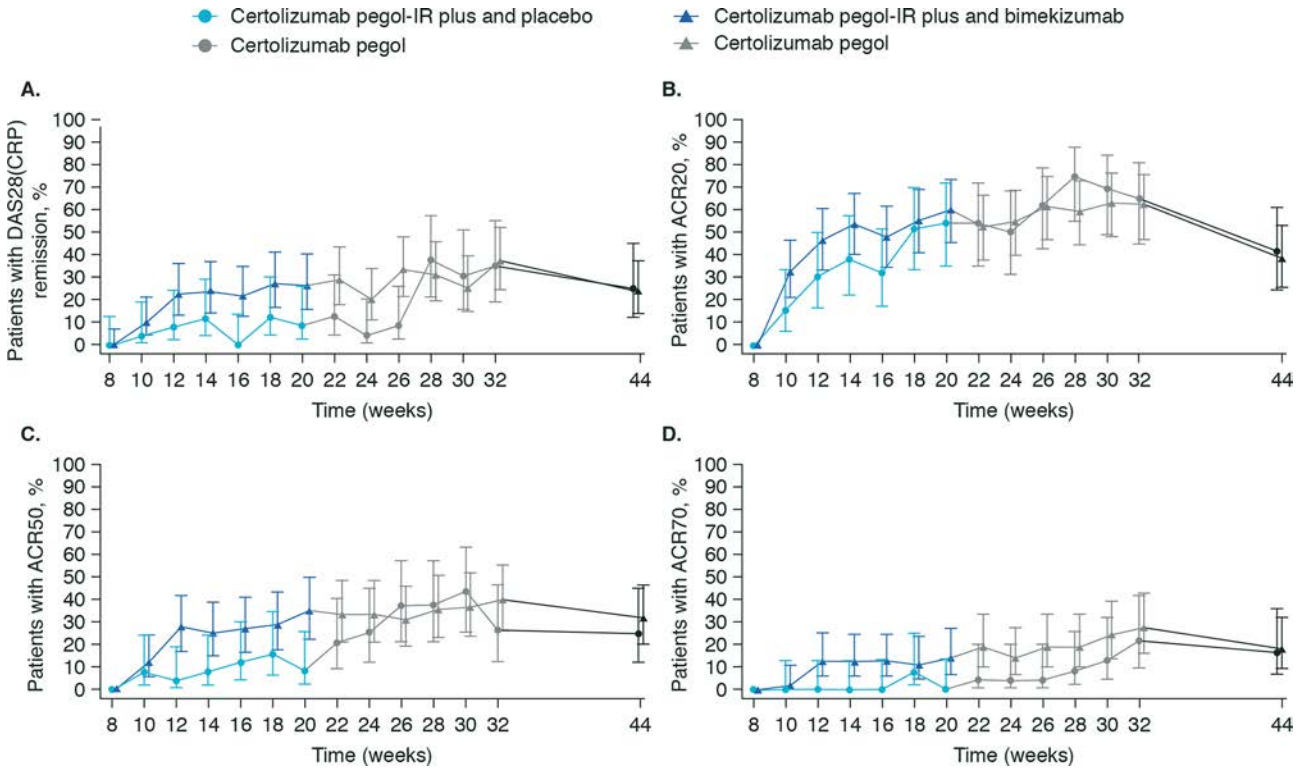

Figure 3 DAS28(CRP) remission by visit (A), percentage of ACR20 (B), ACR50 (C) and ACR70 (D) responders based on Week 8 in the certolizumab pegol-IR plus bimekizumab and certolizumab pegol-IR plus placebo groups. At Week 20, the add-on therapy (bimekizumab or placebo) was withdrawn; certolizumab pegol continued until Week 32. All patients continued certolizumab pegol therapy until Week 32, after which their treatment was determined by their clinician outside of the study protocol. There was a final follow-up visit at Week 44. See online supplementary table S5 for actual data. Error bars represent Wilson's 95% CI. ACR20, ACR50, ACR70, American College of Rheumatology 20%, 50% and 70% improvement criteria; DAS28(CRP), Disease Activity Score 28-joint count (C-reactive protein).

certolizumab pegol-IR plus bimekizumab group; conversely, mean reductions in PtGADA were numerically greater in the certolizumab pegol-IR plus bimekizumab group (online supplementary table S3). Results of additional efficacy endpoints (including Boolean, DAS28(CRP)[3] and CDAI remission) are presented in online supplementary table S4.

The posterior probability of improvement in ACRn in the certolizumab pegol-IR plus bimekizumab group compared with the certolizumab pegol-IR plus placebo group at Week 20 was 70.6%; the estimated posterior mean treatment difference in ACRn was 5.38 (95% CrI -13.8, 25.3). Results of Bayesian analysis of ACR20, ACR50 and ACR70 response are detailed in online supplementary results; percentage of ACR20, ACR50 and ACR70 responders by visit is shown in figure 3, online supplementary table S5 and table 2.

Table 2 DAS28(CRP) and ACR response rates at weeks 20 and 32

	Certolizumab pegol–IR plus bimekizumab (n=52)	Certolizumab pegol–IR plus placebo (n=27)
DAS28(CRP) <3.2, n (%)		
Week 20	21 (45.7)	7 (29.2)
Week 32	26 (60.5)	11 (47.8)
ACR20, n (%)		
Week 20	26 (60.5)	13 (54.2)
Week 32	25 (62.5)	15 (65.2)
ACR50, n (%)		
Week 20	15 (34.9)	2 (8.3)
Week 32	16 (40.0)	6 (26.1)
ACR70, n (%)		
Week 20	6 (14.0)	0
Week 32	11 (27.5)	5 (21.7)

ACR20, ACR50, ACR70, American College of Rheumatology 20%, 50% and 70% improvement criteria.
DAS28(CRP), Disease Activity Score 28-joint count (C-reactive protein).

Safety

Mean durations of exposure for the certolizumab pegol-IR plus bimekizumab group and the certolizumab pegol-IR plus placebo group were similar (80.1 days vs 78.3 days, respectively). Across all parts of the study, the mean duration of exposure for certolizumab pegol was 205.8 days. A greater percentage of patients experienced TEAEs with certolizumab pegol plus bimekizumab compared with certolizumab pegol plus placebo (78.8% (41/52) vs 59.3% (16/27)) (table 3). Severe TEAEs were reported for one patient in each treatment group (one patient in the certolizumab pegol-IR plus bimekizumab group experienced haematoma and psoas abscess; one patient in the certolizumab pegol-IR plus placebo group experienced meningitis). There were no deaths in the certolizumab pegol-IR plus bimekizumab group; one patient in the certolizumab pegol-IR plus placebo group experienced a TEAE resulting in death (meningitis).

The most frequent TEAEs were infections and infestations, with a higher incidence seen with certolizumab pegol plus bimekizumab (50.0% (26/52)) compared with certolizumab pegol plus placebo (22.2% (6/27)). The most common non-serious infections reported by ≥5% of patients in either group were nasopharyngitis, upper respiratory tract infection and pharyngitis

Table 3 TEAEs during certolizumab pegol plus bimekizumab and certolizumab pegol plus placebo treatment

Variable, n (%)*	Certolizumab pegol–IR plus bimekizumab (n=52)	Certolizumab pegol–IR plus placebo (n=27)
Any TEAE	41 (78.8)	16 (59.3)
Serious TEAEs	2 (3.8)	3 (11.1)
Discontinuation due to TEAEs	4 (7.7)	3 (11.1)
Severe TEAEs	1 (1.9)	1 (3.7)
Deaths	0	1 (3.7)
Most common TEAEs by SOC and PT (reported in ≥5% of patients)		
Infections and infestations	26 (50.0)	6 (22.2)
Nasopharyngitis	4 (7.7)	2 (7.4)
Upper respiratory tract infection	3 (5.8)	1 (3.7)
Pharyngitis	3 (5.8)	0 (0.0)
Musculoskeletal and connective tissue disorders	5 (9.6)	7 (25.9)
Rheumatoid arthritis	3 (5.8)	4 (14.8)
Skin and subcutaneous disorders	8 (15.4)	1 (3.7)
Dermatitis allergic	3 (5.8)	0 (0.0)
Gastrointestinal disorders	6 (11.5)	1 (3.7)
Stomatitis	3 (5.8)	0 (0.0)
General disorders and administration site reactions	4 (7.7)	1 (3.7)
Investigations	4 (7.7)	1 (3.7)
Vascular disorders	2 (3.8)	3 (11.1)
Hypertension	0 (0.0)	3 (11.1)
Blood and lymphatic system disorders	3 (5.8)	0 (0.0)
Injury, poisoning and procedural complications	1 (1.9)	2 (7.4)

TEAEs during treatment were defined as an adverse event that started or worsened on or after the first dose of bimekizumab or placebo up to 140 days after the final dose. TEAEs were coded using MedDRA V.19.0.
*n=number of patients reporting at least one TEAE within the SOC/PT.
csDMARD, conventional synthetic disease-modifying antirheumatic drug; PT, preferred term; SOC, system organ class; TEAE, treatment-emergent adverse event.

(table 3). Skin and subcutaneous disorders and gastrointestinal disorders also occurred with a higher incidence in the certolizumab pegol plus bimekizumab group compared with certolizumab pegol plus placebo. Eight patients in the certolizumab pegol plus bimekizumab group reported nine skin and subcutaneous TEAEs, including three of allergic dermatitis, two of rash (both reported by the same patient) and one each of photosensitivity reaction, dermatitis, atopic dermatitis and skin lesion. Six patients reported eight gastrointestinal disorder TEAEs with certolizumab pegol plus bimekizumab, which included three TEAEs of stomatitis, two of dry mouth and one each of diarrhoea, dysphagia and tongue geographic.

Serious infections were experienced by one patient in the certolizumab pegol-IR plus bimekizumab group (psoas abscess 98 days after final bimekizumab dose) and one patient in the certolizumab pegol-IR plus placebo group (meningitis leading to death, 89 days after last placebo dose); both were considered unrelated to study treatment by the investigator.

Treatment-emergent grade 3 low neutrophil values were experienced by three patients (5.8%) in the certolizumab pegol-IR plus bimekizumab group and one patient (3.7%) in the certolizumab pegol-IR plus placebo. In most patients, this was a transient event that resolved and patients remained on treatment; one patient in the certolizumab pegol-IR plus bimekizumab group withdrew from the study. No additional patterns of change in any laboratory parameters were identified.

Overall, the safety profile for certolizumab pegol plus bimekizumab was consistent with previous findings for bimekizumab. The safety profile in the certolizumab pegol responders group (online supplementary results, online supplementary table S6) was also consistent with the known safety profile of certolizumab pegol.[24]

DISCUSSION

This study tested the principle of enhancing an IR to an anti-TNF therapy, in this case certolizumab pegol, by the addition of another treatment, in this case bimekizumab. The primary endpoint for the study was met, with a greater reduction in DAS28(CRP) in the certolizumab pegol-IR plus bimekizumab group at Week 20, compared with the certolizumab pegol-IR plus placebo group. Treatment with certolizumab pegol plus bimekizumab was also associated with a greater percentage of patients achieving DAS28(CRP) remission at Week 20 compared with certolizumab pegol plus placebo. Secondary efficacy outcomes, such as ACR50 and ACR70 at Week 20, also showed evidence for greater benefit with certolizumab pegol plus bimekizumab compared with certolizumab pegol plus placebo.

DAS28(CRP) response in the certolizumab pegol plus bimekizumab group was maintained for an additional 24 weeks of treatment with certolizumab pegol. Clinical response to certolizumab pegol is usually achieved within 12 weeks of treatment[24]; however, the certolizumab pegol-IR plus placebo group showed continued gradual improvement, such that by Week 32 the improvements reached were similar to the certolizumab pegol-IR plus bimekizumab group. Further work is required to determine whether treatment with certolizumab pegol plus bimekizumab for 12 weeks was sufficient to downregulate the inflammatory response to a level that could be maintained once treatment with bimekizumab was withdrawn. In addition, changes to individual components of the DAS28(CRP) suggested that improvements in the certolizumab pegol-IR plus bimekizumab group may have been mostly attributable to changes in PtGADA, with little impact of the combined treatment on SJC or TJC. It is notable that this group had a markedly higher baseline PtGADA score at randomisation.

In contrast to our findings, a recent study found no difference in efficacy with dual inhibition of TNF and IL-17A with ABT-122 compared with inhibition of TNF alone with adalimumab.[25] However, patients in this study were anti-TNF naïve whereas patients in our study demonstrated IR to anti-TNF treatment. The difference in efficacy may also be attributable to the difference in IL-17 inhibition: ABT-122 inhibits IL-17A but bimekizumab inhibits both IL-17A and IL-17F. Any potential gains in efficacy as a result of targeting two inflammatory pathways must be considered in balance with associated risks. Indeed, previous attempts to combine anti-TNF therapy with other modulators of the immune response, for example, etanercept plus abatacept (CTLA-4Ig) and etanercept plus anakinra (IL-1R antagonist), have resulted in increased safety risks, including increased rates of infection in patients with RA.[26 27] The safety profile of certolizumab pegol plus bimekizumab in this study was consistent with that expected in patients receiving certolizumab pegol for RA[21] and patients with psoriasis or psoriatic arthritis receiving bimekizumab.[18 19] There were no unexpected safety findings specifically associated with the combination of certolizumab pegol and bimekizumab. Of note, the incidence of infections and infestations in the certolizumab pegol-IR plus bimekizumab group

was over double that of the certolizumab pegol-IR plus placebo group (50% vs 22.2%). This was not due to an increased rate of any particular AE; nasopharyngitis and upper respiratory tract were the most common infections, with similar incidence in both treatment groups.

There is evidence to suggest that a higher baseline frequency of Th17 cells is associated with poor response to anti-TNF therapy.[5–8] The biology underlying the observed rises in Th17 numbers and IL-17A production after anti-TNF treatment in patients with RA is not fully understood. As noted by Hull *et al*, patients with higher baseline frequencies of circulating Th17 cells may have more IL-17-predominant disease and could therefore obtain greater benefit from the combined inhibition of IL-17A, IL-17F and TNF.[7] However, the study population was not large enough to identify any subpopulations that may have achieved particular benefit from the combination of certolizumab pegol plus bimekizumab. A further limitation of this study was that the effect of bimekizumab treatment alone was not assessed, including the effect of IL-17 inhibition on CRP, independent of clinical activity. In addition, evaluation of response to certolizumab pegol after 8 weeks may have resulted in initiation of additional treatment with bimekizumab in patients who would have met response criteria given additional time. The bimekizumab regimen in this study was significantly less than the maximum human exposure observed at the time of study initiation in patients with mild psoriasis (640 mg).[28] This was to allow for the different study population and any possible drug−drug interactions that might have increased exposure to bimekizumab; the dose regimen used for certolizumab pegol is an approved dose regimen in patients with RA.[24]

A rapid increase in response was achieved with 12 weeks of certolizumab pegol plus bimekizumab in patients with IR to certolizumab pegol. Neutralising both IL-17A and IL-17F in patients with moderate-to-severe RA treated with certolizumab pegol and background csDMARDs did not give rise to unexpected or new safety signals, although the rate of TEAEs was higher with dual inhibition. Overall, these findings support the potential to further explore concomitant neutralisation of multiple pathways in other patient populations where this treatment strategy may provide additional benefits.

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Data availability statement Underlying data from this manuscript may be requested by qualified researchers 6 months after product approval in the USA and/or Europe, or global development is discontinued, and 18 months after trial completion. Investigators may request access to anonymised IPD and redacted study documents which may include raw datasets, analysis-ready datasets, study protocol, blank case report form, annotated case report form, statistical analysis plan, dataset specifications and clinical study report. Prior to use of the data, proposals need to be approved by an independent review panel at www.clinicalstudydatarequest.com and a signed data sharing agreement will need to be executed. All documents are available in English only, for a pre-specified time, typically 12 months, on a password-protected portal.

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EPIDEMIOLOGICAL SCIENCE

Medications associated with fracture risk in patients with rheumatoid arthritis

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ABSTRACT

Objective To examine the fracture risk with use of disease-modifying antirheumatic drugs (DMARDs), statins, proton pump inhibitors (PPIs), opioids, non-opioid analgesics and psychotropic medications in a US-wide observational rheumatoid arthritis (RA) cohort.

Methods Patients with RA without prior fracture from 2001 through 2017 in FORWARD, a longitudinal observational registry, were assessed for osteoporosis-related site fractures (vertebra, hip, forearm and humerus). DMARD exposure was assessed in four mutually exclusive groups: (1) methotrexate monotherapy-reference, (2) tumour necrosis factor-α inhibitors (TNFi), (3) non-TNFi biologics and (4) others. Non-DMARDs and glucocorticoids were classified as current/ever use and based on treatment duration. Fracture Risk Assessment Tool (FRAX) scores estimating for 10-year major osteoporotic fractures were calculated. Cox proportional hazard models stratified by FRAX were used to adjust for confounders.

Results During median (IQR) 3.0 (1.5–6.0) years of follow-up in 11 412 patients, 914 fractures were observed. The adjusted models showed a significant fracture risk increase with use of any dose glucocorticoids ≥3 months (HR (95% CI) for <7.5 mg/day 1.26 (1.07 to 1.48) and for ≥7.5 mg/day 1.57 (1.27 to 1.94)), opioids (for weak: 1.37 (1.18 to 1.59); strong: 1.53 (1.24 to 1.88)) and selective serotonin reuptake inhibitors (SSRIs) (1.37 (1.15 to 1.63)). Fracture risk with opioids increased within 1 month of use (1.66 (1.36 to 2.04)) and with SSRIs >3 months of use (1.25 (1.01 to 1.55)). Statins (0.77 (0.62 to 0.96)) and TNFi (0.72 (0.54 to 0.97)) were associated with reduction in vertebral fracture risk only. PPIs and other psychotropic medications were not associated with increased fracture risk.

Conclusion Use of opioids, SSRIs and glucocorticoids were associated with increased risk of any fracture in patients with RA, whereas statins and TNFi were associated with decreased vertebral fractures.

Key messages

What is already known about this subject?

- Chronic inflammation predisposes patients with rheumatoid arthritis (RA) to fractures, cardiometabolic comorbidities and psychosocial problems which in turn lead to the use of several medications other than disease-modifying antirheumatic drugs such as opioids, non-steroidal anti-inflammatory drugs, antidepressants and statins.
- In the general population opioids, antidepressants, psychotropic medications and proton pump inhibitors have been shown to increase fracture risk while statins have been found to decrease fracture risk. So far, only opioids have been investigated in RA and shown to increase fracture risk.

What does this study add?

- This study demonstrates that fracture risk in patients with RA is also associated with the use of medications in RA such that opioids and selective serotonin reuptake inhibitors antidepressants increase all-site (vertebral and non-vertebral) fracture risk and statins and tumour necrosis factor-α inhibitors decrease vertebral fracture in patients with RA.

How might this impact on clinical practice or future developments?

- Opioid use, which is still frequent in RA despite the presence of effective treatment strategies, should be minimised to avoid fracture risk and other devastating consequences of opioids. A regular review of medications in patients with RA may help deprescription of unnecessary and potentially high risk treatments.
- Given the underuse of statins for cardiovascular disease in RA, the protective association against vertebral fractures may be encouraging for statin prescriptions in patients with RA.

INTRODUCTION

Rheumatoid arthritis (RA) is associated with twofold increased risk of osteoporosis (OP) and fractures across all age groups compared with the general population.[1 2] Osteoporotic fractures can lead to reduced quality of life, disability and increased mortality in patients with RA.[3] The incident osteoporotic fractures and related costs are estimated to exceed 3 billion and US$25 billion per year, respectively, in the US general population by 2025.[4] This growth in fracture burden is worrying in RA due to the already heightened fracture risk, increased life expectancy of patients with the advancements in RA management and suboptimal OP screening and treatment in RA.[5]

Chronic inflammation, physical inactivity and glucocorticoids mediating the increased OP and fracture risk also predispose patients with RA to multiple comorbidities including cardiometabolic, mood, sleep and gastrointestinal disorders, which in turn correlate with disability and mortality.[6–12] To manage comorbidities and pain, many patients

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with RA require medications other than disease-modifying antirheumatic drugs (DMARDs) that might influence fracture development. In the general population, proton pump inhibitors (PPIs), selective serotonin reuptake inhibitors (SSRIs), benzodiazepines, antipsychotics, anticonvulsants, opioids and non-steroidal anti-inflammatory drugs (NSAIDs) have been reported to increase fracture risk[13–17] while statins have been shown to decrease fracture risk.[18] The concomitant use of these medications is quite common and more frequent in patients with RA[19 20] where use of opioids reaches ~40%,[19] statins 25%,[20] antidepressants 30%,[20] NSAIDs 45%–76%,[21] and PPIs ~30%.[22] Despite this, the data regarding these drugs' influence on the fracture risk in RA are very limited and primarily focused on opioids. Studies using administrative data suggested an increased non-vertebral fracture risk with opioids in patients with RA.[23–26] However, they did not include data regarding disease activity, disability, detailed DMARD treatment, body mass index (BMI), physical activity and smoking that can significantly affect fracture risk. There was also no information about patients' baseline fracture risk estimated by WHO Fracture Risk Assessment Tool (FRAX).[27] Furthermore, the fracture risk associated with statins, antidepressants, PPIs and new biological DMARDs (bDMARDs) remain unknown.

Given the important cost, disability and mortality consequences of fractures in RA, it is important to identify modifiable risk factors. Therefore, we performed a cohort study that aimed to assess the fracture risk in patients with RA with the use of statins, PPIs, opioids and non-opioid analgesics, psychotropic medications and DMARDs

PATIENTS AND METHODS

Patients were participants in FORWARD—The National Databank for Rheumatic Diseases, a longitudinal prospective observational study. Participants were primarily recruited from US rheumatologists and were followed with self-report semiannual questionnaires.[28] Patients with RA ≥40 years old who did not have prevalent fracture and completed ≥2 semiannual questionnaires from January 2003 through December 2017 were included.

The primary outcome was incident fractures. By semiannual questionnaires, patients were asked about fractures of the finger, wrist, forearm, humerus, elbow, toe, foot, ankle, lower leg, knee, hip, pelvis, rib, skull and face, cervical, thoracic and lumbar vertebra that developed in the past 6 months. Among these, only the first of the OP-related site fractures, vertebral, hip, forearm and humerus, were included. The secondary outcomes were vertebral and non-vertebral fractures. Follow-up started at cohort entry and continued until the development of the first fracture or censoring at death, loss to follow-up or end of the study.

Fracture risk assessment

We calculated the 10-year probability of a major osteoporotic fracture (MOF) (hip, clinical spine, forearm or humerus) or hip fracture of patients with the US version of FRAX tool (V.4.0) (http://www.shef.ac.uk/FRAX) without bone mineral density (BMD) results.[27 29] This approach uses clinical risk factors (sex, race, age, BMI, previous fracture, parental history of hip fracture, current smoking, glucocorticoid ever use (≥5 mg/day prednisone >3 months), RA, other causes of secondary OP and alcohol consumption (≥3 units/day)) to estimate the 10-year fracture probability. The parental history of hip fracture was unavailable. Therefore, we replaced the missing data by simulations based on the conditional probability of the association of a risk factor with age, BMI and other dichotomous clinical variables by logistic regression as done previously.[5] Using this method, we estimated 5.7% of the patients to have a parental history of hip fracture (online supplementary material). A FRAX score of ≤10% for a 10 year MOF was regarded as low-risk, 10%–20% medium-risk and ≥20% high-risk.

Medication exposure

Treatment exposure was measured at study enrolment and every 6 months by study questionnaires. Glucocorticoid treatment was examined in time-varying dose (prednisone equivalents) and duration-combined categories: not-using (reference), <7.5 mg/day for <3 months, <7.5 mg/day for ≥3 months, ≥7.5 mg/day for <3 months and ≥7.5 mg/day for ≥3 months. We assessed DMARD exposure in four mutually exclusive hierarchical groups: (1) methotrexate monotherapy (reference), (2) tumour necrosis factor-α inhibitors (TNFi) (infliximab, adalimumab, etanercept, golimumab and certolizumab), (3) non-TNFi bDMARD (abatacept, rituximab, tocilizumab, anakinra) and (4) other synthetic DMARDs (sDMARDs). Patients could contribute to different DMARD groups over time when they switched therapy.

Statins, antidepressants, PPIs, opioids, NSAIDs, anticonvulsants, antipsychotics and benzodiazepines were assessed as follows: (1) current use versus non-use, (2) ever-use versus never-use and (3) based on treatment duration with non-use-referent; ≤1 month; 1–3 months and >3 months of use. We categorised antidepressants as SSRIs and non-SSRI antidepressants, opioids as weak and strong opioids and NSAIDs as cyclooxygenase-2 (COX-2) inhibitors and non-selective NSAIDs. Weak opioids included hydrocodone, tramadol, codeine, pentazocine and propoxyphene. Strong opioids included hydromorphone, dihydromorphinone, oxymorphone, butorphanol, methadone, morphine, oxycodone, meperidine and fentanyl.

Covariables

Variables potentially confounding the association between RA and non-RA related medications and fracture risk were adjusted for in multivariable models. Covariables were obtained from self-reported questionnaires. These included age (40–49 years-reference, 50–64 years,≥65 years), sex, location of residence (rural vs urban), education level (years), health insurance (any vs none), BMI in WHO categories (normal weight-reference), smoking history (ever vs never), physical activity, rheumatic disease comorbidity index,[30] prior OP diagnosis, RA duration, patient-reported RA severity and activity measures (individual components of patient activity scale (PAS), Health Assessment Questionnaire (HAQ), pain and patient global scores (0–10)), use of OP medications (bisphosphonates, hormone replacement therapy and teriparatide), number of previous DMARDs, calendar year, all-cause hospitalisations in the past 6 months and annual influenza vaccination as proxy measures of healthcare utilisation. Also, a dichotomous variable representing depression and anxiety assessed by mental component summary (MCS) score of short form-36 (SF-36) (MCS ≤38—probable major depressive/generalised anxiety disorder) was included.[31]

Statistical analysis

Baseline characteristics of patients with RA by incident fractures and medication use were compared. Fracture crude incidence rates were calculated by dividing the number of events per 1000 patient-years of follow-up with 95% CI. For the assessment of

fracture risk, we constructed multivariable Cox proportional hazards models to adjust for above-mentioned confounding factors. We stratified each model according to baseline FRAX 10-year MOF risk category. By using similar multivariable models, we also examined the influence of opioid strength, antidepressant and NSAID types, treatment durations and ever-use of the medications on the fracture risk. In sensitivity analyses, we restricted the analysis to patients with RA who never had an OP diagnosis or OP treatment.

All assessed covariables and medication exposures were time-dependent. Missing covariables were replaced by using multiple imputations by chained equations to create multiple imputed datasets for analyses.[32] All tests were two-sided and considered statistically significant when $p<0.05$. All statistical analyses were performed using Stata V.14.0 (Stata, College Station, Texas, USA).

RESULTS

The study included 11 412 patients with RA with a baseline mean (SD) age of 61.4 (10.8) years and disease duration of 15.6 (12.7) years. Patients who developed fractures were older, more likely to be female, had longer disease duration, higher HAQ, disease activity by PAS, comorbidity and FRAX scores and more frequent glucocorticoid (higher doses and longer durations), antidepressant, PPI and opioid use at baseline than patients who did not (table 1). Baseline characteristics of patients with RA by medication use are shown in the online supplementary table S1.

During 55 482 patient-years (median (IQR) 3.0 (1.5–6.0) years) of follow-up, 914 first-time fractures were observed, yielding a crude incidence rate (95% CI) of 16.5 (15.4–17.6) per 1000 patient-years. Fracture incidence rates were higher in patients receiving glucocorticoids, antidepressants, opioids, antipsychotics and benzodiazepines than patients receiving other medications (table 2).

The fully adjusted models showed a significant fracture risk increase with weak (HR 1.37 (1.18 to 1.59)) and strong opioids (HR 1.53 (1.24 to 1.88)), SSRIs (HR 1.37 (1.15 to 1.63)) and glucocorticoid use of any dose for ≥3 months (HR for <7.5 mg/day 1.26 (1.07 to 1.48); for ≥7.5 mg/day 1.57 (1.27 to 1.94)). In comparison with methotrexate monotherapy, other sDMARDs were associated with decreased fracture risk (HR (95% CI) 0.82 (0.69 to 0.99)). Other medications and bDMARDs did not significantly alter the fracture risk (table 3). Similar to current use, ever-use of opioids (HR 1.45 (1.25 to 1.68)) and SSRIs (HR 1.18 (1.00 to 1.39)) were associated with increased fracture risk compared with never-use (table 4). The fracture risk increase with opioids started even after 1–30 days of use (HR 1.66 (1.36 to 2.04)), which was more prominent than longer-term use (HR for 1–3 months 1.46 (1.20 to 1.78); >3 months 1.35 (1.12 to 1.64)). As for the SSRIs, fracture risk increase started after 3 months of use (HR 1.25 (1.01 to 1.55)) (table 4). Ever-use of other medications and different durations with further assessment of more extended use (≥12 months) were not associated with fracture risk in patients with RA (data not shown).

In the analysis of patients who never had OP or OP treatment (240 incident fractures in 6899 patients), opioids (HR 1.26 (1.01 to 1.72)) and SSRIs (HR 1.86 (1.32 to 2.62)) were again associated with fracture risk increase. However, only strong opioids were associated with fracture risk (HR 1.95 (1.28 to 2.96)). Regarding treatment duration, fracture risk increase with opioids was observed only with short-term (≤1 month) use (HR 1.48 (1.00 to 2.20)), whereas the risk increase with SSRIs started after 1 month of use (HR 1.96 (1.29 to 2.98)).

When we analysed vertebral and non-vertebral fractures separately, we identified 438 vertebral (incidence rate 7.7 (7.0 to 8.4) per 1000 patient-years) and 569 non-vertebral (incidence rate 10.1 (9.3 to 10.9) per 1000 patient-years) first-time fractures. Both vertebral and non-vertebral fracture risks were associated with current use of opioids (table 5). However, opioid-associated risk increase was higher for vertebral fractures than non-vertebral fractures. Again, SSRIs were associated with vertebral fracture risk but not non-vertebral (table 5). Of note, statins (HR 0.77 (0.62 to 0.96)) and TNFi (HR 0.72 (0.54 to 0.97)) were associated with decreased vertebral fracture risk.

DISCUSSION

Osteoporotic fractures are the third greatest cause of mortality in patients with RA, after pulmonary and cardiac disease, and the second cause of disability after depression.[30] Thus, identifying the modifiable risk factors for fractures, particularly medications in RA, is important. Opioids and SSRIs were associated with ~1.5-fold fracture risk increase in patients with RA after stratification according to baseline fracture risk by FRAX. Strong opioids and early treatment (<30 days of use) posed a higher risk than weak opioids and long-term use. SSRI-associated risk increase started after 3 months of use. While glucocorticoid use of any dose for ≥3 months increased fracture risk, TNFi and statins were associated with vertebral fracture risk reduction.

Opioids have been linked with increased fracture risk in the general population.[33 34] Only a few studies examined this in RA through administrative data, and they showed an increased non-vertebral fracture risk with current use of opioids (RR 1.8–4.9).[23–26] Although the risk increase we observed with opioids was similar to previously reported general population results,[34] it was lower than the risk found for patients with RA.[23–25] However, the other studies differed in study population (inclusion of mostly elderly patients with osteoarthritis and RA (~10% RA)), comparators (NSAIDs), outcome (non-vertebral fractures) and lack of baseline fracture risk (FRAX) and clinical data that can influence fracture risk (disease activity, disability, DMARD treatment, BMI, physical activity and smoking).[23–26] Our study also indicated that opioids increase the risk of vertebral fractures more than that of non-vertebral fractures in patients with RA. A similar trend has been demonstrated in the general population,[34] but the only study showing vertebral and non-vertebral fracture risk increase with opioids in RA did not report the quantitative extent.[23]

The most commonly suggested mechanisms for increased fracture risk with opioids are fall risk increase caused by psychomotor effects, influence on bone metabolism through opioid-induced hypogonadism and direct interference with bone formation.[35] Consistent with non-RA studies,[33 34] the greatest fracture risk increase during the first month of opioid treatment suggests that the risk was mediated by increased fall risk rather than bone loss associated with long-term use. This is supported by the fracture risk only increasing with short-term use of opioids in patients who never had OP diagnosis.

Our study also showed that patients with RA receiving SSRIs were more likely to have fractures. The increased fracture risk with SSRIs has been previously reported in several studies in the general population.[13 36] This risk increase with SSRIs has been attributed to increased fall risk and also decreased BMD even in patients without depression.[37 38] Moreover, depression itself has been implicated as a risk factor for falls and fractures.[38] To differentiate the disease and drug effect on fracture

Table 1 Baseline characteristics of patients with rheumatoid arthritis by incident fractures*

	All patients with RA (n=11 412)	No fractures (n=10 498)	Incident fractures (n=914)	P value
Age, years	61.4 (10.8)	61.2 (10.9)	64.8 (10.1)	<0.001
Age distribution, %				<0.001
40–50 years	16.4	17.2	7.9	
51–64 years	46.2	46.6	41.5	
≥65 years	37.4	36.2	50.7	
Women, %	79.9	79.2	86.8	<0.001
Non-Hispanic Caucasians, %	93.4	93.2	94.9	0.119
Education level, years	13.8 (2.2)	13.8 (2.3)	13.7 (2.3)	0.101
Disease duration, years	15.6 (12.7)	15.3 (12.6)	18.3 (13.0)	<0.001
BMI, kg/m^2	28.6 (6.8)	28.7 (6.9)	27.8 (6.4)	<0.001
Rheumatic Disease Comorbidity Index (0–9)	1.8 (1.6)	1.8 (1.6)	2.1 (1.6)	<0.001
Ever-smoked, %	45.9	45.6	49.6	0.020
HAQ (0–3)	1.1 (0.7)	1.0 (0.7)	1.2 (0.7)	<0.001
Patient global score (0–10)	3.5 (2.5)	3.5 (2.5)	3.8 (2.5)	<0.001
Pain score (0–10)	3.7 (2.8)	3.7 (2.8)	4.2 (2.8)	<0.001
PAS (0–10)	3.6 (2.2)	3.5 (2.2)	3.9 (2.2)	<0.001
Methotrexate-ever, %	72.9	72.7	75.5	0.102
TNFi-ever, %	42.1	42.2	41.0	0.733
Non-TNFi bDMARDs-ever, %	9.1	9.2	8.1	0.269
Glucocorticoids-ever	65.4	64.9	70.8	<0.001
Pattern of current glucocorticoid use†, %				<0.001
None	69.3	70	61.8	
<7.5 mg/day for <3 months	3.0	30	3.3	
<7.5 mg/day for ≥3 months	17.9	17.6	21.5	
≥7.5 mg/day for <3 months	2.6	2.5	3.1	
≥7.5 mg/day for ≥3 months	7.2	6.9	10.4	
10 year MOF risk by FRAX, %	14.8 (11.3)	13.9 (10.7)	23.4 (12.9)	<0.001
10 year MOF risk categories by FRAX, %				<0.001
Low (<10%)	40.5	42.7	14.8	
Medium (10%–20%)	30.1	30.1	30.0	
High (>20%)	29.5	27.2	55.2	
Prior diagnosis of osteoporosis, %	21.1	19.9	34.0	<0.001
Osteoporosis specific medications‡, %	31.9	30.7	45.7	<0.001
Statins, %	21.4	21.6	20.2	0.485
Antidepressants, %	17.8	17.4	22.1	<0.001
PPIs, %	26.9	26.5	31.5	<0.001
Opioids, %	25.3	24.7	32.0	<0.001
NSAIDs, %	55.9	55.8	57.2	0.732
Anticonvulsants, %	8.3	8.2	8.6	0.300
Antipsychotics, %	2.2	2.2	2.0	0.565
Benzodiazepines, %	5.7	5.6	6.6	0.110

*The values are presented as mean (SD) unless indicated otherwise.
†As prednisone dose equivalents.
‡Osteoporosis-specific medications included bisphosphonates, hormone replacement therapy, raloxifene and teriparatide.
BMI, body mass index; FRAX, Fracture Risk Assessment Tool; HAQ, Health Assessment Questionnaire; MOF, major osteoporotic fracture; NSAIDs, non-steroidal anti-inflammatory drugs; PPIs, proton pump inhibitors;RA, rheumatoid arthritis; TNFi, tumour necrosis factor-α inhibitors; bDMARDs, biological disease modifying antirheumatic drugs.

risk, we adjusted our models for mood changes measured by SF-36 MCS score. Although we found an increased fracture risk with depression (HR 1.25 (1.06 to 1.46), data not shown), SSRI use was still associated with fracture risk increase. The more prominent risk increase with long-term (>3 months) SSRI use could be caused by decreased BMD rather than an increased fall risk.

To our knowledge, the fracture risk with statins has not been examined in patients with RA. We found that statins were associated with decreased vertebral fracture risk with no effect on non-vertebral fractures. Many observational studies or meta-analyses evaluated the association of statins with OP and fracture risk in the general population.[18 39 40] Most but not all concluded that statin use was associated with an improved BMD and reduced risk of fracture.[18 39 40] The evidence suggests that statins have anabolic and antiosteoclastic effects by inducing proliferation, differentiation and protection of osteoblasts and reducing osteoclastogenesis.[41] Moreover, in patients with RA, statins exert anti-inflammatory effects[42] which might indirectly affect bone health. Thus, it is biologically plausible that the pleiotropic effects of statins may result in reduced fracture risk. It is also possible that the observed associations

Table 2 Crude incidence rates of fractures by medications in patients with rheumatoid arthritis

	No. with fracture/No. exposed	Patient-years	Incidence rates (95% CI)*
All patients	914/11 412	55 482	16.5 (15.4 to 17.6)
DMARD groups			
Methotrexate monotherapy	169/3213	9115	18.5 (15.9 to 21.6)
TNFi	280/5150	18 561	15.1 (13.4 to 17.0)
Non-TNFi bDMARDs	63/1420	3216	19.6 (15.3 to 25.1)
Other DMARDS	402/7129	24 591	16.3 (14.8 to 18.0)
Glucocorticoid use			
None	547/8989	40 282	13.6 (12.5 to 14.8)
<7.5 mg/day for <3 months	10/834	693	14.4 (7.8 to 26.8)
<7.5 mg/day for ≥3 months	220/3387	10 149	21.7 (19.0 to 24.7)
≥7.5 mg/day for <3 months	15/809	660	22.7 (13.7 to 37.7)
≥7.5 mg/day for ≥3 months	121/1948	3616	33.5 (28.0 to 40.0)
Statins	259/4187	15 412	16.8 (14.9 to 19.0)
Antidepressants	236/3110	9536	25.1 (22.1 to 28.5)
SSRIs	185/2369	6971	26.5 (23.0 to 30.6)
Others	84/1339	3663	22.9 (18.5 to 28.4)
PPIs	341/4963	16 820	20.3 (18.2 to 22.5)
Opioids	388/5082	14 100	27.5 (24.9 to 30.4)
Weak opioids	320/4566	12 244	26.1 (23.4 to 29.2)
Strong opioids	116/1483	2885	40.3 (33.5 to 48.2)
NSAIDs	412/7705	26 006	15.8 (14.4 to 17.4)
COX-2 inhibitors	118/2945	7366	16.0 (13.8 to 19.2)
Non-selective NSAIDs	300/6065	18 970	15.8 (14.1 to 17.7)
Anticonvulsants	122/2072	5238	23.3 (19.5 to 27.8)
Antipsychotics	35/643	1364	25.7 (18.4 to 35.7)
Benzodiazepines	75/1223	2930	25.6 (20.4 to 32.1)

*Per 1000 patient-years.
COX-2, cyclo-oxygenase-2; DMARD, disease-modifying antirheumatic drugs; NSAIDs, non-steroidal anti-inflammatory drugs; PPIs, proton pump inhibitors; SSRIs, selective serotonin reuptake inhibitors; TNFi, tumour necrosis factor-α inhibitor; bDMARDs, biological disease modifying antirheumatic drugs.

Table 3 Association of medications with incident fractures in patients with rheumatoid arthritis

	Unadjusted HR (95% CI)	Adjusted HR* (95% CI)
DMARD groups		
Methotrexate monotherapy	Reference	Reference
TNFi	0.79 (0.66 to 0.96)	0.82 (0.67 to 1.00)
Non-TNFi bDMARDs	1.03 (0.77 to 1.37)	0.84 (0.61 to 1.16)
Other DMARDS	0.88 (0.73 to 1.05)	0.82 (0.69 to 0.99)
Glucocorticoid use		
None	Reference	Reference
<7.5 mg/day for <3 months	1.34 (0.72 to 2.51)	1.23 (0.65 to 2.29)
<7.5 mg/day for ≥3 months	1.59 (1.36 to 1.86)	1.26 (1.07 to 1.48)
≥7.5 mg/day for <3 months	2.00 (1.20 to 3.35)	1.66 (0.99 to 2.77)
≥7.5 mg/day for ≥3 months	2.46 (2.02 to 3.00)	1.57 (1.27 to 1.94)
Statins	1.01 (0.88 to 1.17)	0.91 (0.78 to 1.06)
Antidepressants†	2.36 (1.62 to 3.43)	1.32 (1.13 to 1.56)
SSRIs	1.76 (1.50 to 2.07)	1.37 (1.15 to 1.63)
Others	1.42 (1.13 to 1.77)	1.03 (0.81 to 1.30)
PPIs	1.36 (1.19 to 1.55)	0.92 (0.80 to 1.06)
Opioids†	3.78 (2.95 to 4.84)	1.47 (1.27 to 1.71)
Weak opioids	1.90 (1.65 to 2.18)	1.37 (1.18 to 1.59)
Strong opioids	2.66 (2.18 to 3.23)	1.53 (1.24 to 1.88)
NSAIDs†	1.07 (0.67 to 1.71)	1.07 (0.94 to 1.23)
COX-2 inhibitors	1.01 (0.83 to 1.23)	0.99 (0.81 to 1.21)
Non-selective NSAIDs	0.94 (0.82 to 1.08)	1.13 (0.97 to 1.30)
Anticonvulsants	1.47 (1.21 to 1.78)	0.96 (0.78 to 1.17)
Antipsychotics	1.55 (1.10 to 2.17)	1.03 (0.73 to 1.47)
Benzodiazepines	1.61 (1.27 to 2.05)	0.94 (0.73 to 1.20)

*Stratified by FRAX risk categories for MOF and adjusted for age, sex, ethnicity, RA duration, education level, insurance, rural residency, smoking, influenza vaccination, comorbidity index, BMI, HAQ, pain and patient global scores, prior osteoporosis diagnosis, use of osteoporosis specific medications, exercise, mental component score of SF-36, prior csDMARD and bDMARD counts, hospitalisation and calendar year.
†Opioid strength and antidepressant and NSAID types were assessed in a different model using the same covariables.
COX-2, cyclo-oxygenase-2; DMARD, disease-modifying antirheumatic drugs; NSAIDs, non-steroidal anti-inflammatory drugs; PPIs, proton pump inhibitors;RA, rheumatoid arthritis; SSRIs, selective serotonin reuptake inhibitors; TNFi, tumour necrosis factor-α inhibitor; bDMARDs, biological disease modifying antirheumatic drugs.

emerged due to confounding by indication, unmeasured or residual confounding such as BMI, other comorbidities or differences in health behaviours like healthy-adherer effect. Although our study included several covariables associated with fractures and healthcare utilisation, further research is warranted to better understand the effects of statins on OP and fractures in patients with RA.

Our study confirms the glucocorticoid-fracture association and also adds to the existing literature about fracture risk with DMARDs.[10 23 26 43–45] We assessed TNFi and non-TNFi and vertebral and non-vertebral fractures separately, which had not previously been done comprehensively. We showed that TNFi was associated with a significant reduction in vertebral fracture risk compared with methotrexate monotherapy. As chronic inflammation is one of the main reasons for the increased risk of OP and fractures in RA, it is thought that effective anti-inflammatory medication might decrease bone loss and consequently fracture risk. However, consistent with our findings, previous studies mostly using administrative data could not demonstrate a reduction in non-vertebral fracture risk either with TNFi or all bDMARDs assessed together compared with non-bDMARDs.[23 26 43–45] One study examining vertebral fracture risk with TNFi reported no statistical association even though a risk reduction was found in all site fractures with TNFi monotherapy.[43] The discrepancy in the results can be explained by differences in included covariables related to OP and fracture risk, comparison group (non-users) and the TNFi exposure assessment (current or ever).[43]

Given that BMD improvement with TNFi has been shown in both vertebral and non-vertebral sites including hand and femur,[46] it was surprising to find only vertebral fracture risk reduction with TNFi. The reason for this can be potential inclusion of non-vertebral traumatic fractures as we did not have enough data regarding the nature of the activity causing the fracture. Additionally, different pathophysiological and biomechanical mechanisms might be involved in vertebral and non-vertebral fracture aetiology.

Contrary to the general population studies, we did not find any association between fracture risk and PPI or other psychotropic use.[15 16] This might be due to the low number of patients on other psychotropic medications. Although PPI use was common in our cohort, the use of bisphosphonates in this group was also higher than the overall cohort (35% vs 16%) which may explain the lack of association.

Table 4 Risk of incident fractures in patients with rheumatoid arthritis by opioid and SSRI treatment duration

	N events/ N exposure	Adjusted HR* (95% CI)
Opioid use		
Ever vs never use	535/5082	1.45 (1.25 to 1.68)
Treatment duration		
Non-use	379/8526	Referent
≤1 month of use	133/5082	1.66 (1.36 to 2.04)
>1 month and ≤3 months of use	172/3974	1.46 (1.20 to 1.78)
>3 months of use	230/2321	1.35 (1.12 to 1.64)
SSRI use		
Ever vs never use	241/2369	1.18 (1.00 to 1.39)
Treatment duration		
Non-use	673/9983	Referent
≤1 month of use	38/2369	1.00 (0.71 to 1.39)
>1 month and ≤3 months of use	84/1944	1.19 (0.94 to 1.52)
>3 months of use	119/1143	1.25 (1.01 to 1.55)

*Stratified by FRAX risk categories for MOF and adjusted for age, sex, ethnicity, RA duration, education level, insurance, rural residency, smoking, influenza vaccination, comorbidity index, BMI, HAQ, pain and patient global scores, prior osteoporosis diagnosis, use of osteoporosis specific medications, exercise, mental component score of SF-36, prior csDMARD and bDMARD counts, hospitalisation and calendar year.

BMI, body mass index; FRAX, Fracture Risk Assessment Tool; HAQ, Health Assessment Questionnaire; MOF, major osteoporotic fracture; RA, rheumatoid arthritis; SSRI, selective serotonin reuptake inhibitor; bDMARD, biological disease modifying antirheumatic drug; csDMARD, conventional synthetic disease modifying antirheumatic drug.

Table 5 Association of medications with vertebral and non-vertebral fractures in patients with rheumatoid arthritis*

	Vertebral fracture risk	Non-vertebral fracture risk
DMARD groups		
Methotrexate monotherapy	Reference	Reference
TNFi	0.72 (0.54–0.97)	0.93 (0.86–1.25)
Non-TNFi bDMARDs	0.67 (0.42–1.06)	1.04 (0.70–1.53)
Other DMARDS	0.91 (0.70–1.18)	0.80 (0.64–1.01)
Glucocorticoid use		
None	Reference	Reference
<7.5 mg/day for <3 months	1.93 (0.91–4.10)	1.15 (0.51–2.57)
<7.5 mg/day for ≥3 months	1.37 (1.08–1.73)	1.16 (0.94–1.42)
≥7.5 mg/day for <3 months	1.50 (0.67–3.40)	1.76 (0.94–3.32)
≥7.5 mg/day for ≥3 months	2.12 (1.60–2.80)	1.19 (0.89–1.60)
Statins	0.77 (0.62–0.96)	1.04 (0.86–1.25)
Antidepressants†	1.44 (1.15–1.80)	1.15 (0.93–1.42)
SSRIs	1.50 (1.18–1.91)	1.22 (0.97–1.53)
Others	0.98 (0.70–1.36)	0.97 (0.71–1.33)
PPIs	1.04 (0.85–1.27)	0.82 (0.69–1.00)
Opioids†	1.83 (1.48–2.26)	1.29 (1.07–1.56)
Weak opioids	1.61 (1.31–1.98)	1.25 (1.04–1.51)
Strong opioids	1.77 (1.35–2.33)	1.22 (0.92–1.63)
NSAIDs†	1.12 (0.92–1.37)	1.06 (0.89–1.25)
COX-2 inhibitors	0.92 (0.68–1.24)	1.01 (0.78–1.31)
Non-selective NSAIDs	1.23 (1.00–1.51)	1.09 (0.91–1.31)
Anticonvulsants	0.98 (0.74–1.29)	1.08 (0.84–1.40)
Antipsychotics	0.89 (0.53–1.49)	1.18 (0.77–1.83)
Benzodiazepines	1.04 (0.75–1.45)	0.75 (0.53–1.07)

*Stratified by FRAX risk categories for MOF and adjusted for age, sex, ethnicity, RA duration, education level, insurance, rural residency, smoking, influenza vaccination, comorbidity index, BMI, HAQ, pain and patient global scores, prior osteoporosis diagnosis, use of osteoporosis specific medications, exercise, mental component score of SF-36, prior csDMARD and bDMARD counts, hospitalisation and calendar year.

†Opioid strength and antidepressant and NSAID types were assessed in a different model using the same covariables.

COX-2, cyclo-oxygenase-2; DMARD, disease-modifying antirheumatic drugs; NSAIDs, non-steroidal anti-inflammatory drugs; PPIs, proton pump inhibitors; SSRIs, selective serotonin reuptake inhibitors; TNFi, tumour necrosis factor-α inhibitor; bDMARDs, biological disease modifying antirheumatic drugs.

Our study has some limitations. As an observational cohort, the patients were not randomly assigned to the assessed medications. Despite the inclusion of several fracture-related covariables and a patient-reported disease activity measure in the analyses, there might be residual confounding bias due to the unmeasured factors. One important measure we lacked was fall risk, which might explain the association observed for opioids and SSRIs. However, our analyses included factors related to fall risk in RA such as patient-reported disease activity, disability and pain.[47] Also, due to the lack of data about trauma level, some of the traumatic fractures might be misclassified as osteoporotic fractures. Despite that, our fracture incidence rates were comparable to the reported rates in a recent meta-analysis of 25 cohort studies in patients with RA (pooled incidence rates of fragility fractures 15.31 (95% CI 10.43 to 22.47) per 1000 person-years).[48] Additionally, the majority of the vertebral fractures are not clinically recognisable at early stages, and the diagnosis of these fractures requires ≥2 radiographic assessments at appropriate intervals. As we did not have radiographic data, the incidence of vertebral fractures would be higher than our findings.

In conclusion, our study showed that opioids, SSRIs and glucocorticoids were associated with increased vertebral and non-vertebral fracture risk in patients with RA whereas statins and TNFi were associated with decreased vertebral fracture risk. The risk observed with opioids and SSRIs might be related to increased fall risk. In our view, OP, fracture and fall risks in RA and their severe consequences are underestimated. Given the frequent occurrence of chronic pain, mood disorders, polypharmacy and already heightened fracture and fall risks in patients with RA, we suggest a regular review of the necessity of the medications being used. In the opioid epidemic era, to avoid fractures and other devastating consequences, opioid use should be minimised for pain management in RA.

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Patient consent for publication Not required.

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Author note The lead author (the manuscript's guarantor) affirms that the manuscript is an honest, accurate and transparent account of the study being reported; that no important aspects of the study have been omitted and that any discrepancies from the study as planned (and, if relevant, registered) have been explained.

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CLINICAL SCIENCE

Impact of Janus kinase inhibitors on risk of cardiovascular events in patients with rheumatoid arthritis: systematic review and meta-analysis of randomised controlled trials

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ABSTRACT

Objectives To investigate the effect of Janus kinase inhibitors (Jakinibs) on cardiovascular risk in adult patients with rheumatoid arthritis (RA) via a meta-analysis of randomised controlled trials (RCTs).

Methods PubMed, Embase and Cochrane library were thoroughly searched for RCTs reporting safety issues in patients with RA receiving Jakinibs, from inception to October 2018. The primary and secondary outcomes were all cardiovascular events (CVEs) and major adverse cardiovascular events (MACEs)/venous thromboembolism events (VTEs). OR and 95% CI were calculated using the Mantel-Haenszel fixed-effect method.

Results 26 RCTs randomising 11 799 patients were included. No significant difference was observed regarding all CVEs risk following Jakinibs usage in general (OR 1.04 (0.61 to 1.76), p = 0.89), tofacitinib (OR 0.63 (0.26 to 1.54), p = 0.31), baricitinib (OR 1.21 (0.51 to 2.83), p = 0.66), upadacitinib (OR 3.29 (0.59 to 18.44), p = 0.18), peficitinib (OR 0.43 (0.07 to 2.54), p = 0.35) or decernotinib (OR 1.12 (0.13 to 10.11), p = 0.92). Likewise, there was no significant difference for Jakinibs treatment overall regarding occurrence of MACEs (OR 0.80 (0.36 to 1.75), p = 0.57) or VTEs (OR 1.16 (0.48 to 2.81), p = 0.74). Dose-dependent impact of Jakinibs on the risks of all CVEs, MACEs and VTEs was not observed in tofacitinib (5 mg vs 10 mg), upadacitinib (15 mg vs 30 mg), whereas baricitinib at 2 mg was found to be safer than 4 mg in all CVEs incidence (OR 0.19 (0.04 to 0.88), p = 0.03).

Conclusion The existing evidence from RCTs indicated no significant change in cardiovascular risk for Jakinib-treated patients with RA in a short-term perspective, but postmarketing data are sorely needed to ascertain their cardiovascular safety, especially at the higher dose, due to increased risk of thromboembolism events for both tofacitinib and baricitinib at higher dosage.

Key messages

What is already known about this subject?

- Rheumatoid arthritis (RA) has been associated with an increased risk of cardiovascular events (CVEs), including major adverse cardiovascular events (MACEs) and venous thromboembolism events (VTEs).
- Both of two licensed Janus kinase inhibitors (Jakinibs), tofacitinib and baricitinib, are found to probably correlate with a higher risk of thromboembolism events at a higher dose.
- The impact of Jakinib therapies on the risk of CVEs in RA population remains undetermined.

What does this study add?

- The results of largest meta-analysis of 26 randomised controlled trials did not reveal a significant change in the risks of all CVEs, MACEs and VTEs, in patients with RA initiating Jakinib therapies, as compared to placebo.
- During the limited controlled periods, no evidence of dose-dependent impact of tofacitinib and upadacitinib on the risk of all CVEs, MACEs and VTEs was observed, while baricitinib at 2 mg was found to be safer than 4 mg one time a day regarding all CVEs risk.

How might this impact on clinical practice or future developments?

- Based on the best available datasets, this work reveals that Jakinib therapies are not associated with increased cardiovascular risk, but in consideration of an increased cardiovascular risk of 4 mg baricitinib in present study, the US Food and Drug Administration restricts approval to only the low-dose baricitinib, as well as the most recent warning about an increased risk of pulmonary embolism in higher dose tofacitinib, further investigations are urgently needed to confirm long-term cardiovascular safety of Jakinibs, especially at the higher dose.

INTRODUCTION

Patients with rheumatoid arthritis (RA) have a substantially elevated risk of cardiovascular morbidity and mortality, which cannot be entirely explained by traditional cardiovascular risk factors.[1–3] This indicates that RA-specific characteristics, especially systemic inflammation and disease activity, may be associated with increased cardiovascular risk.[4 5] Reaching remission or low disease activity in order to decrease cardiovascular events (CVEs), therefore, is optimally advocated in current-released European League Against Rheumatism (EULAR) recommendations.[6] Disease-modifying antirheumatic drugs (DMARDs), including methotrexate (MTX) and antitumour necrosis factor (anti-TNF), have shown cardioprotective potential in patients with RA, although the evidence

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is inconclusive.[7–10] Irrespective of anti-inflammatory effects, drug-specific mechanisms are also involved in altering cardiovascular outcomes. For instance, glucocorticoids and rofecoxib, which possess potent anti-inflammatory properties, have cardiovascular toxicity.[9 11]

Janus kinase inhibitors (Jakinibs), classified as targeted synthetic DMARDs (tsDMARDs), is being widely investigated in randomised controlled trials (RCTs) and two of them have been subsequently approved for the treatment of RA in a number of countries.[12–14] However, the role of these agents in the modulation of cardiovascular risk remains undetermined.[15 16] Limited evidence suggested that tofacitinib-based therapy positively modifies the risk of cardiovascular disease,[17 18] while a growing body of literature indicated that Jakinibs adversely affect several cardiovascular risk factors (such as serum lipid profile and platelet count) and potentially increase thrombotic risk which directly leads the US Food and Drug Administration (FDA) to restrict approval to only 2 mg baricitinib.[19–22] Additionally, in upadacitinib premarketing trials, major adverse cardiovascular events (MACEs) continuously reported in patients with RA receiving upadacitinib has further generated cardiovascular concerns.[23]

In consideration of currently not well-established cardiovascular safety profile of these agents, we set out to explore the association between Jakinibs therapies and CVEs in adult patients with RA.

METHODS

This article were carried out in line with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses.[24]

Data sources

A systematic search of PubMed, Embase and the Cochrane library databases was performed without language restrictions from inception through 13 October 2018. Search terms comprised rheumatoid arthritis, Jakinibs (trade names, individual drug names, and chemical names) and randomized controlled trial. An example of search strategy is available in online supplementary appendix S1. We also searched Current Index to Nursing and Allied Health Literature (CINAHL), the major annual meetings (American College of Rheumatology, EULAR) in 2016–2018 and reference lists of all included studies for additional studies. Besides, the US National Institutes of Health Ongoing Trials Register (www.clinicaltrials.gov) and relevant FDA files were procured for additional details of clinical trials. Sponsored pharmaceutical companies or the authors were contacted if ambiguity existed.

Study selection

Two investigators (WX and YH) independently screened all titles and abstracts for potential inclusion. Discordances were resolved by a third experienced investigator (ZZ). We included double-blind RCTs that reported adverse events in adult patients with RA receiving at least one dose Jakinibs compared with placebo or two comparable doses of Jakinibs (tofacitinib (5 mg vs 10 mg, two times a day), baricitinib (2 mg vs 4 mg, one time a day), upadacitinib (15 mg vs 30 mg, one time a day)) during the randomised controlled phase. Exclusion criteria included non-randomised design, non-comparative study, healthy volunteers, paediatric patients and abstracts without full-text publication. We excluded one article as the comparator in the study was MTX, an ineligible arm and three articles due to follow-up duration <12 weeks.[25–28] In addition, two studies specifically recruiting elderly (50 years and older) and seropositive patients or patients with bone erosion on radiography were also excluded due to their unrepresentativeness of RA population.[29 30]

Data extraction and outcome measures

Data were independently extracted by two reviewers (WX and YH) with a predesigned template: authors, publication year, sites/countries, trial design, patients' characteristics, treatment regimens and CVEs. The overall number of CVEs during the randomised controlled phase was extracted for patients who received at least one dose of study medication or placebo. For extension RCTs in which treatment assignments were switched (for instance, patients initially treated with placebo switched to a Jakinib), the occurrence of CVEs was documented at switching point (usually 12–24 weeks). The longest randomised period (usually 24–52 weeks) was chosen to compare two dosage arms (tofacitinib (5 mg vs 10 mg two times a day), baricitinib (2 mg vs 4 mg one time a day), upadacitinib (15 mg vs 30 mg one time a day)), and total number of CVEs during eligible period for each dosage was extracted to identify dose-related cardiovascular effect. The Cochrane quality assessment tool for RCTs was used to assess risk of bias.[31]

The primary outcome was the relationship between Jakinibs (all or individual or different dosing) and all CVEs. The secondary outcomes switched to MACEs and venous thromboembolism events (VTEs), including pulmonary embolism (PE) and deep vein thrombosis (DVT). The outcomes of CVEs included angina pectoris, myocardial infarction, congestive heart failure, carotid artery disease, aortic aneurysm, cerebral vascular diseases (stroke and transient ischaemic attack), VTEs and cardiovascular death. MACE was defined as a composite endpoint of myocardial infarction, cerebrovascular accident (ischaemic and haemorrhagic strokes) or cardiovascular death. Two kinds of comparisons were made for both primary and secondary outcomes: (1) all Jakinibs or individual Jakinib versus placebo; (2) comparisons of different dosage (tofacitinib 5 mg vs 10 mg; baricitinib 2 mg vs 4 mg; upadacitinib 15 mg vs 30 mg). In the first comparison, all dosages of study agents were combined.

Statistical analysis

Extracted data were combined using Review Manager (RevMan) software (Cochrane collaboration). OR of CVEs, MACEs or VTE in patients receiving Jakinibs compared with placebo and the same Jakinib with different dosages was calculated by Mantel-Haenszel fixed-effect method. One of sensitivity analyses was conducted with the Peto method to examine whether analysis methods had influence on the results.[32] Additional sensitivity analysis was performed after excluding four exclusively Japan-based studies or the studies without an independent cardiovascular committee. Forest plots were constructed to summarise the OR estimates and their 95% CI. Heterogeneity across studies was measured by χ^2 test ($p<0.05$ was regarded statistically significant) and I^2 statistics (significant heterogeneity, $I^2 >50\%$; insignificant heterogeneity, $I^2 <40\%$). Funnel plot analysis was used to detect the potential publication bias.

RESULTS

In total, 26 citations comprising 11 799 patients met predefined criteria, as summarised in figure 1.[33–57] The included RCTs, covering a range of 19–281 (median 88) centres, were international (6–35 countries), except 4 from Japan.[33 42 48 53] Most of the eligible trials were about tofacitinib,[33–42] baricitinib[43–48] and upadacitinib.[49–52] Other Jakinibs,

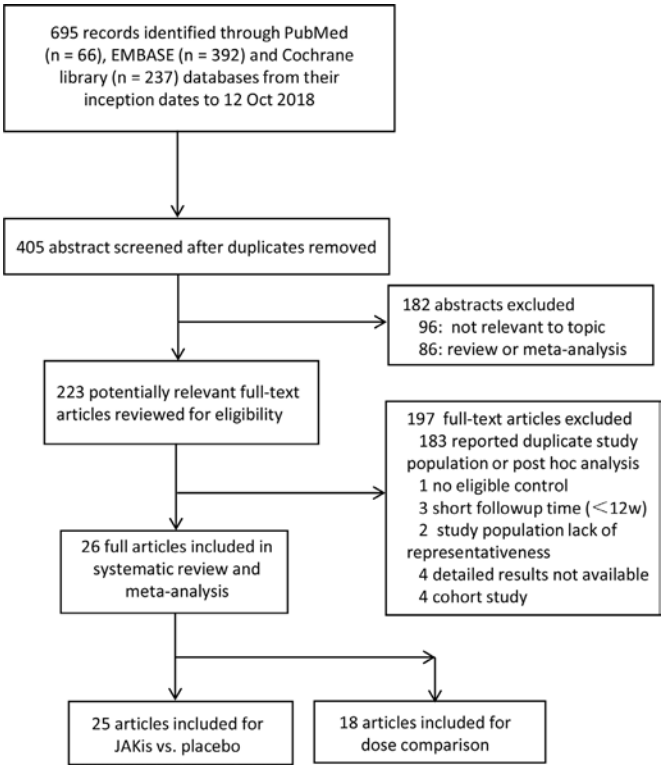

Figure 1 Preferred Reporting Items for Systematic Reviews and Meta-Analyses flow chart of included randomised controlled trials.

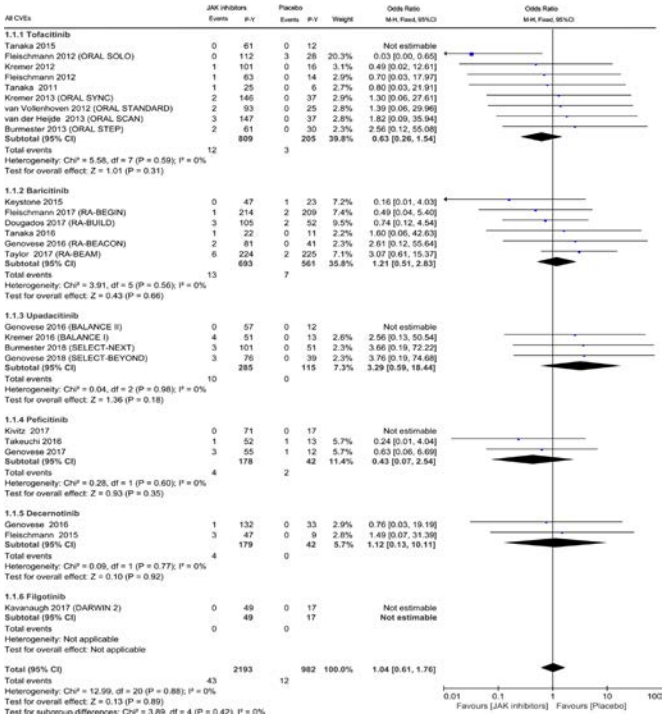

Figure 2 OR of all cardiovascular events (CVEs) in patients treated with Janus kinase (JAK) inhibitors compared with placebo in randomised controlled trials using the Mantel-Haenszel (M-H) fixed-effect method. P-Y, patient–year.

peficitinib[53 54] decernotinib[55 56] and filgotinib,[57] were relatively less reported. Of these, 14 were phase II trials[33 34 36 42 43 48–50 53–57] and 12 were phase III trials.[35 37–41 44–47 51 52] Baseline characteristics of patients were generally comparable with regard to age, sex composition, disease duration and disease activity across most of arms and studies (online supplementary table S1).

Of included studies, 25 were eligible for the comparison of Jakinibs and placebo and 18 were eligible for the comparison of low and high dosages. The available duration for Jakinibs compared with placebo and different-dose comparisons ranged from 12 to 52 (median 12) weeks and 12 to 108 (median 24) weeks, respectively (online supplementary table S2). The numbers of CVEs (MACEs/VTEs) during the controlled phase of these studies were summarised in online supplementary table S2. The crude incidence rates of CVEs (MACEs/VTEs) in Jakinibs and placebo group were, respectively, 1.961 (0.593/0.547) and 1.222 (0.305/0.305) per 100 patient years. Regarding the dose comparison, the crude incidence rates of CVEs (MACEs/VTEs) in low-dose and high-dose arms were 1.367 (0.365/0.228) and 1.425 (0.445/0.267) per 100 patient years, respectively.

Meta-analysis

For comparison of Jakinibs against placebo, pooled analysis of 25 trials indicated no statistically significant difference in all CVEs risk (OR=1.04, 95% CI 0.61 to 1.76, p=0.89) (figure 2). Considered separately, statistical differences remained undetectable regarding the occurrence of all CVEs for tofacitinib (OR=0.63, 95% CI 0.26 to 1.54, p=0.31), baricitinib (OR=1.21, 95% CI 0.51 to 2.83, p=0.66), upadacitinib (OR=3.29, 95% CI 0.59 to 18.44, p=0.18), peficitinib (OR=0.43, 95% CI 0.07 to 2.54, p=0.35) and decernotinib (OR=1.12, 95% CI 0.13 to 10.11, p=0.92) (figure 2). Concerning MACEs, there was no statistically significant correlation either between all Jakinibs in general (OR=0.80, 95% CI 0.36 to 1.75, p=0.57) or individual agent compared with placebo (figure 3). The statistical heterogeneity across all intergroup and intragroups analysis was very low, allowing for combination of trial results using the fixed-effect method (figures 2 and 3). Subanalysis of VTEs showed a trend towards higher rates in Jakinibs-treated patients than placebo, but the difference did not reach statistical significance (OR=1.16, 95% CI 0.48 to 2.81, p=0.74) (figure 4). A notable variation was observed in three groups of Jakinibs (tofacitinib OR=0.17, 95% CI 0.03 to 1.05, p=0.06; baricitinib OR=2.33, 95% CI 0.62 to 8.75, p=0.21; upadacitinib OR=1.77, 95% CI 0.20 to 16.00, p=0.61). Additionally, separate meta-analyses of PE and DVT showed no increased difference in Jakinibs overall, relative to placebo (OR=0.91, 95% CI 0.30 to 2.77, p=0.87 for PE; OR=1.18, 95% CI 0.35 to 3.94, p=0.79 for DVT) (online supplementary figure S1).

Considering the potential increased risk of VTEs in higher dose of Jakinibs, we conducted dose comparisons for tofacitinib, baricitinib and upadacitinib.[58 59] For pairwise comparisons of tofacitinib 5 mg against 10 mg, upadacitinib 15 mg against 30 mg, no significant results were identified in the risk of all CVEs (tofacitinib OR=1.02, 95% CI 0.56 to 1.87, p=0.94; upadacitinib OR=2.61, 95% CI 0.80 to 8.50, p=0.11 for upadacitinib). Conversely, baricitinib at dose of 2 mg appeared to be safer than 4 mg based on randomised controlled datasets (OR=0.19, 95% CI 0.04 to 0.88, p=0.03) (figure 5). We found very low level of heterogeneity across all included RCTs ($\chi 2=16.82$; df=14; p=0.27; $I^2=17\%$), but a high heterogeneity was detected among the three kinds of Jakinibs ($\chi 2=7.07$; df=2; p=0.03; $I^2=71.7\%$) (figure 5). Additional analyses of MACEs and VTEs showed no dose-dependent effect in general (OR=0.86, 95% CI 0.38 to 1.92, p=0.71 for MACEs; OR=0.91, 95% CI 0.36 to 2.25, p=0.83 for VTEs) (online supplementary figures S2, S3). Concretely, a comparable impact of two dosages of tofacitinib was observed, while patients receiving 2 mg baricitinib tended

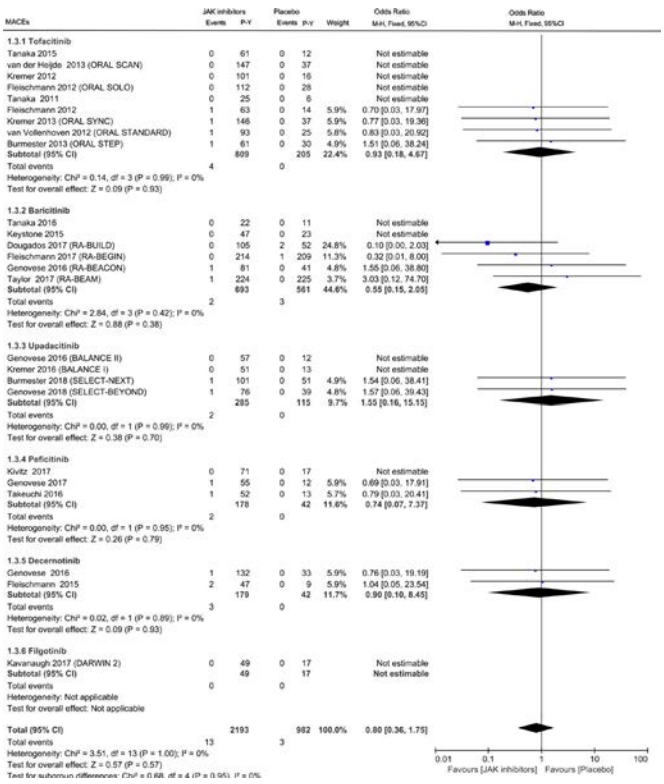

Figure 3 OR of major adverse cardiovascular events (MACEs) in patients treated with Janus kinase (JAK) inhibitors compared with placebo in randomised controlled trials using the Mantel-Haenszel (M-H) fixed-effect method. P-Y, patient–year.

to have lower occurrences of MACEs and VTEs than those receiving 4 mg one (OR=0.20, 95% CI 0.01 to 4.18, p=0.30 for MACEs; OR=0.23, 95% CI 0.02 to 2.17, p=0.20 for VTEs). In comparison with 30 mg upadacitinib, the ORs (95% CI) of MACEs and VTEs in 15 mg dosage were 0.71 (0.14–3.65) and 4.36 (0.47–40.57), respectively. Results of the separate analyses of PE and DVT risk in lower dose Jakinibs in comparison with higher dose one were shown in online supplementary figure S4 (OR=0.85, 95% CI 0.27 to 2.67, p=0.78 for PE; OR=1.01, 95% CI 0.25 to 4.16, p=0.99 for DVT).

The sensitivity analyses using the Peto method or excluding four Japan-based studies showed similar results for main comparisons (online supplementary figures S5, S6). After we specifically included the studies with an independent cardiovascular committee, a numerically higher, but statistically non-significant risk of all CVEs in patients with RA receiving Jakinibs (OR=1.79, 95% CI 0.85 to 3.77, p=0.13) was observed (online supplementary figure S7).

Risk of bias assessment

We found that 13 RCTs (50%) adequately reported the generation of random sequence and 15 RCTs (65%) had adequate descriptions of concealed allocation. Blinding of participants, personnel, and outcome assessor was performed in all RCTs. Incomplete outcome data were well balanced in 23 RCTs (88%). Twelve RCTs (46%) had an independent cardiovascular safety adjudication committee. Of 23 RCTs, patient baseline characteristics in all intervention groups were well balanced (online supplementary table S3). For the Mantel-Haenszel (M-H) fixed-effect and Peto methods, funnel plot analysis showed no evidence of publication bias in all comparisons (figure 6 and online supplementary figure S8).

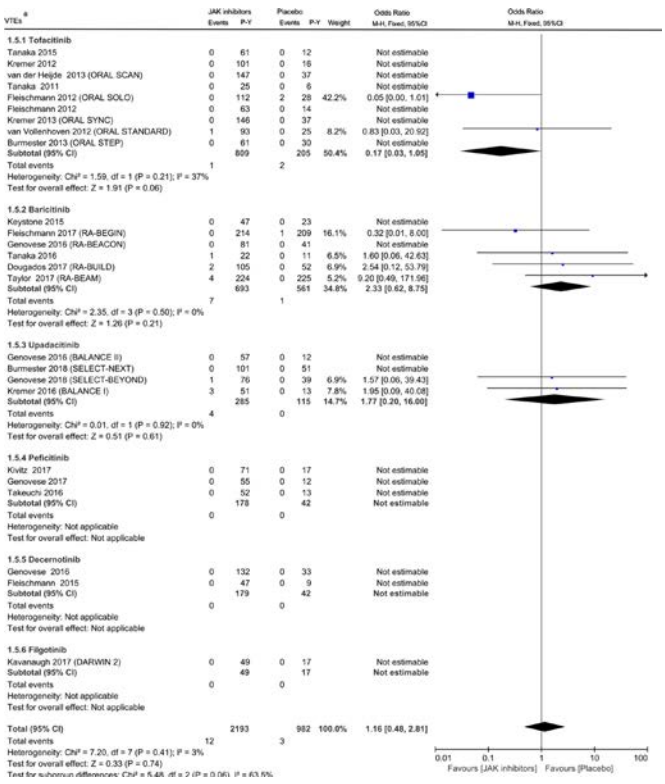

Figure 4 OR of venous thromboembolism events (VTEs) in patients treated with Janus kinase (JAK) inhibitors compared with placebo in randomised controlled trials using the Mantel-Haenszel (M-H) fixed-effect method. P-Y, patient–year. *In consideration of the US Food and Drug Administration warning about greater risk of thromboembolic events in the higher dose of 10 mg tofacitinib, relative to both the 5 mg regimen and antitumour necrosis factor therapy, the subanalysis of the risk of VTEs between JAK inhibitors and placebo or low-dose and high-dose regimens of tofacitinib, baricitinib, as well as upadacitinib was performed.

DISCUSSION

To our knowledge, this is the first meta-analysis exploring the relationship between Jakinib therapies and cardiovascular risks

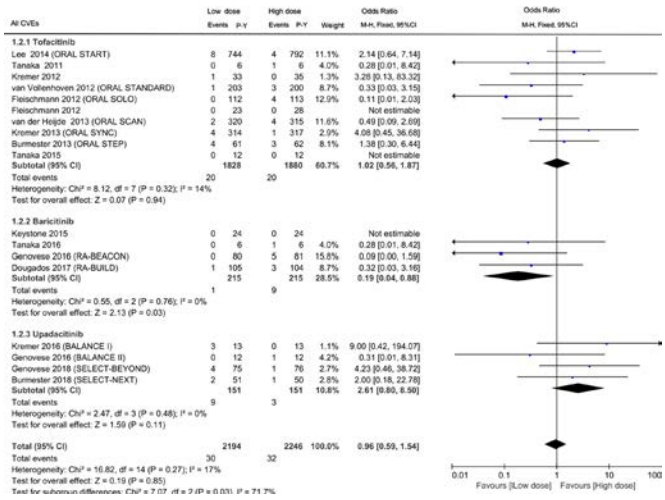

Figure 5 OR of all cardiovascular events (CVEs) in patients treated with different dosages of Janus kinase (JAK) inhibitors in randomised controlled trials using the Mantel-Haenszel (M-H) fixed-effect method. P-Y, patient–year.

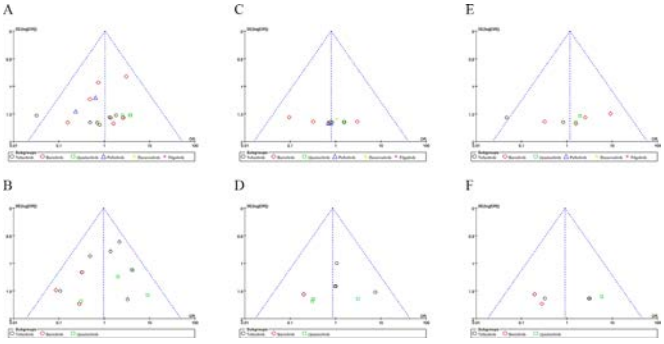

Figure 6 Funnel plots for the meta-analysis of occurrence of all cardiovascular events (a) Janus kinase (JAK) inhibitorsversus placebo (b) Different dosages comparison; major adverse cardiovascular events (c) JAK inhibitors versus placebo (d) Different dosages comparison; and venous thromboembolism events (e) JAK inhibitorsversus placebo (f) Different dosages comparison.

in RA population. According to our results, short-term use of Jakinibs does not increase the risk of CVEs, relative to placebo. Furthermore, there was no adequate evidence of dose-dependent impact on the occurrence of CVEs regarding tofacitinib 5 mg against 10 mg, upadacitinib 15 mg against 30 mg, but baricitinib at 2 mg appeared to be a safer treatment option against 4 mg one.

Tofacitinib and baricitinib have been approved for treating RA, but there are residual uncertainties regarding viral infection, malignancy, as well as cardiovascular safety. A recent meta-analysis assessed the hazard of developing malignancies or serious infections in patients with RA receiving Jakinibs treatment.[58] ORs of 2.48 (95% CI 0.76 to 8.11) and 1.39 (95% CI 0.21 to 9.11) alarmed safety signals regarding malignancy and serious infection risk. Regarding cardiovascular safety, only one pooled analysis suggested that tofacitinib treatment might be associated with low incidence of CVEs,[17] but the reliability was limited by uncertain comparability of participants' characteristics and non-comparative control. In this present meta-analysis, ORs of 1.04 (95% CI 0.61 to 1.76) for CVEs, 0.80 (95% CI 0.36 to 1.75) for MACEs, 1.16 (95% CI 0.48 to 2.81) for VTEs, generally indicated a 'neutral' role of short-term Jakinibs treatment in cardiovascular outcomes. Separately viewed, tofacitinib, the first approved tsDMARD, seemed to be safe for both of all CVEs, MACEs and VTEs risk. Tofacitinib 5 mg is recommended as the optimum dose for the RA treatment after balancing the dose-related safety risks and efficacy benefits. As outlined, two dosing regimens of tofacitinib appeared to produce almost the same cardiovascular safety profile. However, the preliminary analysis of an ongoing safety trial enrolling elder (≥50) patients with RA with at least one cardiovascular risk factor showed the risk of PE to be fivefold higher in 10 mg tofacitinib compared with anti-TNF therapy, and approximately threefold higher than tofacitinib in previous clinical programme. Additionally, all-cause mortality in 10 mg arm was higher compared with 5 mg one or anti-TNF comparator.[59 60] In this safety trial, the risk-benefit profile of 5 mg tofacitinib and anti-TNF remains appropriately balanced, which are consistent with the results of Desai *et al*.[61] This trial will be completed by the end of 2019 and is expected to provide additional information. Baricitinib both at 2 mg and 4 mg doses have been approved for the treatment of RA in various countries worldwide, except USA with 2 mg permitted only. Based on randomised controlled datasets, baricitinib showed a trend towards increased risk of all CVEs, though statistically insignificant. Subanalyses revealed that the elevation was largely driven by VTEs rather than MACEs. Notably, there was an imbalance for the occurrence of VTEs in the baricitinib clinical programme. All VTEs occurred in patients receiving 4 mg baricitinib, in contrast to none in those receiving 2 mg. Pairwise comparisons of baricitinib 2 mg and 4 mg regimens suggested that 2 mg appeared to be a far safer therapeutic option in the consideration of cardiovascular outcomes. In FDA update documents including both controlled and uncontrolled datasets from four pivotal trials and associated long-term extension study (cut-off date of 1 April 2017), there were 5 and 34 VTEs in the baricitinib 2 mg and 4 mg, respectively, accompanied by a numerically lower incidence rate of VTEs in 2 mg baricitinib than in 4 mg (0.397 vs 0.584 per 100 patient years).[62] As the duration of treatment exposure and numbers of VTEs were relatively small, future investigations are desired to quantify whether cardiovascular and thromboembolic risk is drug related and further dose related. Another promising Jakinibs, upadacitinib, appear to be associated with possible increased cardiovascular risk, despite no statistical significance. Paired comparisons showed 15 mg did not seem to be safer than 30 mg regarding all CVEs risk, but the interpretation of these findings is challenging given that the limited exposure is insufficient to draw a definite conclusion now. Concerning the remaining Jakinibs, no particular red signal in CVEs was alarmed based on the limited datasets.

On the other hand, accumulating evidence has indicated the positive correlation between well-controlled inflammation and improved cardiovascular outcomes in patients with chronic inflammation,[63–65] including RA.[7–10] Several agents, such as MTX and anti-TNF, have been indicative of cardioprotection in RA population. However, it is still largely open to discussion that tsDMARDs-based therapy serves as friend or foe in cardiovascular outcomes in patients with RA given unfavourable alterations in cardiovascular risk factors and the limitation in current safety datasets. In light of these findings of the present study and FDA recent safety warning, cardiovascular risk assessment such as age, hypertension, prior cardiovascular disease and medication patterns should be considered before initiation of high-dose Jakinibs treatment. Besides, an independent cardiovascular safety endpoint adjudication committee reviewing all potential cardiovascular and thromboembolic events should be routinely established in subsequent Jakinibs clinical programme. To develop a more thorough understanding of the role of these small molecules in cardiovascular outcomes, both continued surveillance of emerging data and the exploration of underlying mechanisms are sorely required.

Recent findings indicated that racial disparities occur in tsDMARDs-based strategies regarding the incidence of several side effects. Higher rate of herpes zoster infection in Japanese patients than overall populations is an example.[66] However, access to ethnicity-level or patient-level data was not granted by any of the study sponsors. Four cases of CVEs (including one MACEs) reported in five Japanese population-based trials indicated cardiovascular safety in Asian population need to be taken seriously.[33 42 48 53 67]

Limitations may exist when the findings of our meta-analysis are interpreted. First, most of included trials had a relatively short duration of randomised controlled phase (12–104 weeks), which possibly influenced the performance of this meta-analysis to detect the relationship of Jakinibs to CVEs risk. But both Peto and Mantel-Haenszel fixed-effect methods were applied in the present meta-analysis, which strengthened the statistical power. Secondary, the background therapy (such as non-steroidal anti-inflammatory drugs and oral corticosteroid) may influence the results, but the randomisation producing similar

treatment patterns and background cardiovascular risk among arms could minimise the potential bias, to some extent. Besides, patient selection process of clinical trials may indirectly influence the background cardiovascular risk, such as excluding elderly patients who are at increased risk of CVEs, although the included RCTs did not explicitly restrict the patients at elevated cardiovascular risk. This may consequently limit the generalisability of the results.

In conclusion, based on the best available evidence from RCTs, the present meta-analysis indicates that neither individual Jakinib nor all Jakinibs in general significantly influences cardiovascular outcomes in adult patients with RA. Meanwhile, baricitinib at 4 mg seems to be associated with a higher occurrence of CVEs, relative to 2 mg dose. These results should be interpreted specifically in the context of limited duration of follow-up in RCTs. In consideration of these findings in present study, FDA restricts approval to only the low-dose baricitinib, as well as the most recent warning about an increased risk of PE with higher dose tofacitinib, continuous postmarketing surveillance of emerging data is urgently needed to comprehensively clarify the association of Jakinibs and cardiovascular outcomes in RA population.

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Contributors ZZ conceived the study, participated in its design and coordination, and critically revised the manuscript. WX had full access to all of the data collection, analysis, interpretation and drafted the manuscript. YH, XS, YF and SX were study investigators and contributed to the process of data collection. All authors read and approved the final manuscript.

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Patient consent for publication Not required.

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TRANSLATIONAL SCIENCE

Association of response to TNF inhibitors in rheumatoid arthritis with quantitative trait loci for *CD40* and CD39

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ABSTRACT

Objectives We sought to investigate whether genetic effects on response to TNF inhibitors (TNFi) in rheumatoid arthritis (RA) could be localised by considering known genetic susceptibility loci for relevant traits and to evaluate the usefulness of these genetic loci for stratifying drug response.

Methods We studied the relation of TNFi response, quantified by change in swollen joint counts (ΔSJC) and erythrocyte sedimentation rate (ΔESR) with locus-specific scores constructed from genome-wide assocation study summary statistics in 2938 genotyped individuals: 37 scores for RA; scores for 19 immune cell traits; scores for expression or methylation of 93 genes with previously reported associations between transcript level and drug response. Multivariate associations were evaluated in penalised regression models by cross-validation.

Results We detected a statistically significant association between ΔSJC and the RA score at the *CD40* locus (p=0.0004) and an inverse association between ΔSJC and the score for expression of CD39 on CD4 T cells (p=0.00005). A previously reported association between CD39 expression on regulatory T cells and response to methotrexate was in the opposite direction. In stratified analysis by concomitant methotrexate treatment, the inverse association was stronger in the combination therapy group and dissipated in the TNFi monotherapy group. Overall, ability to predict TNFi response from genotypic scores was limited, with models explaining less than 1% of phenotypic variance.

Conclusions The association with the CD39 trait is difficult to interpret because patients with RA are often prescribed TNFi after failing to respond to methotrexate. The CD39 and *CD40* pathways could be relevant for targeting drug therapy.

Key messages

What is already known about this subject?

- To date, no strong associations of individual genetic loci with response to tumour necrosis factor inhibitors (TNFi) in rheumatoid arthritis (RA) have been identified, despite recent large efforts based on conventional genome-wide association studies and a crowdsourcing initiative.

What does this study add?

- We introduced a new methodological approach for localising genetic effects by using genotypic risk scores based on known genetic loci for related traits and likely biomarkers.
- We identified two genetic loci strongly associated with TNFi response in RA and demonstrated that the genetic determinants of TNFi response are different to the known susceptibility loci for RA.

How might this impact on clinical practice or future developments?

- Measurements of expression of *CD40* and CD39 and their corresponding pathways could be relevant for targeting drug therapy in RA.
- Our new methodological approach could be useful for localising genetic effects in traits for which assembling large sample sizes is not feasible, such as drug response.

INTRODUCTION

Biologic therapies have transformed the outlook for rheumatoid arthritis (RA). However, for the most commonly used class of agent, tumour necrosis factor inhibitors (TNFi), there is substantial variability in response to treatment among patients with RA.[1] This has spurred efforts to discover predictors of response and more generally to understand how to subtype this heterogeneous disease to predict which therapies will work.[2 3]

Genome-wide association studies (GWAS) of response to TNFi have shown that common single

nucleotide polymorphisms (SNPs) explain an estimated 40% and 50% of the variance of change in swollen joint counts (SJC) and erythrocyte sedimentation rate (ESR), respectively; however, no strong associations with individual SNPs have been detected.[4] Thus, as with many complex phenotypes, the genetic architecture of response to TNFi is likely to be polygenic with many small genetic effects.[5] In this situation, the sample size required to learn a predictive model is very large—up to 10 cases per variable[6]—and it may not be feasible to assemble such large sample sizes for studying response to a single drug or drug class.

It has been suggested that improving prediction of complex clinical outcomes may be possible by incorporating information about the genetics of relevant traits in the prediction model.[7 8] One such approach is to use publicly available summary GWAS results of relevant traits to compute genotypic scores, which can then be used as variables ('features') from which to build predictive models. By harnessing the genetic profiles of intermediate traits, these scores aggregate the effects of individual SNPs into larger regional or whole-genome effects. Relevant traits can include diseases, biomarkers and gene transcription levels. For polygenic traits such as RA, for which multiple genetic susceptibility loci have been identified, we can construct locus-specific scores allowing us to examine the extent to which drug response is related to genetic heterogeneity of the disease.

In the current study, we incorporated available genetic information on susceptibility to RA,[9] immune cell traits from a publicly available bioresource[10] and expression or methylation of genes implicated in response to TNFi treatment in RA.[11] The genotypic scores associated with these intermediate traits were then tested for association with response to TNFi; by reducing the number of hypotheses being explored, the thresholds for claiming statistical significance are relaxed, which could help identify useful predictors.

MATERIALS AND METHODS

Cohorts

We used a sample of 2938 individuals of European ancestry for whom complete clinical and GWAS data were available. This sample comprised individuals from a pre-existing international collaboration formed to study the genetics of response to TNFi agents[12] and individuals recruited to the Biologics in Rheumatoid Arthritis Genetics and Genomics Study Syndicate (BRAGGSS) after 2013.[4]

Table 1 shows sample sizes, phenotypes and clinical variables for each of the data collections used in this study. All participants provided informed consent, and institutional review board/ethics approvals were in place as described in Cui *et al*[12] and Massey *et al*.[4]

Definition of response to TNFi treatment

In RA, response to treatment is quantified by change in the Disease Activity Score (DAS), which depends on four measurements: ESR, SJC, tender joint count (TJC) and patient global health assessment rated on a visual analogue scale (GHVAS). Previous work has shown that only the SJC and ESR measurements have evidence of non-zero heritability[4] and correlate significantly with synovitis quantified by ultrasound or MRI.[13 14] Since TNFi were developed to control synovitis, we used the two objective components of the DAS (ESR and SJC) as primary outcomes for evaluating genetic effects and the two subjective components (TJC and GHVAS) and the composite score (DAS28-ESR4) as

Table 1 Sample information per cohort.

	BRAGGSS	DREAM	EIRA	ReAct	WTCCC	Other*	Total
Sample size	954	764	283	258	556	123	2938
Sex, female %	76	68	74	77	77	82	74
Concomitant non-biologic DMARD %	85	74	74	50	73	97	76
TNF inihibitor							
Adalimumab	416	441	47	258	64	29	1255
Certolizumab	34	0	0	0	0	0	34
Etanercept	293	66	97	0	246	19	721
Golimumab	17	0	0	0	0	0	17
Infliximab	194	138	139	0	246	75	792
Baseline disease activity, mean (SD)							
DAS28-ESR4	6.3 (1.0)	5.4 (1.2)	5.3 (1.2)	5.8 (1.0)	6.7 (0.9)	5.6 (1.1)	6.0 (1.2)
ESR	36.2 (26.2)	27.7 (21.3)	32.4 (22.9)	31.2 (21.4)	45.0 (28.8)	30.2 (21.5)	34.6 (25.4)
SJC	10.1 (6.1)	10.3 (5.5)	9.2 (6.0)	9.9 (5.1)	11.8 (6.4)	10.5 (6.3)	10.4 (6.0)
TJC	15.4 (7.3)	10.0 (7.4)	8.3 (6.0)	13.1 (6.5)	17.0 (7.3)	10.7 (6.3)	13.2 (7.8)
GHVAS	71.0 (19.2)	62.3 (22.1)	56.1 (23.3)	59.7 (20.8)	72.6 (18.1)	59.3 (23.9)	66.1 (21.4)
6-month disease activity, mean (SD)							
DAS28-ESR4	3.7 (1.6)	3.6 (1.3)	3.5 (1.4)	3.7 (1.4)	4.2 (1.5)	3.9 (1.6)	3.8 (1.5)
ESR	22.8 (22.0)	18.1 (16.9)	20.0 (17.3)	18.6 (17.0)	27.6 (24.9)	22.2 (19.7)	21.8 (20.7)
SJC	3.0 (4.0)	4.8 (4.4)	3.4 (3.8)	3.6 (3.6)	4.0 (4.7)	5.0 (4.9)	3.8 (4.3)
TJC	5.0 (6.3)	3.7 (4.6)	3.6 (4.9)	4.7 (5.5)	6.3 (6.6)	5.1 (6.1)	4.7 (5.8)
GHVAS	37.2 (25.2)	34.5 (21.7)	34.4 (25.2)	31.8 (25.9)	37.1 (25.0)	31.8 (26.6)	35.5 (24.5)

* 'Other' displays aggregate sample characteristics for collections with sample size <100

BRAGGSS, Biologics in Rheumatoid Arthritis Genetics and Genomics Study Syndicate; DAS, Disease Activity Score; DMARD, disease-modifying antirheumatic drug; DREAM, Dutch Rheumatoid Arthritis Monitoring Registry; EIRA, Swedish Epidemiological Investigation of Rheumatoid Arthritis; ESR, erythrocyte sedimentation rate; GHVAS, global health assessment rated on a visual analogue scale; ReAct, French Research in Active Rheumatoid Arthritis; SJC, swollen joint count; TJC, tender joint count; TNF, tumour necrosis factor; WTCCC, Wellcome Trust Case Control Consortium.

secondary outcomes. For each outcome, a baseline measurement was taken before initiation of TNFi treatment, and a follow-up measurement was taken between 3 and 6 months after initiation of TNFi treatment. The measurements for each component were transformed in accordance to the DAS28-ESR4 formula (see online supplementary methods). Response was modelled as the difference between the baseline and the follow-up measurement.

Genotypic risk scores

We used the GENOSCORES platform (https://pm2.phs.ed.ac.uk/genoscores/) to compute genotypic risk scores for the intermediate traits. GENOSCORES is a database of published SNP to trait associations from a large number of well-powered GWAS, including GWAS of disease traits, biomarkers, gene expression and methylation. The database is accompanied by a software package implemented in R that can be used to compute genotypic risk scores and run downstream statistical analyses in cohorts with SNP data.

We queried the GENOSCORES database for genetic associations with RA risk,[9] 149 heritable immune cell traits reported by Roederer *et al*,[10] and whole-blood expression and methylation for 93 genes reported in a recent meta-analysis[11] as differentially expressed before treatment between responder and non-responder patients with RA treated with TNFi. GWAS summary statistics reported by Westra *et al*[15] and Gusev *et al*[16] were used for expression quantitative trait loci (eQTLs). GWAS summary statistics reported by Gaunt *et al*[17] were used for methylation quantitative trait loci (mQTLs).

GWAS summary statistics for each intermediate trait were filtered at p value$<10^{-5}$. SNPs were then split into trait-associated regions, with regions defined as genomic loci containing at least one SNP with p value$<10^{-7}$. Only 19 of the immune cell traits had a corresponding trait-associated region. SNPs not assigned to a region were discarded. For each trait-associated region, a genotypic score was computed as a sum of SNP genotypes, $\mathbf{g}$, weighted by the effect size estimates, β (log OR for binary traits, regression slope for quantitative traits) and adjusted for linkage disequilibrium. The regional score, s_{itr}, for an individual i, trait t and region r was computed as: $s_{itr} = \beta_{tr}^T R_r^{-1} \mathbf{g}_{ir}$, where R_r denotes the SNP–SNP correlation matrix in genomic region r.

Additional details about the GENOSCORES platform, the score computation and the specific regional scores used in this study are given in online supplementary materials (see online supplementary methods, online supplementary tables S1–S5, online supplementary figure S1).

Predictive modelling

To evaluate genetic prediction of response to TNFi, we compared a model with clinical covariates only to a model with clinical covariates and genotypic scores for each type of intermediate trait. To avoid numerical instabilities, we removed highly correlated scores prior to fitting a model (see online supplementary methods). The number of filtered regional scores for each type of intermediate trait is shown in table 2.

We expected that only a subset of genotypic scores would be relevant for prediction of response to TNFi, and thus used a hierarchical shrinkage prior for the score coefficients. We implemented the prediction models in STAN[18] using a horseshoe prior distribution and performed inference with Markov chain Monte Carlo sampling.[19 20] To rank the importance of genotypic scores in a model, we applied projection predictive variable selection, an approach that projects posterior draws from the high-dimensional model to lower dimensional subspaces.[21]

Table 2 Prediction of response to TNFi using penalised regional genotypic scores for different types of intermediate traits

Intermediate trait type	No of regional scores	No of filtered scores	Prediction of ΔSJC (%)	Prediction of ΔESR (%)
Rheumatoid arthritis	37	37	5.3 (0.26)	−1.6 (0)
Immune cell traits	508	470	−0.7 (0)	2.9 (0.17)
eQTLs	94	87	3.4 (0.16)	2.9 (0.17)
eQTLs and mQTLs	268	228	2.5 (0.11)	1.6 (0.09)

Prediction performance is quantified by the difference in test log-likelihood (in nats) between a model with clinical covariates and genotypic scores and a model with clinical covariates only and by the per cent of phenotypic variance explained (in parenthesis). Results from 10-fold cross-validation.
eQTL, expression quantitative trait loci; mQTL, methylation quantitative trait loci.ESR, erythrocyte sedimentation rate; SJC, swollen joint count; TNFi, tumour necrosis factor inhibitors;

We used a statistical model with the following clinical covariates: measurements for the four DAS components before initiation of TNFi treatment, gender, whether the patient was concomitantly treated with any non-biologic disease-modifying antirheumatic drugs (DMARDs), cohort (which is also a proxy for country), genotyping array and the 10 first principal components computed from the genotypic data of the full data set. We used individuals with complete measurements in the statistical analyses of each TNFi response outcome (see online supplementary table S6).

Evaluation of prediction

We used two measures to quantify improvement in prediction: the difference in log-likelihood between a model with clinical covariates and genotypic scores and a model with clinical covariates only (measured in natural log units (nats)); and the per cent of residual variance explained by the genotypic scores. Both measures were computed on the testing data from 10-fold cross-validation on the full data set.

For readers who prefer a frequentist interpretation, the asymptotic equivalence of model choice by cross-validation and Akaike's information criterion (AIC)[22] implies that a p value of 0.01 for comparison of nested models is equivalent to a difference in test log-likelihood of 2.3 nats (likelihood ratio of 10) for models differing by one extra parameter. The large sample size of this study means that small robust increments in predictive performance can be detected.

Univariate associations

For models with a test log-likelihood difference of at least two nats, we further examined the univariate associations between genotypic scores and the TNFi response outcomes. We used the full data set to test the univariate association and included the same clinical covariates as in the multivariate prediction, in addition to a score.

For genotypic scores significantly associated with TNFi response at the Bonferroni-corrected p value threshold, we compared the estimated effects among groups receiving different TNFi agents. We considered etanercept, adalimumab and infliximab, where a large enough sample size was available. Additionally, we tested if the associations held when we adjusted

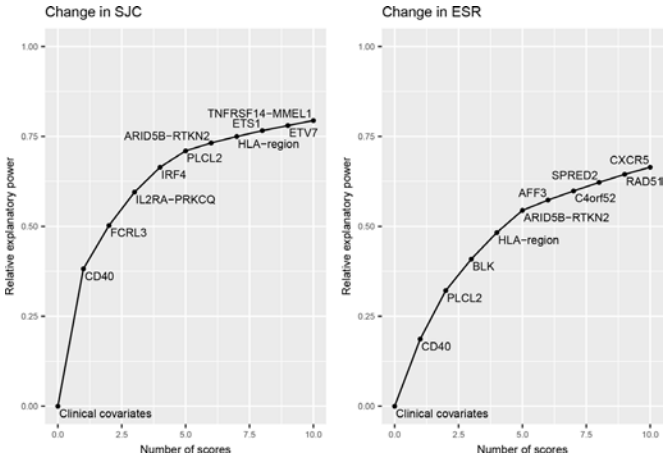

Figure 1 Contribution of top 10 RA regional scores to prediction of response to TNFi phenotypes, starting from a model containing only clinical covariates. The curve gradually converges to one with the addition of all remaining scores. ESR, erythrocyte sedimentation rate; RA, rheumatoid arthritis; SJC, swollen joint count; TNFi, tumour necrosis factor inhibitors.

for additional covariates: anticitrullinated protein antibodies (ACPA) status and smoking status. These covariates have been reported to influence TNFi response but were only available for a third of the samples, and thus were not included in the full models.

A diagram of the statistical analysis pipeline is given in online supplementary figure S2.

RESULTS

In the following sections, we focus on the results for the primary TNFi response outcomes. The results for secondary outcomes are discussed in the online supplementary results (see online supplementary table S7).

RA genotypic scores

Prediction of response to TNFi as quantified by ΔSJC improved by including the regional genotypic scores for RA risk in a penalised regression model (table 2). The test log-likelihood increased by 5.3 nats, suggesting that some of the genetic drivers for RA are also influencing response to TNFi; however, the absolute improvement in prediction was small, with less than 1% of phenotypic variance being explained by the RA genotypic scores.

The regional score at the *CD40* locus had the highest explanatory power for both response phenotypes (figure 1). The direction of the effect was consistent for response as measured by both phenotypes, with higher RA load at the *CD40* locus being associated with better TNFi response. The univariate association of the RA score at the *CD40* locus with ΔSJC passed the p value threshold corrected for the number of RA scores and two response phenotypes (table 3).

The regional score for RA at the *CD40* locus was correlated with a cis-acting eQTL score for *CD40* expression in whole blood (correlation=0.65) and a cis-acting mQTL score for methylation of *CD40* in whole blood (correlation=−0.70). The strongest association between response to TNFi and genotypic scores at the *CD40* locus was with the score for RA (table 3). The estimated effect did not change when we stratified by TNFi agent and when we adjusted for ACPA and smoking status (see online supplementary tables S8 and S9).

Table 3 Univariate associations between response phenotypes and regional genotypic scores of interest

Response phenotype	Genetic score	Coefficient	P value
Genotypic scores at the CD40 locus			
ΔSJC	RA score at *CD40*	0.07	0.0004
ΔSJC	*CD40* eQTL	0.06	0.002
ΔSJC	*CD40* mQTL	−0.05	0.009
ΔESR	RA score at *CD40*	0.05	0.01
ΔESR	*CD40* eQTL	0.03	0.06
ΔESR	*CD40* mQTL	−0.03	0.07
Genotypic scores for immune cell traits at the ENTPD1 locus			
ΔSJC	CD39 on CD4 T	−0.07	5e−05
ΔSJC	mDC:%32+	−0.02	0.2
ΔSJC	CD8:%39+	−0.06	0.001
ΔSJC	CD4:%Treg(39+)	−0.07	0.0001
ΔSJC	CD4:%Treg(39+73-)	−0.07	0.0001
ΔSJC	CD4:%Treg(39+73+)	−0.07	0.0002
ΔSJC	NKeff:%314−158a+	−0.04	0.02
ΔSJC	CD4 T:%CD39+CD38+PD1−	−0.07	8e−05
ΔESR	CD39 on CD4 T	−0.003	0.9
ΔESR	mDC:%32+	−0.01	0.5
ΔESR	CD8:%39+	−0.008	0.7
ΔESR	CD4:%Treg(39+)	−0.004	0.8
ΔESR	CD4:%Treg(39+73−)	−0.004	0.8
ΔESR	CD4:%Treg(39+73+)	0.006	0.8
ΔESR	NKeff:%314−158a+	0.04	0.04
ΔESR	CD4 T:%CD39+CD38+PD1−	−0.004	0.8

The coefficients are the effect sizes of the standardised score on the standardised phenotype.
eQTL, expression quantitative trait loci; mTQL, methylation quantitative trait loci.ESR, erythrocyte sedimentation rate; RA, rheumatoid arthritis; SJC, swollen joint count;

Genotypic scores for immune cell traits

Prediction of response to TNFi as measured by ΔESR improved by adding penalised genotypic scores for immune cell traits in the model (table 2). In univariate analyses, the regional scores for a number of immune cell traits at the *ENTPD1* locus (which codes for CD39) had suggestive associations with ΔSJC (table 3). The association of ΔSJC with the genotypic score for 'CD39 on CD4 T cells' at the *ENTPD1* locus passed the p value threshold corrected for the number of immune cell trait scores tested (470) and two response phenotypes. This score was correlated with the cis-acting eQTL score for *ENTPD1* (correlation=0.65). Higher score for 'CD39 on CD4 T cells' at the *ENTPD1* locus was associated with worse TNFi response as quantified by ΔSJC. There was no association between ΔESR and genotypic scores at the *ENTPD1* locus (table 3) and no association between either ΔESR or ΔSJC and a genotypic score for cell subset frequency of CD73, which is the second ectonucleotidase involved in adenosine production in regulatory T cells (see online supplementary results).

Previously, Peres *et al*[23] showed that low expression of CD39 on peripheral regulatory T cells was associated with worse response to methotrexate (MTX) in patients with RA. We note that the direction of the effect is reversed, but this is not unlikely since we considered response to TNFi. To further investigate the effect of 'CD39 on CD4 T cells' expression on TNFi response, we performed two analyses stratified by concomitant treatment. Information on whether a patient was receiving a concomitant non-biologic DMARD was available for

Table 4 Univariate association between ΔSJC and genotypic score for the expression of 'CD39 on CD4 T cells' at the *ENTPD1* locus stratified by concomitant treatments

Patient group	Coefficient (SE)	P value	Sample size
All samples, adjusted for concomitant DMARD	−0.07 (0.02)	5e−05	2922
All samples, no adjustment for DMARD	−0.08 (0.02)	2e−05	2922
Samples not on concomitant DMARD	−0.02 (0.04)	0.5	691
Samples on concomitant DMARD	−0.09 (0.02)	1e−05	2231
Samples on concomitant MTX	−0.1 (0.03)	0.002	958

The coefficients are the effect sizes of the standardised score on the standardised phenotype. The phenotype is adjusted for covariates: baseline DAS components, gender, cohort, genotyping array, 10 genetic principal components.
DAS, Disease Activity Score; DMARD, disease-modifying antirheumatic drug; MTX, methotrexate; SJC, swollen joint count.

all samples, while information on whether a patient was specifically receiving concomitant MTX treatment was available for a subset of patients from the BRAGGSS cohort. Table 4 shows the effect of the genotypic score for expression of 'CD39 on CD4 T cells' on ΔSJC for patients receiving TNFi treatment stratified by concomitant treatment with either any non-biologic DMARD (top) or specifically with MTX (bottom). The effect became stronger in the groups receiving concomitant treatment and attenuated in the group receiving TNFi monotherapy; the CIs among all groups overlapped. Similarly, we did not detect statistically significant differences when we stratified by TNFi agent and when we adjusted for ACPA status (see online supplementary tables S8 and S9).

eQTL and mQTL scores

Prediction of response to TNFi as quantified by both ΔSJC and ΔESR improved by adding eQTL and mQTL scores of implicated genes in a penalised regression model (table 2). Of the 93 genes reported in Kim *et al*[11] as differentially expressed between responders and non-responders to TNFi in RA, 54 genes had at least one eQTL, 54 genes had at least one mQTL and 36 had both. The test log-likelihood increased by 3.4 and 2.9 nats for ΔSJC and ΔESR, respectively, by adding the eQTL scores in the model. We did not see a further improvement by adding genotypic scores for mQTLs.

DISCUSSION

In the largest international study of TNFi response to date, we have shown how using methods that leverage information from relevant intermediate traits can identify predictors of TNFi response. In a recent crowdsourced effort to use machine learning to construct a predictor of response to TNFi in RA, including SNP genotypes did not improve prediction beyond that obtained with clinical covariates alone.[24] Genotypic prediction of psychiatric disorders and related phenotypes has been shown to improve by exploiting genetic correlations among multiple related traits,[7 25] and methods have been extended to incorporate polygenic scores for multiple traits.[8]

In the current study, we have combined these approaches and implemented them in a newly developed platform, called GENOSCORES, which contains GWAS data for multiple traits and automates construction of genotypic scores. For polygenic traits with multiple trait-associated loci, such as RA, locus-specific scores can be constructed to examine how genetic heterogeneity of the intermediate trait can influence the trait of interest. Our approach reduces the dimensionality of the prediction task from about 2 million common SNPs to a few hundred or a few thousand genotypic scores, depending on how relevant traits are selected. The score constructions are a type of feature engineering, a task commonly used in machine learning applications.

Understanding the pathogenic mechanisms that initiate and perpetuate RA could give rise to informative biomarkers of prognosis, therapeutic response and toxicity.[26] However, in agreement with earlier studies,[27] we did not find strong predictors of TNFi response among alleles linked to the development of RA. A strength of the current study is the large sample size which allowed us to detect small robust increments in predictive performance. Our methodological approach was to first establish the predictive value for a set of genetic markers using a multivariate model and then to examine univariate associations between each marker and the outcome. Using this approach, we showed that a model including RA scores led to a small robust improvement in prediction, with the regional score at the *CD40* locus driving the predictive signal.

Higher RA risk at the *CD40* locus, higher *CD40* transcription and lower *CD40* methylation were associated with better TNFi response. CD40 is a transmembrane protein which belongs to the TNF receptor superfamily, critically important in modulating immune-(auto-immune) responses.[28] CD40 is expressed by B cells and antigen-presenting cells (APCs), whereas CD40L is induced on CD4+ T cells following T-cell antigen receptor (TCR) with major histocompatability complex (MHC) molecule interaction. Engagement of the CD40–CD40L axis leads to B cell activation, proliferation and (auto)-antibody production, while activation of APCs by CD40L on CD4+ T cells induces upregulation of CD80, CD86, MHC class I and MHC class II, as well as secretion of proinflammatory cytokines such as interleukin (IL)-12, IL-23 and TNF-α.[29 30]

The risk allele associated with RA is associated with elevated *CD40* expression in whole blood.[31] As high *CD40* expression is associated with elevated TNF-α production and *CD40* and *CD40L* transcripts are increased in the disease tissue in both early and established disease,[32] it is not surprising that patients with the *CD40* risk allele respond better to TNFi therapies.

Overall, if there are genetic loci that predict TNFi response, these are mostly different to the known RA risk loci. However, we note that patients who receive TNFi therapy are likely to have more severe disease and to have failed on other treatments. It is therefore possible that our study sample has been selected with respect to genetic load for RA, thus limiting the heterogeneity in genetic RA risk profiles compared with a sample of newly diagnosed cases.

The genotypic score for the expression of the ectonucleotidase CD39 on CD4 T cells was inversely associated with TNFi response. The SNPs contributing to this score are in the *ENTPD1* gene which encodes CD39. In stratified analyses, the inverse association with response was stronger in the groups receiving TNFi concomitantly with MTX or another non-biologic DMARD compared with the group receiving TNFi monotherapy and was stronger in the group receiving infliximab compared with the groups receiving adalimumab or etanercept. The CIs of the estimated effects among all groups overlapped. This effect on response to TNFi agents is in the opposite direction to the association reported between low expression of CD39 on regulatory T cells and resistance to MTX in RA.[23]

Interpreting the association between drug response and the CD39 trait is difficult both epidemiologically and mechanistically.

The reasons for this being that in the UK and most European countries TNFi are usually prescribed only after patients have had a poor response to MTX and, unless of intolerance, always in combination with MTX. Therefore, these patients are likely to represent a selected group; this, together with the almost universal use of combination MTX/TNFi therapy, hinders progress in dissecting the potential mechanisms responsible for the divergence. Nonetheless, as RA is a highly heterogeneous disease, it is plausible to speculate that different cellular and molecular networks may be involved in driving diverse immune/inflammatory responses in different patients to different drugs. For example, as mentioned above, the poor response to MTX has been associated with low CD39 expression by regulatory T cells, while increased CD39 expression has been reported to be important in the expansion of Th17 cells driven by IL-6 and TGF-β via Stat3 and Gfi-1 transcription factors.[33] In turn, the expansion of Th17 cells has been reported to be associated with incomplete response to TNFi.[34] It remains to be established whether measurements of CD39/*ENTPD1* expression or genotype may be useful in the choice of MTX, TNFi agent or concomitant treatment as first-line therapy for patients who need a DMARD.

Using previously reported associations between transcripts and TNFi response to select relevant genes, we have shown evidence that eQTL scores for these genes contain information that predicts TNFi response, even though the proportion of variance explained was low and no single genes associated with response could be identified.

Improved genomic prediction of treatment response requires measuring response more precisely to capture the molecular in addition to the clinical phenotype. The detected associations between genotypic scores and TNFi response were not the same for the two measures of response—change in SJC and change in ESR—suggesting that the two measures reflect different aspects of disease activity affected by TNFi. To derive refined measures of drug response, large data sets with multiple inflammatory biomarkers, joint imaging and clinical variables before and after treatment are needed.

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Competing interests None declared.

Patient and public involvement statement This study was conducted as part of Work Stream 2 of the MATURA Consortium (http://www.matura.whri.qmul.ac.uk/what_is_matura.php). A patient advisory group was established in 2014 when the MATURA project commenced. The group meets regularly to: Ensure MATURA strategy is maintaining relevance, accountability and direction by embedding patients and members of the public within the decision making processes. Determine what level of confidence in tests, and what type of tests, would be acceptable to patients for treatment decisions. Maximise patient recruitment to research studies by increasing awareness through patient groups. Readily obtain patients' perspective on grant applications related to stratified medicines for RA.Facilitate the dissemination of the results from MATURA research, for instance by producing lay summaries of papers in conjunction with the researchers (http://www.matura.whri.qmul.ac.uk/news.php).

Patient consent for publication Not required.

Ethics approval This study used anonymised data for human subjects from an international collaboration of 13 studies originally published in PLOS Genetics 2013;9:e1003394. All participants provided informed consent and institutional review board and ethics approvals were in place for each of the studies and described in the original publication.

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CLINICAL SCIENCE

Identification of rare coding variants in *TYK2* protective for rheumatoid arthritis in the Japanese population and their effects on cytokine signalling

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ABSTRACT

Objective Although genome-wide association studies (GWAS) have identified approximately 100 loci for rheumatoid arthritis (RA), the disease mechanisms are not completely understood. We evaluated the pathogenesis of RA by focusing on rare coding variants.

Methods The coding regions of 98 candidate genes identified by GWAS were sequenced in 2294 patients with RA and 4461 controls in Japan. An association analysis was performed using cases and controls for variants, genes and domains of TYK2. Cytokine responses for two associated variants (R231W, rs201917359; and R703W, rs55882956) in *TYK2* as well as a previously reported risk variant (P1004A, rs34536443) for multiple autoimmune diseases were evaluated by reporter assays.

Results A variant in *TYK2* (R703W) showed a suggestive association ($p=5.47\times10^{-8}$, OR=0.48). We observed more accumulation of rare coding variants in controls in *TYK2* ($p=3.94\times10^{-12}$, OR=0.56). The four-point-one, ezrin, radixin, moesin (FERM; 2.14×10^{-3}, OR=0.66) and pseudokinase domains (1.63×10^{-8}, OR=0.52) of TYK2 also showed enrichment of variants in controls. R231W in FERM domain especially reduced interleukin (IL)-6 and interferon (IFN)-γ signalling, whereas P1104A in kinase domain reduced IL-12, IL-23 and IFN-α signalling. R703W in pseudokinase domain reduced cytokine signals similarly to P1104A, but the effects were weaker than those of P1104A.

Conclusions The FERM and pseudokinase domains in TYK2 were associated with the risk of RA in the Japanese population. Variants in *TYK2* had different effects on cytokine signalling, suggesting that the regulation of selective cytokine signalling is a target for RA treatment.

Key messages

What is already known about this subject?

- Multiethnic genome-wide association studies have identified more than 100 loci associated with the risk of rheumatoid arthritis (RA), but the majority of causal genes remain to be identified.
- The analysis of rare coding variants is an alternative method to identify likely causal genes owing to their functional impact and strong effect sizes.

What does this study add?

- The accumulation of rare coding variants was found in *TYK2*, and the four-point-one, ezrin, radixin, moesin (FERM) domain and pseudokinase domain of TYK2 showed associations with RA.
- Each variant in the FERM and pseudokinase domains of TYK2 reduced interleukin (IL)-6, IL-12 and IL-23 signalling, but these variants had different effects on interferon (IFN)-α and IFN-γ signalling.

How might this impact on clinical practice or future developments?

- Based on our findings in the functional analysis of *TYK2* variants, selective regulation of TYK2 domains might become new therapies for RA.

INTRODUCTION

Rheumatoid arthritis (RA) is a complex autoimmune disease with a prevalence of 0.5%–1.0% worldwide.[1] Twins studies have indicated that RA has a genetic basis, with a heritability of approximately 50%–65%.[2 3] This suggests that the identification of causal genes for the disease can improve our understanding of the molecular mechanism underlying RA.

Multiethnic genome-wide association studies (GWAS) have identified more than 100 loci associated with the risk of RA other than human leukocyte antigen (HLA).[4 5] However, most single nucleotide polymorphisms (SNPs) are non-coding, and some loci are in regulatory regions of neighbouring target genes and mediate subtle changes in expression.[4–10] These risk genes are strongly enriched in T-cell regulation signalling pathway,[11 12] the nuclear factor-kappa B signalling pathway,[13] or the Janus kinase (JAK)-signal transducers and activators of transcription (STAT) signalling pathway,[4] suggesting that functional changes in these pathways are important for the pathogenesis of RA. Indeed, several drugs targeting these pathways (such as abatacept for T-cell regulation signalling pathway,[14] and tofacitinib[15] and baricitinib[16] for JAK-STAT pathway) have been used in clinical settings and have dramatically improved the treatment of RA. However, some patients are still refractory to these therapies, and further studies of detailed underlying mechanisms are needed.

The majority of common variants identified by the GWAS have moderate effects on the disease (OR=~1.2) and account for a small proportion of disease heritability.[4] Thus, the analysis of rare coding variants is an alternative method to identify

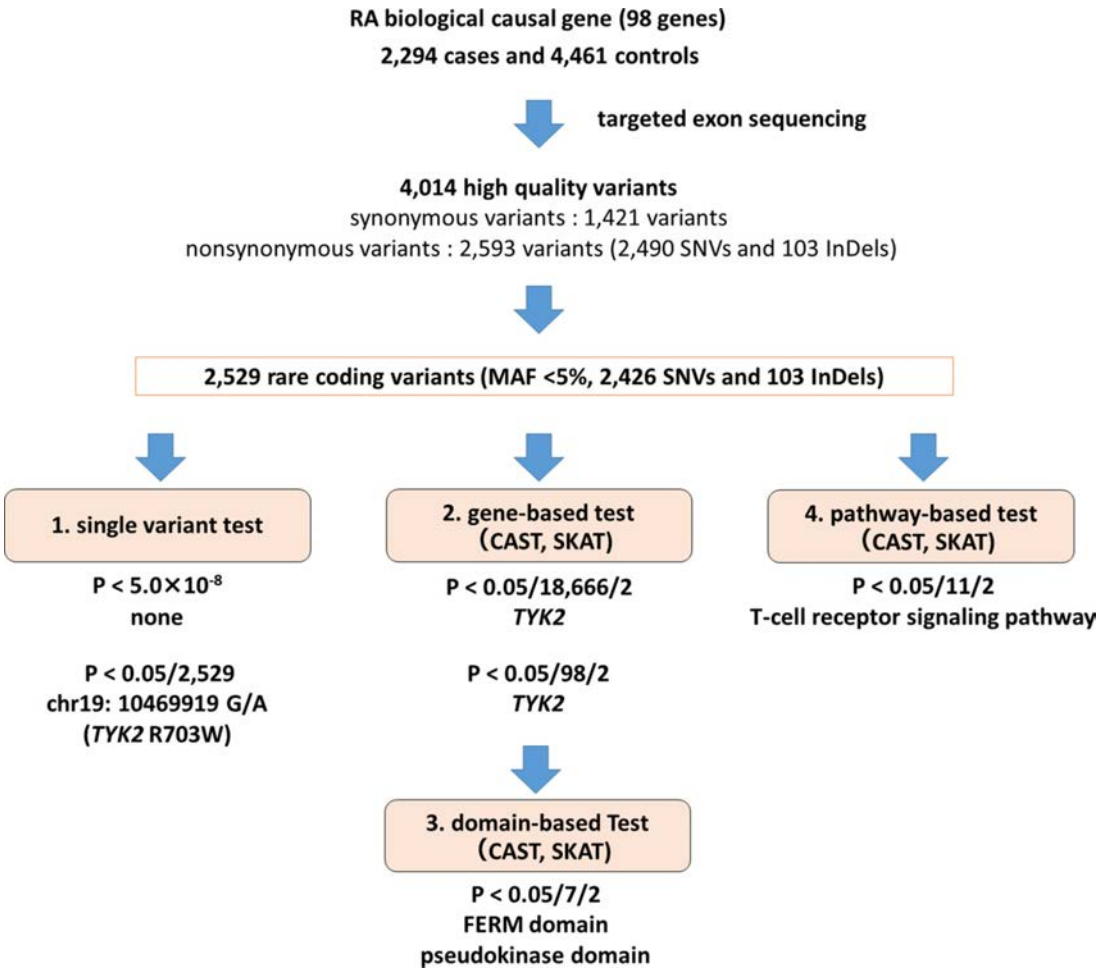


Figure 1 Description of the study design. We performed resequencing of 98 candidate genes identified by GWAS in 2322 cases and 4517 controls by multiplex PCR-based target exon sequencing. After quality control, we identified 4014 variants. We analysed data from rare coding variants (MAF <5%) within targeted genes using four different analyses. CAST, cohort allelic sums test; FERM, four-point-one, ezrin, radixin, moesin; GWAS, genome-wide association studies; MAF, minor allele frequency; RA, rheumatoid arthritis; SKAT, sequence kernel association test; SNV, single nucleotide variants; .

likely causal genes owing to their functional impact and strong effect sizes.[17] This strategy worked well to pinpoint likely causal genes in other diseases, for example, *IFIH1* in type 1 diabetes,[18] interleukin (IL)-23 receptor in inflammatory bowel disease,[19] *MTNR1B* in type 2 diabetes,[20] *PLD3* in Alzheimer's disease,[21] *CARD9* and *RNF186* in ulcerative colitis,[22] and multigenic modules in Crohn's disease.[23] Several studies have applied this strategy to RA and have reported associations between RA and *IL2R*,[24] *VSTM1*[25] and the mitochondrial respiratory chain pathway.[26] To identify rare variants in genes with relevant biological functions,[24 25] large sample sizes are required to detect their signals.

In this study, we evaluated the pathogenesis of RA by focusing on rare coding variants (including missense, splice site, nonsense and frameshift variants) in 98 candidate genes determined by bioinformatics methods after a multiethnic GWAS.[4] We resequenced the coding regions of 98 genes in 2294 cases and 4461 controls and evaluated the accumulation of rare variants in these genes and domains of TYK2. We found the significant accumulation of rare coding variants in *TYK2* and T-cell receptor signalling pathway, and evaluated cytokine responses for two associated variants in *TYK2* according to the accumulation of rare variants in domains.

MATERIALS AND METHODS

Samples

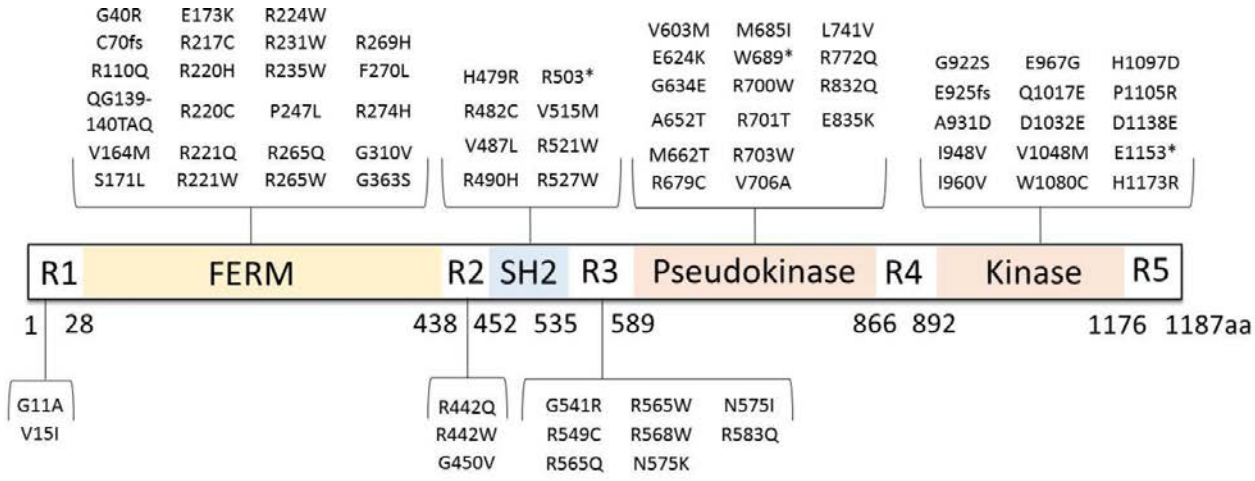
In total, 2322 RA cases and 4517 controls were collected from BioBank Japan samples.[27 28] Details of the samples are summarised in online supplementary information.

Gene selection

In total, 377 genes have been identified in 101 RA-associated loci by a meta-analysis of multiethnic GWAS.[4] All 98 candidate genes previously identified by established bioinformatics methods based on missense variants, cis-expression quantitative trait locus (eQTL), PubMed text mining, protein–protein interactions, haematological cancer somatic mutations, associated knockout mouse phenotypes and molecular pathway analysis were analysed. All coding regions registered in the consensus coding sequence (CCDS) database Release 15[29] and 2 base pair (bp) of exon–intron boundaries were included. The total sequence length was 176 335 bp.

Association analysis

The study design is shown in figure 1. The multiplex PCR-based target sequencing method was described in our previous study[30] (details in the online supplementary information). Association



domains	number of variants	frequency (%; Case)	frequency (%; Control)	CAST		SKAT
				P value	OR (95% CI)	P value
R1	2	0.1	0.1	0.696	1.46 (0.21-8.63)	0.554
FERM	23	3.2	4.8	2.14E-03	0.66 (0.49-0.86)	1.99E-03
R2	3	0.1	0.2	0.727	0.56 (0.06-2.92)	0.607
SH2	8	0.3	0.7	0.056	0.45 (0.17-1.05)	0.090
R3	8	0.3	0.5	0.328	0.62 (0.22-1.50)	0.388
Pseudokinase	16	4.0	7.4	1.63E-08	0.52 (0.41-0.66)	1.88E-08
R4	-					
Kinase	15	0.4	0.6	0.159	0.55 (0.22-1.25)	0.230
R5	-					

Figure 2 Rare coding variants in *TYK2* and results of domain-based association analysis. Top: Schematic variant sites in the TYK2 structure. TYK2 is 1187 amino acid residues and consists of nine regions including four structural domains, that is, region 1 (R1), the FERM domain; R2, SH2 domain; R3, pseudokinase (Pseudokinase) domain; R4, C-terminal kinase (Kinase) domain; and R5.* indicates stop codon. Bottom: Domain-based analysis of the accumulation of rare coding variants in *TYK2*. $P=3.57\times10^{-3}$ was the threshold for significance in the domain-based association analysis (red letters). CAST, cohort allelic sums test; SKAT, sequence kernel association test; FERM, four-point-one, ezrin, radixin, moesin.

studies were performed with only rare coding variants (including missense, splice site, nonsense and frameshift variants) that had a minor allele frequency (MAF) of <5% using 2×2 Fisher's exact tests. $P=5\times10^{-8}$ was the threshold for genome-wide significance, and $p>5\times10^{-8}$ but $p<1.98\times10^{-5}$ (0.05/2529) indicated a suggestive association.

In the gene-based association analysis, the accumulation of rare coding variants in either cases or controls was estimated by the cohort allelic sums test (CAST).[31] The sequence kernel association test (SKAT)[32] was also used to consider the direction and magnitude of variant effects by the accumulation of rare coding variants (Efficient and Parallelizable Association Container Toolbox (EPACTS) v.3.2.6). $P=1.34\times10^{-6}$ (0.05/18 666/2) was the threshold for significance according to the number of genes in CCDS Release 15 and $p=2.45\times10^{-4}$ (0.05/98/2) was the threshold for a suggestive association. Domain-based tests were performed by dividing TYK2 amino acid residues into nine regions (see figure 2 and online supplementary information). Region 4 (R4) and R5 had no variants and were omitted from the analysis. A p value of 3.57×10^{-3} (0.05/7/2) was the threshold for an association.

We extended gene-based analysis to pathway-based analysis. Eleven pathways were obtained for 98 candidate genes in a Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis using DAVID V.6.8 (Database for Annotation, Visualization, and Integrated Discovery; https://david.ncifcrf.gov/)[33 34] with the Fisher's exact test ($p<0.05$) and a false discovery rate q-value of <0.050.[35] The accumulation of rare coding variants in each pathway was estimated using CAST and SKAT, with a threshold of $p=2.27\times10^{-3}$ (0.05/11/2).

Luciferase reporter assay

Two variants with the lowest p values, R231W (rs201917359) and R703W (rs55882956), in two domains with significant associations by a domain-based analysis in TYK2 were selected. The effects of the two variants on cytokine responses were evaluated by reporter assays for STAT signalling pathways. For comparison, P1104A (rs34536443) in the C-terminal kinase domain, which is already reported to be associated with susceptibility to multiple autoimmune diseases, was also analysed.[36–38] We used adherent cells in the luciferase assays to obtain robust results for its high transfection efficiency. The hepatocellular carcinoma cell line, HepG2 (American type culture collection (ATCC)), was used for the evaluation of IL-6, interferon alpha (IFN-α) and interferon gamma (IFN-γ) signalling for its constitutive expression of receptors for these cytokines. The embryonic kidney cell line, HEK-293FT (Thermo Fisher Scientific), was used for the evaluation of IL-12 and IL-23 by cotransfecting these cytokine receptors. We transfected TYK2 expression

vectors (pcDNA3.1D, Thermo Fisher Scientific), pGL4.24 vectors (Luc2P/minP, Promega) cloned with STAT response elements, and the pGL4.74 vector (hRluc/TK, Promega) with internal control into cells using Lipofectamine 3000 (Thermo Fisher Scientific). No significant difference was observed in the transfection efficiency among *TYK2* variants as evaluated by flow cytometry (data not shown). After 18 hours of incubation, the cells were stimulated with IFN-α (1000 U/mL, PBL Assay Science) or IFN-γ (0.1 ng/mL, PeproTech), IL-6 (10 ng/mL, PeproTech), IL-12 (10 ng/mL, PeproTech) and IL-23 (10 ng/mL, PeproTech), for 4 hours (combinations of cytokines, cells and vectors are described in online supplementary table S1). These cytokines were chosen for the involvement of TYK2 in their signalling.[39] Cells were collected and luciferase activity was measured by Dual-Luciferase Reporter Assays according to the manufacturer's protocol (Promega). The relative luciferase activity was obtained by dividing firefly luciferase activity by hRluc activity and then normalising to the mock transfectant without stimulation, by setting its value as 1. Differences in cytokine signal transduction between wild-type and variant *TYK2* were evaluated by Student's t-tests.

Patient and public involvement

This research was done without patient involvement.

RESULTS

Summary of sequencing results and variant calling

After 28 of 2322 cases and 56 of 4517 controls were excluded due to the low coverage rate or poor quality of genome, the overall coverage rate was 99.3% (online supplementary table S2), and the average coverage depth was 1143. We identified 1421 synonymous variants and 2593 coding variants (2490 single nucleotide variants (SNVs) and 103 InDels). Finally, an association analysis was performed using 2529 rare coding variants with MAF <5%.

Association analysis

In the single variant association analysis, 35 variants had a nominal association ($p<0.05$) and 1 variant, R703W in *TYK2*, had a suggestive association with RA after Bonferroni correction (MAF in cases: 1.4%; MAF in controls: 2.8%; $p=5.47\times10^{-8}$, OR=0.48; online supplementary table S3).

We performed a gene-based association analysis of the 98 RA candidate genes by CAST and SKAT, only using coding variants. A total of 14 genes had a nominal association ($p<0.05$), and only *TYK2* showed significant genome-wide association (table 1 and online supplementary table S4). The carrier frequency of variants in *TYK2* was significantly higher in the controls than RA cases by CAST (8.3% in cases and 14.0% in controls, $p=3.94\times10^{-12}$, OR=0.56). *TYK2* also showed a significant association with RA by SKAT ($p=1.46\times10^{-9}$). Since our samples observed the differences in sex ratio and age between cases and controls, we performed four stratified gene-based analyses of rare variants in *TYK2* to clarify these effects on disease risk. In online supplementary table S5, each analysis shows a very similar OR, although p values differ because of the different number of samples. It suggests that the contributions of rare coding variants in *TYK2* would be independent of sex and age. In addition to R703W, three rare variants in *TYK2* had a nominal association with RA (R231W, $p=1.33\times10^{-3}$, OR=0.54; R220H, p=0.04, OR=infinity (Inf); V164M, p=0.04, OR=Inf).

We repeated the analysis using seven regions, including four domains of TYK2 (figure 2). The four-point-one, ezrin, radixin, moesin (FERM) domain and pseudokinase domain showed significant enrichment ($p<7.14\times10^{-3}$). The FERM domain had 23 variants and the carrier frequency was significantly higher in controls than in RA cases (3.2% in cases and 4.8% in controls; $p=2.14\times10^{-3}$, OR=0.66). The pseudokinase domain had 16 variants and the carrier frequency was also significantly higher in controls than in RA cases (4.0% in cases and 7.4% in controls; $p=1.63\times10^{-8}$, OR=0.52). The FERM domain and pseudokinase domains also showed a significant association with RA by SKAT ($p=1.99\times10^{-3}$ and $p=1.88\times10^{-8}$, respectively).

Cytokine responses of *TYK2* SNPs

To reveal the influence of phenotype-associated domains on TYK2 function, we focused on the FERM domain and pseudokinase domain in the TYK2 gene. We performed reporter assays of the most highly significant variant in each domain (R231W in the FERM domain and R703W in the pseudokinase domain) as well as the previously reported risk variant P1004A for multiple autoimmune diseases in the kinase domain (figure 3). In no stimulation with a cytokine, all transfectants significantly reduced each STAT response than wild-type except for R703W mutant

Table 1 Gene-based tests of rare coding variants associated with rheumatoid arthritis (p<0.05)

Gene	Variants (n)	Frequency (%, case)	Frequency (%, control)	CAST		SKAT
				P value	OR (95% CI)	P value
TYK2	75	8.3	14.0	3.94E-12	0.56 (0.47 to 0.66)	1.46E-09
PTPRC	47	8.5	6.2	7.27E-04	1.40 (1.15 to 1.70)	6.45E-04
TNFRSF14	18	13.2	10.6	2.51E-03	1.27(1.09 to 1.49)	1.62E-03
CCL21	5	0.3	0.0	3.87E-03	7.80 (1.55 to 75.34)	0.065
ERBB3	59	8.5	10.4	0.0154	0.80 (0.67 to 0.96)	0.055
ICAM1	36	1.7	1.0	0.0177	1.73 (1.09 to 2.75)	0.194
NFKBIE	34	8.4	6.8	0.0236	1.24 (1.03 to 1.51)	6.82E-03
RASGRP1	22	3.3	2.4	0.0263	1.41 (1.03 to 1.92)	0.071
FCER1G	4	2.3	1.5	0.0312	1.52 (1.03 to 2.23)	0.027
ZNF594	63	7.4	6.1	0.0375	1.24 (1.01 to 1.52)	0.221
PTPN11	9	0.4	0.2	0.0393	2.79 (0.96 to 8.63)	0.021
CFLAR	12	2.0	2.8	0.0418	0.70 (0.49 to 0.99)	0.068
SH2B3	43	12.8	11.6	0.1438	1.12 (0.96 to 1.31)	2.47E-03
IRF5	22	1.1	0.9	0.5149	1.19 (0.69 to 2.01)	0.022

CAST, the cohort allelic sums test; SKAT, the Sequence kernel association test.

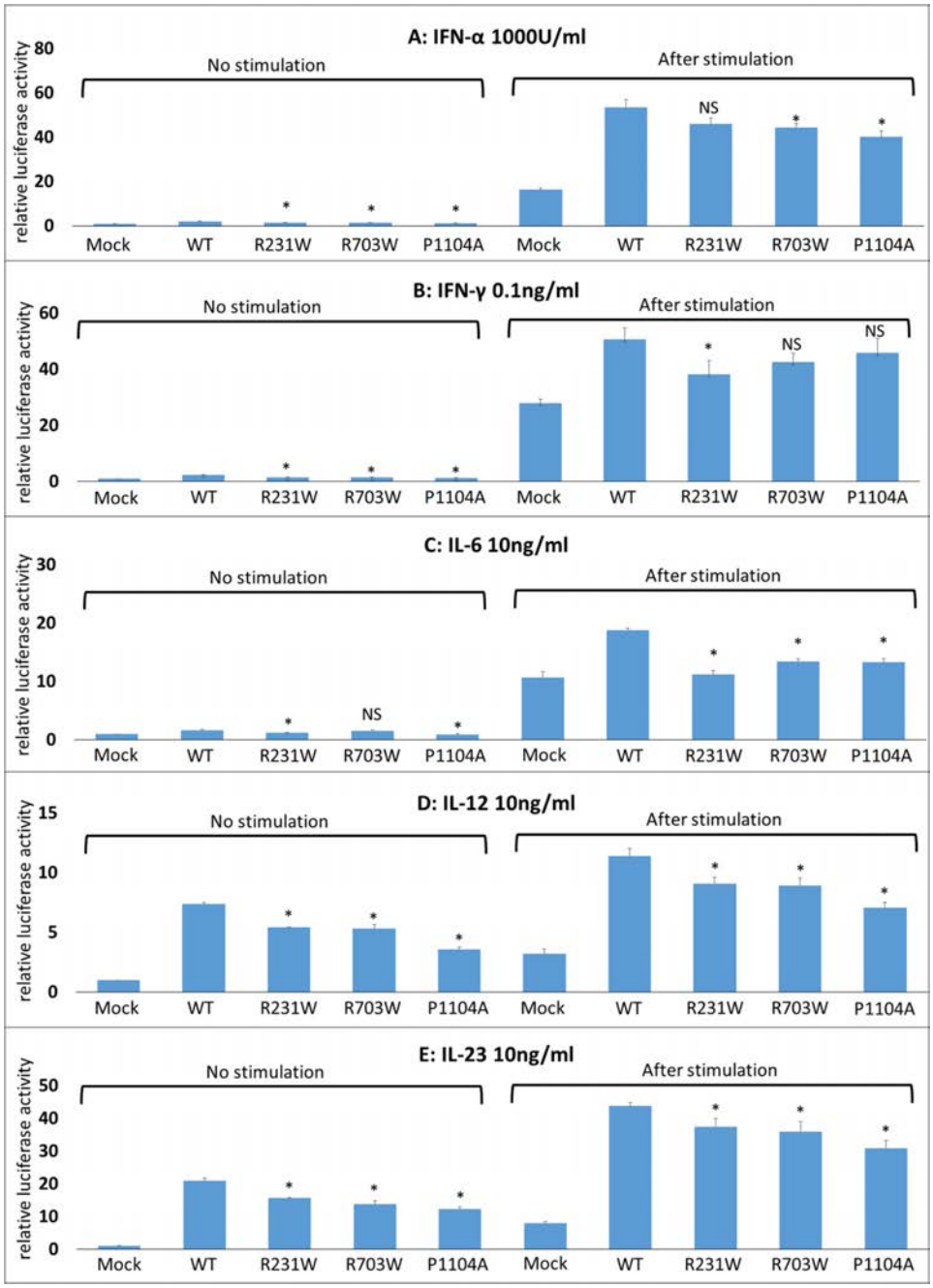


Figure 3 Functional evaluation of the effects of *TYK2* variants on cytokine signalling transduction. The effects of variants were examined using luciferase vectors containing STAT response elements (GAS2 for STAT1, SIE for STAT3 and STAT4 for STAT4) in HepG2 or HEK-293FT cells. The combination of cytokines, cells and vectors is described in online supplementary information. Transfected TYK2 isoforms (R231W, rs201917359; R703W, rs55882956; and P1104A, rs34536443) were evaluated by stimulation with IFN-α (A), IFN-γ (B), IL-6 (C), IL-12 (D) and IL-23 (E). Means and SD are shown for four technical replicates. The experiment was independently repeated three times, and representative data are shown. *P<0.05 by Student's t-test versus wild-type (WT) with Bonferroni correction (six tests for each cytokine). IFN-α, interferon alpha; IL-6, interleukin 6; NS, not significant; STAT, signal transducers and activators of transcription.

with sis-inducible element (SIE) response element. After stimulation with each cytokine, P1104A significantly reduced all cytokine responses except IFN-γ. R231W reduced IFN-γ ($p=0.04$), IL-6 ($p=4.03\times10^{-6}$), IL-12 ($p=8.94\times10^{-3}$) and IL-23 signal transduction ($p=0.02$) compared with that of the wild-type but did not significantly reduce IFN-α signal transduction ($p=0.10$). R703W reduced cytokine signals similarly to P1104A (IFN-α, $p=0.03$; IL-6, $p=8.33\times10^{-6}$; IL-12, $p=9.75\times10^{-3}$; IL-23, $p=0.02$), but the effects were weaker than those of P1104A. Among the three variants, R231W mostly reduced IL-6 and IFN-γ signalling, whereas P1104A mostly reduced IL-12, IL-23 and IFN-α signalling.

Pathway-based association analysis

Table 2 shows that 5 of 11 pathways were significantly associated with RA, that is, the JAK-STAT signalling pathway, measles, T-cell receptor signalling pathway, Epstein-Barr virus infection and herpes simplex infection by CAST and SKAT. Since four pathways included *TYK2*, which showed significant associations

Table 2 Pathway-based tests of rare coding variants in rheumatoid arthritis

		All variants						Omitting *TYK2* variants					
					CAST		SKAT				CAST		SKAT
Pathway term	Gene	Variants (n)	Frequency (%, case)	Frequency (%, control)	P value	OR (95% CI)	P value	Variants (n)	Frequency (%; Case)	Frequency (%, control)	P value	OR (95% CI)	P value
JAK-STAT signalling pathway	*TYK2, CSF3, CSF2, IL3, IL2RB, STAT4, IL2RA, IL20RB, IL6R, IL21, IFNGR2, PTPN11, IL2*	232	23.0	28.2	3.12E-06	0.76 (0.67 to 0.85)	1.49E-07	157	15.9	16.6	0.466	0.95 (0.82 to 1.09)	0.547
Measles	*TYK2, IRAK1, IL2RB, IL2RA, FCGR2B, HSPA6, CDK6, TNFAIP3, TRAF6, CDK4, IFNGR2, CDK2, CD28, IL2*	291	28.5	33.2	8.69E-05	0.80 (0.72 to 0.9)	3.45E-07	216	22.1	22.4	0.829	0.99 (0.87 to 1.11)	0.548
T-cell receptor signalling pathway	*CSF2, PTPRC, NFKBIE, RASGRP1, CTLA4, CDK4, TEC, IL2, CD28*	179	22.6	18.9	4.24E-04	1.25 (1.10 to 1.42)	8.82E-05						
Epstein-Barr virus infection	*TRAF1, TYK2, IRAK1, ICAM1, XPO1, NFKBIE, HSPA6, CD40, TRAF6, TNFAIP3, CDK2*	284	28.6	31.2	0.025	0.88 (0.79 to 0.99)	1.87E-09	209	22.0	20.3	0.122	1.10 (0.97 to 1.25)	0.019
Herpes simplex infection	*TRAF1, TYK2, C1QBP, C5, CASP8, TNFRSF14, TRAF6, IFNGR2, CDK2, PTPN11*	253	38.7	41.0	0.059	0.91 (0.82 to 1.01)	1.65E-09	178	33.1	31.3	0.137	1.09 (0.97 to 1.21)	0.011
Cell adhesion molecules	*ICAM1, PTPRC, ICAM3, CD2, CTLA4, CD40, CD226, ICOSLG, CD28*	190	21.1	19.2	0.071	1.12 (0.99 to 1.27)	2.23.E-03						
Primary immunodeficiency	*PTPRC, UNG, RAG1, AIRE, RAG2, CD40*	169	17.1	15.4	0.074	1.13 (0.9 to 1.30)	1.61.E-03						
Tuberculosis	*IRAK1, CASP10, CYP27B1, FCGR2B, CASP8, FCER1G, FCGR2A, TRAF6, IFNGR2*	140	13.5	13.0	0.568	1.05 (0.90 to 1.22)	0.591						
Haematopoietic cell lineage	*CSF3, IL3, CSF2, IL2RA, CD2, IL6R, CD5*	121	19.3	19.7	0.698	0.97 (0.86 to 1.11)	0.767						
Nuclear factor-kappa B signalling pathway	*TRAF1, IRAK1, ICAM1, CFLAR, CCL21, CCL19, CD40, TRAF6, TNFAIP3, ATM*	287	25.5	25.9	0.724	0.98 (0.87 to 1.1)	0.111						
Cytokine–cytokine receptor interaction	*CSF3, CSF2, IL3, IL2RB, IL2RA, CCL19, TNFRSF14, IL6R, CD40, IL21, TNFRSF9, CCR6, IL20RB, CXCR5, CCL21, IFNGR2, IL2*	214	31.0	30.7	0.760	1.02 (0.91 to 1.14)	0.018						

CAST, cohort allelic sums test;JAK-STAT, Janus kinase-signal transducers and activators of transcription; SKAT, sequence kernel association test.

in the gene-based test, we repeated the analysis for these four pathways after excluding *TYK2*. The four pathways did not show associations with RA. The T-cell receptor signalling pathway included nine genes (not *TYK2*), and *PTPRC*, *NFKBIE* and *RASGRP1* had nominal associations with RA ($p<0.05$; online supplementary table S6). Cell adhesion molecules and primary immunodeficiency pathways were significantly associated by only SKAT.

DISCUSSION

We performed resequencing of 98 biological candidate genes in 2322 cases and 4517 controls by multiplex PCR-based target sequencing. We identified 2529 rare coding variants. We found the significant accumulation of rare coding variants in *TYK2*. In the domain-based analysis of *TYK2*, the FERM domain and pseudokinase domain showed associations with RA. Both R231W and R703W in the two domains of TYK2 reduced IL-6, IL-12 and IL-23 signalling, but these variants had different effects on IFN-α and IFN-γ signalling. We also found the significant accumulation of rare coding variants in the T-cell receptor signalling pathway.

TYK2 is a member of the JAK family of proteins, which also includes JAK1, JAK2 and JAK3. A previous study has indicated that three common variants, I684S, A928A and P1104A, in *TYK2* are protective in the pathogenesis of RA,[36 40] but the accumulation of rare missense variants in *TYK2* has not been significant.[24–26 40] Our results showed that rare coding variants in *TYK2* protected against the onset of RA in the Japanese population. We observed two protective variants that showed a nominal association with RA in *TYK2*, namely R703W (MAF=2.4%) and R231W (1.3%). These variants were very rare in non-Finnish European data from the Exome Aggregation Consortium (0.11% and 0.0035%, respectively).[41] Therefore, although associated variants in *TYK2* differ between European and Japanese populations, rare coding variants determine the individual risk of developing RA in both populations.

TYK2 is essential for cytokine signal transduction and has pleiotropic functions in host responses to infection[39] and tumour surveillance.[42] In domain-based association analysis, we found that rare coding variants accumulated significantly in both the FERM domain and pseudokinase domain and that they were protective against the onset of RA. The FERM domain mediates stable association with receptor domains and involved in kinase activity regulation.[43 44] The pseudokinase domain has a canonical kinase domain that lacks catalytical function despite binding ATP[45] and is also important for regulating the activity of the kinase domain.[46 47] We further investigated whether the effects of these variants on cytokine signal transduction differ depending on their domains. Interestingly, the variants in the FERM domain (R231W), pseudokinase domain (R703W) and kinase domain (P1104A) had effects on different cytokines, suggesting that the domain influences the effects of variants. This was supported by the results of previous studies showing that the effects of *TYK2* variants on type I IFN signalling differ in their domain.[37 48 49] Moreover, the effects of I684S (pseudokinase domain) and P1104A on IL-6, IL-12 and type I IFN signalling differ in several types of immune cells.[36–39 50]

The pathway-based analysis suggests that the dysfunction of T-cell receptor signalling increased the risk of RA. GWAS have shown that more than 60 RA risk loci are strongly enriched in the regulation of the T-cell activation.[4 11] Our findings confirmed the importance of T-cell receptor signalling in the pathogenesis of RA. Among the nine genes in the T-cell receptor signalling pathway, *PTPRC*, *NFKBIE* and *RASGRP1* showed nominal associations. Although the functions of these three genes are not well known in the pathogenesis of RA, some studies indicate that these genes were related to the function of the T-cell receptor. RASGRP1 induces positive and negative selection with the received signal from T-cell receptor in double-positive T-cell.[51] The function of *PTPRC* is shown to exhibit both positive and negative regulatory roles on T-cell receptor (TCR) signalling by dephosphorylating lymphocyte-specific protein tyrosine kinase.[52] Our results suggest that the dysfunction of these genes might increase the risk of RA; further studies are needed to clarify the function of these genes in the pathogenesis of RA. On the other hand, cell adhesion molecules and primary immunodeficiency pathways were significantly enrichment of rare coding variants by only SKAT. The result suggests these pathways are associated with RA, but further studies are also needed for the pathogenesis because SKAT evaluates different directions and magnitude of each variant effect.

In this study, we explored rare coding variants in 2294 cases and 4461 controls, and identified that only *TYK2* showed genome-wide significant enrichment of rare coding variants. We performed power analyses with G*Power software (v.3.1.9.4)[53] to investigate this limited contribution of rare variants to the genetic aetiology of RA. We set the same number of samples (case: 2294; controls: 4461) and the threshold of genome-wide significance at $p=1.34\times10^{-6}$. In order to detect moderate effects (OR=2) of rare variants, carrier frequency of rare variants needs to be 5% or more. In this study, we observed 20 of 99 genes showing a carrier frequency higher than 5%. However, only *TYK2* showed genome-wide significant results. This means the effect of rare coding variants in the 19 other genes is weaker. This suggests that rare exonic variants in these genes are unlikely to play a role in the genetic aetiology of RA in Japanese people, a finding that is concurrent with previous studies of RA in European[24] and Korean populations.[5] On the other hand, there is another contribution of the sequencing study. This study showed rare coding variants in *TYK2* could affect cytokine signalling differently. The previous sequencing studies also identified *IL2RA* and *IL2RB*[24]and *TYK2*,[40] which led to the pathogenesis of RA and other related inflammatory diseases. Therefore, it might suggest that sequencing studies will contribute to the identification of a new mechanism more than explaining the missing heritability.

This study has limitations. Although we used adherent cells to obtain robust results in luciferase assays, the findings should be confirmed in *TYK2* mutation carriers of the disease-relevant cells, such as primary lymphocytes. However, since our observation for P1104A in HEK-293FT and HepG2 cells was similar to those of other studies with respect to TYK2 function by cytokine stimulation,[36–38] our results likely reflect TYK2 function by cytokine stimulation. Other limitations included the assumption that all rare coding variants influence the phenotype in the same direction with the same effect size.[31] Coding variants may increase or decrease protein function with various effect sizes.[54] However, SKAT, which considers the direction and magnitude of effects, showed similar associations, suggesting this limitation did not substantially impact the results of this study.

In conclusion, targeted sequencing of biological candidate genes identified by GWAS revealed that rare variants in the FERM and pseudokinase domains of TYK2 and the T-cell receptor signalling pathway were involved in the risk of RA. Based on the findings in the functional analysis of *TYK2* variants, selective regulation of TYK2 domains might become new therapies for RA.

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Contributors TM and YM designed the study and drafted the manuscript. TM and YM performed the resequencing and analysed these data. YK conducted the

reporter assays and revised the manuscript. MK, KM and KY critically reviewed the manuscript.

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Competing interests None declared.

Patient consent for publication Not required.

Ethics approval All subjects provided written informed consent for participation in the study as approved by the ethical committee of each institutional review board. The study was approved by the ethics committee at the RIKEN Center for Integrative Medical Sciences (accession number 17-17-16(3)).

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Data availability statement Data are available upon reasonable request.

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CLINICAL SCIENCE

T cell receptor β repertoires as novel diagnostic markers for systemic lupus erythematosus and rheumatoid arthritis

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ABSTRACT

Objective T cell receptor (TCR) diversity determines the autoimmune responses in systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) and is closely associated with autoimmune diseases prognosis and prevention. However, the characteristics of variations in TCR diversity and their clinical significance is still unknown. Large series of patients must be studied in order to elucidate the effects of these variations.

Methods Peripheral blood from 877 SLE patients, 206 RA patients and 439 healthy controls (HC) were amplified for the TCR repertoire and sequenced using a high-throughput sequencer. We have developed a statistical model to identify disease-associated TCR clones and diagnose autoimmune diseases.

Results Significant differences were identified in variable (V), joining (J) and V-J pairing between the SLE or RA and HC groups. These differences can be utilised to discriminate the three groups with perfect accuracy (V: area under receiver operating curve > 0.99). One hundred ninety-eight SLE-associated and 53 RA-associated TCRs were identified and used for diseases classification by cross validation with high specificity and sensitivity. Disease-associated clones showed common features and high similarity between both autoimmune diseases. SLE displayed higher TCR heterogeneity than RA with several organ specific properties. Furthermore, the association between clonal expansion and the concentration of disease-associated clones with disease severity were identified, and pathogen-related TCRs were enriched in both diseases.

Conclusions These characteristics of the TCR repertoire, particularly the disease-associated clones, can potentially serve as biomarkers and provide novel insights for disease status and therapeutical targets in autoimmune diseases.

Key messages

What is already known about this subject?

- Clonal expansion and a reduction in diversity of the T cell receptor (TCR) repertoire have been identified in a small series of systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA) patients. However, the clinical implication of this variation in the TCR repertoire still remains unclear.

What does this study add?

- We conducted the most comprehensive and quantitative analysis on TCR beta repertoires to date, including data from 877 SLE patients, 206 RA patients and 439 healthy controls (HC).
- We found significant differences in variable (V), joining (J) and V-J pairing between the SLE or RA and HC groups and developed a random forest model to classify the SLE, RA and HC using V and V-J genes with perfect accuracy.
- We identified 198 SLE-associated and 53 RA-associated TCR clones which can discriminate between SLE, RA and HC with very high specificity and sensitivity and found that disease-associated clones correlated with clinical features.

How might this impact clinical practice or future developments?

- These findings contribute to understanding the immunological aetiology and clinical heterogeneity of SLE and RA, as well as provide a biomarker for the diagnosis of SLE and RA and a target for disease treatment.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a prototypic autoimmune disorder, characterised by an excessive production of autoantibodies, immune complex formation and T cell infiltration into tissues and causes organ damage.[1] Rheumatoid arthritis (RA) is an organ-specific autoimmune disease that is characterised by chronic synovitis inflammatory and bone erosion.[2] T cells play an essential role in SLE and RA pathogenesis. Autoreactive T cells have been observed in the peripheral blood (PB) and various organs of patients with SLE and RA, where

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they are activated to secret inflammatory cytokines and provide help to reactive B cells.[3–9]

In T cell receptors (TCRs), the high diversity is generated by genomic rearrangement of the variable (V), diversity (D) and joining (J) regions, along with palindromic and random nucleotide additions, which is crucial for understanding of adaptive immunity in health and disease.[10–12] In previous studies, expansion of the partial TCRs and reduction of TCR repertoire diversity were observed in the PB of SLE and RA patients, as well as a correlation between PB T cell expansions or spectra type skewing with disease activity.[13–16] However, finite TCR sequences were obtained because of the limited techniques. Recently, although high-throughput sequencing of the TCR repertoire was used in SLE and RA,[17–19] the small number of patient samples in these studies limited their findings. Whether the TCR repertoire or TCR clones can be used for biomarkers of SLE or RA remains unclear.

In this work, we provided the most comprehensive, quantitative and unrestricted immunogenetical landscape of the T cell receptor repertoire in PB samples from SLE, RA patients and healthy controls (HC) subjects, and identified novel biomarkers for the accurate diagnosis and monitor of SLE and RA.

METHODS

Sample collection and preprocess

This study was approved by the ethics committees and institutional review board of the Second Xiangya Hospital of Central South University, and all study participants signed a written informed consent. SLE and RA patients were recruited from the outpatient clinics and ward in the Second Xiangya Hospital of Central South University, Xiangya Hospital of Central South University and Chenzhou No.1 People's Hospital. The HCs had no history of cancer, cardiovascular diseases, autoimmune diseases or known infectious diseases, and were collected from the Health Examination Centre of Second Xiangya Hospital. The baseline characteristics of all samples and the clinical information analysed in this study are presented in online supplementary table S1.

TCR repertoire sequencing

TCR repertoire was prepared from genomic DNA and the details of library construction, sequencing, data processing and the method of identifying disease's specific clones were described in online supplementary note and table S2.

STATISTICS

A statistical analysis was performed using R software (V.3.4.1). A non-parametric test (Mann-Whitney-Wilcoxon test) was used to compare the difference between two groups. For the multiple group comparison, analysis of covariance was initially used. Correction for multiple tests was performed using false discovery rate method.[20]

RESULTS

The overall TCR repertoire diversity and the expansion of public TCR clones in autoimmune diseases

The sample size of the current study is at least ten times larger than any previous researches regarding autoimmune diseases, though the rare-fraction analysis showed that the TCR clone diversities in HC and RA were less than saturation (figure 1A). Overall, 7.5, 2.7 and 8.1 million unique TCR clones were identified in SLE, RA and HC, respectively, and limited TCR clones were shared (figure 1B). Compared with the HCs, both the SLE and RA patients showed a decreased Shannon entropy, and the value in SLE were the smallest (figure 1C), which implies the existence of expanded TCR clones in the repertoire of typical autoimmunity. The accumulative frequencies of the top 100 or 50 TCR clones in the autoimmune disease patients were significantly higher than that in the HCs (figure 1C, online supplementary figure S1A, B), and the patients had more abundant clones (figure 1C). These comparisons indicate the higher clonality of autoimmunity.

TCR clones shared by more than one individual are generally considered as public. We referred the publicity of a public clone to the number of individuals who share the clone. Rare fraction analysis showed the increment trend of the public clone number with the growth of the sample size (figure 1A). Compared with the total TCR clones, the sharing of public clones among the three groups was higher, which implies the selection advantage of public clones (figure 1B). The public clones in both SLE and RA, were significantly more expanded than their counterparts in the HCs with matched publicity (figure 1D). Moreover, clones with higher publicity (eg, publicity >50) had higher clonal frequencies (figure 1D). As public clones were reported to have biological implications with antigens,[21] the above results imply the higher-frequency clones in SLE or RA, which are shared by more individuals and induce their lower diversity, may contribute to the aetiopathology of these autoimmune diseases.

The gene usage comparison and its classification performance

Numerous genes showed significant differential usages between diseases and health (adjusted $p<0.05$, figure 2A,B). The V-J pairings were more evenly distributed in RA compared with SLE and HCs (figure 1C, online supplementary figure S2). Genes and gene combinations was more discrepant between SLE and RA (figure 2C). Based on these observations, SLE can be classified from HCs using V genes with an area under receiver operating curve (AUC) of 99.63%, and the performance improved to 99.85% by V-J gene pairing. RA was classified from the HC group with AUCs of 99.78% and 99.56% with V genes and V-J pairing, respectively. SLE and RA may also be accurately separated with AUCs of 99.99% and 99.97% using V and V-J genes. Utilising J gene usage alone showed a poorer performance of 93.22% (figure 2D). Taking into account the abundance of unique sequences did not improve the overall performance of classification (online supplementary figure S3). Different discrimination models, such as linear discriminant analysis, also generated a high classification accuracy with an AUC of 99.67% between the SLE and HC groups (online supplementary figure S4A). To examine whether phenotypical differences, such as age and gender, contribute to the classification, we randomly selected samples with the same age and gender composition from the SLE and HC groups, and the AUC still achieved 99.49% (online supplementary figure S4B).

In silico identification of autoimmune disease-associated TCRs and application in disease diagnosis

Previous studies have emphasised the importance and antigenic association of public TCRs in various diseases.[21] We also identified that TCR clones with a higher publicity in SLE and RA had higher clonal frequencies compared with HCs (figure 1D). Importantly, by mapping the clones to manually curated TCR specificity databases,[22–24] a clear trend showed that clones with high publicity had a higher probability of being identified in

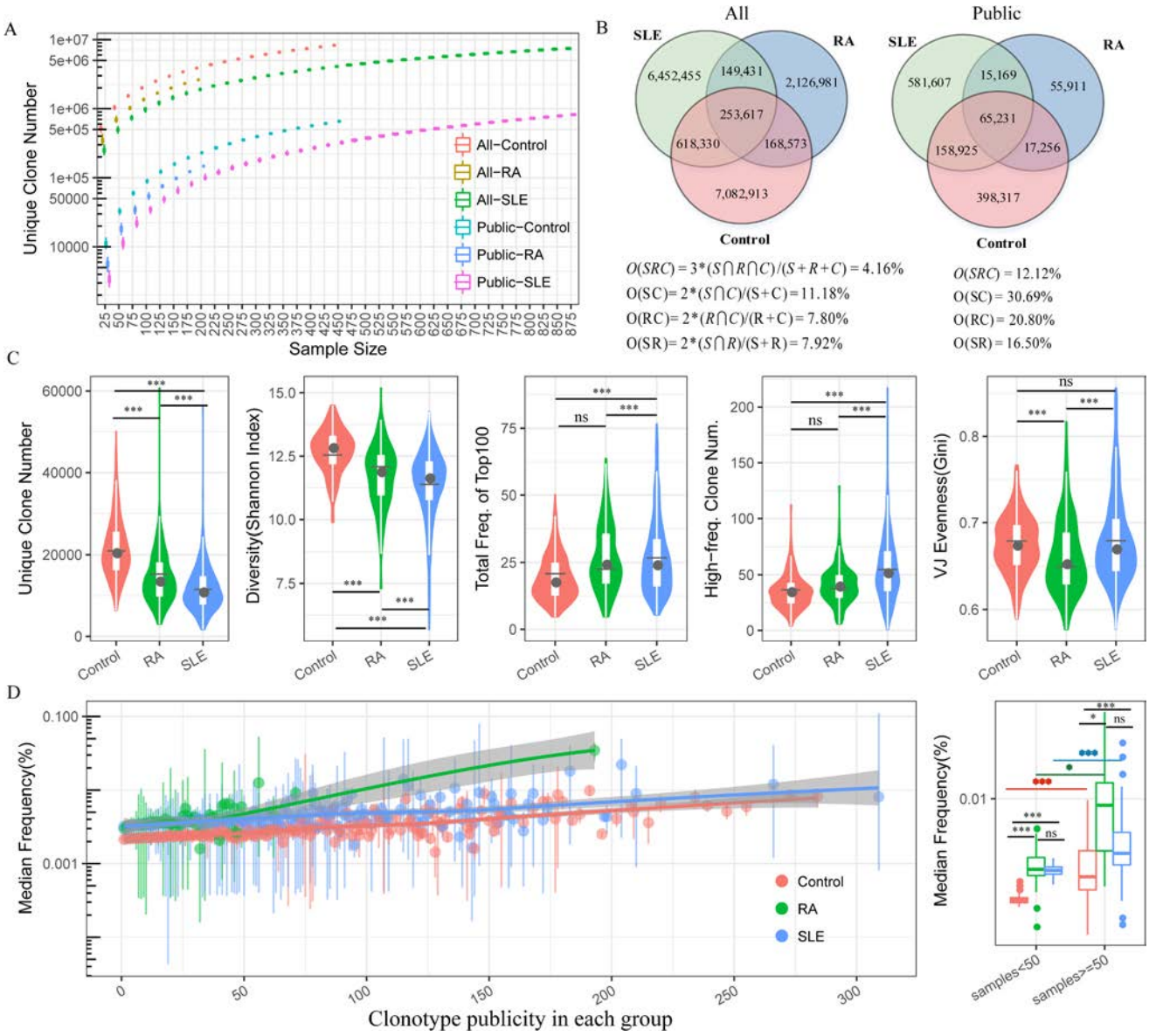

Figure 1 Overall statistics of TCRβ repertoires for autoimmune patients and HC samples. (A) Rarefaction analysis for three groups. Each time, a certain amount of subsamples was selected randomly from the total samples to calculate the unique clone number and public clone (observed in least two subjects) number. For each sample size, the process of subsampling was repeated 50 times. (B) Amount of overlap for all unique clones and the public clones among the three groups. The lower texts show the formula and results of the overlapped rate. (C) Comparison of overall TCRβ repertoire diversity indices among the three groups (ANCOVA test and multiple comparisons test). The dot is the median and the grey line is the average value after being corrected by the covariate age. A high-frequency clone indicates the frequency is greater than 0.1%. (D) Relationship between clone incidence in each group and clone's frequency. The dot in the left panel is the clone median frequency, and the line shows the +/-SE of the frequencies. The clone median frequencies were divided into two groups for comparison (Mann-Whitney test). ANCOVA, analysis of covariance; freq., frequency; HC, healthy controls; J, joining; num., number; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; V, variable.

the database (online supplementary figure S5). Therefore, we assumed that the autoimmune disease-associated or specific TCR clones have higher publicities and clonal frequencies than HCs. With the strength of our large sample size, we have developed an in silico pipeline to identify all disease-associated TCRs in SLE and RA (online supplementary note, figure 3A). The distribution of the p values, relative risks and false discovery rates for all TCR clones in the patients were plotted and evaluated (online supplementary figure S6). According to the performance of the leave-one-out cross validation with a set of p value thresholds, including the AUC, accuracy and cross-entropy loss, an appropriate p value cut-off was used to determine the disease-associated clones (online supplementary figure S7). Finally, we identified 198 SLE-associated TCR clones ($p<5e-4$, online supplementary table S3) and 53 RA-associated clones ($p<1e-3$, online supplementary table S4). To exclude the possibility that some of the associated TCR clones were misidentified due to sequence cross contamination, a subset of samples was randomly selected, and replicated library preparation and sequencing were performed. The numbers of disease-associated TCRs in replicated samples were not reduced, ruling out a random cross contamination (online supplementary figure 8). These TCRs were disease specific or shared by significantly more patients (figure 3B). Three hundred twenty-seven TCRs have been reported before[22–24] to have SLE specificity with 156 found in our SLE repertoires, but none have been identified

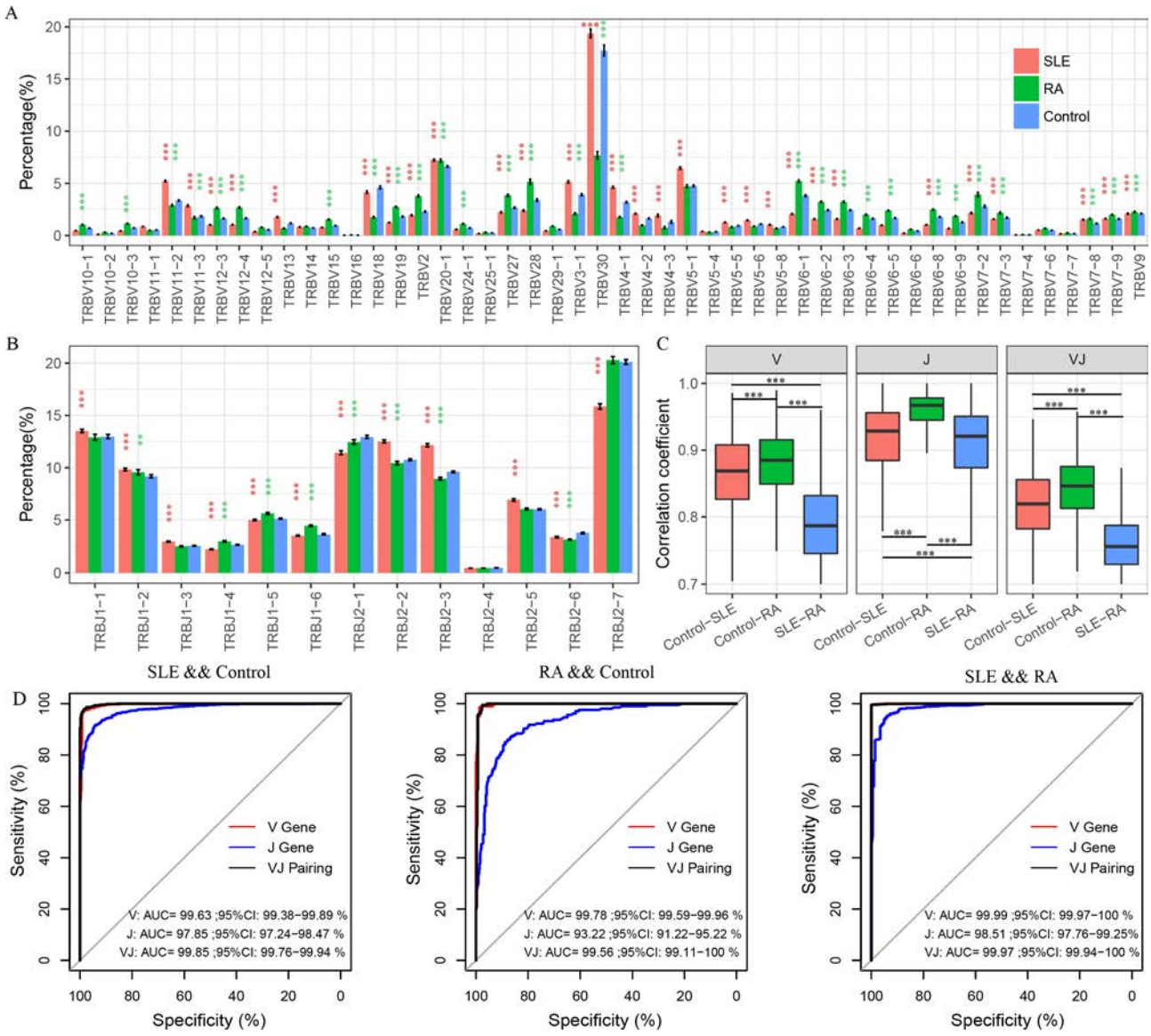

Figure 2 The differential analysis of gene usage in TCRβ repertoires. (A) Vβ gene usage distribution. (B) Jβ gene usage distribution. The error bar in (A) and (B) is the SE. The red asterisk represents the p value between SLE and HCs, and the blue asterisk represents the p value between RA and HCs. Only the genes for which the average frequency is greater than 1% display the p values (Mann-Whitney test, corrected by false discovery rate). (C) Spearman correlation of Vβ gene, Jβ gene and V-J pairings between any two samples that belong to different groups. (D) ROC curves showing the performance of classification for two groups using the Vβ gene, Jβ gene and V-J pairings by 10-fold cross validation. The area under receiver operating curve and 95% CI are shown in the bottom box. AUC, area under receiver operating curve; HCs, healthy controls; J, joining; RA, rheumatoid arthritis; ROC, receiver operating characteristic; SLE, systemic lupus erythematosus; TCR, T cell receptor; TRBJ, TCR beta chain joining; TRBV, TCR beta chain variable; V, variable.

to be SLE-associated in our study (figure 3B). Intriguingly, disease associated TCRs are significantly correlated with disease activity in both SLE and RA, reflecting the reliability of our *in silico* identification, which will be discussed later.

The major purpose of the current study is to validate whether autoimmune disease-associated TCRs are capable to be diagnostical biomarkers. Using these TCR clones, we developed a machine learning model to classify SLE, RA and HCs (online supplementary note). In the leave-one-out cross-validation test, the AUC of the classification reached 94.27% between SLE and HC. The diagnostical accuracy was even higher for RA with an AUC of 96.71%. Discrimination between SLE and RA was more effective with the AUC achieving 96.78% (figure 3C).

Features of autoimmune disease-associated TCR clones

We subsequently examined the features of these disease-associated TCR clones in SLE and RA. We found the four clones with the highest publicities were SLE-associated TCRs, and two of them were also associated with RA (figure 4A). The clone with the highest publicity in RA was both an RA and SLE-associated clone (online supplementary figure S9A). We also found that both the SLE- and RA-associated TCRs had apparently higher frequencies than the other unrelated clones (figure 4B). It has been reported that shorter TCR complementarity-determining region 3 (CDR3) were recombined to confer susceptibility in type 1 diabetes.[25] Our data indicated that the lengths of autoimmunity-associated TCR CDR3s, particularly in SLE, were significantly shorter than unrelated clones (figure 4C).

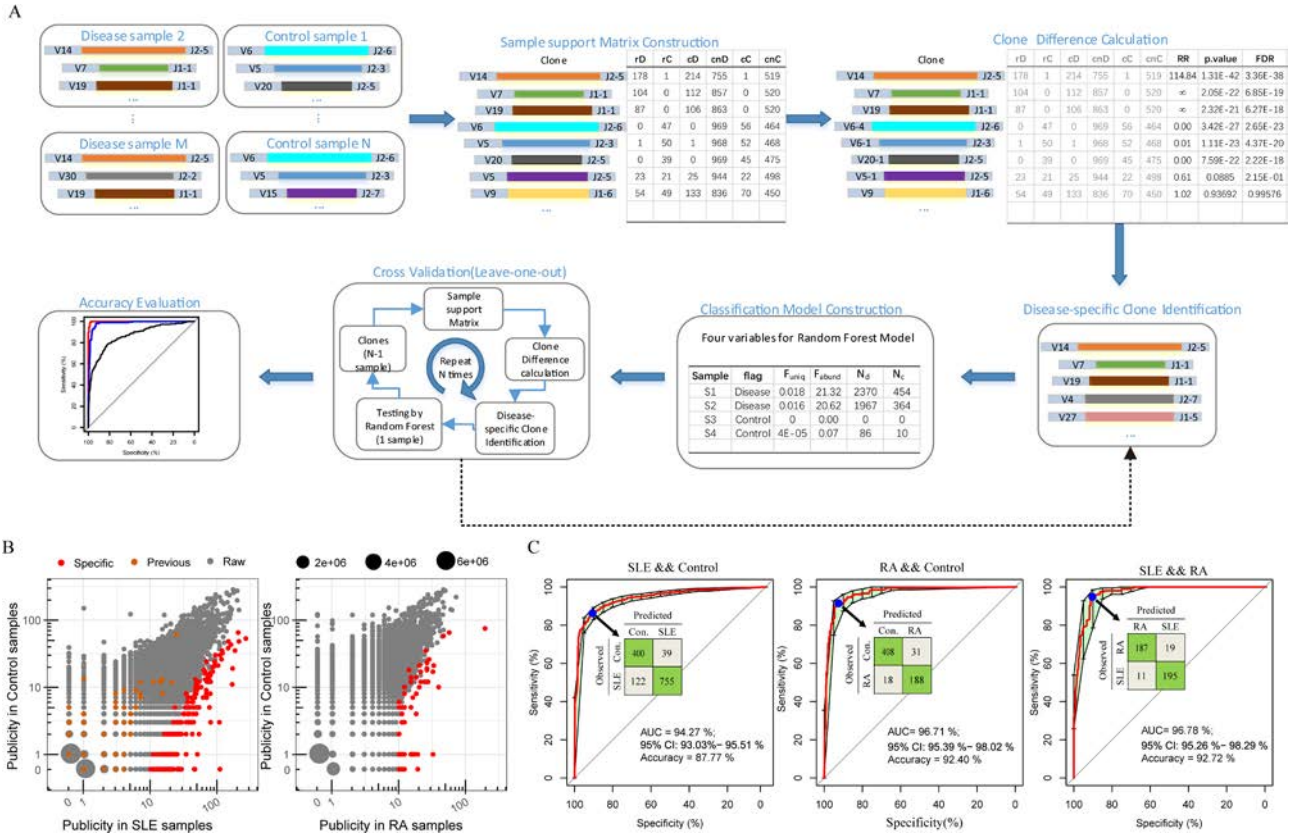

Figure 3 Disease-associated clone identification and disease detection using these clones. (A) Bioinformatic pipeline for identification of disease-associated clones and the process of disease classification. (B) Publicity of TCRβ clone in disease and HC samples. The size of the spot in each position represents the abundance of the clone. Disease-associated clones identified in this study are represented by red dots. Previously, reported SLE-reactive TCRβ clones are represented by brown dots. All other clones are shown in grey. (C) ROC curves showing the classification performance of a classifier for two groups by leave-one-out cross validation. The blue dot in each line is closest to the top-left part of the plot with perfect sensitivity or specificity. The inset depicts the classification results under the best accuracy. *rC*, clone's supporting sample numbers in control. *rD*, clone's supporting sample numbers in disease. cC, corrected supporting sample numbers in control. cD, corrected supporting sample numbers in disease. cnC, total samples in control - cC. cnD, total samples in control - *cD*. RR, relative risk. FDR, false discovery rate. F_{uniq}, poportion of unique specific clones presented in the sample. F_{abund}, proportion of total specific clones presented in the sample. N_d, accumulated corrected supporting sample number (in disease group) of specific clones found in the sample. N_c, accumulated corrected supporting sample number (in control group) of specific clones found in the sample. HC, healthy control; RA, rheumatoid arthritis; ROC, receiver operating characteristic; SLE, systemic lupus erythematosus; TCR, T cell receptor.

We subsequently demonstrated the shorter CDR3 length was mostly attributed to the reduced probabilities of longer insertions in the junction regions for the disease-associated clones (online supplementary figure S9B, C). A set of V and J gene usage of the disease-associated TCR clones have been found to be over represented in SLE and RA (figure 4D). The motif analysis in CDR3 indicated that SLE- and RA-associated clones shared more motifs than other unrelated clones, and these motifs were more prevalent than motifs derived from unrelated clones (figure 4E). To examine the similarities of these disease-associated TCR clones, the Levenshtein distances was used to cluster the clones. We found disease-associated clones were significantly enriched in large clusters, which was not the case for other randomly selected unrelated clones (figure 4F,G). We also used a published method Grouping of Lymphocyte Interactions by Paratope Hotspots (GLIPH) that integrates both naïve T cell receptor sequences and motif analysis to cluster the clones and plotted the connection among them.[26] As expected, the SLE-associated TCRs were well connected and clustered, thereby illustrating the high sequence similarity among these clones. The RA-associated TCR clones were not clustered as well by this method (figure 4H). We also demonstrated our SLE- and RA-associated TCR sequences had significantly higher similarities within each group than sorted naïve T cells in the published TCR data[26] (figure 4I).

In the 53 RA-associated TCRs, 32 (60.4%) clones can be found in SLE patients, and 46 of 198 (23.2%) SLE-associated TCRs can be found in RA. Among them, we determined 12 TCR clones were both RA- and SLE-associated TCRs (figure 4J). Moreover, for the 32 RA associated TCRs found in SLE, 81.3% (26/32) had higher publicity in SLE than HC, implying their potential association with SLE. In contrast, 76.1% (35/46) SLE associated TCRs found in RA showed similar trend (figure 4J). These findings indicate the universal autoimmunity for disease associated TCRs. Disease-associated clones in RA and SLE tended to highly use similar V genes, such as V7-7 or V6-4 (figure 4D). Moreover, RA- and SLE-associated clones were clustered in the same large cluster, including nine clones associated with both RA and SLE, which indicates the high similarity among these clones (figure 4F). Taken together, this evidence supports the existence of disease-associated TCR clones in the entire autoimmune disease spectrum.

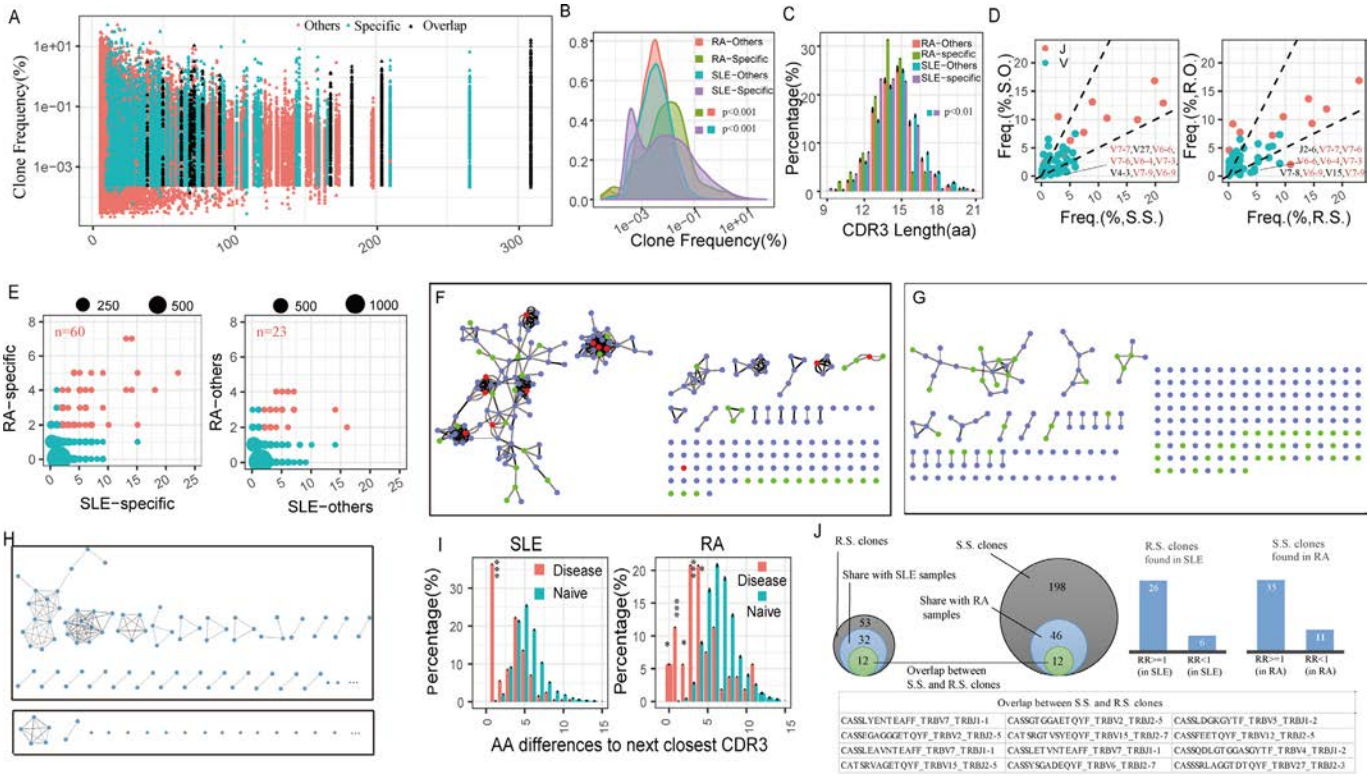

Figure 4 Characteristic analysis of SLE- and RA-associated TCRβ clones. (A) All SLE TCRβ clone frequencies are shown with their incidences in SLE samples. The SLE-specific clones overlapped with RA-specific clones are represented by black triangles. The other SLE-specific clones are represented by blue triangles. The left SLE clones are shown in red triangles. The results of comparison between SLE/RA specific clones and unrelated control clones are shown in (B) (C) (D) (E) (F) and (G). The SLE unrelated clones were randomly selected from all SLE clones with the exception of the SLE specific clones, and RA unrelated clones were selected by the same method. The number of unrelated clones is the same as the disease specific clones and were sampled with replacement for 100 times. (B) Clone frequency distribution (Kolmogorov–Smirnov test). (C) CDR3 amino acid length distribution (Kolmogorov–Smirnov test). The error bar for other unrelated clones is the SE of 100 repeated samples. (D) Vβ gene and Jβ gene usage. The dashed lines were drawn with the slope 0.5 and 2 separately. S.O., SLE other clones. s.s., SLE specific clones. R.O., RA other clones. R.S., RA specific clones. (E) Shared motif between SLE and RA specific clones (left Panel) and SLE and RA other clones (right Panel). Shared motif observed in both SLE and RA groups; at least two samples were marked in red, and the number in the left top corner shows the amount of motif in red. (F) SLE and RA specific clone clustering using Levenshtein distance. (G) SLE and RA other clone clustering using Levenshtein distance. Two CDR3s are clustered together in (I) and (J) if the Levenshtein distance is less than 3. Dot represents a CDR3 and CDR3s in a cluster were connected by a line. Red dot, shared by both SLE and RA. Blue dot, RA clones. Purple dot, SLE clones. (H) SLE-specific clones (top Panel) and RA-specific clones (bottom Panel) were clustered by the tool GLIPH. The dot represents a clone, and two dots are connected if they share a significant motif (compared with naïve clones) or have a similar CDR3 region. (I) Minimum Hamming distance of clones in disease-specific clones compared with equal-sized randomly sampled naïve T cell receptor clone pool. The error bar in the figure is the SE of 100 repeated random samples of naïve clones (X^2 test). (J) comparison of SLE and RA specific clones. R.S., RA-specific. s.s., SLE-specific. CDR3, complementarity-determining region 3; freq. frequency; J, joining; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; TCR, T cell receptor; V, variable.

The divergence and heterogeneity of the TCR repertoire in autoimmune diseases

The correlation of the V, J gene usage and V-J pairing were the lowest in the SLE patients, which indicates the high heterogeneity of TCR gene usage in SLE patients. However, these metrics in RA patients were even higher than in the HC samples, which implies RA exhibits higher homogeneity in this respect (figure 5A). We then measured the clonal overlap between every two groups and found the numbers in SLE and RA were smaller than in the HCs, with SLE being the smallest (figure 5B). Furthermore, the publicities of public clones were substantially smaller in SLE and RA than in HC (figure 5C,D). The above evidences support the high heterogeneity in autoimmune diseases, particularly in SLE. Hierarchical clustering on all samples by the public TCR clones also revealed higher heterogeneity in SLE and RA though no apparent clusters appear (figure 5E-G).

The clinical implication of the TCR features and enrichment of pathogen-related TCRs in autoimmunity

We subsequently investigated whether the TCR repertoire properties were correlated with the clinical features of these autoimmune diseases. In SLE, the Gini index of the V-J gene combinations and the content of SLE-associated clones were positively correlated with the disease activity measured by the SLE Disease Activity Index (SLEDAI) (figure 6A,C, online supplementary figure S10A). The content of SLE-associated TCR clones and the total unique clone number were correlated with the complement C3 and C4 measurements in SLE, respectively. Furthermore, the content of SLE-associated clones, the total frequency of the top 100 clones and the Gini index of V and J gene pairing were also correlated with the number of damaged organs (figure 6A, online supplementary figure S10A). Additionally, we found some medications such as steroids could

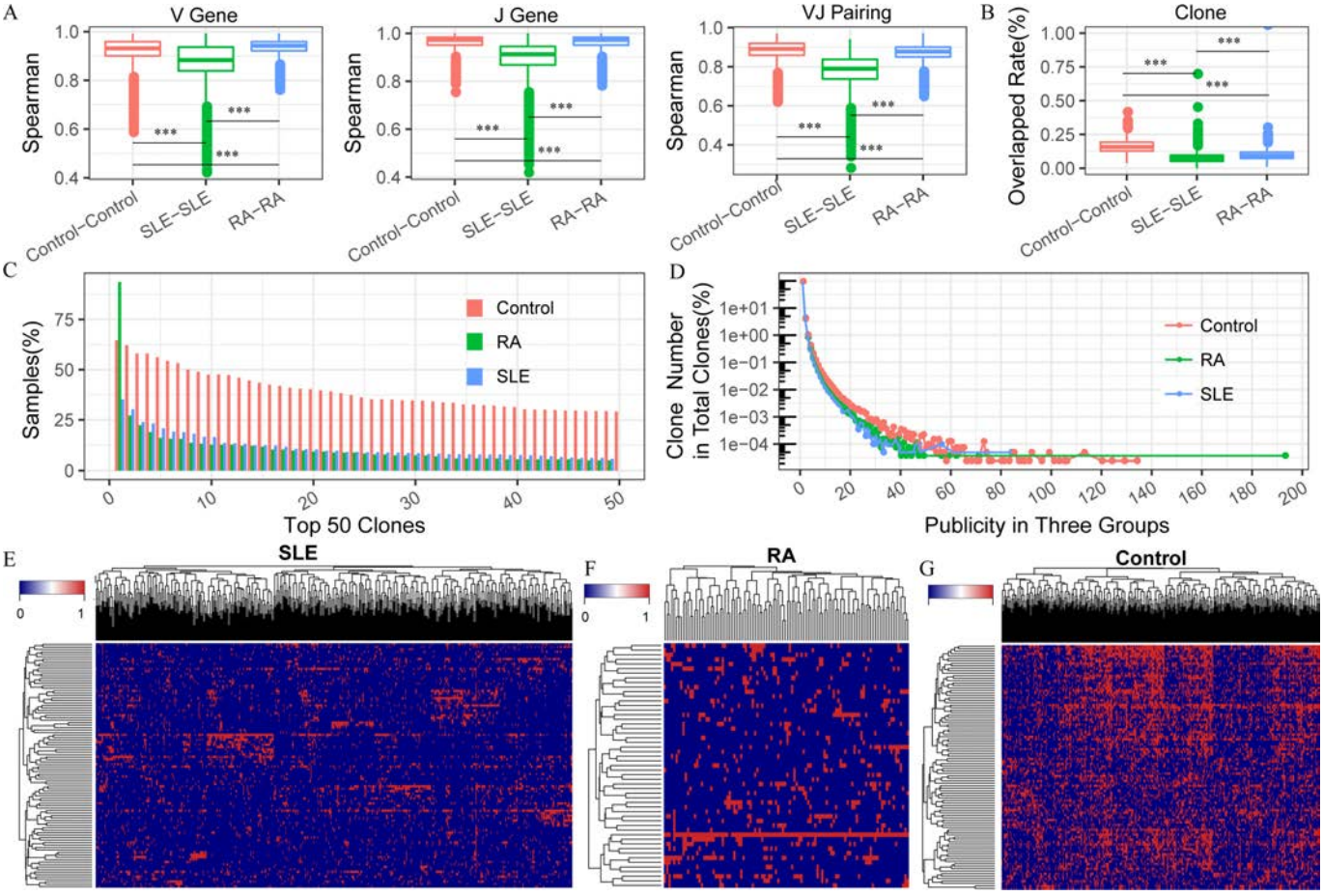

Figure 5 Heterogeneity of TCRβ repertoire in autoimmune diseases. (A) Spearman correlation of Vβ gene, Jβ gene and V-J pairings between any two Subjects (Mann-Whitney test). (B) Overlapped rate (Jaccard Index) of TCRβ clone repertoire between any two Subjects (Mann-Whitney test). (C) Sample incidences of the top 50 clones for three groups. (D) Percentage of clone number in total clones are shown with the clone's publicity. (E) Top 100 (ranking by publicity) SLE specific clones (y-axes) distributed among all SLE samples (x-axes). The red indicates the clone was observed in the sample, and the blue indicates the clone was not observed in the sample. (F) All 56 RA specific clone distributions among all RA samples. (G) Top 100 clones (ranking by publicity) distributed in HC samples. HC, healthy control; J, joining; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; TCR, T cell receptor; V, variable.

lower the diversity through increasing expanded-steroids clone frequencies in SLE (online supplementary figure S10A).

It is reported that organ-specific factors, such as tissue resident cells, may contribute to inflammation and tissue injury in SLE.[27] Therefore, we assessed whether organ specific autoantigens may drive the selection of TCR clones with specific organ lesions. Our data showed that patients with skin damage tended to have more similar TCR repertoires, but no clear evidence supported the TCR selection of other specific organ lesions. Intriguingly, patients without organ lesions shared closest repertoires (online supplementary figure 11A, B). Blood system involved patients tended to have more divergent repertoires (online supplementary figure 11A-C). We also identified a significantly shorter CDR3 length in patients with blood system involvement; however, the contribution of deletion or insertion was obscure (online supplementary figure11D-F). Notably, the total frequency of SLE-associated TCR clones was the highest in kidney damaged patients, which implies a more active state (online supplementary figure 10A).

In RA, we also identified a higher clonal expansion and lower diversity, measured by the Shannon entropy, the unique clone number and the number of expanded clones in high ESR and C-reactive protein patients, which could indicate disease severity in RA (figure 6B). Intriguingly, in antinuclear antibody (ANA) positive patients, we identified a higher repertoire diversity measured by the unique clone number and the lower expansion of RA-associated TCRs (figure 6B,D and online supplementary figure S10B. In both autoimmune diseases, we found disease-associated clones were more correlated with the clinical features than the overall diversity indices, which further validated the reliability of disease-associated clone identification (figure 6A,B).

Astonishingly, by comparing the appearance of annotated TCR sequences from manually curated TCR specificity databases TBAdb,[22] VDJdb[23] and McPAS-TCR[24] in autoimmune disease samples with that in HCs, autoimmunity displayed a significant enrichment in several categories with pathogen identified at most. SLE harboured significantly more T cells targeting influenza and Epstein-Barr virus (EBV), and tuberculosis specific T cells are enriched in both SLE and RA. Furthermore, we observed the prevalence of multiple sclerosis (MS), allergy and cancer-related T cells in SLE (figure 6E,F).

DISCUSSION

We have developed an in silico pipeline to test and identified substantial disease-associated TCRs in SLE and RA. Of note, published SLE-associated TCRs either unexist or have low publicity or clonal frequencies in our data. This finding is not

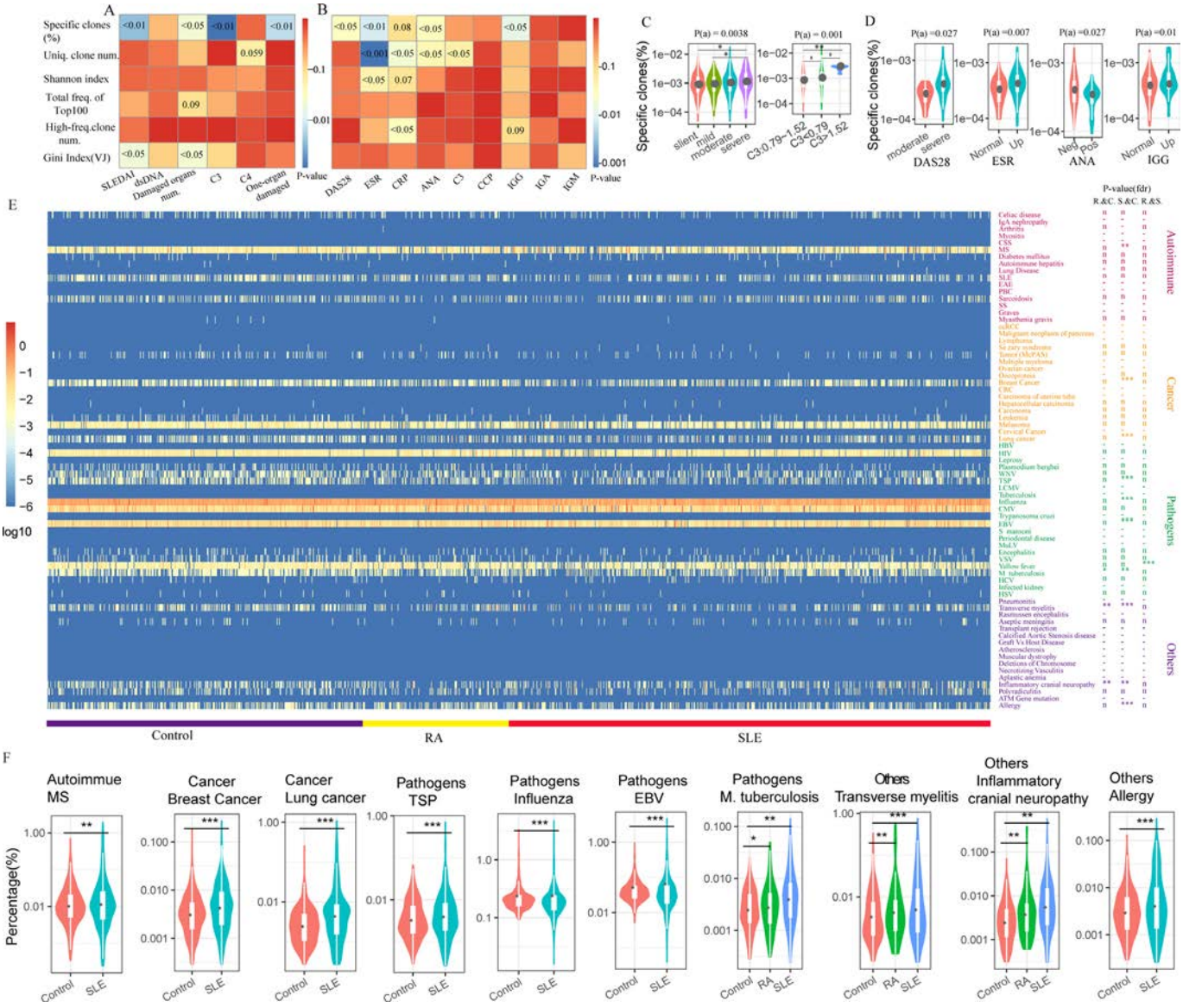

Figure 6 Clinical association analysis and TCRβ repertoire's annotation by manually curated TCR specificity databases. The heatmap shows p values between the repertoire diversity indices (ANOVA test and multiple comparisons test) or contents of disease-specific clones (ANOVA test and multiple comparisons test) and the clinical information in SLE (A) and RA (B). (C) percentage of SLE specific clones in patients with different SLEDAI and C3. (D) Percentage of RA specific clones in patients with multiple clinical factors. (E) TCRβ repertoire's annotation for each sample (x-axes) by databases TBAdb, VDJdb, McPAS-TCR. The colour presents the percentage of clones that were found in the database in the sample's total unique clone number. The right panel shows the p values between two groups (Mann-Whitney test, corrected by false discovery rate). R, RA. S, SLE, C, HC. -, test is not available due to small dataset. (F) Violin plots illustrating the percentage of annotated clones for significantly different groups in (E). MS, multiple sclerosis. ANOVA, analysis of variance; DAS28, disease activity score 28; ESR, erythrocyte sedimentation rate; HC, healthycontrol; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; TCR,T cell receptor.

unexpected, and the major reasons include antigenic TCR privacy, cross reactivity with different antigens and lack of TCRα pairing information, which have been carefully discussed in cytomegalovirus (CMV) infection.[28] In our study, other possibilities should also be raised, such as different human leukocyte antigen (HLA) backgrounds and mostly the extreme heterogeneous autoantigenic exposure in SLE, as shown in our results. Using the identified SLE- or RA-associated TCRs, we can classify SLE or RA from HCs with very high accuracy, as well as between SLE and RA. These findings indicate that TCR can be a better biomarker than traditional serum immunological markers, such as anti-ANA antibody and anti-double stranded DNA (anti-dsDNA) antibody. Although the phenomenon of epitope spreading further diversifies the TCR response from its original epitopes to newer epitopes over time, our finding suggests an immune response exists to a limited set of common autoantigens in SLE or RA patients, which may provide help in developing targeted therapy and vaccinations for SLE and RA.

The interplay between autoimmunity and infections has been discussed for many years but remains elusive. There is evidence that viruses or other infection agents, such as EBV and CMV, could trigger autoimmunity and drive pathogenesis. Several mechanisms could serve as explanations to their co-occurrence, such as molecular mimicry to stimulate the cross activation of T cells or dysregulated activation of the host immune system.[29 30] Our discovery of the enrichment of pathogen specific T cells in autoimmune diseases is in line with clinical observations and supplements the evidence from a brand new perspective. As we

showed in this study, given the sharing of associated T cells in both SLE and RA, it is not surprising TCRs targeting other autoimmune-related phenotypes, such as MS or allergy, are more prevalent in SLE. The enrichment of cancer-related TCRs may be explained by the cross reactivity of self-antigens, as indicated by the association between autoimmunity and cancer.[31]

In summary, our large-scale work moves a step forward in demonstrating the clinical utility of TCR repertoire sequencing to assist diagnosis, treatment and potentially early detecting the autoimmune diseases.

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Correction notice This article has been corrected since it published Online First. The fourth author's name has been corrected.

Contributors QL, XL, WZ and MZ designed the study, analysed the data and wrote the manuscript. LF, JW, LW, LL, SW, LL, XW, TL, YZ, ZW, MF and HP performed TCR sequencing and data analysis. LL, SL, ZW, YL, YY, JJ, YT, BZ, XG, CH, QL, XL, JC, FL, GL, HZ, HL, ZX JL, YL, HY, HL and HW collected the samples and information of patients and healthy subjects. QL, XL, HY and JW supervised the study.

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Data availability statement The study data have been made publicly available at pan immune repertoire database (PIRD, https://db.cngb.org/pird/),[22] which is located in China National GeneBank (CNGB). The project ID in PIRD are P18081001, P18081101 and P18080801.

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TRANSLATIONAL SCIENCE

Combined genetic and transcriptome analysis of patients with SLE: distinct, targetable signatures for susceptibility and severity

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ABSTRACT

Objectives Systemic lupus erythematosus (SLE) diagnosis and treatment remain empirical and the molecular basis for its heterogeneity elusive. We explored the genomic basis for disease susceptibility and severity.

Methods mRNA sequencing and genotyping in blood from 142 patients with SLE and 58 healthy volunteers. Abundances of cell types were assessed by CIBERSORT and cell-specific effects by interaction terms in linear models. Differentially expressed genes (DEGs) were used to train classifiers (linear discriminant analysis) of SLE versus healthy individuals in 80% of the dataset and were validated in the remaining 20% running 1000 iterations. Transcriptome/genotypes were integrated by expression-quantitative trail loci (eQTL) analysis; tissue-specific genetic causality was assessed by regulatory trait concordance (RTC).

Results SLE has a 'susceptibility signature' present in patients in clinical remission, an 'activity signature' linked to genes that regulate immune cell metabolism, protein synthesis and proliferation, and a 'severity signature' best illustrated in active nephritis, enriched in druggable granulocyte and plasmablast/plasma–cell pathways. Patients with SLE have also perturbed mRNA splicing enriched in immune system and interferon signalling genes. A novel transcriptome index distinguished active versus inactive disease—but not low disease activity—and correlated with disease severity. DEGs discriminate SLE versus healthy individuals with median sensitivity 86% and specificity 92% suggesting a potential use in diagnostics. Combined eQTL analysis from the Genotype Tissue Expression (GTEx) project and SLE-associated genetic polymorphisms demonstrates that susceptibility variants may regulate gene expression in the blood but also in other tissues.

Conclusion Specific gene networks confer susceptibility to SLE, activity and severity, and may facilitate personalised care.

Key messages

What is already known about this subject?

- Previous DNA microarray gene expression studies have identified gene signatures involved in systemic lupus erythematosus (SLE) such as those linked to granulocytes, pattern recognition receptors, type I interferon and plasmablasts.

What does this study add?

- A more comprehensive profiling of the 'genomic architecture' of SLE by combining genetic and transcriptomic analysis by next-generation RNA sequencing.
- SLE has three distinct gene signatures: *susceptibility*, *activity* and *severity* signatures, the last best illustrated in nephritis which is enriched in 'druggable' granulocyte and plasmablast/plasma cell pathways.
- Patients with SLE exhibit perturbed mRNA splicing in genes enriched in immune system and interferon signalling pathways.
- Blood transcriptome discriminates SLE versus healthy individuals with high accuracy and can distinguish active versus inactive/low disease activity states.
- DNA polymorphisms that confer susceptibility to SLE regulate gene expression not only in the blood but also in multiple other tissues, which may explain the multiorgan involvement in SLE.

How might this impact on clinical practice?

- Characterisation of the 'genomic architecture' of SLE provides additional clues to the understanding of the systemic nature of the disease, its marked heterogeneity and novel targets of therapy and biomarkers for diagnosis/monitoring.

INTRODUCTION

Genome-wide expression analyses provide an unbiased approach to investigate complex diseases such as systemic lupus erythematosus (SLE). Previous microarray studies have identified gene signatures involved in SLE pertaining to granulocytes, pattern recognition receptors, type I interferon (IFN) and other cytokines, and plasmablasts.[1–5] Notwithstanding, these results have not been associated with clinically defined disease outcomes or correlated with genetic data in a systematic way.

We combined genotype and RNA-sequencing data to comprehensively profile the blood transcriptome in

142 patients compared with healthy individuals, after controlling in silico for cellular heterogeneity. We define distinct '*susceptibility*', '*activity*' and '*severity*' disease signatures, enriched in druggable pathways. Also, patients with SLE have perturbed mRNA splicing in immune system and interferon signalling genes. Based on transcriptome differences, we discriminate SLE versus healthy individuals with high accuracy and distinguish active versus inactive/low disease activity states. Finally, by integrating genotypes from our SLE cohort and using expression-quantitative trait loci (eQTLs) from the Genotype Tissue Expression (GTEx) project,[6] we demonstrate that SLE-susceptibility polymorphisms regulate gene expression in the blood and also in other tissues.

METHODS

See also online supplementary methods.

Experimental design and patient characteristics

We conducted a cross-sectional study of SLE and age/sex-matched healthy individuals recruited from the participating Rheumatology and Blood Transfusion Units, respectively. History of biopsy-proven nephritis, classification criteria, serum autoantibodies, disease activity (physician global assessment, SLEDAI-2K[7]), definitions of Lupus Low Disease Activity State and remission,[8] Lupus Severity Index (LSI)[9] and use of medications were evaluated by standardised protocol. Participants gave informed consent and all procedures were approved by the local institutional review boards. All assays were performed in whole blood (PaxGene RNA tubes for mRNA extraction, EDTA tubes for DNA extraction), and patients withdrew lupus medications for 12 hours prior to sampling.

Genotyping

Individuals were genotyped with the Illumina HumanCoreExome-24 array, phased with SHAPEIT[10] and imputed to the 1000 Genomes Project Phase III using IMPUTE2,[11] yielding ~6.9 million variants.

RNA sequencing, mapping, quantifications and quality control

RNA libraries were prepared with the Illumina TruSeq kit and were sequenced on Illumina HiSeq2000. Paired-end reads (49 bp) were mapped to the GRCh37 reference human genome using GEM mapper.[12] Exon quantification was performed on GENCODEv15. After quality control (online supplementary figure S1), we obtained quantifications for ~21 000 genes in 142 patients and 58 healthy volunteers (online supplementary table S1).

Differential gene expression and pathway enrichment analysis

DESeq2[13] was used to call differentially expressed genes (DEGs) including GC content, RNA integrity, study centre, insert size mode, age, gender, amount of RNA to construct the library and plate number as technical covariates. Statistical significance was set at 5% false discovery rate (FDR; Benjamini-Hochberg). We correlated gene expression with the SLEDAI-2K assigning −1 value for healthy individuals. Functional enrichment gene-set analysis for KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways and GO (Gene Ontology) terms was performed.[14]

Cell type estimation-deconvolution

We used CIBERSORT[15] to estimate the proportion of blood immune cell subsets for each individual. Cell-specific DEGs were identified by introducing as additional covariates the estimated cell proportions and the *disease×cell proportion* interaction term, and obtaining p values for every gene for the interaction term. For every cell type, we estimated the proportion of true positives from the enrichment of significant p values ($\pi 1$ statistic).[16]

Disease classification

Linear discriminant analysis (LDA) was performed introducing DEGs as features. We divided our dataset into training (80%) and validation (20%) and run 1000 iterations. For each iteration, we identified DEGs between SLE and healthy individuals in the training set, which were used to build the LDA classifier. Each classifier was tested for its sensitivity and specificity to discriminate SLE versus healthy in the validation set.

Effect of genetic variation on gene expression

Genotypes obtained from our cohort were assessed for their effect on blood gene expression by eQTL mapping.[17 18] Genetic ancestry was accounted by using the first three principal components (PCs) obtained from EIGENSTRAT (online supplementary figure S1E).[19] To measure the impact of SLE GWAS (genome-wide association study) polymorphisms on SLE blood gene expression and across different tissues, we used eQTL data from our SLE cohort and GTEx.[6] Co-localisation was assessed by the Regulatory Trait Concordance (RTC) score (>0.9)[20] and calculated the shared probabilities (>0.9) that a SLE GWAS polymorphism and the eQTL tag the same functional variant.[21]

RESULTS

Patients with SLE demonstrate widespread transcriptome perturbations

We found 6730 DEGs in SLE versus healthy individuals (online supplementary figure S2A, online supplementary table S2). Novel and previously identified pathways were implicated such as the IFN and *NOD-like receptor* signalling (online supplementary figure S2B–D, online supplementary figure S3A). Our DEGs overlapped significantly with DEGs in paediatric SLE versus healthy counterparts[2] ($p=10^{-165}$, Fisher's exact test) (online supplementary figure S3B), denoting marked aberrancies in SLE blood transcriptome.

IFN signature is robust in SLE and is present across various immune cell types

We used CIBERSORT[15] to estimate the proportions in the blood of different immune cells and confirmed previously reported differences in SLE versus healthy individuals (figure 1A).[22–26] By controlling for these data, we defined a global signature independent of cell variation that comprised 1613 DEGs (online supplementary table S3). The IFN and other pathways such as *p53 signalling* were prominent and independent of cell composition (figure 1B–C), underscoring a critical role in disease pathogenesis. By interrogating the *interferome database*,[27] we found both type I and type II IFN response.

We next explored cell-specific gene perturbations by a statistical model that fits the proportions of immune cells as covariates and a (*SLE vs healthy*)×*proportion* interaction term for every cell type. We tested for the significance of the interaction term and quantified for every cell type the proportion of true positives estimated from the enrichment of significant p values ($\pi 1$ statistic) (figure 2A).[16] In naive and memory B cells, $CD4^+$ memory resting T cells, $CD8^+$ T cells and neutrophils, $\pi 1$ exceeded zero indicating that varying proportions of these cells correlate with divergent effects on gene expression in SLE versus healthy individuals. By introducing treatment with corticosteroids as an additional covariate, $\pi 1$ for neutrophils decreased from 0.067 to 0.034, still the distribution of p values

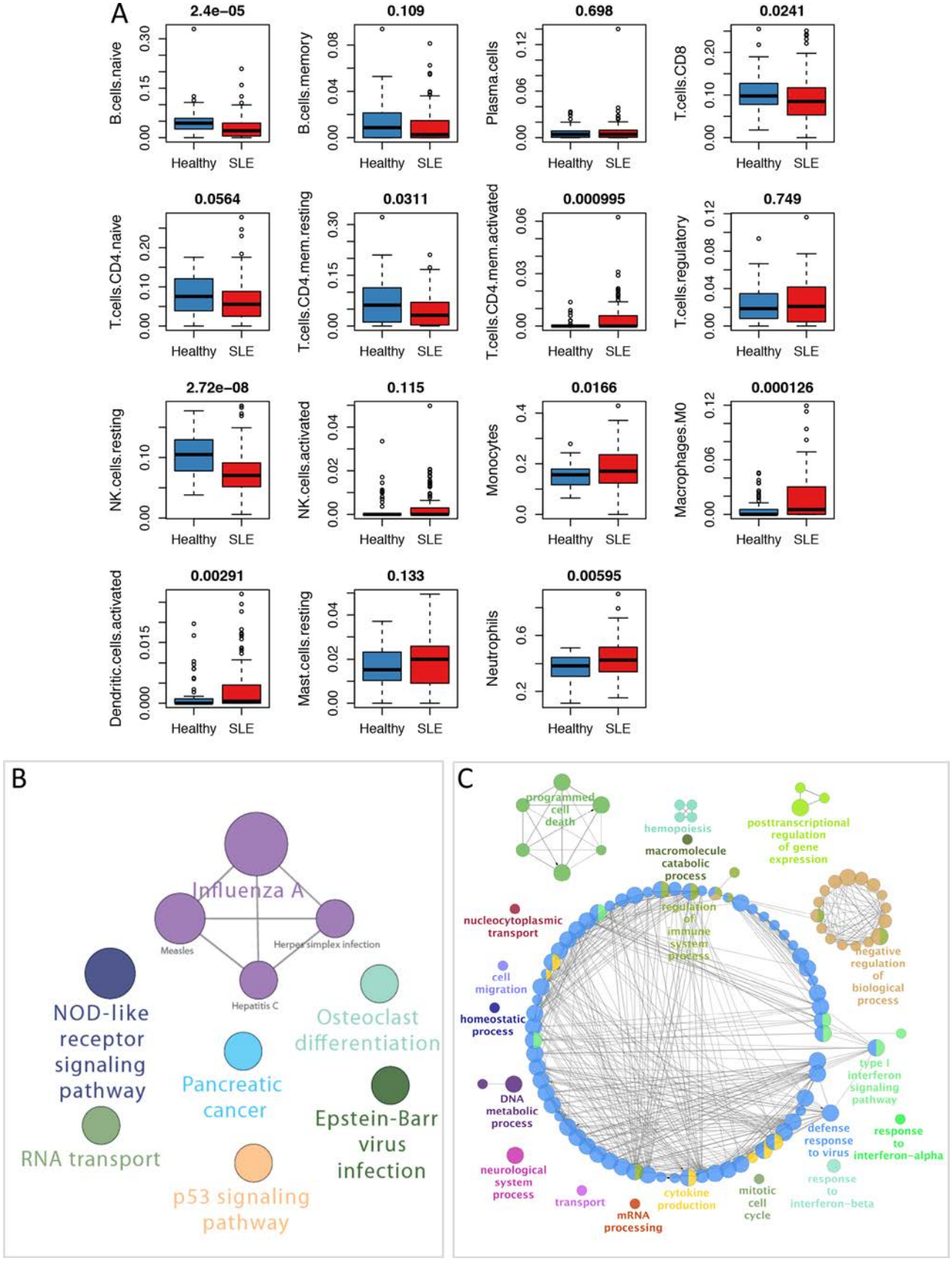


Figure 1 Blood transcriptome deconvolution in systemic lupus erythematosus (SLE) and enriched pathways independent of cell composition. (A) Estimated proportions of different immune cell subsets in healthy and SLE individuals. For every cell type, the Mann-Whitney U test p value comparing healthy vs SLE individuals is displayed on top. (B) Pathway enrichment analysis with all differentially expressed genes (SLE vs healthy) after correcting for the proportion estimates of cell types. (C) Mechanistic map of the biological regulation (GO terms enrichment) in SLE after correcting for cell type proportion estimates.

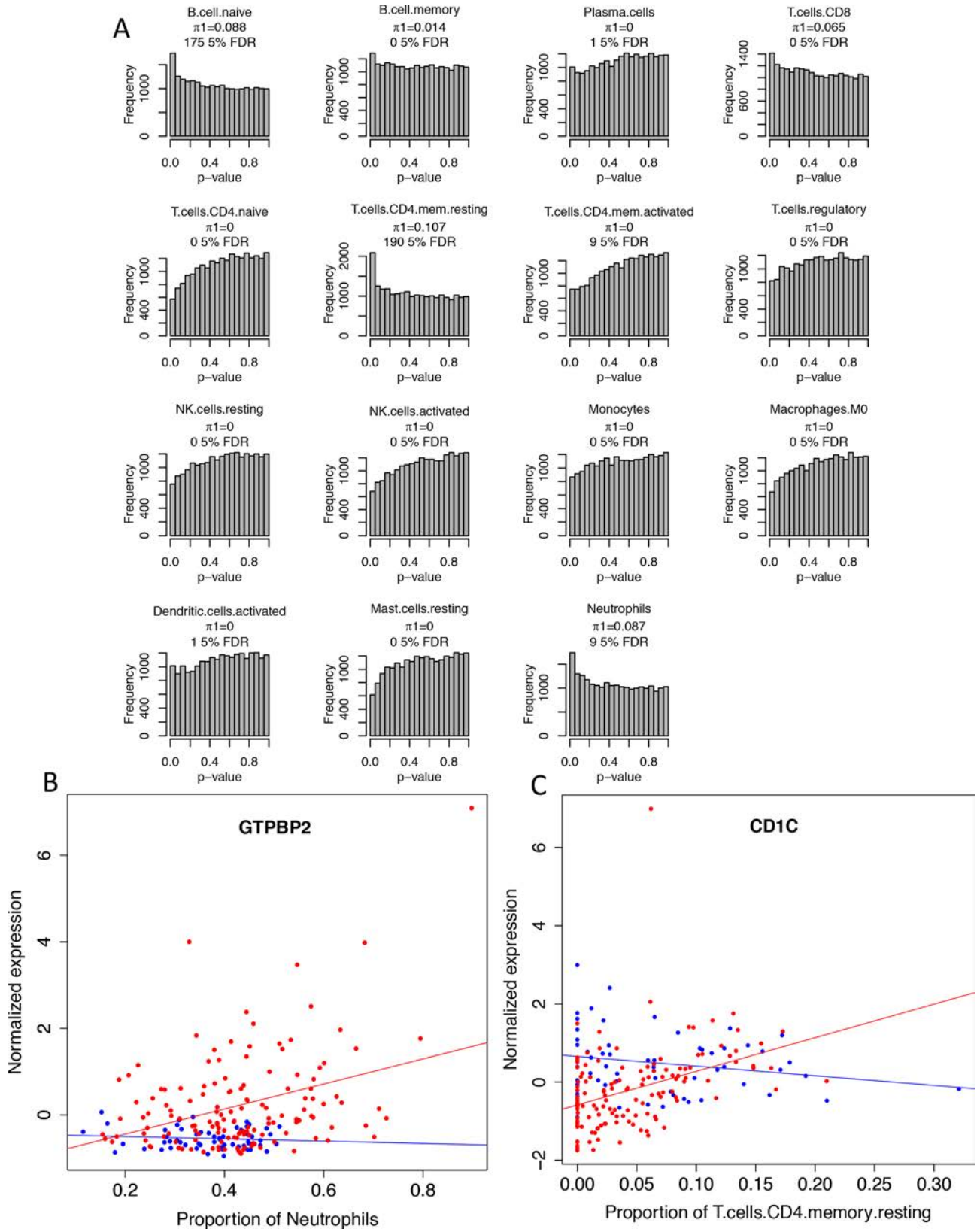
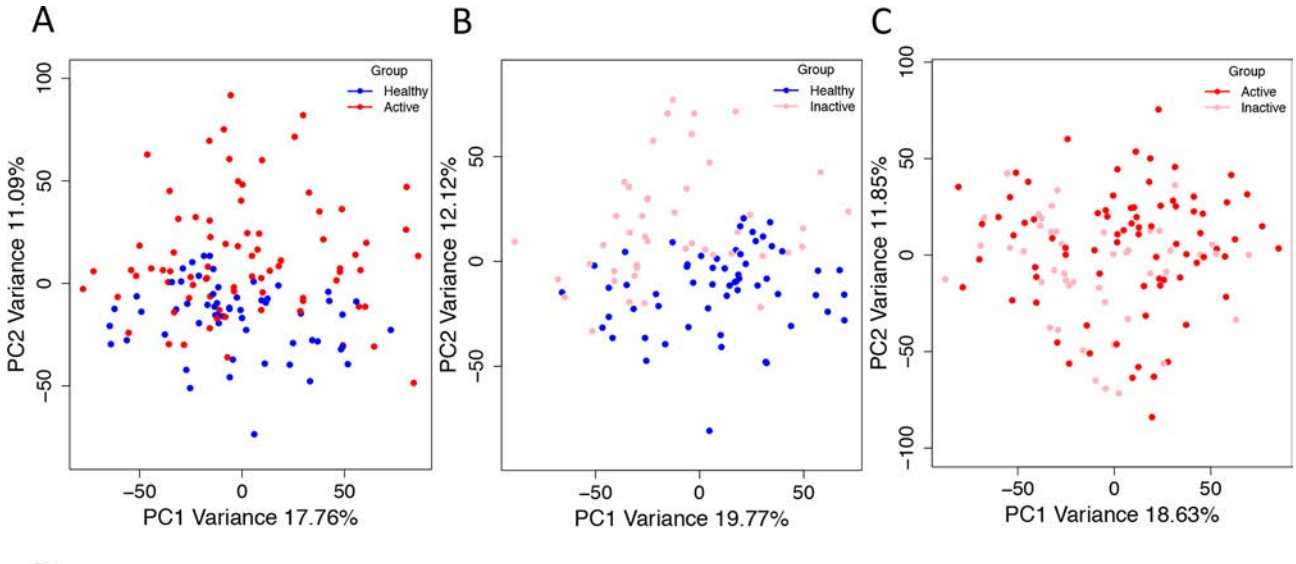
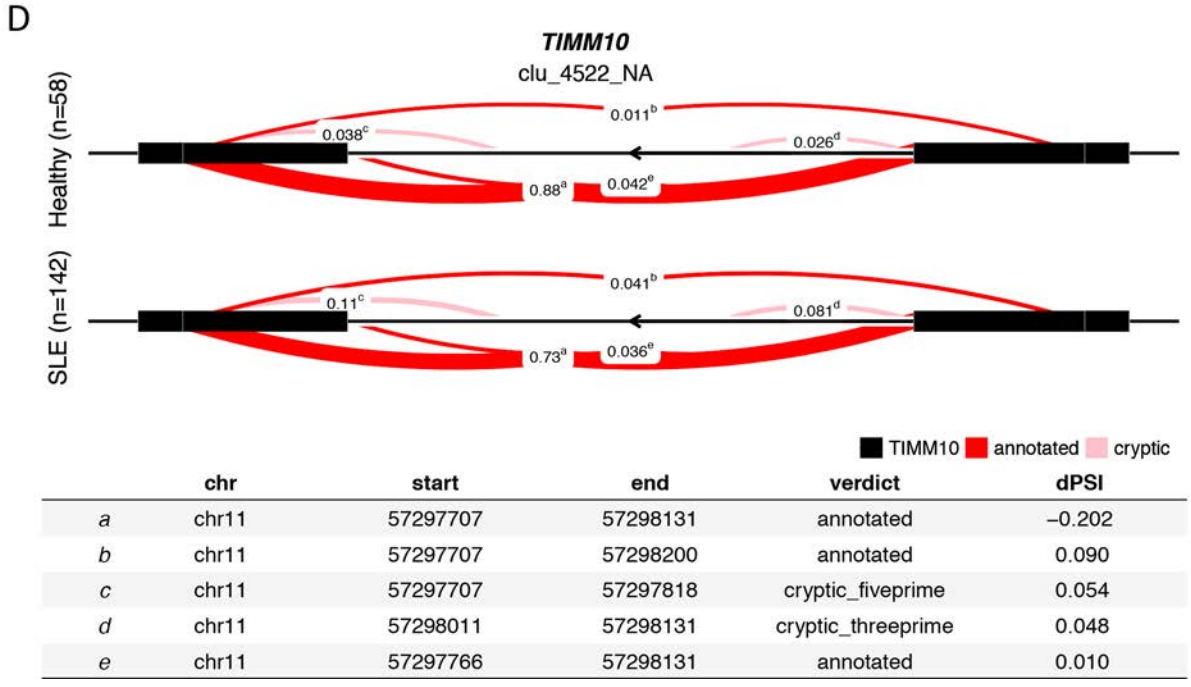


Figure 2 Cell-specific gene expression perturbations in patients with systemic lupus erythematosus (SLE). (A) Histogram of p values for the *disease* (*SLE vs healthy*)×*proportion* interaction term reveals cell type–specific gene expression effects for SLE. For every cell type, the proportion of estimated true positives (π1) and the number of significant genes at 5% false discovery rate (FDR) is shown. (B) Disease (SLE vs healthy) by estimated neutrophil proportion interaction for the gene *GTPBP2*. *x*-axis indicates the estimated proportion of circulating neutrophils while *y*-axis indicates the normalised *GTPBP2* expression. Red dots indicate patients with SLE while blue dots indicate healthy individuals. (C) Disease (SLE vs healthy) by estimated $CD4^+$ T-cell memory resting proportion interaction for the gene *CD1c*. *x*-axis indicates the estimated proportion of circulating $CD4^+$ T-cell memory resting cells while *y*-axis indicates the normalised *CD1c* expression. Red dots indicate patients with SLE while blue dots indicate healthy individuals.





	chr	start	end	verdict	dPSI
a	chr11	57297707	57298131	annotated	−0.202
b	chr11	57297707	57298200	annotated	0.090
c	chr11	57297707	57297818	cryptic_fiveprime	0.054
d	chr11	57298011	57298131	cryptic_threeprime	0.048
e	chr11	57297766	57298131	annotated	0.010

Figure 3 Blood transcriptome variation in systemic lupus erythematosus (SLE) vs healthy individuals and differential mRNA splicing. (A) Principal component analysis (PCA) of whole transcriptome between healthy and active SLE individuals. The two first principal components (PC1, PC2) are plotted. PC2 can differentiate the two groups implying differences in gene expression. (B) PCA between healthy and inactive SLE individuals. PC2 can differentiate the two groups indicating that even in disease remission, the transcriptome of patients with SLE is different compared with healthy individuals. (C) PCA between active and inactive patients with SLE. The first two PCs do not differentiate the two groups suggesting the absence of significant differences in gene expression between them. (D) We characterised alternative splicing events by focusing on intron excisions. A cluster is defined as set of overlapping spliced junctions or introns. For each cluster, we calculated the PSI (percentage splicing intron) coverage and then normalised it as a fraction of the total counts. Each splicing event is plotted as a line connecting its start and end coordinates of the intron with a thickness proportional to the displayed normalised count value. Differential splicing is measured in terms of the difference in the per cent spliced in dPSI. Illustratively, in the case of *TIMM10*, the splicing event 'a' is more frequent in healthy (88%) as compared with SLE (73%) individuals; therefore, the difference in PSI (SLE vs healthy; dPSI) is −0.202.

was left-skewed suggesting the existence of neutrophil-specific effects (online supplementary figure S4). Illustratively, increasing neutrophils correlated positively with *GTPBP2* in SLE but not in healthy individuals (figure 2B). The same trend was observed for the correlation between $CD4^+$ memory T cells and *CD1c* (figure 2C). Notably, GTPBP2 regulates type I IFN production,[28] and CD1c mediates the presentation of modified peptides to T cells.[29]

Distinct transcriptional signatures define susceptibility to SLE

Remission of disease activity is a therapeutic target in SLE,[8] but whether this is mirrored by transcriptome changes remains unknown. We determined a '*susceptibility*' signature that persists in the absence of disease activity following treatment. Principal component analysis (PCA) discriminated transcriptome in clinically active, inactive (clinical SLEDAI-2K=0) patients and healthy individuals (figure 3A–C). Inactive SLE

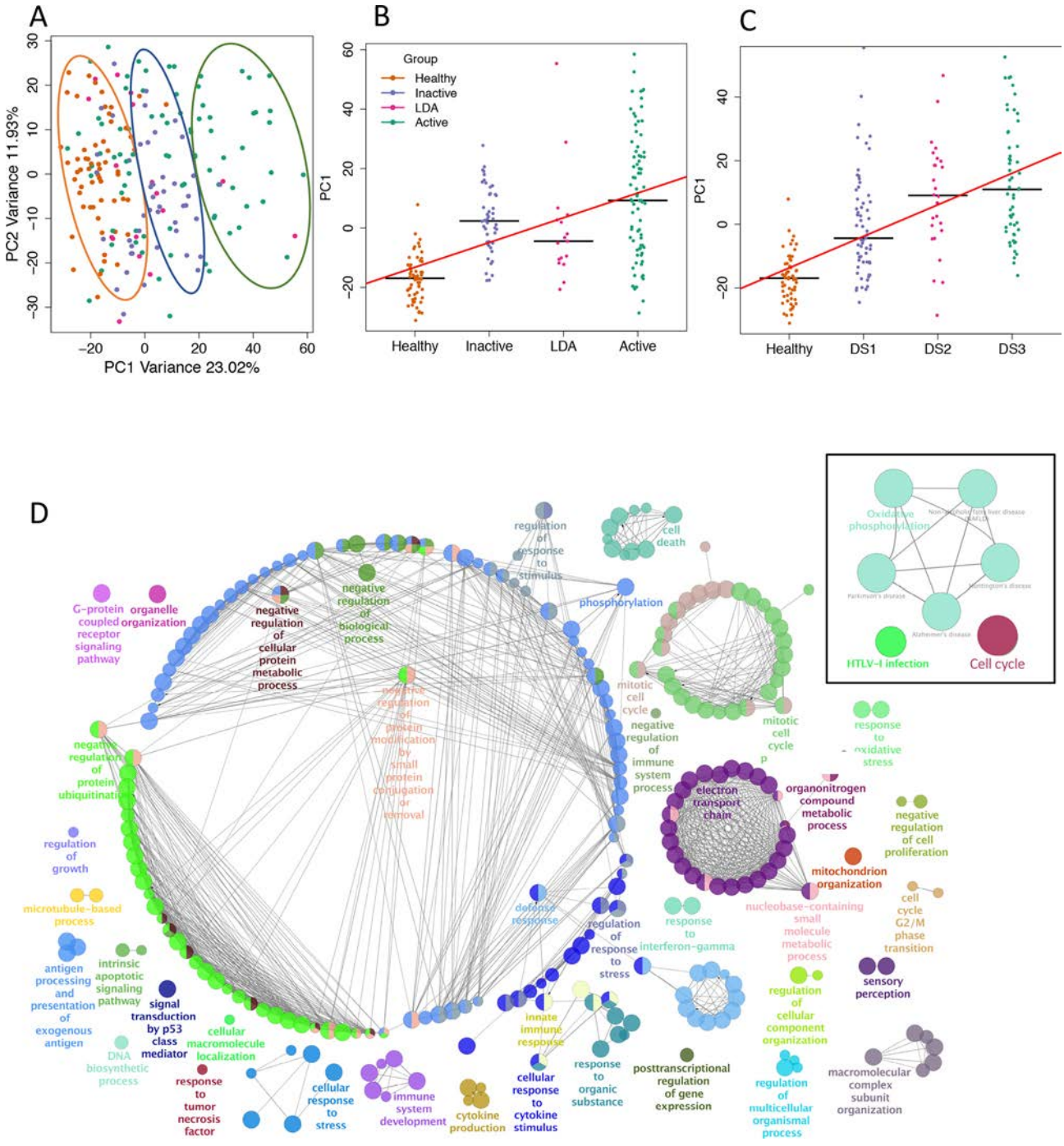


Figure 4 Transcriptomic index correlates with systemic lupus erythematosus (SLE) activity and severity. (A) Principal component analysis (PCA) of 3690 differentially expressed genes (DEGs) significantly correlated with the SLEDAI-2K index (modified to include a fixed score of −1 for healthy individuals). The first two PCs are plotted. PC1 captures SLE activity states, thus separating different groups of individuals namely healthy individuals, patients with SLE at remission, low disease activity (LDA) state and active disease. (B) Jitter plot of PC1 weights according to SLE activity states. For each group, the median value is plotted. The regression line is plotted in red ($p=5.86\times10^{-17}$). (C) Jitter plot of PC1 weights according to SLE severity. Patients with SLE were stratified into three equal-sized groups based on the distribution of Lupus Severity Index scores (lowest to 33rd percentile score; 33rd to 66th percentile score; 66th to highest score), thus creating three distinct groups of increasing disease severity (DS1 to DS3). For each group, the median value is plotted. The regression line is plotted in red ($p=1.02\times10^{-23}$). (D) KEGG pathway enrichment analysis of 3690 DEGs (inset on the right side of the plot) with prominent *oxidative phosphorylation* and *cell cycle* pathways. Functionally grouped networks of GO terms are shown on the right. Multiple biological aberrations are captured by differences in gene expression based on different levels of SLE activity/severity.

were differentiated from healthy but not from active SLE, denoting persistently deregulated gene expression despite remission. We next took the intersection of DEGs in healthy versus active SLE (online supplementary table S4) and healthy versus inactive SLE (online supplementary table S5) that are not DEGs in active versus inactive SLE (online supplementary table S6), to reach 2726 DEGs which comprise the '*susceptibility*' disease signature. These genes were enriched in *regulation and response of the immune system* processes (online supplementary figure S5A–C) suggesting persistence of immune activation. We also

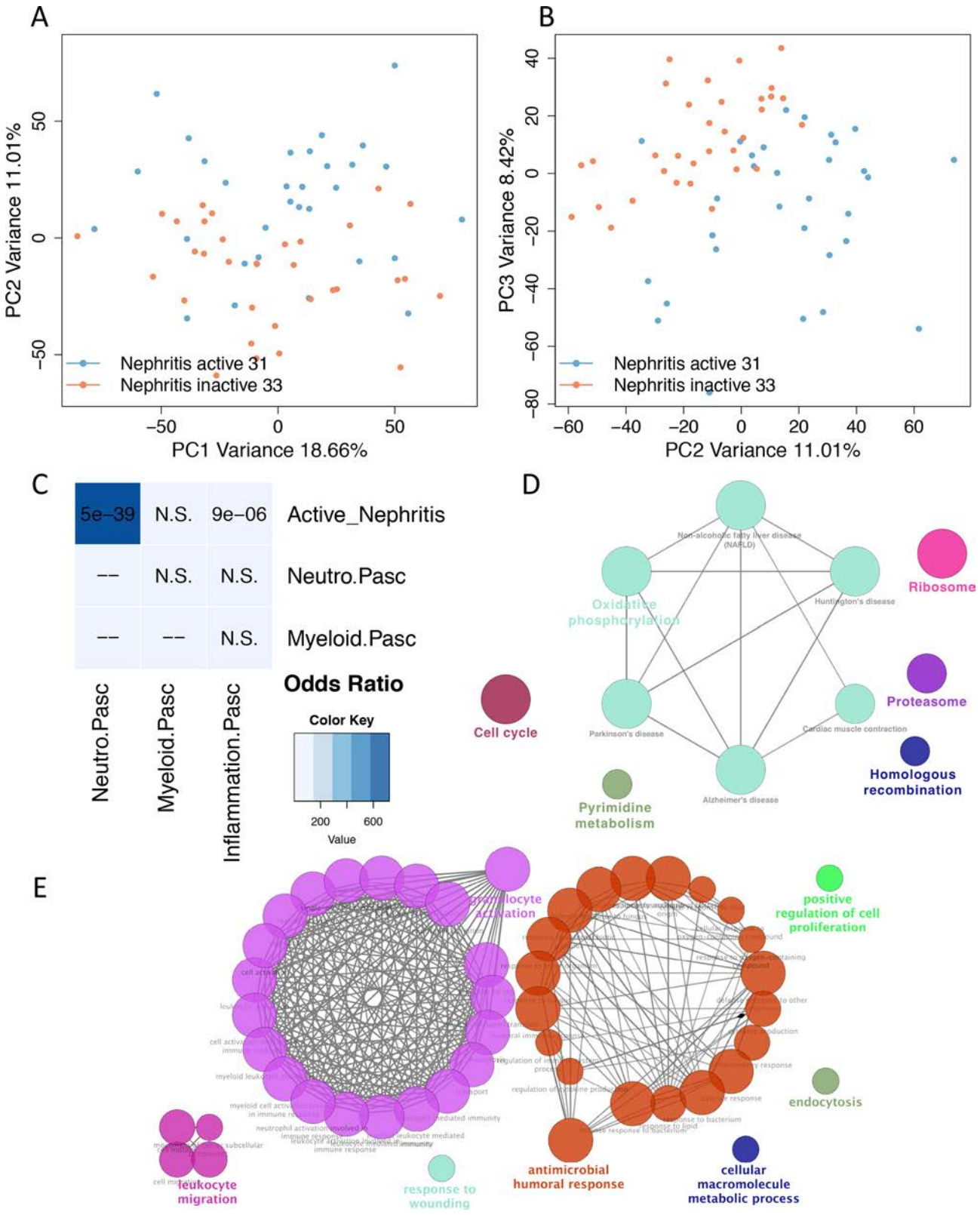


Figure 5 Transcriptome analysis of lupus nephritis reveals prominent neutrophil and humoral response signatures. (A) Principal component analysis (PCA) of blood gene expression between active lupus nephritis and lupus nephritis in remission. PC1 and PC2 are plotted on *x*-axis and *y*-axis, respectively. PC2 is differentiating the two groups. (B) PC2 and PC3 are plotted on *x*-axis and *y*-axis, respectively. PC3 is also differentiating the two groups suggesting that PC2 and PC3 capture different transcriptome/biological aberrations involved in active lupus nephritis. (C) Comparison of neutrophil gene signature with data from a paediatric systemic lupus erythematosus (SLE) study (Banchereau and Pascual; *Cell* 2016). Fisher's exact p values of overlap are plotted in accordance with the ORs denoted as heatmap. (D) Pathway enrichment analysis of the intersection of differentially expressed genes (DEGs) in active nephritis vs inactive SLE with those in active vs inactive SLE. (E) Functionally grouped networks of enriched GO term categories were generated for the 136 DEGs (5% false discovery rate) between patients with SLE with active nephritis vs those with activity from other organs. The main enriched terms are *granulocyte activation* and antimicrobial *humoral response*.

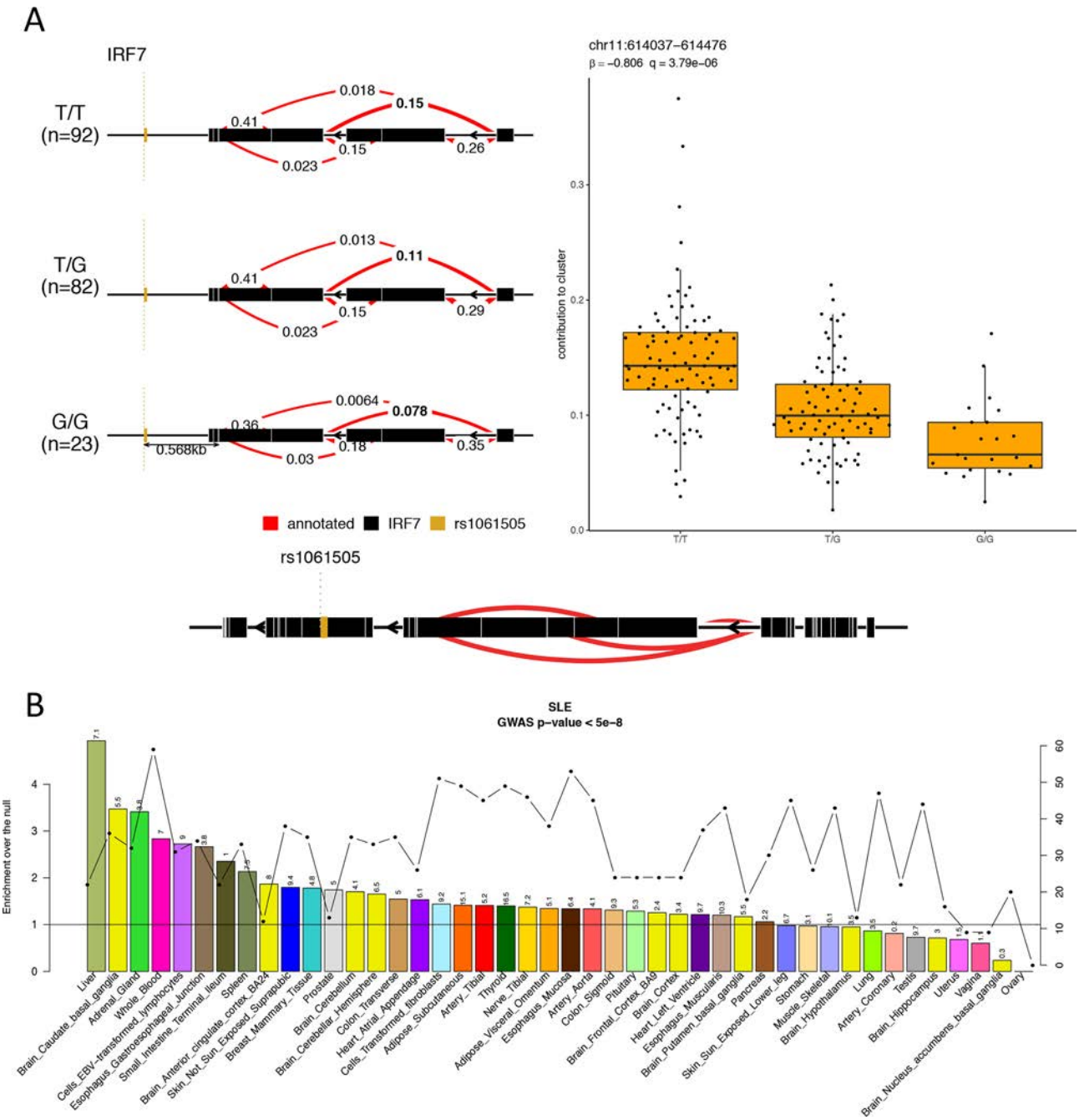


Figure 6 Systemic lupus erythematosus (SLE)-susceptibility variants regulate gene expression in blood and non-blood tissues. (A) Splicing QTL example for the gene *IRF7*. Individuals with SLE carrying the TT genotype show higher contribution of the same splicing event to the intron cluster compared with individuals carrying the GG genotype. (B) To address the impact of SLE GWAS (genome-wide association study) polymorphisms on gene expression across different tissues, we used the eQTL data in 44 tissues from GTEx[6] and calculated the tissue-sharing probabilities of eQTLs and the probabilities that a SLE GWAS polymorphism and the eQTL tag the same functional effect. On the *primary y*-axis, the enrichments-over-the-null per tissue are plotted as bars; on the *secondary y*-axis, the number of SLE GWAS variants that colocalise with eQTLs per tissue are plotted as dotted line. The horizontal black line indicates the null. On top of each bars are the –log10 Benjamini-Hochberg–corrected p values for the enrichments.

quantified alternative mRNA splicing events and found 84 genes with differential splicing (5% FDR) between SLE and healthy individuals (figure 3D, online supplementary table S7), enriched in *immune system* and *type I interferon signalling* pathways. Twenty-six out of 84 genes were also differentially expressed between SLE and healthy individuals after correcting for cell counts (online supplementary table S8). By comparing patients with clinically inactive SLE but evidence for serological activity (high anti-dsDNA, low complement) against those who are clinically and serologically inactive, we found no DEGs, corroborating clinical studies showing comparable prognosis for these two groups.[30]

SLE activity signature is enriched in oxidative phosphorylation

Active disease in SLE may result in organ damage.[31] We selected the DEGs from the inactive versus active SLE comparison that are not in the '*susceptibility*' signature. A total of 365 DEGs were identified, which were enriched for *oxidative phosphorylation*, consistent

with the alterations in mitochondrial mass and membrane potential in lupus T cells[32] and the enhanced oxidative stress.[33] Other enriched pathways included *ribosomes* and *cell cycle*. Together, active SLE is linked to perturbed expression of genes regulating metabolism, protein synthesis and proliferation of blood immune cells. The '*activity*' signature persisted after controlling for serological activity/autoantibodies or treatments, therefore suggesting it could be of potential use for monitoring patients with SLE, although further validation in longitudinal studies will be required. To further corroborate this, we found significant overlap ($p=10^{-5}$, online supplementary figure S3B) between our DEGs (inactive vs active disease) with the respective DEGs in paediatric SLE.[2]

Blood transcriptome as a biomarker of SLE severity

Patients with SLE demonstrate marked variability in clinical outcome and there is an unmet need for biomarkers that reflect disease severity and the underlying molecular mechanisms. We assessed transcriptome according to varying degrees of the SLEDAI-2K index.[7] By performing PCA (figure 4A) on the identified 3690 DEGs (online supplementary table S9), we defined PC1 (explaining 23% of the transcriptome variance) as a new variable summarising the expression properties of genes that recapitulate SLE activity (figure 4B) and severity (LSI-defined) (figure 4C). PC1 clustered closely patients with clinical remission and low disease activity, consistent with evidence that both these states have a favourable outcome.[8] Pathway analysis showed enrichment in *oxidative phosphorylation* and *cell cycle* (figure 4D), similar to the '*activity*' signature. Network enrichment revealed signatures of protein ubiquitination, electron transport chain, protein phosphorylation, cell cycle, defence response and regulation of response to stress (figure 4D), all of which have been linked to SLE.[33–35] These results suggest that multiple molecular pathways determine SLE progression/severity; in this context, blood transcriptome may serve as biomarker for clinically defined outcomes.

Neutrophil activation and humoral response signatures correlate with renal involvement in SLE

SLE can affect multiple tissues, but the molecular basis of this heterogeneity remains elusive. Comparison of active versus inactive patients with nephritis revealed extensive gene expression differences with 1496 DEGs (figure 5A–B). To discern the transcriptome basis for kidney disease, we compared patients with active nephritis versus those with activity from other organs (online supplementary table S10) and found 136 DEGs (online supplementary table S11) enriched in *granulocyte activation* and *antimicrobial humoral response* (figure 5E), consistent with the role of neutrophils[36–38] and of plasmablasts/plasma cells.[39 40] We aligned our results with those in paediatric lupus nephritis and confirmed the presence of *neutrophil gene signature* ($p=5\times10^{-39}$) (figure 5C). Next, we took the intersection of DEGs in active nephritis versus inactive SLE (1375 DEGs) with those in active versus inactive SLE (377 DEGs); 305 genes were common, suggesting a stepwise progression of transcriptome alterations from inactive to active non-renal and active renal SLE. These genes were enriched in oxidative phosphorylation, ribosome, proteasome (online supplementary table S12), cell cycle and pyrimidine metabolism pathways (figure 5D).

Gender differences in gene expression in patients with SLE patients

SLE exhibits a striking gender bias with women affected more frequently than men, yet the latter suffering from more severe disease.[41] We examined for gender-biased gene expression specifically in SLE by taking the non-overlapping DEGs in male versus female SLE (Bonferroni significant) and male versus female healthy (90% FDR threshold to increase specificity) individuals. Six genes had perturbed gender-biased expression in SLE (online supplementary figure S6A), two of which (*SMC1A*, *ARSD*) escape X-chromosome inactivation. *SMC1A*[42] demonstrated the strongest gender difference (online supplementary figure S6A–B), and this was confirmed in purified $CD14^{+}$ monocytes (online supplementary figure S6C). Although preliminary, these results provide candidate genes for further studies.

Blood transcriptome discriminates SLE versus healthy individuals

SLE diagnosis can be challenging especially at early stages.[43] We asked whether we could classify individuals based on their gene profile by LDA using DEGs as features. We divided our cohort into training (80%) and validation (20%) and run 1000 iterations. By sampling different individuals in each iteration, we inserted perturbations in our model therefore building multiple LDA classifiers to account for SLE heterogeneity. Using a median 5438 DEGs, we obtained a median diagnostic accuracy of 87.5% (85.7% sensitivity, 91.7% specificity) in the validation set (online supplementary figure S7). This finding needs further confirmation and testing against lupus-like control diseases.

SLE genetic causality may arise from blood and non-blood tissues

Considering the extended genomic perturbations in patients with SLE, we asked to what extent they may be genetically determined by combining RNA-seq with genotype data and mapping eQTLs in our dataset. We found 3142 *cis*-eQTLs with highly significant eQTLs clustering close to the transcription start site of the genes (online supplementary figure S8A–B), in accordance with their putative regulatory effects. Approximately 17.5% of the DEGs (SLE vs healthy) had an eQTL. Co-localisation analysis (RTC method)[20 21] with GWAS SLE variants revealed nine genes where both the GWAS and the eQTL tag the same variant, namely *UBE2L3*, *HLA-DRB5*, *RP11-356I2.3*, *BLK*, *FAM167A*, *NADSYN1*, *RP11-660L16.2*, *ALDH2* and *ALDH18A1*, thus implicating them in SLE pathogenesis.

To detect disease-specific eQTLs, we compared our SLE eQTLs with those from a larger blood RNA-seq study (n=384 healthy) to gain statistical power.[44] SLE and control eQTLs replicated well ($\pi1=0.89$, online supplementary figure S8C), suggesting the lack of disease specificity. Although it is possible that many of the SLE eQTLs are common eQTLs found also in healthy individuals, this result could be due to insufficient power and/or the need to assay cell subtypes. We analysed active and inactive patients with SLE and found 1072 and 539 eQTLs, respectively. The replication rate between the two groups was 0.854 and 0.921 ($\pi1$ measurement), respectively, again suggesting no specificity according to the disease status.

We next performed splicing-QTL analysis for the SLE individuals by calculating intron excision ratios,[45] and found 777 splicing-QTLs. Notably, 13 of the 26 genes with both perturbed expression and splicing in SLE (online supplementary table S8) had a splicing-QTL, suggesting underlying genetic effects (figure 6A).

Finally, we examined whether SLE-associated genetic variation may regulate gene expression in non-blood tissues. We used SLE GWAS signals and eQTLs from 44 tissues,[6] and employed the RTC method[21] to normalise the GWAS-eQTL probabilities with the tissue-sharing estimates of the eQTLs to determine relevant tissue(s). Notably, SLE-associated polymorphisms regulated gene expression not only in the blood but also in other tissues,

particularly the liver followed by basal ganglia and adrenal gland (figure 6B), suggesting that SLE genetic susceptibility may affect multiple tissues. The finding of liver as a causal tissue concords with our result that disease activity correlates with changes in metabolism genes.[46]

DISCUSSION

Our study provides a comprehensive characterisation of gene signatures in adult SLE associated with susceptibility and clinically relevant outcomes. Moreover, we explore the genetic regulation of transcriptome and provide novel insights regarding SLE pathogenesis.

By transcriptome deconvolution, we estimated the proportions of blood immune cell subsets. We confirmed previously reported perturbations in the abundancies of various cells in SLE,[22–26] which collectively accounted for 75% of blood DEGs. Type I IFN and *p53 signalling* represented two robust signals unaffected by the cell composition, suggesting their critical involvement in disease pathogenesis.

Our analysis for cell-specific effects in gene expression might provide unique insights regarding perturbed molecular and/or genetic mechanisms in SLE. Illustratively, it can be hypothesised that there is a molecular mechanism that keeps *GTPBP2* levels stable across increasing peripheral blood neutrophils in healthy individuals, but this is disturbed in SLE (figure 2B). Alternatively, a genetic mechanism such as a disease-specific eQTL might operate in neutrophils and regulate *GTPBP2*. Together, differences in SLE blood transcriptome may be driven by both altered abundances of circulating cells and cell-specific gene regulation.

Our data revealed a '*susceptibility*' signature pertaining to regulation and response of the immune system that persists even when the disease has remitted. This emphasises the role of immune cell activation in SLE but also implies that existing therapies fail to restore immune aberrancies. Notably, patients with SLE exhibited perturbed splicing pattern in several genes implicated in immune system and interferon signalling, raising the possibility that proteins with aberrant amino acid sequence and/or function are produced.[47] We also defined an '*activity*' signature enriched in cell metabolism/oxidative phosphorylation genes. These data corroborate studies implicating mitochondrial function and aerobic glycolysis in regulation of immune cells relevant to SLE[48] and could be explored therapeutically.

SLE typically follows a waxing–waning course, necessitating patient monitoring to optimise outcomes. We generated a novel transcriptome index that correlates with SLE activity and severity (figure 4A–C). Notably, this index distinguished patients with active disease versus remission or low disease activity,[8] and could be further exploited as biomarker.

Lupus nephritis represents a distinct disease subset with increased morbidity and treatment complications. We detected extensive transcriptome perturbations in active nephritis with prominent granulocyte and plasmablasts/plasma cell signatures, a finding confirmed in paediatric patients with SLE.[2] Notably, blockade of neutrophil extracellular traps formation,[49] and of plasma cells by proteasome[50] or Bruton's tyrosine kinase[51] inhibitors have shown evidence of efficacy in murine lupus nephritis.

SLE diagnosis is often based on the acumen of experienced physicians due to its clinical heterogeneity.[41 43] We found that blood transcriptome had high specificity and sensitivity in discriminating SLE versus healthy individuals, suggesting it might serve as a diagnostic aid. Further studies including also control diseases are required to support these findings.

We assessed how genetic variation correlates with gene expression in SLE by integrating genotype and RNA-seq data. We found numerous *cis*-eQTLs which, however, were not disease specific. Moreover, our splicing-QTL data corroborate previous results,[52] further emphasising the genetic contribution to the disease. Besides immune activation, the function of tissues may be critical in determining disease outcome. To this end, we used SLE GWAS data and eQTLs from the GTEx tissues. Top causal tissues included liver followed by basal ganglia, adrenal gland and whole blood. Interestingly, mTORC1-dependent liver mitochondrial dysfunction has been implicated in murine lupus[46] although further mechanistic studies are needed to disentangle these findings.

Our study has also certain limitations. First, the lack of longitudinal data to assess intra-individual changes in transcriptome. Second, although our sample size was adequate to characterise subgroup differences, still it lacked sufficient power to detect SLE-specific eQTL signals. Finally, as we studied predominantly Caucasians, our findings may not be generalised to other ethnic groups.

Conclusively, by studying transcriptome differences in SLE and healthy individuals, we describe distinct susceptibility and activity/severity signatures. Our data further illustrate the molecular heterogeneity of the disease and may facilitate the development of novel biomarkers and therapies.

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Contributors NIP carried out the analyses. NIP and GKB drafted the manuscript, with contributions from all authors. GKB recruited and took care of patients, collected blood samples, extracted RNA and DNA. HO performed the RTC analysis. LRP and DB prepared the RNA-seq libraries and CH processed the RNA-seq and genotyping data. IG contributed in recruitment of patients and healthy individuals, and extracted clinical data from the medical charts. DK isolated immune cell subsets from peripheral blood samples and performed RT-PCR studies. MGT, MT, CP, AF, AR and PS contributed patient samples and participated in the analyses of data. DTB and ED conceived and oversaw the study and the writing. All authors read and approved the final manuscript for publication.

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Data availability RNA-seq, genotype, clinical and processed data (summary statistics and expression files) have been deposited at the European Genome-phenome Archive (EGA), which is hosted by the EBI and the CRG, under the accession number EGAS00001003662.

Competing interests None declared.

Patient consent for publication Obtained.

Ethics approval All procedures were approved by the local institutional review boards.

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TRANSLATIONAL SCIENCE

Lupus-associated atypical memory B cells are mTORC1-hyperactivated and functionally dysregulated

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ABSTRACT

Objectives A population of atypical memory B cells (AtMs) are greatly expanded in patients with active lupus, but their generation and pathophysiological roles are poorly defined. The aim of this study was to comprehensively characterise lupus AtMs with a purpose to identify therapeutic clues to target this B cell population in lupus.

Methods Peripheral B cell subsets were measured by flow cytometry. Sorting-purified B cell subsets were subject to RNA sequencing and functional studies. Plasma cytokines and secreted immunoglobulins were detected by Luminex or ELISA. In situ renal B cells were detected by multiplexed immunohistochemistry.

Results $CD24^{-}CD20^{hi}$ AtMs were strongly increased in two Chinese cohorts of patients with treatment-naïve lupus. Gene expression profile indicated that B cell signalling and activation, lipid/saccharide metabolism and endocytosis pathways were abnormally upregulated in lupus AtMs. In addition, the mammalian target of rapamycin complex 1 (mTORC1) pathway was remarkably activated in lupus AtMs, and blocking mTORC1 signalling by rapamycin abolished the generation of $T\text{-}bet^{+}$ B cells and terminal differentiation of lupus AtMs. Furthermore, lupus AtMs displayed a dysfunctional phenotype, underwent accelerated apoptosis, poorly co-stimulated T cells and produced proinflammatory cytokines. Interestingly, lupus AtMs were in a paradoxically differentiated status with markers pro and against terminal differentiation and enriched with antinucleosome reactivity. Finally, AtMs were accumulated in the kidneys of patients with lupus nephritis and associated with disease severity.

Conclusions These findings demonstrated that mTORC1-overactivated lupus AtMs are abnormally differentiated with metabolic and functional dysregulations. Inhibiting mTORC1 signalling might be an attractive option to target AtMs and to improve therapeutic effectiveness in patients with lupus.

Key messages

What is already known about this subject?

► $T\text{-}bet^{+}CD11c^{+}$ atypical memory B cells (AtMs) are greatly expanded in lupus and implicated in the pathogenesis of systemic lupus erythematosus (SLE).

What does this study add?

► We find that the mTORC1 pathway is highly activated in lupus AtMs and plays a critical role in the generation and terminal differentiation of these cells. Importantly, inhibiting mTORC1 signalling by rapamycin blocks $T\text{-}bet^{+}$ B cell generation and the terminal differentiation of AtMs.

► Our study also demonstrates that AtMs are aberrantly differentiated, metabolically abnormal and functionally dysregulated. In addition, the abundance of AtMs both in the blood and in the kidneys of patients with lupus nephritis reflects the disease activity, indicating that AtMs could be a novel biomarker for patients with lupus.

How might this impact on clinical practice or future developments?

► Targeting AtMs by inhibiting mTORC1 signalling pathway could be an attractive option to improve therapeutic effectiveness in patients with SLE.

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease triggered by loss of self-tolerance and the resultant autoreactive cellular and humoral immune responses, leading to a striking heterogeneity of clinical manifestations and organ dysfunction.[1 2] B cell abnormality plays a central role in the development of SLE by contributing to the overproduction of autoantibodies, cytokines and augmented presentation of autoantigens to T cells.[3 4]

The B cell compartment is highly distorted in patients with active SLE. Transitional B cells and plasmablasts are greatly increased, while non-switched memory B cells are decreased in active SLE.[5–7] In addition, a population of atypical memory B cells (AtMs) sharing similar phenotypes and identified as $CD19^{hi}$,[8] $CD19^{hi}CD21^{lo}$,[5 9] $CD27^{-}IgD^{-}$[10 11] are expanded in active SLE. Increased frequency of these AtMs has been associated with high disease activity and disease-specific autoantibodies such as anti-Smith (Sm) antibody,[8 10] suggesting that these

cells are associated with disease development. Phenotypically similar B cells were also expanded in other autoimmune diseases like primary Sjoegren's syndrome, systemic sclerosis[5] and infectious diseases like HIV infection,[12] hepatitis C virus infection[13] and malaria.[14] Thus, an environment of chronic inflammation seems to promote the generation of these B cells.

Recent studies have revealed that human AtMs specifically express transcription factor (TF) T-bet and integrin CD11c[15–17] which are closely linked to a population of B cells named age-associated B cells (ABCs) in aged female mice and young lupus-prone mice.[18 19] Murine ABCs are T-bet$^+$CD11c$^+$ and enriched with antichromatin autoantibodies which play an essential role in lupus development.[16 19] Furthermore, T-bet is necessary and sufficient for the generation of murine CD11c$^+$ ABCs[20] and deletion of T-bet in B cells causes a loss of CD11c$^+$ ABCs and the amelioration of disease in lupus-prone mice.[16 21] These studies demonstrated a common pathogenic role of T-bet$^+$CD11c$^+$ B cells in both human lupus and murine lupus models.

The identification of a population of atypical memory T-bet$^+$CD11c$^+$ B cells in patients with SLE raises the possibility that targeting this population may open new therapeutic opportunities for this complex disease, as the efficacies of B cell depletion therapy by targeting whole B cells in patients with SLE are highly variable.[1 22] Particularly, it was reported that an expanded population of CD19^{hi} AtMs in patients with SLE predicts a poor clinical response to rituximab treatment.[8] Although several factors including Toll-like receptor (TLR) 7, interferon gamma (IFN-γ), interleukin (IL) 21 have been implicated in the differentiation of AtMs,[19 20 23 24] no clinically targetable pathway has been identified so far. Thus, to identify the key pathway leading to their generation and to develop feasible strategies to block their development represent an urgent need in lupus B cell study.

In this study, we aim to perform a comprehensive study of lupus AtMs at molecular, cellular, functional and clinical levels, with a purpose to uncover the unknown nature of AtMs and provide clues to target these cells in clinics.

RESULTS

AtMs are increased in patients with lupus and correlated with disease activity

We have analysed peripheral B cell subsets in a Chinese population of patients with new-onset treatment-naïve SLE by flow cytometry and observed that a population of CD24$^-$CD20^{hi} B cells was significantly increased in patients with SLE compared with patients with new-onset rheumatoid arthritis (RA) and healthy donors (HDs) (mean±SEM, 7.54%±0.87%, range 0.19%–25.3%; 3.07%±0.45%, range 0.67%–10.6%; 1.56%±0.15%, range 0.39%–3.68%; respectively) (figure 1A,B) (Detailed characteristics of the three groups were listed in online supplementary table 1). Phenotypical analysis revealed that the CD24$^-$CD20^{hi} B cells expressed high levels of CD11c, T-bet and memory marker CD95, but didn't express CD21, while the expression of the classical memory marker CD27 was variable (figure 1C), indicating that the CD24$^-$CD20^{hi} B cells were corresponding to a population of AtMs identified in SLE[10 16 17] as well as in chronically infectious diseases[12 14] and the healthy elderly.[25]

We then assessed the clinical relevance of these lupus AtMs. We found that the frequency of circulating AtMs was positively correlated with Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) Scores, titres of serum antinucleosome antibody (ANUA) and anti-dsDNA, while negatively correlated with serum complement 3 (C3) and C4 (figure 1D and online supplementary figure 1A). We further showed that the frequency of AtMs was much higher in lupus nephritis (LN) compared with those patients without renal involvement (figure 1E), and was negatively correlated with blood haemoglobin concentration (online supplementary figure 1B). Furthermore the frequency of lupus AtMs was positively correlated with a panel of proinflammatory cytokines including IL-6, IL-18, IFN-α and IFN-γ in plasma (online supplementary figure 1C). We confirmed the correlation between AtMs and disease activity in a second cohort of 19 patients with new-onset lupus (online supplementary table 2). Either defined as CD24$^-$CD20^{hi} or CD11c$^+$CD24$^-$CD20^{hi}, the frequencies of both AtMs were positively correlated with SLEDAI Scores and negatively correlated with serum C3 concentrations (online supplementary figure 1D–F).

Twenty-three patients from the first cohort who have received standard therapy were followed up for 24 weeks (online supplementary tables 3 and 4). The frequencies of AtMs were rapidly decreased 4 weeks after treatment and maintained at relatively low levels thereafter (figure 1F). Strikingly, we observed a significant increase of AtMs in a subgroup of eight patients who experienced relapses or showed resistance to the treatment at week 24 (figure 1G). These results suggest that AtMs could be a potential biomarker to monitor disease activity and predict disease flare.

Lupus AtMs exhibit a unique gene expression profile with BCR activation and metabolic dysregulation

To explore the molecular features of lupus AtMs, we performed an RNA-seq-based gene expression profiling of AtMs and classical memory B cells (CMs) from six patients with new-onset SLE, as well as CMs from five HDs. Principal component analysis separated the three memory B cell populations apart (figure 2A). Venn diagrams showed that 578 genes were significantly upregulated and 384 genes were significantly downregulated in lupus AtMs compared with lupus CMs and healthy CMs, respectively (figure 2B). Gene clustering analysis classified the differentially expressed genes into two major clusters: lupus AtMs high and low expressions (figure 2C). With gene ontology terms as the reference, gene set enrichment analysis (GSEA)[26] identified three pathways were predominantly upregulated in lupus AtMs compared with CMs: B cell signalling and activation, lipid and saccharide metabolism and endocytosis (figure 2D,E and online supplementary figure 2A). By contrast, RNA/protein synthesis-related pathways were dampened in lupus AtMs (online supplementary figure 2B).

Consistent with the above pathway analysis, we observed that lupus AtMs displayed heightened B cell receptor (BCR) signalling (pSyk(Y348), pSyk(Y352), pBTK(Y551/511) and pPLCγ2(Y759)) compared with lupus CMs at steady state (figure 2F,G). However, following BCR cross-linking, lupus CMs rapidly upregulated phosphorylated BCR signalling molecules while limited increase was observed in AtMs (online supplementary figure 2C). This BCR responding feature is similar to that observed in the AtMs from malaria-infected patients[27] which indicates that AtMs are not intrinsically highly responding to BCR stimulation.

mTORC1 pathway is highly activated and implicated in the differentiation of AtMs

The increased BCR signalling in lupus AtMs at steady state prompted us to check the downstream signalling pathways. GSEA and gene differential analysis showed that the PI3K pathway was mobilised in lupus AtMs (figure 2D, online supplementary figure 3). Strikingly, using the HALLMARK database, GSEA identified that mTORC1 pathway, a key target of the PI3K/Akt pathway, was highly activated in lupus

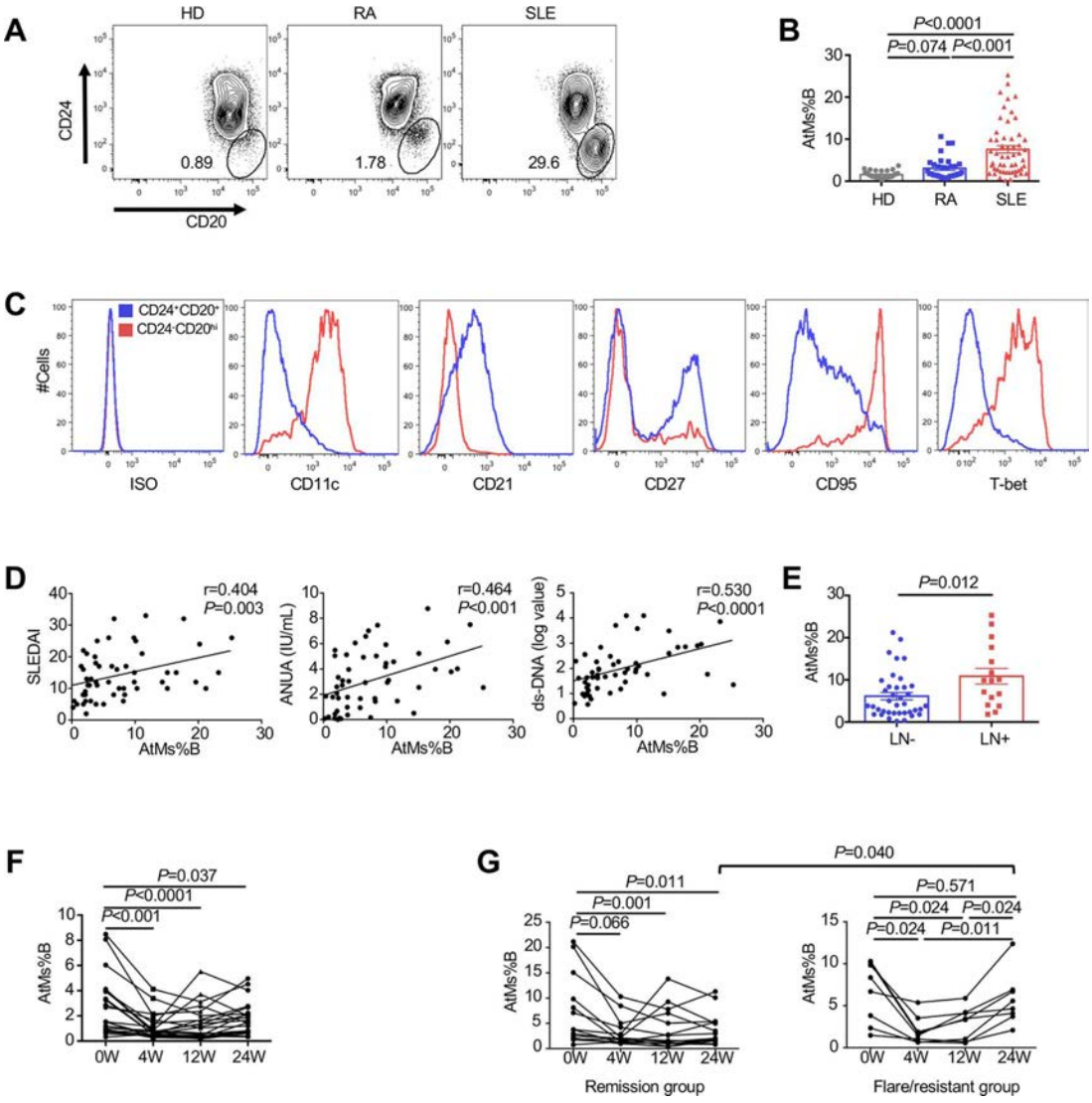

Figure 1 Atypical memory B cells (AtMs) were increased in the blood of patients with active SLE. (A) Flow cytometry analysis showed the frequency of circulating $CD24^{-}CD20^{hi}$AtMs among total B cells (AtMs%B) in healthy donor (HD), patient with RA and patient with new-onset SLE. (B) Comparison of circulating AtMs%B among HD (n=30), patient with RA (n=33) and patient with new-onset SLE (n=54). (C) Flow cytometry analysis of differentially expressed markers between peripheral $CD24^{+}CD20^{+}$ B cells and $CD24^{-}C20^{hi}$ B cells in patients with active SLE. For each marker, one representative plot out of three to four was shown. (D) Correlations between circulating AtMs%B and SLEDAI Scores, titres of serum ANUA and anti-dsDNA of patients with new-onset SLE (n=54). (E) Comparison of circulating AtMs%B between patients with new-onset lupus nephritis (LN) (n=16) and without LN (n=38). (F) Changes of circulating AtMs%B in patients with new-onset SLE before and after treatments (at weeks 0, 4, 12 and 24, n=23). (G) Changes of circulating AtMs%B in patients with new-onset SLE in the remission group (n=15) and flare/resistant group (n=8) before and after treatments (at weeks 0, 4, 12 and 24), and comparison of circulating AtMs%B between two groups at week 24. Error bars indicated mean±SEM. *P* values were determined by Kruskal-Wallis test with Dunn's multiple comparisons test (B), Spearman's rank correlation (D), Mann-Whitney test (E and G) and Friedman test with Dunn's multiple comparisons test (F and G). ANUA, antinucleosome antibody; SLE, systemic lupus erythematosus.

AtMs compared with CMs (figure 3A). Consistently, we observed increased phosphorylations of mTOR(Ser2448) and S6(Ser235/236) in lupus AtMs, indicating activation of the mTORC1 pathway[28 29] in these cells. By contrast, the phosphorylation level of Akt(Ser473), a marker of mTORC2 activation,[28] didn't differ between AtMs and CMs in patients with SLE (figure 3B,C).

Next we investigated whether the mTORC1 pathway is involved in the differentiation of $T\text{-}bet^{+}$ AtMs. We chose an in vitro differentiation protocol by using IFN-γ and a TLR7 agonist R848, as both IFN-γ and TLR7 signalling were implicated in the differentiation of $T\text{-}bet^{+}$ AtMs.[19 23 30] The results showed that the combination of IFN-γ and R848 had a synergistic effect on mTORC1 activation reflected by the increased frequency of $p\text{-}S6^{+}$ (phosphorylated S6(Ser235/236)) cells and the generation of $CD11c^{+}T\text{-}bet^{+}$ and $CD11c^{-}T\text{-}bet^{+}$ B cells (figure 3D–F). Interestingly, nearly all $CD11c^{+}T\text{-}bet^{+}$ and $CD11c^{-}T\text{-}bet^{+}$ B cells were $p\text{-}S6^{+}$ while the frequency of $p\text{-}S6^{+}$ cells within $CD11c^{-}T\text{-}bet^{-}$ B cells was significantly reduced (online supplementary figure 4A,B). The results suggest that T-bet expression in B cells could be associated with mTORC1 activation.

To further explore this connexion, we tested the effect of Rapamycin, a specific inhibitor of mTORC1,[31] on the differentiation of $T\text{-}bet^{+}$ B cells. Consistent with an early report,[32] Rapamycin decreased the viability and blocked the proliferation of IFN-γ/R848-stimulated B cells (online supplementary figure 4C-F). In gated live B cells, rapamycin

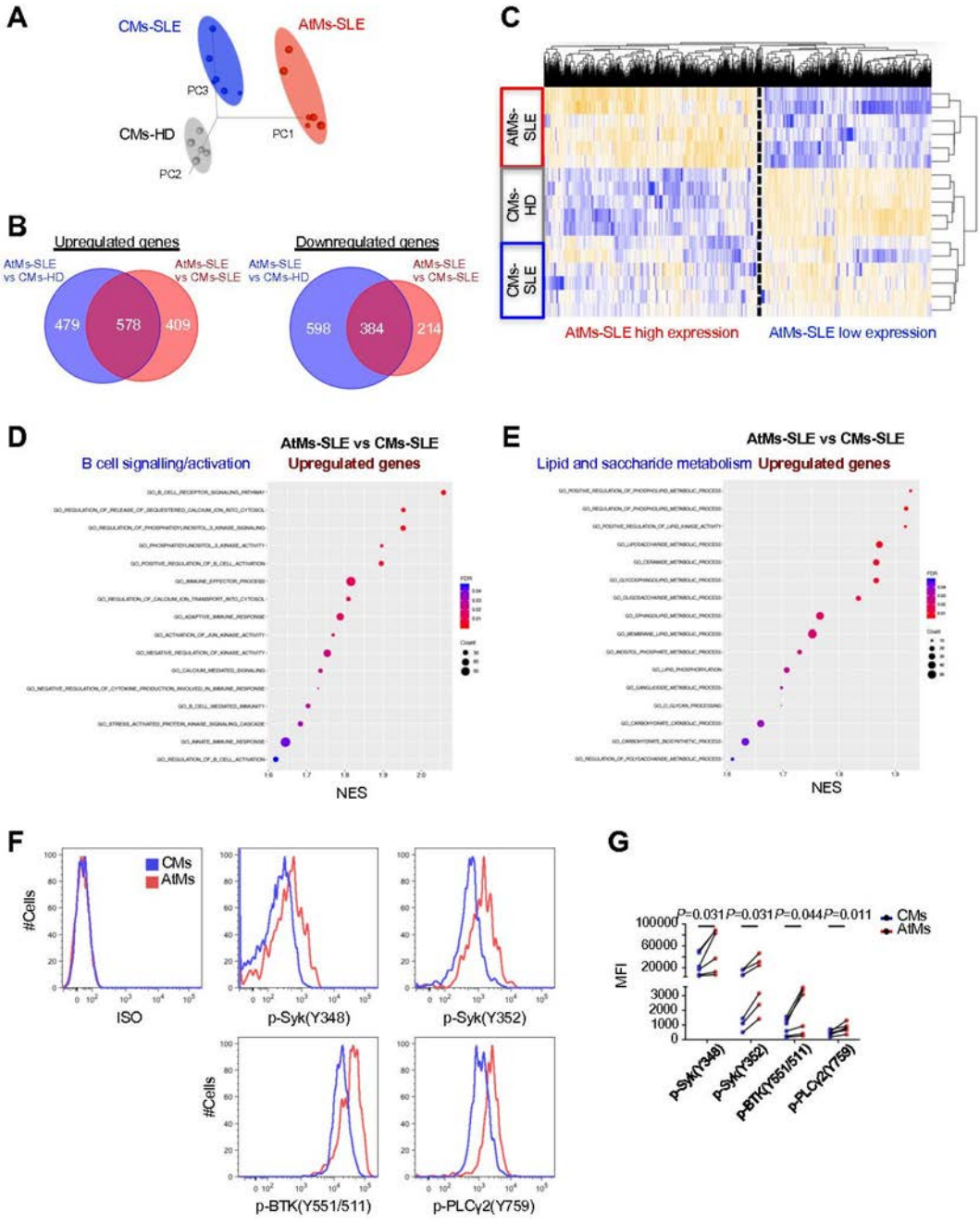

Figure 2 Lupus atypical memory B cells (AtMs) displayed a unique gene expression profile from CMs. (A) Principal component analysis (PCA) of the RNA-Seq data showed the differential gene expression profiles of CMs from HD (n=5), CMs and AtMs from patients with new-onset SLE (n=6). (B) Venn diagrams of RNA-Seq differentially expressed genes (DEGs) of AtMs-SLE compared with CMs-SLE or CMs-HD. (C) Unbiased hierarchical clustering was performed to show the 2688 DEGs from AtMs-SLE, CMs-SLE and CMs-HD. The selection criterion was fold change (FC) >2, *P* value adj <0.05 between any two populations. (D–E) Gene set enrichment analysis (GSEA) identified the pathways of B cell signalling/activation (D) and lipid and saccharide metabolism (E) were highly enriched in AtMs-SLE compared with CMs-SLE based on gene expression data from RNA-seq. (F–G) Flow cytometry analysis (F) and accumulated data (G) of the differentially expressed markers of BCR signalling at baseline between AtMs and CMs from same patients with SLE (n=6). *P* values were determined by paired *t* test (G). CMs, classical memory B cells; HD, healthy donor; NES, normalised enrichment score; PC1, Principal Component 1; PC2, Principal Component 2; PC3, Principal Component 3; ISO, isotype; SLE, systemic lupus erythematosus.

strongly decreased the frequencies of p-S6$^+$, as well as CD11c$^+$T-bet$^+$ and CD11c$^-$T-bet$^+$ B cells (figure 3E). Rapamycin also strongly inhibited T-bet expression at mRNA level in stimulated B cells (figure 3F). To differentiate whether the inhibition of mTORC1 activation and T-bet expression by rapamycin is due to the inhibition of proliferation, we focused on non-proliferating B cells. The results showed that the phosphorylation of S6(Ser235/236) and T-bet expression were not dependent on proliferation and rapamycin strongly decreased the frequencies of p-S6$^+$ and T-bet$^+$ B cells in gated live non-proliferating B cells (online supplementary figure 4G,H). Collectively, these studies reveal a potential link of mTORC1 activation and the differentiation of T-bet$^+$ B cells.

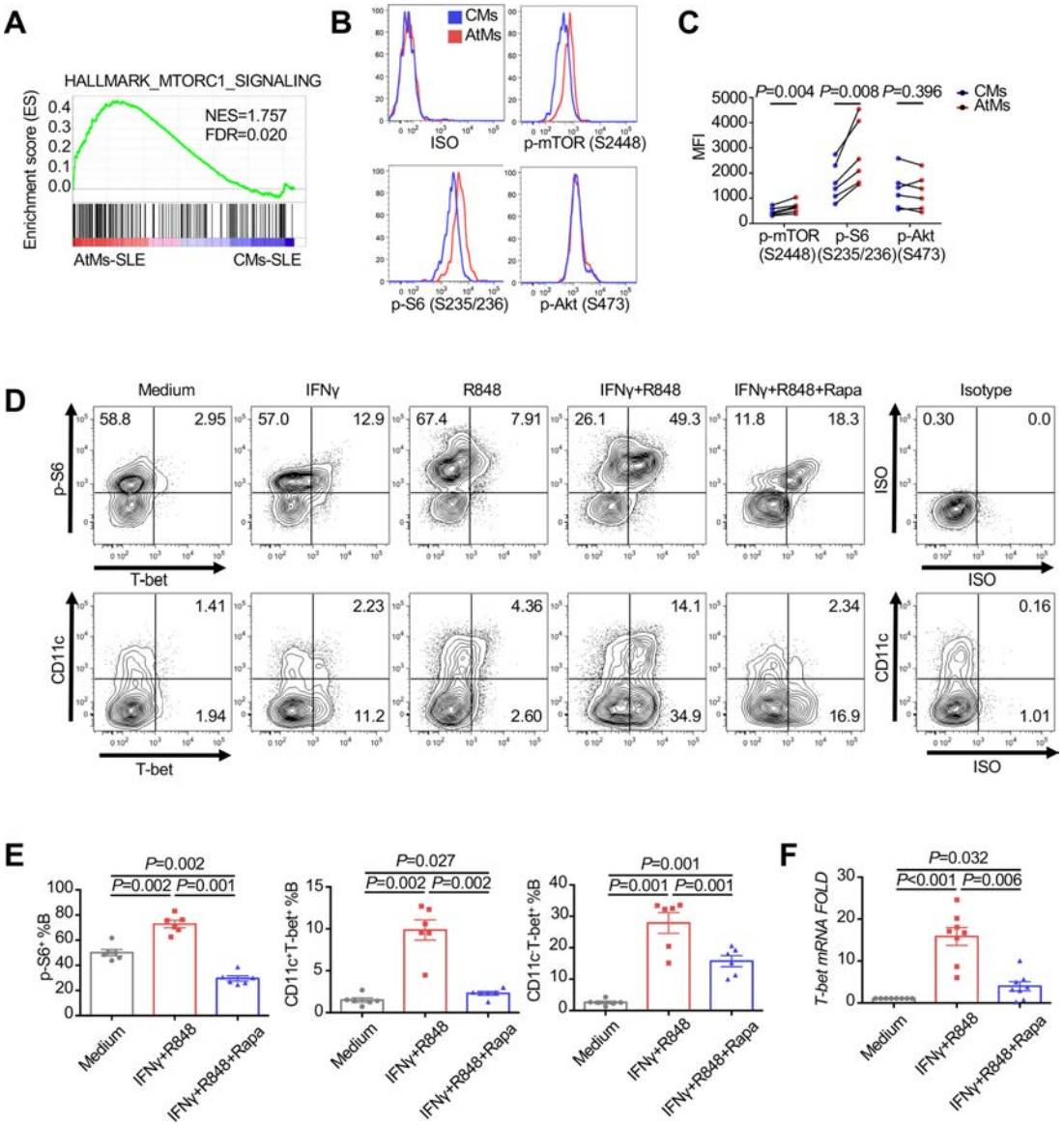

Figure 3 mTORC1 signalling pathway was implicated in the differentiation of atypical memory B cells (AtMs). (A) Gene set enrichment analysis (GSEA) identified that the mTORC1 signalling pathway was enriched in lupus AtMs compared with CMs. (B) Differential expression of mTOR signalling-related phosphorylated proteins between AtMs and CMs from the same patient with SLE, analysed by flow cytometry. One representative experiment out of four was shown. (C) Comparison of mean fluorescence intensities (MFI) of the mTOR signalling pathway-related phosphorylated proteins between AtMs and CMs from the same patients with SLE (n=6). (D–E) Representative flow cytometry plots (D) and accumulated data (E) of the frequencies of p-S6^{+}, CD11c^{-}T-bet^{+} and CD11c^{+}T-bet^{+} B cells generated from stimulated healthy donor B cells (n=6). For D, B cells were stimulated with IFNγ and R848 alone or in combination in the absence or presence of 0.1 nM rapamycin (rapa). For E, B cells were stimulated with the combination of IFNγ and R848 in the absence or presence of 0.1 nM rapamycin. (F) T-bet mRNA levels were measured in stimulated B cells for 6 hours (n=8). The stimulation conditions were the same as in (E) and the concentration of rapamycin was 10 nM. Error bars indicated mean±SEM. *P* values were determined by paired *t* test (C) and one-way analysis of variance (ANOVA) with Holm-Sidak's multiple comparisons test (E and F). CMs, classical memory B cells; NES, normalised enrichment score; FDR, false discovery rate; ISO, isotype; mTOR, the mammalian target of rapamycin; SLE, systemic lupus erythematosus.

Lupus AtMs are enriched with antinucleosome reactivity despite impaired terminal differentiation

There have been inconsistent reports for the terminal differentiation potentials of AtMs, with evidence of normal[17 33] or resistant[12 27 34] to plasma cell differentiation. Our RNA-seq data showed that lupus AtMs expressed higher levels of B cell identity genes including *PAX5*, *BCL6* and *AICDA*, and higher amounts of plasma cell-related genes like *IRF4*, *XBP1* and *SLAMF7* (figure 4A). At the protein level, AtMs expressed high levels of PAX5, BCL6 and IRF4 (figure 4B). PAX5 and BCL6 are TFs to maintain B cell identity while IRF4 is considered to promote plasma cell differentiation,[35] thus AtMs seem to be in an aberrant state of differentiation.

Then we tested the terminal differentiation capacity of sorted lupus AtMs and CMs with a strong stimulating protocol (cytosine-phosphate-guanidine (CpG) 2006/IL-2/IL-10) for 7 days. We found that lupus AtMs displayed a compromised capacity to differentiate into CD27^{hi}CD38^{hi} plasmablasts (figure 4C,D) and to secrete immunoglobulins compared with CMs (figure 4E). Interestingly, after normalisation to the same concentrations, the

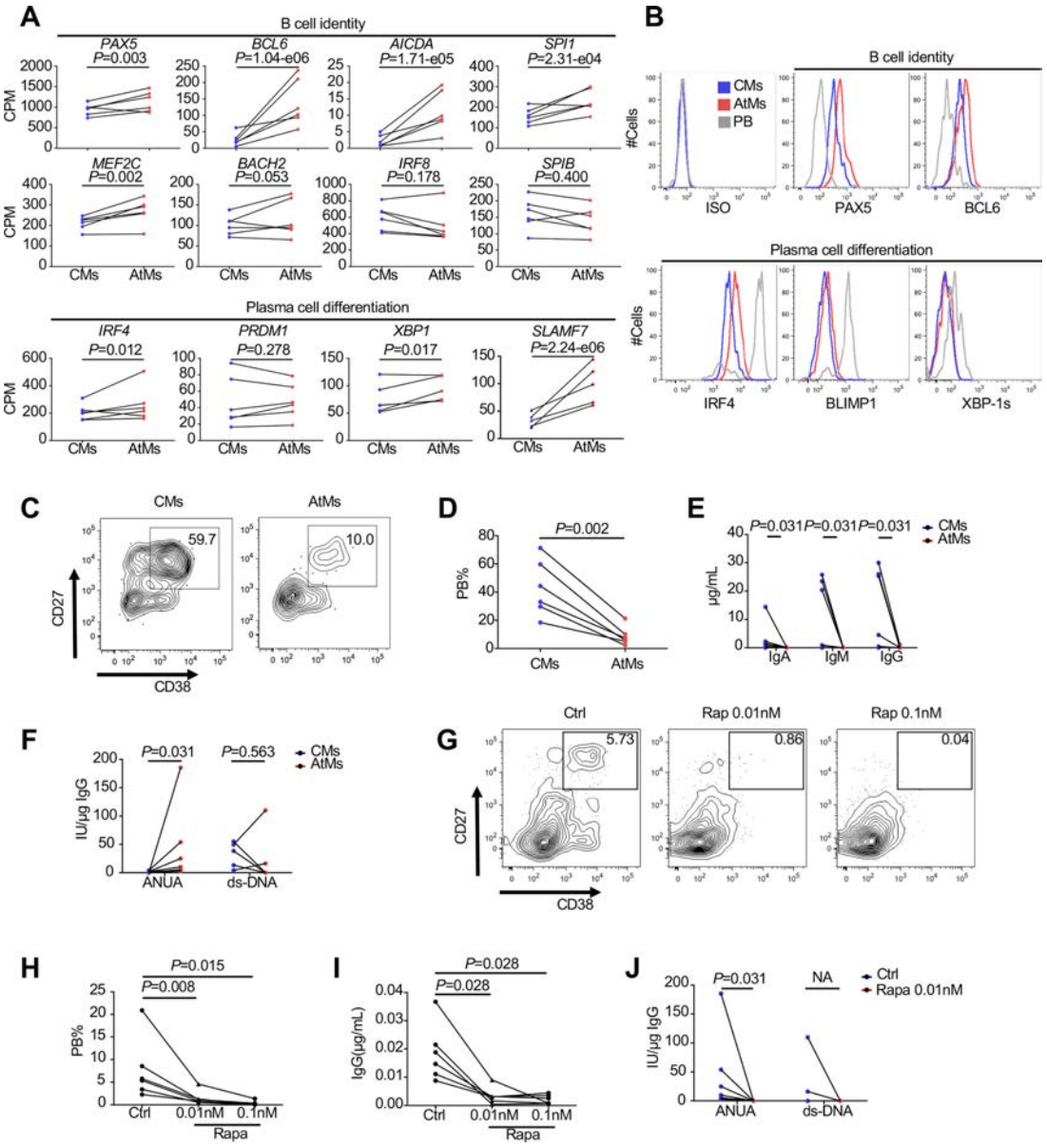

Figure 4 AtMs exhibited altered ability of antibody secretion. (A) RNA-Seq data were used to compare the expression profiles of B cell identity and plasma cell differentiation-related genes between AtMs and CMs from active patients with SLE (n=6). (B) Flow cytometry analysis to show B cells and plasma cell differentiation-related transcription factors between CMs and AtMs from the same patients with SLE. One representative experiment out of four was shown. (C–D) Representative flow cytometry plots (C) and accumulated data (n=6) (D) to show the capacity of lupus AtMs and CMs to differentiate into $CD27^{hi}CD38^{hi}$ plasmablasts (PB) following the stimulation of CpG +IL-2+IL-10 for 7 days. Shown were the frequencies among live cells. (E–F) Comparison of the concentrations of secreted immunoglobulin IgA, IgM and IgG (E) or ANUA and anti-dsDNA levels based on the normalised IgG concentration (F) between AtMs and CMs from patients with SLE (n=6). (G–H) Representative flow cytometry plots (G) and accumulated data (H) (n=6) to show the capacity of lupus AtMs to differentiate into $CD27^{hi}CD38^{hi}$ plasmablasts following the stimulation of CpG +IL-2+IL-10 in the absence or presence of 0.01 nM and 0.1 nM rapamycin (rapa) for 7 days. (I–J) Changes in the concentration of IgG (I), ANUA and anti-dsDNA levels (J) secreted by AtMs-SLE after adding rapamycin (n=6). For A, Limma+voom (paired samples test) was used for differential analysis and *P* values were adjusted by the Benjamini-Hochberg multiple test. *P* values were determined by paired *t* test (D), Wilcoxon signed-rank test (E,F,J), one-way analysis of variance (ANOVA) with Holm-Sidak's multiple comparisons test (H), and Friedman test with Dunn's multiple comparisons test (I). ANUA, antinucleosome antibody; AtMs, atypical memory B cells; CMs, classical memory B cells; ISO, isotype; SLE, systemic lupus erythematosus.

secreted IgG from AtMs showed an enrichment of ANUA, but not anti-dsDNA antibodies compared with that of CMs (figure 4F). It was reported that mTORC1 signalling was important for plasma cell differentiation and antibody secretion;[32 36] we found that rapamycin significantly blocked plasmablast generation, IgG and ANUA production from stimulated AtMs (figure 4G–J). Thus, the above studies indicate that lupus AtMs represented a unique B cell population aberrantly differentiated and enriched with antinucleosome reactivity. Furthermore, inhibiting the mTORC1 pathway could effectively block their terminal differentiation and the capacity to secrete autoantibodies.

Lupus AtMs are molecularly and phenotypically dysfunctional

We observed a panel of genes encoding inhibitory receptors (eg, *PDCD1*, *FCRL3*, *ILIRB1*, *ILIRB2*) linked to dysfunction

or exhaustion in T cells[12 27] were highly expressed in lupus AtMs (online supplementary figure 5A), and the dysfunctional phenotype was confirmed at the protein levels for most markers detected (online supplementary figure 5B). Furthermore, markers of B cell development and activation also showed aberrations at molecular and protein levels (online supplementary figure 5A and 6).

TFs play a decisive role in coordinating cell fate and functions, however, the TF network of AtMs is not clear. We found that lupus AtMs expressed a similar TF gene profile to dysfunctional T cells,[37 38] including increased expression of *BATF, FOXO1, IKZF2, NFATc2, MAF* and decreased expression of *MAML2, ZEB2, ZBTB20* and *TCF7* (online supplementary figure 5C). The enhanced expressions of NFATc2, BATF, FOXO1 and HELIOS were confirmed by flow cytometry (online supplementary figure 5B). Interestingly, we also found that lupus AtMs expressed higher levels of HIF1α and c-Myc (online supplementary figure 5B), two TFs associated with T cell dysfunction[39 40] and metabolism.[41 42] Thus, the dysfunctional phenotype of lupus AtMs could be linked to a unique metabolic status.

Lupus AtMs are apoptosis-prone, defective for proinflammatory cytokine production and poorly co-stimulate T cells to proliferate

Next we investigated the survival capacity of lupus AtMs. GSEA identified an apoptotic gene signature for lupus AtMs (figure 5A) and several antiapoptotic genes *BCL2, PIM1, PIM2* and *PIM3* were all decreased in AtMs compared with CMs (online supplementary figure 7A). The downexpression of BCL2 was confirmed at the protein level in AtMs by flow cytometry (online supplementary figure 7B). Consistent with these data, we found that the sorted lupus AtMs displayed significantly less survival advantage and higher frequency of active caspase-3-positive cells than CMs following overnight culture (figure 5B,C).

The RNA-seq data showed that lupus AtMs expressed lower proinflammatory molecules *IL6* and *Tumor Necrosis Factor (TNF)* and higher anti-inflammatory molecules *IL10* and *Transforming Growth Factor Beta-1 (TGFB1)* (online supplementary figure 7C). We found that lupus AtMs showed much reduced capacity to produce proinflammatory cytokine IL-6 and TNF-α following polyclonal stimulations (figure 5D and online supplementary figure 7D).

B cells also play an active role in co-stimulating T cells.[4] Compared with lupus CMs, AtMs exhibited a significantly decreased capacity to co-stimulate $CD4^+$ T cells to proliferate, as checked by carboxyfluorescein succinimidyl ester (CFSE) dilution (figure 5E), which was accompanied with a diminished survival of AtMs (figure 5F). These results suggest that lupus AtMs are apoptosis-prone, defective for proinflammatory cytokine production and poorly provide co-stimulatory signals to T cells under the current experimental conditions.

AtMs are heavily infiltrated into the kidneys of patients with LN

Finally we performed an in situ study of AtMs in renal tissues of patients with LN by multiplexed immunohistochemistry (figure 6A and online supplementary figure 8A). The renal tissues from patients with LN (detailed characteristics are in online supplementary table 5) showed a tendency of increased infiltration of $CD20^+$ B cells compared with controls (online supplementary figure 8B). Interestingly, $CD20^+$T-bet^+ AtMs were abundantly detected in the renal tissues of patients with LN compared with controls for both frequency and density (figure 6B). Consistent with the data from peripheral AtMs, we observed that T-bet^+ AtMs from renal tissues of patients with LN displayed significantly higher levels of phosphorylated S6(Ser235/236) than T-bet^- B cells (figure 6C,D).

The frequency of AtMs in renal tissues was positively correlated with that in peripheral blood (figure 6E), suggesting that peripheral AtMs frequency may predict the degree of renal AtMs infiltrations. Furthermore, the frequency of renal AtMs was significantly higher in a group with high renal SLEDAI Scores (12~16) than the group with low renal SLEDAI Scores (0~4) (figure 6F). When different pathological types were taken into consideration, type IV LN showed a significantly higher frequency of renal AtMs than that of type III+V (figure 6G). Moreover, the density of renal AtMs was positively correlated with SLEDAI Scores, serum anti-dsDNA titres, urine proteins and renal function indicators while negatively correlated with serum C3 and albumin levels (online supplementary figure 8C,D). These results clearly demonstrated that the degree of renal AtM infiltration is closely associated with disease activity and renal dysfunction, hinting that infiltrated AtMs may directly play a pathogenic role in situ.

DISCUSSION

The identification of AtMs with a phenotype of $CD19^{hi}CD21^{lo/-}$ in lupus can be dated back to 2002.[9] Since then, a series of studies have reported phenotypically similar B cells to be expanded in patients with lupus.[5 8–11 17 23 33] By integrating phenotypical, molecular, functional and histological approaches, the current study adds new evidence that AtMs are abnormally differentiated with metabolic and functional dysregulation, and associated with disease activity. Particularly, we have identified that the mTORC1 pathway is highly activated in lupus AtMs and potentially involved in their generation and terminal differentiation.

In mice, it has been shown that ABCs (murine AtMs) arose from follicular B cells[18] and a lot of factors contributed to their generation, including TLR7, IFN-γ, BCR, IL-21, CD40 and major histocompatibility complex (MHC) II molecules.[19 20 23 24] Similarly, studies in humans also implicated TLR7/TLR9, IFN-γ and IL-21 in the generation of T-bet^+ AtMs-like B cells.[17 23 33] However, to our understanding, the key signalling pathway leading to their generation has not been unveiled. Interestingly, we identified that the mTORC1 pathway was particularly elevated in human lupus AtMs and blocking this pathway inhibited the differentiation of T-bet^+ AtMs-like B cells. Furthermore, the mTORC1 pathway is known to be critical for plasma cell differentiation and antibody production.[43] We confirmed this point by showing that rapamycin strongly decreased the antibody secretion from stimulated lupus AtMs. Given the promising results from a few clinical trials to use rapamycin (sirolimus) to treat patients with lupus,[44 45] our current study hints that some effect of rapamycin could be via inhibiting the generation and terminal differentiation of AtMs.

AtMs expressing a panel of dysfunctional/inhibitory markers have been reported in HIV[12] and malaria[27] infections, as well as in patients with lupus.[17 33] We extended these studies by demonstrating that lupus AtMs expressed dysfunctional markers, and shared a TF profile similar to dysfunctional T cells.[37 38] Interestingly, lupus AtMs also upregulate the two metabolism-related TFs HIF1α and c-Myc,[41 42] which may be linked with the upstream mTORC1 activation and general metabolic aberrations, raising an important question to be addressed in future study.

There is some inconsistence about the capacity of AtMs to undergo terminal differentiation. Several studies have shown

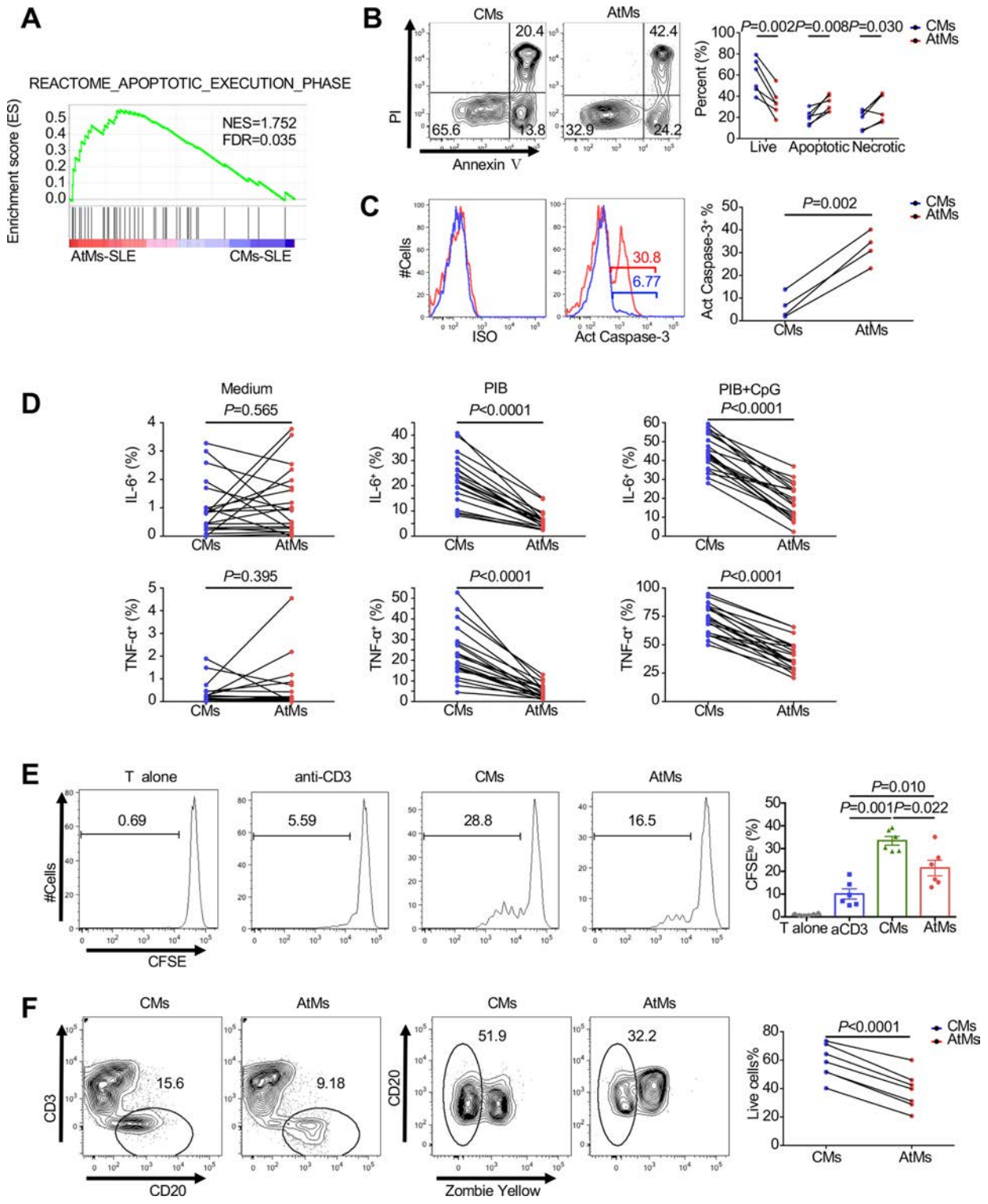

Figure 5 AtMs exhibited increased apoptosis potential, defective for proinflammatory cytokine production and impaired ability to co-stimulate T cells. (A) Gene set enrichment analysis (GSEA) indicated that the apoptotic execution phase pathway was enriched in lupus AtMs compared with CMs. (B) Representative flow cytometry plots and accumulated data showed the survival, apoptosis and necrosis rates of lupus AtMs and CMs cultured for 18–20 hours in vitro (n=6). (C) Flow cytometry analysis and accumulated data to show the differentially expressed active caspase-3 between AtMs and CMs from the same patients with SLE after incubated at 37°C for 20 hours (n=4). (D) Accumulated data showed the capacity of AtMs and CMs from active patients with SLE (n=20) to produce IL-6 and TNF-α. PBMCs were stimulated with PIB or PIB plus CpG for 5 hours and then the cytokines were detected by flow cytometry. (E) Representative flow cytometry plots (left) and accumulated data (right) showed the ability of AtMs and CMs to co-stimulate $CD4^+$ T cells to proliferate in vitro (n=6). (F) Representative flow cytometry plots (left) and accumulated data (right) to show the survival rate of CMs and AtMs co-clutured with $CD4^+$ T cells (n=6). Error bars indicated mean±SEM. *P* values were determined by paired *t* test (B–D, F) and one-way analysis of variance (ANOVA) with Holm-Sidak's multiple comparisons test (E). AtMs, atypical memory B cells; CMs, classical memory B cells; NES, normalised enrichment score; FDR, false discovery rate; PI, Propidium Iodide; ISO, isotype; PIB, PMA+Ionomycin+Brefeldin A; CpG, cytosine-phosphate-guanidine; CFSE, carboxyfluorescein succinimidyl ester; PBMCs, peripheral blood mononuclear cells; SLE, systemic lupus erythematosus.

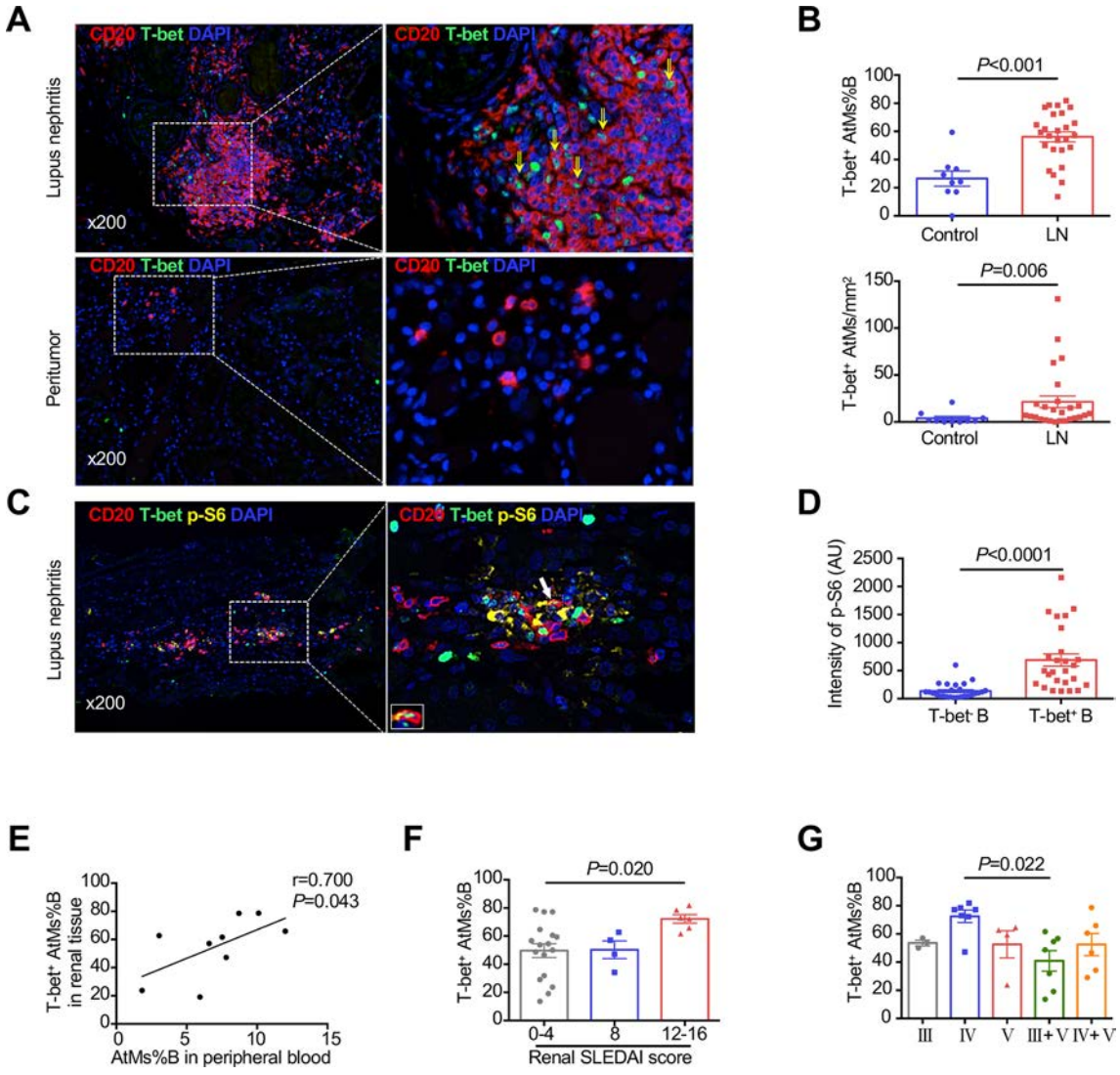

Figure 6 T bet$^+$ AtMs infiltrated into the renal tissues of patients with lupus nephritis (LN). (A) Multiplexed IHC was applied to detect the CD20$^+$T-bet$^+$ AtMs in renal tissues of patients with LN. Peritumor renal tissue was used as control. (B) Comparison of the frequency (T-bet$^+$ AtMs%B) and density of T-bet$^+$ AtMs in renal tissues of LN (n=27) and peritumor renal tissues (n=10). (C) T-bet$^+$ B cells showed higher expression of p-S6 than T-bet$^-$ B cells in renal tissues of patients with LN. (D) Comparison of the fluorescent intensity of p-S6 in 26 T-bet$^-$ and 26 T-bet$^+$ B cells in renal tissues from three patients with LN. (E) Correlation between T-bet$^+$ AtMs%B in renal tissues and AtMs%B in peripheral blood from the same patients with LN (n=9). (F) Patients with LN were divided into three groups based on renal SLEDAI Scores and the frequency of renal T-bet$^+$ AtMs was compared among the three groups (n=17, 10, 6, respectively). (G) Comparison of the frequency of renal T-bet$^+$ AtMs among different pathological types of patients with LN (type III (n=3), type IV (n=7), type V (n=4), type III +V (n=7) and type IV +V (n=6)). Error bars indicated mean±SEM. *P* values were determined by Mann-Whitney test (B and D), Spearman's rank correlation (E) and Kruskal-Wallis test with Dunn's multiple comparisons test (F and G). AtMs, atypical memory B cells; AtMs%B, frequency of AtMs among total B cells; AU, arbitrary unit; IHC, immunohistochemistry.

the compromised capacity of AtMs to undergo terminal differenation[12 27 34] and we obtained similar results. By contrast, in one lupus study, following the stimulation of TLR7/IL-21/IL-10, although lupus AtMs did show a proliferation defect compared with CMs, they produced comparable amount of IgG on a per cell basis.[33] Using activated T cells as the stimulus, Wang *et al* found similar frequencies of antibody-secreting cells and IgG secretion from lupus AtMs and CMs.[17] While the difference from the above studies can be ascribed to the different experimental conditions, it should be mentioned that lupus AtMs are in a paradoxically differentiated status: AtMs express high levels of both B cell identity genes *SPI1*, *MEF2C*, *PAX5* and *BCL6*, as well as plasma cell-driving genes *IRF4*, *XBP1* and *SLAMF7*. Our tentative explanation is that AtMs are in fact dragged by two opposing differentiation forces. Most likely AtMs are not essentially poised to plasma cell differentiation themselves, however, they may be efficiently mobilised into plasma cell pool under certain pathogenic conditions like lupus flare.

Our and other studies have shown that lupus AtMs are highly enriched with antinuclear autoantibodies, including Sm, Nucleosome and Chromatin.[8 16 17 19] It's interesting to ask how these pathogenic antibodies are generated and diversified in vivo. The isotype non-switched lupus AtMs (named activated naive B cells) showed higher IgM mutation rates than those of naïve B cells,[46] indicating that a mechanism of antibody mutation is working for non-switched AtMs. For switched AtMs, the IgG mutation rates were lower than those of switched memory B cells from the same patients with lupus.[33] Together with lower expressions of CXCR4 and CXCR5 on AtMs, the above evidence suggests that AtMs are a population of extrafollicular B cells independent of

germinal centre origin. Intriguingly, we found that lupus AtMs express high levels of *AICDA*, which encodes activation-induced cytidine deaminase (AID), a molecule critical for somatic mutation and immunoglobulin isotype switching.[47] We propose that AtMs are uniquely differentiated from a population of self-reactive naïve B cells[48] in the context of chronic stimulation. High expression of PAX5 and BCL6 will ensure B cell identity in order for AID to perform its function and generate autoantibodies with sufficient affinity. In the context of disease flare, lupus AtMs could be abundantly expanded and mobilised into plasma cells to produce high levels of pathogenic autoantibodies.

In summary, the current study reveals new molecular and functional features of lupus AtMs. Particularly, as mTORC1 is required for both the generation and the terminal differentiation of AtMs, targeting mTORC1 pathway may represent an alternative strategy to the current lupus therapies, especially for those patients refractory to conventional regimens.

METHODS

Details of methods are available in online supplementary materials section.

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Contributors CW and SG performed experiments and analysed data. SS, ML, TF and TL performed experiments. QF, QG, SC, FC, ZC, YL and PH analysed data. XC and YH provided reagents. QG, NS, CB and XZ designed the research. CW, QF and XZ wrote the manuscript.

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CLINICAL SCIENCE

Anti-Ku syndrome with elevated CK and anti-Ku syndrome with anti-dsDNA are two distinct entities with different outcomes

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ABSTRACT

Objective To refine the spectrum of anti-Ku-associated disease, a condition that is equivocally described by current diagnostic criteria for connective tissue diseases.

Methods Among 42 consecutive patients harbouring anti-Ku antibodies, subgroups with similar phenotypes and prognosis were delineated without an a priori diagnosis using hierarchical clustering analysis of the cumulative clinico-biological features recorded during the follow-up. Features present at baseline that most efficiently predicted the outcomes were then identified using a sensitivity–specificity sum maximisation approach.

Results Clinico-biological features were clustered into three groups. Glomerulonephritis and ILD, the two fatal complications in this cohort, were unequally distributed between the three clusters that additionally differed on six clinico-biological features.

Among features present at baseline, elevated serum level of creatine kinase (CK) and anti-dsDNA antibodies were generally mutually exclusive and most efficiently predicted the cluster belonging at last follow-up. Anti-Ku patients with elevated CK had a 22-fold higher risk of ILD while anti-Ku patients with anti-dsDNA antibodies had a 13-fold higher risk of glomerulonephritis

Conclusion "Anti-Ku with elevated CK" syndrome and "anti-Ku with anti-dsDNA" syndrome represent two distinct entities that are important to recognise in order to best tailor patient care.

Key messages

What is already known about this subject?

► Autoantibodies are useful biomarkers when they delineate subgroups of patients with homogeneous manifestations and prognosis. In this regard, the usefulness of anti-Ku, a rare autoantibody, is not yet established.

What does this study add?

► We demonstrate herein that anti-Ku patients with anti-dsDNA and anti-Ku patients with elevated creatine kinase (CK) are two distinct subgroups, rarely overlapping, with significant implications for patient care. Glomerulonephritis frequently occurred in anti-Ku patients with anti-dsDNA at baseline, whereas anti-Ku patients with elevated CK at baseline frequently developed interstitial lung disease.

How might this impact on clinical practice or future developments?

► "Anti-Ku with elevated CK" syndrome and "anti-Ku with anti-dsDNA" syndrome represent two distinct entities that are important to recognise in order to best tailor patient care.

INTRODUCTION

Autoantibodies are useful biomarkers when they delineate subgroups of patients with homogeneous manifestations and prognosis. In this regard, the usefulness of anti-Ku, a rare autoantibody, is not yet established.

On the one hand, anti-Ku antibodies have been associated with various clinical manifestations and subsequent diagnoses which require distinct management. These diagnoses include myositis, systemic sclerosis (SSc), systemic lupus erythematosus (SLE), mixed connective tissue disease (MCTD), Sjögren's syndrome (SS) and rheumatoid arthritis (RA). In addition, a proportion of anti-Ku patients are moreover not classifiable either because they do not match available criteria (undifferentiated connective tissue disease (UCTD)) or because they conversely match criteria for several connective tissues diseases (overlap syndrome).[1–4]

On the other hand, data have indicated that, in the setting of a given condition, anti-Ku-related disease may represent an entity in its own right. Indeed, in SSc patients, anti-Ku-antibodies have been linked with myositis and arthritis but negatively associated with fingertip ulcers.[5 6] Accordingly, myositis patients with anti-Ku antibodies have a higher risk of lung involvement but lower risk of associated cancer,[2 7] this distinct phenotype being additionally associated with a distinct genotype.[8] In SLE, anti-Ku antibodies are common in African but rare in white-American patients,[9 10] although whether this translates at the clinical level remains unanswered.

In light of the above, we endeavoured to refine the spectrum of anti-Ku-associated disease by identifying subgroups of anti-Ku-positive patients with similar clinico-biological features and prognosis, without an a priori diagnosis. This enabled us to

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overcome the heterogeneity of diagnoses yielded by available criteria for connective tissue diseases.

METHODS

Patients

Patients with anti-Ku antibodies (positive line immunoassay, verified by immunodiffusion) were identified in the database of the University Hospital of Strasbourg which includes data recorded from consecutive inpatients and outpatients over the 1995–2018 period. Our hospital hosts the national accredited public referral centre for rare autoimmune diseases (Centre de Référence des Maladies Auto-immunes Rares).

Serological data

All sera from patients with suspected connective tissue disease were tested for immunofluorescence on HEp-2 cells (Zeus Scientific, Branchburg, New Jersey, USA). Titres ≥1/160 were considered positive. The antinuclear antibody (ANA) pattern originally associated with anti-Ku is nuclear fine speckled (AC-4 as defined by the ICAP[11]) without nucleoli staining.[12] However, homogeneous (AC-1), nuclear fine speckled with nucleoli (AC-4) and nuclear large/coarse speckled (AC-5) staining patterns hampered the assessment of this ANA pattern. Thus, all sera that yielded AC-1, AC-4 or AC-5 ANA patterns were tested for anti-Ku antibodies at a serum dilution of 1/150 using line immunoassay (ANA10DIV-24, D-Tek, Mons, Belgium). Positivity was confirmed by immunodiffusion (Auto I.D. ref 6050 Immuno Concept). Anti-SSA/Ro60, anti-SSB/La, anti-Sm, anti-RNP, anti-Scl70, anticentromere and anti-Jo1 antibodies were investigated using line immunoassay (D-Tek). Anti-dsDNA antibodies (Kallestad Anti-dsDNA Microplate EIA, Bio-Rad, Hercules, California, USA), anticitrullinated peptide/protein antibodies (Euro Diagnostica, Malmö, Sweden) and rheumatoid factors (in-house assay) were detected by ELISA. Anticardiolipin antibodies were detected by fluorescence enzyme immunoassay (ThermoScientific, Waltham, Massachusetts, USA), anti-β2GPI antibodies were detected by QUANTA-Lite (Werfen, Artarmon, Australia) and lupus anticoagulant activity was detected according to the guidelines of the International Society of Thrombosis and Hemostasis.[13]

CLINICAL DATA

The prospective public referral centre for rare autoimmune diseases registry database was used to summarise the presenting clinical features during follow-up.

- Elevated serum levels of creatine kinase (CK) was defined by levels above the upper limit of normal.[14]
- Dysphagia was defined as pharyngeal and/or oesophageal signs when eating and/or drinking (eg, difficulty swallowing solid and/or liquids, food sticking in throat, coughing while eating).
- Pulmonary involvement was defined by interstitial lung disease (ILD) on high resolution CT scan of the chest, sufficient for the diagnosis as previously defined.[15]
- Arthralgia was defined by inflammatory joint pain.
- Renal involvement was defined and categorised as follows: (1) renal crisis: new onset of blood pressure >150/85 mm Hg, with decrease in renal function[16] or (2) glomerulonephritis: proteinuria (>0.5 g/24 hours)[17] without alternative cause and/or kidney biopsy demonstrating immune-mediated glomerulonephritis.
- Cutaneous involvement was diagnosed and categorised as lupus rash, skin thickening, telangiectasia, dermatomyositis rash and/or mechanic's hand ascertained by experienced physicians.
- Haematological involvement consisted of cytopenia on two blood samples, without other causes than the connectivitis.
- Thrombotic manifestations included arterial or venous thrombosis.
- Echocardiography was used to screen for pulmonary hypertension.[18]
- Serositis was defined as pericarditis and/or pleural effusion.

Diagnoses were systematically retrospectively reviewed, using American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) criteria for SSc, myositis, SLE, RA and SS.[19] MCTD and antiphospholipid syndrome (APLS) were respectively diagnosed using criteria proposed by Alarcón-Segovia *et al*[20] and Miyakis *et al*.[13] When symptoms recorded during the entire follow-up did not match available criteria for a connective tissue disease, the patient was classified as UCTD.

Statistical analyses

Detailed description of the statistical methods used in this study is available in the Supplementary Material.

RESULTS

A total of 47 anti-Ku-positive patients were identified (eg, representing), 188 out of 34 486 sera tested for anti-Ku after ANA pattern screening. Complete data were available in 42 cases. The study flowchart is illustrated in online supplementary figure S1.

Clinical features are summarised in table 1. Patients most frequently matched the criteria for SLE, myositis and primary Sjögren's syndrome. However, a third of the patients were equivocally classified with available criteria since 5 (12%) met the criteria for several connective tissue diseases (even after exclusion of secondary SS and APLS) while 7 (17%) had UCTD.

During the 63.7 months of follow-up (range 12–226), three patients (7%) died: one from complications of lung transplantation for ILD, one from pulmonary infection during cyclophosphamide treatment for ILD and one from complications of terminal renal failure due to glomerulonephritis. In addition, another patient was on chronic haemodialysis after extramembranous glomerulonephritis relapse on a kidney-graft.

Given that anti-Ku-positive patients were heterogeneously classified with available criteria, hierarchical clustering was performed on 28 clinico-biological data (table 1) in order to identify subgroups with similar clinico-biological features and prognosis. Three clusters were identified (figure 1A).

Importantly, glomerulonephritis and ILD, the two fatal complications in this series, were differentially distributed between these three groups: lung involvement was exclusively present in cluster no 1 while renal involvement was 6-fold and 14-fold higher in cluster no 3 comparatively to clusters numbers 1 and 2 ($p<0.0001$) (figure 1C). The three clusters also significantly differed on six other clinico-biological parameters including elevated CK level and muscle weakness, which were more frequent in cluster no 1, while lupus rash, cytopenia, positive anti-dsDNA and positive anti-RNP were more frequent in cluster no 3 (table 1).

In a sensitivity–specificity sum maximisation approach (figure 1D), among signs present at baseline examination, elevated CK was the most powerful parameter for identifying patients from cluster no 1 (sensitivity 93%; specificity 96%) while presence of anti-dsDNA antibodies was the most powerful criterion for identifying patients from cluster no 3 (sensitivity 89%; specificity 94%).

Table 1 Clinical characteristics of anti-Ku patients in cluster numbers 1–3

		Cluster			
	(n=42)	C1 (n=15)	C2 (n=18)	C3 (n=9)	P value
Demographic data					
Age, years (range)	39.1 (11.3–74.6)	60.5 (22.6–74.3)	39.1 (17.5–74.6)	17.9 (11.3–64.2)	0.29
Sex (F/M)	35/7	13/2	14/4	8/1	1.0
Geographical origin (Caucasia/Africa)	31/11	11/4	14/4	6/3	1.0
Organ involvements (ever)					
Arthralgia	35 (83)	11 (73)	15 (83)	9 (100)	1.0
Raynaud's phenomenon	18 (43)	9 (60)	6 (33)	3 (33)	1.0
Increased CK	15 (35)	**14 (93)**	**1 (6)**	**0**	**<0.001**
Muscle weakness	8 (19)	**8 (53)**	**0**	**0**	**0.0039**
Dysphagia	6 (14)	6 (40)	0	0	0.056
ILD*	13 (31)	**13 (87)**	**0**	**0**	**<0.001**
Renal involvement†	10 (24)	**2 (13)**	**1 (6)**	**7 (78)**	**0.0036**
Cytopenia	13 (31)	**3 (20)**	**1 (6)**	**9 (100)**	**<0.001**
Serositis	8 (19)	3 (20)	1 (6)	4 (44)	1.0
Lupus rash	9 (21)	**1 (7)**	**0**	**8 (89)**	**<0.001**
Telangiectasia	2 (5)	2 (13)	0	0	1.0
Sclerodactyly	2 (5)	2 (13)	0	0	1.0
Skin thickening proximal to the MCP	1 (2)	1 (7)	0	0	1.0
Mechanic's hand	3 (7)	3 (20)	0	0	1.0
Thrombosis	9 (21)	5 (33)	2 (11)	2 (22)	1.0
Neuropathy	4 (9)	1 (7)	0	3 (33)	0.66
Myocarditis	1 (2)	1 (7)	0	0	1.0
Autoantibodies (ever)					
Anti-dsDNA‡	10 (24)	**2 (13)**	**0**	**8 (89)**	**<0.001**
Anti-SSA/Ro60	13 (31)	5 (33)	3 (17)	5 (56)	1.0
Anti-SSB/La	3 (7)	0	1 (6)	2 (22)	1.0
Anti-Sm	2 (5)	0	0	2 (22)	0.81
Anti-RNP	5 (12)	**0**	**0**	**5 (56)**	**<0.001**
Rheumatoid factor	13 (31)	4 (27)	9 (50)	0	1.0
ACPA	5 (12)	1 (7)	3 (17)	1 (11)	1.0
Antiphospholipid biology§	7 (17)	1 (7)	1 (6)	5 (56)	0.064
Matched CTD criteria (at last follow-up)					
SLE	8 (19)	**1 (7)**	**0**	**7 (78)**	**<0.001**
Myositis	10 (24)	**10 (67)**	**0**	**0**	**<0.001**
SS¶	15 (36)	4 (27)	9 (50)	2 (22)	1.0
pSS	11 (26)	2 (13)	9 (50)	0	0.27
MCTD	5 (12)	**0**	**0**	**5 (56)**	**<0.001**
SSc	2 (5)	2 (13)	0	0	1.0
RA	4 (10)	0	4 (22)	0	1.0
APLS**	4 (10)	1 (7)	1 (6)	2 (22)	1.0
UCTD	7 (17)	2 (13)	5 (28)	0	1.0
>1 CTD	11 (26)	4 (27)	1 (6)	6 (67)	0.12
>1 CTD (after exclusion of sSS and APLS)	5 (12)	2 (13)	0	3 (33)	1.0
Associated cancer††	4 (10)	1 (7)	2 (11)	1 (11)	1.0

C1 to 3: clusters 1, 2 and 3 were identified by multiple correspondence analyses of the 28 cumulative clinico-biological features of the 42 anti-Ku patients.
Statistically significant results are in bold.
*CT scan patterns were NSIP (n=8), UIP (n=2), OP (n=2); median vital capacity was 66% (range 20–124) and transfer factor of the lung for carbon monoxide was 57% (range 15–94).
†Renal involvement consisted of glomerulonephritis in all cases. Kidney biopsy (available in eight patients) revealed lupus glomerulonephritis class IV (n=5), extracapillary glomerulonephritis (n=2) and proliferative glomerulonephritis (n=1).
‡The titre of anti-dsDNA was 146 UI/L (56–300).
§Antiphospholipid biology consisted of the presence of one or more of the following laboratory criteria, on two or more occasions, at least 12 weeks apart: titre of anticardiolipin antibodies (IgG and/or IgM isotype)>40 GPL or MPL (n=4); anti-β2GPI antibodies (IgG and/or IgM isotype)>40 GPL or MPL (n=1) and/or lupus anticoagulant activity (n=4).
¶Patients with sSS were listed both by sSS and by their primary connective tissue disease.
**APLS was secondary in all cases (no patient had primary antiphospholipid syndrome).
††Associated cancer consisted of cancer diagnosed within 3 years (before or after) the diagnosis of idiopathic inflammatory myopathies.
ACPA, anticitrullinated peptide antibodies; APLS, antiphospholipid syndrome;CK, creatine kinase; CTD, connective tissue disease; GPL, IgG Phospholipid Units; ILD, interstitial lung disease;MCP, metacarpophalangeal joint; MCTD, mixed connective tissue disease;MPL, IgM Phospholipid Units; NSIP, non specific interstitial pneumonia; OP, organizing pneumonia; RA, rheumatoid arthritis; RNP, ribonucleoprotein; SLE, systemic lupus erythematosus; SS, Sjögren's syndrome; SSc, systemic sclerosis;UCTD, undifferentiated connective tissue disease; UIP, usual interstitial pneumonia; pSS, primary Sjögren's syndrome; sSS, secondary Sjögren's syndrome.

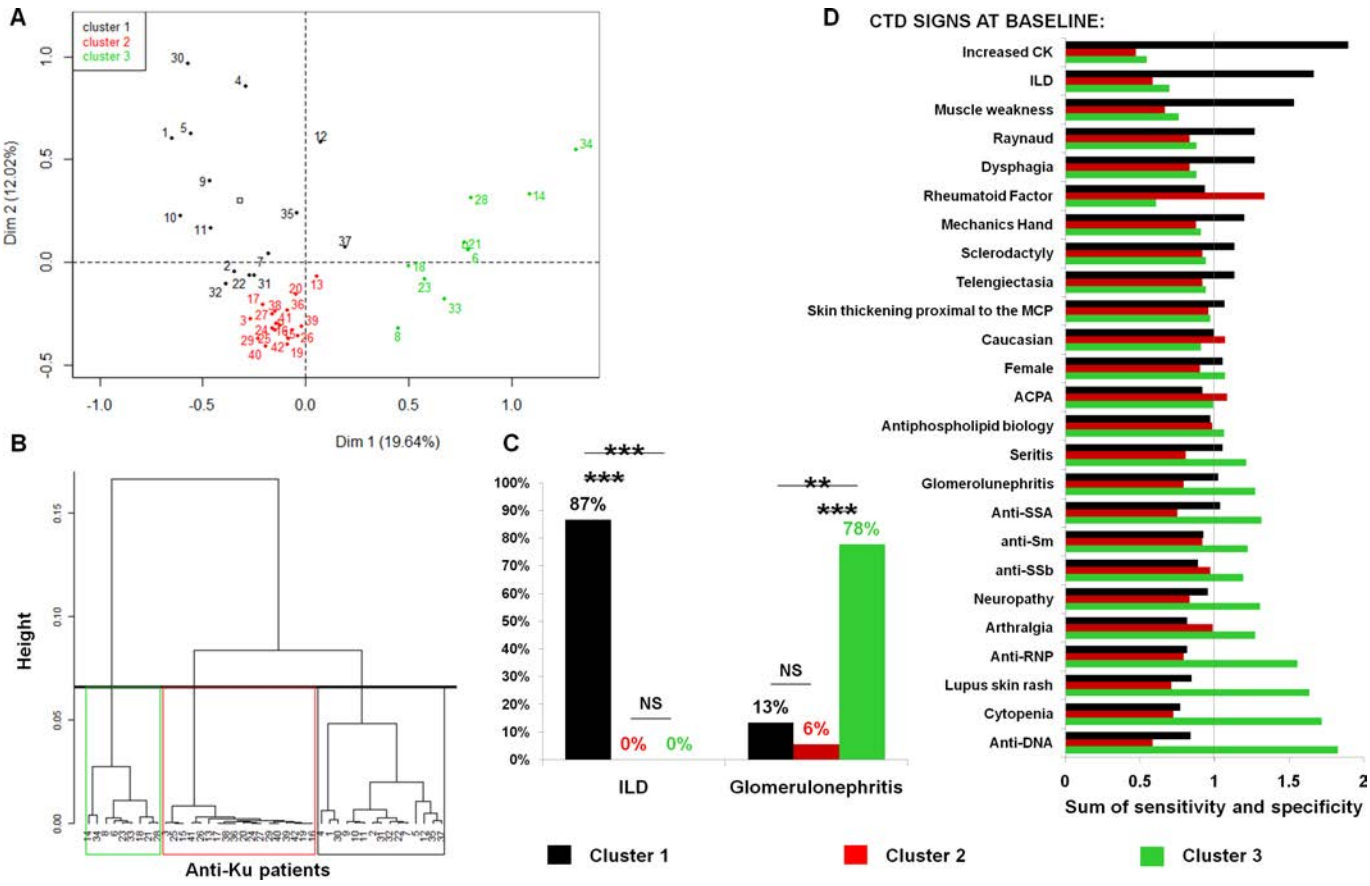

Figure 1 (A) Factorial map of the 42 anti-Ku patients plotted in a multidimensional Euclidean space according to the results of the multiple correspondence analyses of their 28 cumulative clinico-biological features. Each patient is represented by a dot coloured according to the cluster to which she/he belongs (see also partitioning in B). The first two dimensions (DIMs) cumulatively explained 31.7% of the total variance. (B) Dendrogram generated using Euclidean distance in the first three DIMs of the factorial map and the ward agglomerative method. The bold vertical line indicates the height of the fusion into clusters and the x-axis indicates the patients (n=42) at the bottom of the dendrogram. Coloured lines indicate the partitioning of the dendrogram used to delineate the clusters (see the Methods section). (C) Proportion of glomerulonephritis and ILD, the two fatal complications in this series, in the three clusters. (D) Diagnostic performance (assessed by the sum of sensitivity and specificity) of clinico-biological features present at baseline for the diagnosis of cluster numbers 1–3. ACPA,anticitrullinated peptide antibodies; ANA, antinuclear antibodies; CK, creatine kinase; ILD, interstitial lung disease; MCP, metacarpophalangeal joints; RNP, ribonucleoprotein. **, ***, NS: p value <0.005;<0.001 and >0.05, respectively.

Moreover, as shown in table 2, patients with elevated CK at baseline had a higher risk of ILD (relative risk 21.6; 95% CI 2.2 to 266.1) while patients with anti-dsDNA antibodies at baseline had a higher risk of renal involvement (relative risk 12.8; 95% CI 1.3 to 32.2).

DISCUSSION

The present findings demonstrate that patients with anti-Ku antibodies can be clustered into three subgroups with distinct phenotypes and outcomes, easily distinguishable at first evaluation by simple testing, namely: CK serum-level measurement and anti-dsDNA antibodies testing. Anti-Ku patients with elevated CK have a high risk of ILD whereas patients with anti-dsDNA antibodies have a high risk of glomerulonephritis.

This study benefited from a large cohort with regard to the rarity of anti-Ku antibodies.[7] To overcome recruitment bias, patients were identified on the basis of laboratory screening of both inpatients and outpatients from all departments of our institution. To improve the specificity of anti-Ku testing, a combination of immunofluorescence, LIA assay and immunodiffusion was used. Patient charts, along with extensive follow-up, were systematically reviewed. Thus, our data are likely to provide an accurate reflection of the entire spectrum of anti-Ku-related diseases. A potential limitation is that the status for the most recent autoantibodies associated with myositis was not available in all anti-Ku patients with elevated CK.

In accordance with previous findings, our patients with anti-Ku antibodies were not efficiently categorised using currently available criteria for connective tissue diseases. Indeed, our anti-Ku patients initially accounted for a total of seven different CTD diagnoses, which frequently overlapped, while other patients were not categorisable (UCTD). Yet, separate previous studies had indicated that anti-Ku patients with anti-dsDNA rarely had myositis,[4] and that anti-Ku patients with myositis frequently had ILD.[2] We demonstrate herein that anti-Ku patients with anti-dsDNA and anti-Ku patients with elevated CK are two distinct subgroups, rarely overlapping, with significant implications for patient care. Furthermore, in contrast with previous reports based on a single department,[10] our data from unbiased recruitment highlight that glomerulonephritis frequently occurred in anti-Ku patients with anti-dsDNA at baseline and even resulted in terminal renal failure in two cases, one of which was fatal. On the other hand, anti-Ku patients with elevated CK at baseline frequently developed ILD which resulted in a fatal complication

Table 2 Clinical characteristics of anti-Ku patients with versus without increased CK and with versus without anti-DNA

	Increased CK (at first evaluation)			Anti-dsDNA (at first evaluation)		
	Yes, n=15 (%)	No, n=27 (%)	P value	Yes, n=10 (%)	No, n=32 (%)	P value
Demographic data						
Age, years (range)	49.2 (22.6–73.0)	30.6 (11.3–74.6	1.0	18.0 (11.3–68.1)	48.1 (17.5–74.6)	1.0
Sex (F/M)	13/2	22/5	1.0	9/1	26/6	1.0
Geographical origin (Caucasia/Africa)	11/4	20/7	1.0	7/3	24/8	1.0
Organ involvements (ever)						
Increased CK	–	–	–	2 (20)	13 (40)	1.0
ILD*	**12 (80)**	**1 (4)**	**<0.001**	1 (10)	12 (38)	1.0
Arthralgia	12 (80)	23 (85)	1.0	10 (100)	25 (78)	1.0
Raynaud's phenomenon	9 (60)	9 (33)	1.0	3 (30)	15 (47)	1.0
Muscle weakness	**8 (53)**	**0**	**0.0074**	2 (20)	6 (19)	1.0
Dysphagia	6 (40)	0	0.22	1 (10)	5 (16)	1.0
Sclerodactyly	2 (13)	0	1.0	0	2 (6)	1.0
Skin thickening proximal to the MCP	1 (7)	0	1.0	0	1 (3)	1.0
Telangiectasia	2 (13)	0	1.0	0	2 (6)	1.0
Mechanic's hand	3 (20)	0	1.0	1 (10)	2 (6)	1.0
Lupus rash	1 (7)	8 (30)	1.0	**8 (80)**	**1 (3)**	**<0.001**
Thrombosis	5 (33)	4 (15)	1.0	3 (30)	6 (19)	1.0
Myocarditis	1 (7)	0	1.0	0	1 (3)	1.0
Neuropathy	1 (7)	3 (11)	1.0	3 (30)	1 (3)	1.0
Serositis	2 (13)	6 (22)	1.0	4 (40)	4 (13)	1.0
Glomerulonephritis†	2 (13)	8 (30)	1.0	**8 (80)**	**2 (6)**	**<0.001**
Cytopenia	3 (20)	10 (37)	1.0	**9 (90)**	**4 (13)**	**<0.001**
Autoantibodies (ever)						
Anti-dsDNA‡	2 (13)	8 (30)	1.0	---	---	---
Anti-SSA/Ro60	5 (33)	8 (30)	1.0	4 (40)	9 (28)	1.0
Anti-SSB/La	0	3 (11)	1.0	1 (10)	2 (6)	1.0
Anti-Sm	0	2 (7)	1.0	2 (20)	0	1.0
Anti-RNP	0	5 (19)	1.0	4 (40)	1 (3)	0.16
Rheumatoid factor	5 (33)	8 (30)	1.0	0	13 (41)	0.60
ACPA	2 (13)	3 (11)	1.0	1 (10)	4 (12)	1.0
Antiphospholipid biology§	1 (7)	6 (22)	1.0	5 (50)	2 (6)	0.33
Matched CTD criteria (at last follow-up)						
Myositis	**10 (67)**	**0**	**<0.001**	2 (20)	8 (25)	1.0
SLE	1 (7)	7 (26)	1.0	**8 (80)**	**0**	**0.001**
MCTD	0	5 (19)	1.0	4 (40)	1 (3)	0.16
SS¶	4 (27)	11 (41)	1.0	2 (20)	13 (41)	1.0
pSS	2 (13)	9 (33)	1.0	0	11 (34)	1.0
SSc	2 (13)	0	1.0	0	2 (6)	1.0
RA	1 (7)	3 (11)	1.0	0	4 (12)	1.0
APLS**	1 (7)	3 (11)	1.0	2 (20)	2 (6)	1.0
UCTD	1 (7)	6 (22)	1.0	0	7 (22)	1.0

Statistically significant results are in bold.

*CT scan patterns were NSIP (n=8), UIP (n=2), OP (n=2); median vital capacity was 66% (range 20–124) and transfer factor of the lung for carbon monoxide was 57% (range 15–94).

†Kidney biopsy (available in eight patients) revealed lupus glomerulonephritis class IV (n=5), extracapillary glomerulonephritis (n=2) and proliferative glomerulonephritis (n=1).

‡The titre of anti-dsDNA was 146 UI/L (56–300).

§Antiphospholipid biology consisted of the presence of one or more of the following laboratory criteria, on two or more occasions, at least 12 weeks apart: titre of anticardiolipin antibodies (IgG and/or IgM isotype)>40 GPL or MPL (n=4); anti-β2GPI antibodies (IgG and/or IgM isotype)>40 GPL or MPL (n=1) and/or lupus anticoagulant activity (n=4).

¶Patients with sSS were listed both by sSS and by their primary connective tissue disease.

**APLS was secondary in all cases (no patient had primary antiphospholipid syndrome).

ACPA, anticitrullinated peptide antibodies; APLS, antiphospholipid syndrome;CK, creatine kinase; ILD, interstitial lung disease; MCTD, mixed connective tissue disease;pSS, primary Sjögren's syndrome; RA, rheumatoid arthritis; table 1 for other abbreviations.RNP, ribonucleoprotein; sSS, secondary Sjögren's syndrome;SS, Sjögren's syndrome; SLE, systemic lupus erythematosus; SSc, systemic sclerosis;UCTD, undifferentiated connective tissue disease.

in two cases. Although patients in this last subgroup more frequently exhibited signs suggestive of SSc, only two matched the ACR–EULAR criteria for SSc even after a long follow-up. In addition, despite having elevated CK, one-third did not match the ACR–EULAR criteria for myositis. Moreover, it has been reported that even when fulfilling SSc and/or myositis criteria,

patients with anti-Ku antibodies have a distinct phenotype comparatively to anti-Ku-negative patients.[5–8] Altogether, the above data indicate that "anti-Ku with elevated CK" syndrome and "anti-Ku with anti-dsDNA" syndrome represent two distinct entities that are important to recognise in order to best tailor patient care.

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TRANSLATIONAL SCIENCE

Molecular signature characterisation of different inflammatory phenotypes of systemic juvenile idiopathic arthritis

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ABSTRACT

Objectives The International League of Associations for Rheumatology classification criteria define systemic juvenile idiopathic arthritis (SJIA) by the presence of fever, rash and chronic arthritis. Recent initiatives to revise current criteria recognise that a lack of arthritis complicates making the diagnosis early, while later a subgroup of patients develops aggressive joint disease. The proposed biphasic model of SJIA also implies a 'window of opportunity' to abrogate the development of chronic arthritis. We aimed to identify novel SJIA biomarkers during different disease phases.

Methods Children with active SJIA were subgrouped clinically as systemic autoinflammatory disease with fever ($SJIA^{syst}$) or polyarticular disease ($SJIA^{poly}$). A discovery cohort of n=10 patients per SJIA group, plus n=10 with infection, was subjected to unbiased label-free liquid chromatography mass spectrometry (LC-MS/MS) and immunoassay screens. In a separate verification cohort ($SJIA^{syst}$, n=45; $SJIA^{poly}$, n=29; infection, n=32), candidate biomarkers were measured by multiple reaction monitoring MS (MRM-MS) and targeted immunoassays.

Results Signatures differentiating the two phenotypes of SJIA could be identified. LC-MS/MS in the discovery cohort differentiated $SJIA^{syst}$ from $SJIA^{poly}$ well, but less effectively from infection. Targeted MRM verified the discovery data and, combined with targeted immunoassays, correctly identified 91% ($SJIA^{syst}$ vs $SJIA^{poly}$) and 77% ($SJIA^{syst}$ vs infection) of all cases.

Conclusions Molecular signatures differentiating two phenotypes of SJIA were identified suggesting shifts in underlying immunological processes in this biphasic disease. Biomarker signatures separating SJIA in its initial autoinflammatory phase from the main differential diagnosis (ie, infection) could aid early-stage diagnostic decisions, while markers of a phenotype switch could inform treat-to-target strategies.

Key messages

What is already known about this subject?
- Insights into the biological basis of systemic juvenile idiopathic arthritis (SJIA) increasingly support the existence of an initial autoinflammatory phase of the disease offering a therapeutic 'window of opportunity'.
- Early initiation of effective treat-to-target strategies may change the course of SJIA (Still's disease) and prevent the development of a chronic polyarticular disease phenotype.

What does this study add?
- We identified molecular signatures discriminating SJIA phenotypes and separating SJIA from infection.
- Diagnostic biomarkers could aid early-stage therapeutic decisions in initial SJIA phases, while markers of a phenotype switch could inform treat-to-target strategies.

How might this impact on clinical practice or future developments?
- These findings may have future implications for improving SJIA classification criteria and for the development of precision medicine.

INTRODUCTION

Systemic juvenile idiopathic arthritis (SJIA, or Still's disease) is an autoinflammatory disorder of unknown pathogenesis that accounts for 10%–15% cases of juvenile idiopathic arthritis (JIA).[1] The International League of Associations for Rheumatology (ILAR) criteria for JIA classification define SJIA by the presence of quotidian fever at onset for a minimum of 2 weeks, a transient rash and chronic arthritis.[2] Early in disease, arthritis is minimal or absent, which complicates establishing the diagnosis when features also fitting differential diagnoses such as infections dominate. However, early diagnosis is key to initiate effective treat-to-target approaches.[3 4] In a subgroup of patients, stable remission status can be reached rapidly (monophasic disease),[5] though in most cases SJIA progresses to become recurrent or persistent. Recent findings on pathophysiology suggest a biphasic model of SJIA.[6 7] Innate immune dysregulation is present early with systemic features of an autoinflammatory disorder, while adaptive immunity is thought to dominate later phases with destructive joint disease. Therefore, initial immune dysregulation induced by unknown triggers may then drive autoimmune arthritis. A genetic association of SJIA with major histocompatibility complex class II specific allele HLA-DRB1*11 also suggests an autoimmune component.[8]

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Table 1 Clinical and laboratory characteristics of sampled patients

	Discovery cohort, n=30			Verification cohort, n=106		
	SJIA^{syst}	SJIA^{poly}	Infection	SJIA^{syst}	SJIA^{poly}	Infection
Patients, n	10	10	10	45	29	32
Gender (M:F)	4:6	3:7	4:6	30:15	11:18	11:21
Age at diagnosis, years (IQR)	n.a.	n.a.	6 (7)	5 (9)	4 (6)	6 (9)
Age at sample, years (IQR)	7 (5)	15 (6)	6 (7)	9 (8)	11 (5)	6 (9)
Phenotype at sampling, n						
Fever	10	0	10	40	0	27
Arthralgia	7	5	4	25	9	7
Arthritis	4	10	n.a.	14	29	n.a.
Active joints (mean)	1.3 (0–3)	3.6 (3–13)	n.a.	2 (0–5)	6 (5–23)	n.a.
Exanthema	6	0	3	18	0	6
Serositis	1	0	1	9	0	1
Hepatosplenomegaly	4	0	4	9	0	2
Phenotype after sampling						
Monophasic:chronic	7:3	0:10	n.a.	26:19	5:24	n.a.
Medication at sampling						
Antibiotics	1	0	5	0	2	16
NSAID	4	2	2	13	7	6
Methotrexate	2	5	0	6	16	0
Biologics	0	0	0	0	0	0
Steroid	3	2	0	15	12	2
Markers of inflammation at sampling						
ESR (mm/h)	95 (55)	5 (11)	60 (56)	80 (67)	11 (17)	68 (39)
Missing ESR, n	1	1	3	7	3	16
CRP (mg/dL)	11 (7.3)	0.2 (1.4)	13 (5.6)	12.8 (17.9)	0.4 (1.8)	9.7 (8.0)
Missing CRP, n	0	0	0	1	0	4
WCC (×10^9/L)	21 (12)	7 (5.3)	11.8 (7.0)	19 (10)	8 (4)	12 (14)
Missing WCC, n	0	0	0	1	0	4

Values are median (IQR) unless otherwise specified.
CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; NSAID, non-steroidal anti-inflammatory drug; SJIA^{poly}, systemic juvenile idiopathic arthritis with polyarthritis but systemic disease; SJIA^{syst}, SJIA with fever/systemic disease; WCC, white cell count; n.a., not available.

Early diagnosis and recognition of phenotypes could therefore be key to initiate effective treat-to-target–based management strategies during a window of opportunity and also to prevent the progression of SJIA into a more aggressive chronic arthritis phenotype. A revision of the ILAR criteria to facilitate the implementation of targeted and personalised management strategies is a current initiative.[3 9 10]

There is an unmet need for laboratory tests to diagnose and monitor SJIA disease activity.[11] Differential gene expression profiles in peripheral blood mononuclear cells and cytokine signatures in serum have been investigated in new-onset and active versus inactive SJIA.[12] Notably, several immune mediators including interleukin-1 (IL-1), IL-18 as well as the S100 proteins S100A12 and S100A8/A9 (MRP8/14, myeloid related protein 8/14) might be important players in the pathogenesis of SJIA.[13–15] S100A12 and MRP8/14 are highly elevated in SJIA compared with infection or other causes of fever of unknown origin[16–18] and are predictive for subclinical inflammatory activity and disease flares.[19–21]

We aimed to identify molecular markers of systemic-autoinflammatory and chronic-polyarticular SJIA phenotypes by applying unbiased label-free proteomics and multiplex immunoassays in a discovery cohort and targeted candidate biomarker analyses in a verification cohort.

PATIENTS AND METHODS

Patients

Serum samples were collected between 2009 and 2012 from 136 paediatric patients with SJIA or infections (clinically diagnosed and/or confirmed by serology/microbiology; see online supplementary table S1) attending various hospitals in Germany. In 2015 to 2016, treating clinicians retrospectively reported whether patients with SJIA developed a predominantly systemic or (poly)arthritic phenotype and a monophasic or chronic disease course. Patients were subgrouped as classical autoinflammatory (SJIA^{syst}) or chronic articular-dominant (SJIA^{poly}) based on the overall clinical disease course. Patients with SJIA^{poly} had a polyarthritic course (≥5 joints affected) and active arthritis at sampling, but no fever, rash, serositis, splenomegaly or generalised lymphadenopathy. In contrast, patients with SJIA^{syst} had a systemic course with fever, rash, serositis, splenomegaly, lymphadenopathy, elevated acute phase reactants or white cell count and <5 joints involved at time of sampling. No patient fulfilled the criteria for a diagnosis of macrophage activation syndrome (MAS).[22]

Ten patients each with either SJIA^{syst}, SJIA^{poly} or infection (total n=30) formed the 'discovery cohort'. Another 106 patients (45 SJIA^{syst}, 29 SJIA^{poly} and 32 infections; table 1) formed the 'verification cohort'. Patients were not included in both cohorts. At sampling, all patients had clinically active disease (eg, active joints or fever) and laboratory signs of inflammation (eg, elevated acute phase reactants). In the verification cohort, disease duration was significantly shorter in patients with SJIA^{syst} (median 0.1 years; range, 0.1–8.3) compared with SJIA^{poly} (median 4.7 years; range, 0.1–10; p=0.001). Patients using biological treatments at time of sampling were excluded. The study overview is shown in online supplementary figure S2.

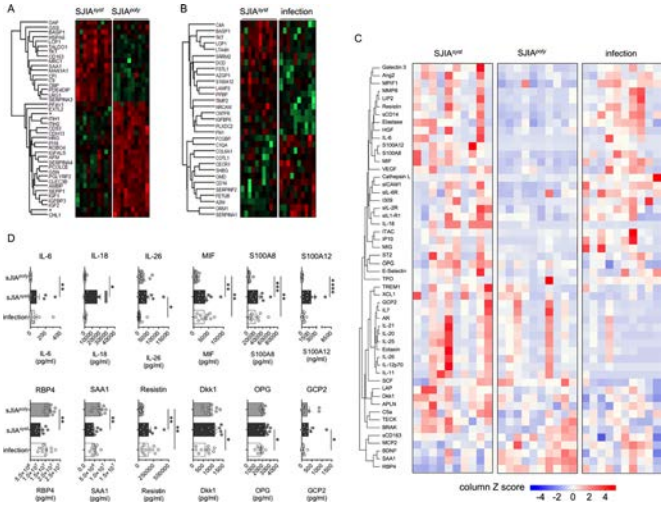

Figure 1 Proteomic and immune assay analyses in the discovery cohort. Serum was analysed by LC-MS/MS in a discovery cohort with the differences between the phenotypes shown as follows: (A) SJIA with systemic disease (SJIA^{syst}, n=10) vs SJIA with polyarticular disease (SJIA^{poly}, n=10) and (B) SJIA with systemic disease (SJIA^{syst}, n=10) vs infections (n=10). Heatmaps are shown with red squares representing overexpressed proteins and green representing lower expression (*indicates peptides without protein assignment). (C) Sera of identical patients as in (A) and (B) were analysed by 150-plex bead array Luminex assay. Analytes differentiating between groups with a p value <0.08 (see online supplementary table S4) are shown as heatmap based on Spearman rank correlation and average linkage clustering. S100A12 levels in respective sera were determined by ELISA and data are included in the heatmap. (D) Top performing serum markers based on significant separation of SJIA^{syst} from SJIA^{poly} or infections are shown as box-and-whisker plots (10th–90th percentile). Black full circles indicate outliers. Data were analysed by Kruskal-Wallis test and corrected for multiple comparisons, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

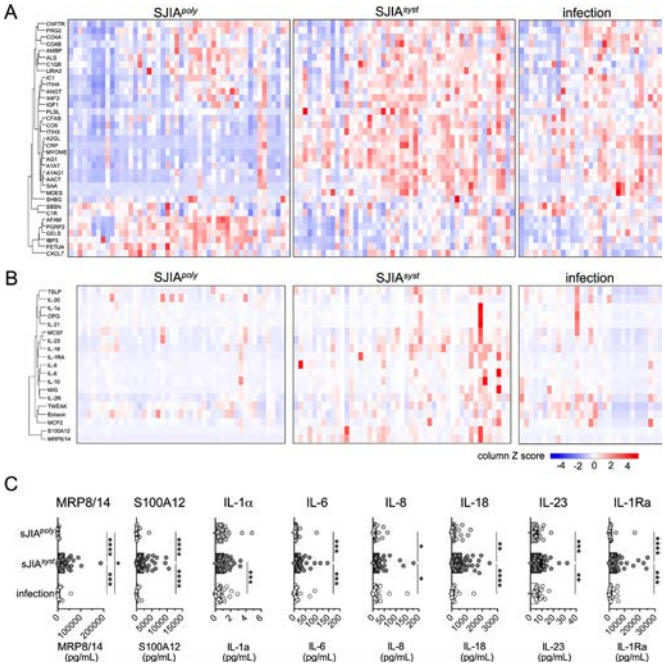

Figure 2 Proteomic and immune assay analyses in the verification cohort. (A) Heatmap of MRM peak area data for peptides derived from the indicated proteins as detected in sera of patients with systemic juvenile idiopathic arthritis with systemic (SJIA^{syst}, n=46) or polyarticular disease (SJIA^{poly}, n=47) or infections (n=32) based on Spearman rank correlation and average linkage clustering. (B) Heatmap of serum marker concentrations by bead array assay in cohorts as described in (A) based on Spearman rank correlation and average linkage clustering. (C) Box-and-whisker plots (10th–90th percentile) of top performing serum markers. Black full circles indicate outliers. Data were analysed by Kruskal-Wallis test and corrected for multiple comparisons, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Serum collection

Samples were collected in routine clinical settings. A 20 min centrifugation of the blood sample (collected in a serum gel tube) was performed within 2 hours of collection and the serum directly separated from the cell pellet. Serum was aliquoted into 1.5 mL or 2 mL Eppendorf tubes and posted at room temperature, to be stored at −80°C on arrival after the measurement of serum S100 proteins. Discovery cohort samples were subjected to unbiased label-free proteomics using liquid chromatography mass spectrometry (LC-MS/MS) and a broad multiplexed immunoassay screen (Luminex). Multiple reaction monitoring MS (MRM-MS) and targeted Luminex assays were performed on verification cohort samples. All samples were analysed for S100A12 and MRP8/14 by ELISAs (figures 1 and 2).

Proteomic analysis

Serum for LC-MS/MS analysis was prepared by depletion of the 14 most highly abundant proteins using a MARS Hu-14 affinity depletion column (Agilent Technologies, P/N 5188-6557) and visually confirmed using SDS chromatography as previously described.[23] Depleted samples were further concentrated by centrifugation and then subjected to in-solution tryptic digestion followed by desalting using C18 zip tips (online supplementary methods S3). Technical reference samples were included. Samples were stored at −80°C before analyses using a Q-Exactive mass spectrometer.[24] LC-MS/MS data were analysed by MaxQuant (V.1.4.1.3) with protein identifications generated using Andromeda.[25] Qualitative analysis was performed using PEAKS Studio (BSI) and Spectrum Mill (Agilent). Statistical analyses of the LC-MS/MS data were performed using Perseus (V.1.4.1.3).

MRM analysis of the verification cohort

To evaluate the candidate biomarkers identified during discovery, a unique MRM assay was generated, incorporating as many candidate proteins as possible (online supplementary methods S3).

Bead array assays and ELISA

In the discovery cohort, a total of 150 immune-related proteins (online supplementary table S4) were screened by a bead-based (Luminex) multiplex panel.[26] Proteins with little or no variation between samples (less than 75% unique values) or proteins with low expression levels were excluded. In the verification cohort, a customised panel of 17 cytokines was designed using commercially available Luminex analytes (eBioscience): interleukin (IL) 1 alpha (IL1α), IL1 receptor antagonist (IL1RA), IL2 receptor (IL-2R), IL6, IL8, IL10, IL18, IL20, IL21, IL23, macrophage colony stimulating factor, osteoprotegerin, thymic stromal lymphopoietin, monocyte chemotactic protein 2, eotaxin, TNF-like weak inducer of apoptosis (TWEAK) and monokine induced by gamma interferon.[24 27 28] S100A12 and MRP8/14 concentrations were measured in all serum samples

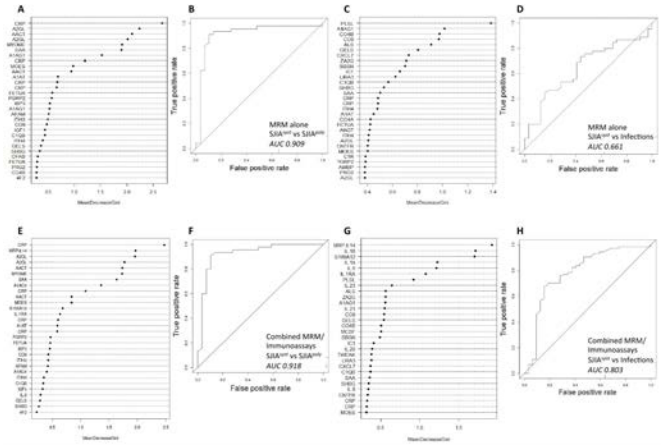

Figure 3 Predictive power of biomarker signatures. The accuracy of either the proteomic MRM panel alone (upper panels A–D) or a combination of MRM and immunoassays Luminex/ELISA (lower panels E–H) was analysed using a Random Forest model with 'leave-one-out cross-validation' statistical method. In the top rank list of the Random Forest models, the same protein could be identified by multiple peptides. Based on the ranked markers, receiver operating characteristic (ROC) curves were plotted for different comparisons. The analyses were performed for comparison groups SJIA^{syst} vs SJIA^{poly} (A, B or E, F, respectively) and for the differentiation of SJIA^{syst} vs infections (C, D or G, H, respectively). The ROC curve and area under the curve (AUC) are shown.

using standardised in-house ELISAs as previously reported.[29 30] Both assays included reference internal control sera with established cut-off values and were performed blinded to the patient characteristics.

Data analysis

Random Forest models were used to discriminate between the clinical groups (Random Forest package in R V.4.3.2). Reported performance measures were cross-validated in all models using leave-one-out cross-validation. Measurements of accuracy included the classification rate (percentage of total cases correctly classified by the model) and the area under the receiver operating characteristic (ROC) curve (AUC), calculated using the pROC package in R V.3.4.4. The Random Forest model provides a measure of importance of each variable contributing towards the overall performance, calculated using the Gini decrease in impurity (higher decrease in impurity means the variable is more predictive). In each model, the overall importance of variables was taken as the average decrease in the Gini decrease in impurity over each of the *n* random forest models run (n=number of samples in a given cohort). Kruskal-Wallis test, corrected for multiple comparisons, was applied to compare biomarkers between the groups. Data are shown as median (IQR), unless otherwise specified. Network analyses were performed using the GeneMANIA platform by applying Gene Ontology network weighting based on biological processes.[31]

RESULTS

Clinical and laboratory characteristics of the cohorts

Clinical characteristics and laboratory markers of participants are detailed in table 1. Patients with infection had similarly high levels of C-reactive protein (CRP), WCC and erythrocyte sedimentation rate as patients with SJIA^{syst}. Frequencies of fever and exanthema were highest in SJIA^{syst} followed by infection and lowest in SJIA^{poly}. Patients with infection and SJIA^{poly} had similar frequencies of joint pain and hepatosplenomegaly, which were lower than in SJIA^{syst}.

LC-MS/MS proteomic analysis in the discovery cohort

Univariate analysis revealed 41 differentially expressed proteins between SJIA^{syst} and SJIA^{poly} and 31 non-overlapping proteins differing between SJIA^{syst} and infections (total 72 candidate markers). Heat maps showing upregulated and downregulated proteins indicated good separation of the groups (figure 1). Some markers that discriminated groups in the immunoassay panel (eg, SAA1, S100A12 and sCD163) were also discovered by unbiased proteomics and thus acted as independent confirmation.

Proteomic analysis in the verification cohort

Of the 72 differentially expressed proteins from the LC-MS/MS discovery phase, 48 could be included in the MRM assay. Univariate and multivariate analysis of the data confirmed the presence of protein signatures capable of differentiating SJIA^{syst} from SJIA^{poly} and separately SJIA^{syst} from infection (figure 2A). The top 30 performing peptides and their recognised proteins for each analysis are shown in the variable importance plot in figure 3A–D. Interestingly, a CRP peptide was the top-ranked peptide for SJIA^{syst} versus SJIA^{poly} and also featured in the top 30 for SJIA^{syst} versus infection. CRP featured more than once in the list due to the inclusion of several different peptides in the assay panel. Analyses using the Random Forest model distinguished the SJIA^{syst} phenotype from SJIA^{poly} with 91% accuracy (sensitivity 86%, specificity 93%; table 2). Patients with SJIA^{syst} were less well distinguished from those with infection (accuracy, 62%; sensitivity, 38%; specificity, 80%). ROC curves showed a good differentiation of the two SJIA phenotypes with an AUC of 0.91 (95% CI 0.83 to 0.99), which outperformed SJIA^{syst} versus infection (AUC 0.66, 95% CI 0.54 to 0.79).

Inflammatory markers analysed by immune assays

In the discovery cohort, Luminex and ELISA analyses showed differences between the groups (figure 1). However, sample size limited statistical analysis. Therefore, a verification panel including commercially available analytes that were discriminative in the discovery cohort or in published literature, allowing a semitargeted reproducible approach, was designed. Univariate analyses of the best-performing individual markers in differentiating SJIA^{syst} and SJIA^{poly} measured by ELISA and Luminex are shown in figure 2B and levels of most important markers per group are plotted in figure 2C. Variable importance plots ranking each of the markers tested within the combined analysis revealed that MRP8/14, IL18 and S100A12 were the most important variables for differentiating SJIA^{syst} from infection, whereas MRP8/14, S100A12 and IL-1Ra were the top variables differentiating SJIA^{syst} from SJIA^{poly} (online supplementary figure 5). The ROC curve showed a good differentiation of the two SJIA phenotypes (AUC 0.90, 95% CI 0.81 to 0.98), similar to the discrimination of SJIA^{syst} and infection (AUC 0.84, 95% CI 0.75 to 0.93).

Lastly, to compare the performance of known biomarkers, ROC analyses of the single markers MRP8/14, S100A12, IL18 and ferritin were performed in the verification cohort (summarised in online supplementary figure S6 and online supplementary table S7). For the distinction between SJIA^{syst} versus infection, the single markers S100A12 (AUC 0.81) and MRP8/14 (AUC 0.82) performed almost as well as the immune assay panel (AUC 0.84). The best discriminator between SJIA^{syst}

Table 2 Accuracy of the proteomic marker panels

	SJIA^{syst} vs SJIA^{poly}			SJIA^{syst} vs infection		
	MRM	Immune assays	Combined	MRM	Immune assays	Combined
Sensitivity	0.86	0.76	0.86	0.80	0.69	0.69
Specificity	0.93	0.91	0.93	0.38	0.82	0.82
Accuracy	0.91	0.85	0.91	0.65	0.77	0.77
PPV	0.89	0.85	0.89	0.80	0.73	0.73
NPV	0.91	0.85	0.91	0.57	0.79	0.79
LR+	14.33	8.44	14.33	1.28	4.06	4.06
LR–	0.15	0.26	0.15	0.53	0.39	0.39
AUC	0.909 (0.828–0.990)	0.895	0.918	0.661	0.840	0.803

AUC, area under the curve; LR–, negative likelihood ratio; LR+, positive likelihood ratio;MRM, multiple reaction monitoring; NPV, negative predictive value; PPV, positive predictive value; SJIA^{poly}, systemic juvenile idiopathic arthritis with polyarthritis but systemic disease;SJIA^{syst}, SJIA with fever/systemic disease.

and SJIA^{poly} was MRP8/14 (AUC 0.93), which was also ranked high in the variable importance plots of the multiplex analyses.

Accuracy of multimodular analysis combining proteomic and immune assays

The top 30 variables in the combined multimarker panels comprising MRM, ELISA and Luminex were ranked (figure 3E–H). The top five discriminating biomarkers for SJIA^{syst} versus SJIA^{poly} were CRP, leucine-rich alpha-2-glycoprotein (A2GL), MRP8/14, alpha-1-antichymotrypsin (AACT) and myomegalin (myome); for SJIA^{syst} versus infection, MRP8/14, IL18, S100A12, IL1α and IL6. MRP8/14 was the only marker featuring in the top five of both panels. Eleven biomarkers were common to both panels: CRP, MRP8/14, AACT, serum amyloid A (SAA), alpha-1-acid glycoprotein 1 (A1AG1), moesin (MOES), S100A12, IL1RA, gelsolin (GELS), IL6 and sex hormone–binding globulin. In the combined model, distinction between SJIA^{syst} versus SJIA^{poly} was possible with an overall accuracy of 90.5% with 67/74 cases correctly identified (AUC 0.93). Accuracy of the SJIA^{syst} versus infection model was 76.6% and 59/77 cases were correctly classified (table 2). The combined multimodal panel (MRM and immunoassay) improved the distinction between SJIA^{syst} and SJIA^{poly}, but not SJIA^{syst} from infections, with immunoassays outperforming MRM.

To better understand potential relationships between the diverse identified markers, network analyses based on Gene Ontology biological process annotations using the GeneMANIA platform (figure 4 and online supplementary figures S8, S9) were performed. Serum marker panels identified by the combined Random Forest models to discriminate SJIA^{syst} from SJIA^{poly} (figure 3E) or SJIA^{syst} from infections (figure 3G) revealed multiple associations between each other as well as additional proteins that were not in the analysed panels (figure 4A,B). The top performing markers (mean Gini decrease >0.5) of both Random Forest model panels associated best with members of the IL1 signalling pathway, namely IL1R2 (figure 4). The association with IL1 signalling appeared more pronounced with markers in the panel differentiating SJIA^{syst} from SJIA^{poly}, with IL1R2 further linking to IL1β and IL1R1 (figure 4B), which was not pronounced for the separation of SJIA^{syst} from infections.

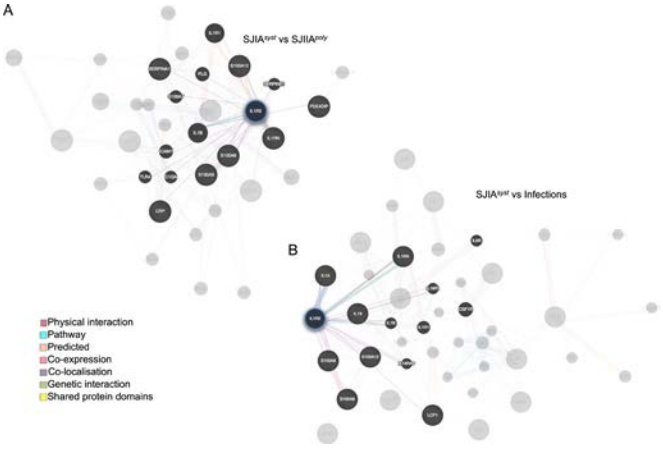

Figure 4 Association of identified discriminating serum markers. Plots show GeneMANIA-generated networks seeded with the proteins identified by Random Forest analysis discriminating SJIA^{syst} from SJIA^{poly} (A) and SJIA^{syst} from infections (B) above a mean Gini decrease cut-off of 0.5. Seeded markers are depicted as hatched circles of uniform size, while those that were added as relevant based on gene ontology biological process annotations are depicted as solid circles. Circle size is proportional to the number of interactions. The most relevant identified associations are highlighted.

DISCUSSION

Using proteomic analyses and immunoassays, signatures of serum proteins that distinguish two clinical phenotypes of SJIA and help differentiate autoinflammatory SJIA from infections were discovered. Proteomics has been relatively underused in paediatric rheumatology with most studies focusing on synovial fluid protein expression in JIA.[32 33] Proteomic analyses have, however, identified serum protein profiles of SJIA and revealed biomarkers for monitoring response to therapy in SJIA.[28 34] Clinical heterogeneity is a well-recognised feature of JIA and assumed to have a biological basis,[35 36] with differential PBMC gene expression profiles found in patients with SJIA versus non-systemic JIA.[12] Serum cytokine profiles have so far predominantly focused on discriminating active and inactive SJIA, or predicting response to treatment. With that regard, specifically RNA expression studies have been performed.[37–39] However, recent analyses failed to show distinct transcriptional profiles that could be attributed to diverse subphenotypes of SJIA.[39 40]

This study is the first aiming to systematically discriminate SJIA phenotypes with proteomic biomarkers. Correct discrimination of SJIA^{syst} from SJIA^{poly} was achieved in over 90% of cases using any of the three identified biomarker panels (MRM alone, ELISA/Luminex alone and combined). Our study included a number of markers with reported potential value for the diagnosis of SJIA.[41–43] Of these, IL18 is not regularly measured due to technical limitations in performing bioassays, while the routine use of IL6 is still limited for various reasons.[44 45] In the MRM assay, peptides of S100A12, MRP8/14 and the bead assay–measured

cytokines were below the limit of detection for MRM and were therefore excluded from panels. Lack of available analytes and/or low sensitivity are limitations of proteomic analyses and may explain the variation in results from the different approaches. The use of the combined approach could overcome this to some extent. However, our analyses of MRP8/14 and S100A12 by immunoassays show that these single analytes, partially already in routine care, perform very well as surrogate markers.

Interestingly, a number of markers in our combined panel that discriminated SJIA^{syst} from SJIA^{poly} were also identified in a published panel of markers that differentiated flare from quiescent SJIA.[28] The common markers were alpha-2-macroglobulin (A2M or A2GL), alpha-1-acid glycoprotein 1 (A1AG1 or AGP1), serpin a3/alpha-1-antichymotrypsin (AACT), GELS, SAA, MRP8/14 and CRP, which as part of the full panel published by Ling *et al* also differentiated SJIA from acute febrile illnesses. Of these biomarkers, A1AG1, GELS, SAA, MRP8/14 and CRP featured in both the SJIA^{syst} versus SJIA^{poly} and the SJIA^{syst} versus infection comparisons. Kininogenin (KLKB1), a high molecular weight protein which plays a role in the pathogenesis of inflammatory reactions, was previously identified by MS and also featured among the top gene ontology associated markers in the SJIA^{syst} from SJIA^{poly} network analysis performed here.[28]

A differentiation of phenotypes is not currently included in SJIA classification criteria. As knowledge of underlying immunological processes increases, leading to new treatment strategies,[46] it is important that treat-to-target approaches are supported by reliable biomarkers. The primary target is disease remission, and the available data support the hypothesis of a therapeutic window of opportunity in the autoinflammatory phase of the disease.[4] Our patients with clinically discriminated SJIA phenotypes had significantly different disease durations, which itself supported the biphasic model of SJIA. It is therefore important to start therapy early, which requires timely diagnosis before chronic arthritis develops. Recent data show that about half of the patients treated as SJIA do not have arthritis and therefore do not fulfil the ILAR classification criteria,[47] resulting in SJIA often being a diagnosis of exclusion. Here, the tested biomarker panel can help the earlier differentiation of SJIA versus infections. Another important aspect of treat-to-target protocols is the monitoring of the therapeutic response to check for necessary treatment adaptation. Phenotype switches occurring during the clinical course may require a corresponding therapy adjustment. The identification of underlying immunological imbalances could be facilitated by biomarker panels as described here.

Our study has a number of limitations. The sample size was relatively small. The quality of some samples may have been suboptimal for unbiased proteomic profiling, although clinically useful diagnostic biomarkers should be robust and stable.[48] Multiple preanalytical factors are thought to affect results, including the handling, shipping and storage of samples.[49] However, internal evaluation of the impact of freeze–thawing samples for MRM analysis and tryptic digestion of proteins before proteomic analysis showed that any preanalytical proteolysis had no effect on the final measurements (data not shown).

CONCLUSION

In summary, differing biomarker profiles between two phenotypes of SJIA were identified, strengthening the biological basis for subphenotypes in SJIA. Moreover, separate panels discriminating patients with SJIA^{syst} from those with infections were established. Biomarker panels were measurable using MRM, ELISA and Luminex assays or a combination of these, which improved the accuracy of the discrimination of SJIA^{syst} from infection, but not the discrimination of SJIA^{syst} from SJIA^{poly}, which already performed very well with single-platform panels.

The identified protein signature of SJIA versus infections can help to establish an early diagnosis. The discrimination of SJIA subphenotypes may improve the understanding of the pathophysiology underlying different disease phases and courses, which may inform future treat-to-target strategies. Future work could include biomarker measurements at specific time points including at diagnosis and flare as well as in established phenotype switches in a larger cohort.

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Contributors DF, OF and SRP designed and planned the study. FG, DH, CP, CK, SdR, AM, MJ, NC and SRP performed experiments. FG, AM, MJ, BH, CP, CK, EL, SJV, SdR, DF and SRP analysed data. FG, AM, MJ, BH, NC, MM-G, CK, ML, EL, SJV, SdR, OF, DH, SRP and DF participated in data interpretation and discussion. All authors were involved in writing the manuscript and all made substantial contributions to the content and approved the final manuscript. Collaborators provided clinical data and samples (full details listed in online supplementary material).

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CLINICAL SCIENCE

Two weeks versus four weeks of antibiotic therapy after surgical drainage for native joint bacterial arthritis: a prospective, randomised, non-inferiority trial

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ABSTRACT

Objective The optimal duration of postsurgical antibiotic therapy for adult native joint bacterial arthritis remains unknown.

Methods We conducted a prospective, unblinded, randomised, non-inferiority study comparing either 2 or 4 weeks of antibiotic therapy after surgical drainage of native joint bacterial arthritis in adults. Excluded were implant-related infections, episodes without surgical lavage and episodes with a follow-up of less than 2 months.

Results We enrolled 154 cases: 77 in the 4-week arm and 77 in the 2-week arm. Median length of intravenous antibiotic treatment was 1 and 2 days, respectively. The median number of surgical lavages was 1 in both arms. Recurrence of infection was noted in three patients (2%): 1 in the 2-week arm (99% cure rate) and 2 in the 4-week arm (97% cure rate). There was no difference in the number of adverse events or sequelae between the study arms. Of the overall 154 arthritis cases, 99 concerned the hand and wrist, for which an additional subgroup analysis was performed. In this per-protocol subanalysis, we noted three recurrences: one in the 2-week arm (97 % cure); two in the 4-week arm (96 % cure) and witnessed sequelae in 50% in the 2-week arm versus 55% in the 4-week arm, of which five (13%) and six (13%) needed further interventions.

Conclusions After initial surgical lavage for septic arthritis, 2 weeks of targeted antibiotic therapy is not inferior to 4 weeks regarding cure rate, adverse events or sequelae and leads to a significantly shorter hospital stay, at least for hand and wrist arthritis.

Trial registration number NCT03615781.

Key messages

What is already known about this subject?

- The treatment of a septic arthritis requires a combination of at least one lavage/debridement and a long-lasting antibiotic administration.
- Usually, this initial antibiotic administration is parenteral during the first 2 weeks.

What does this study add?

- According to our randomised controlled trial, and at least for hand and wrist septic arthritis, total postsurgical antibiotic therapy can be limited to 2 weeks.
- Likewise, the initially systemic (and often empirical) parenteral antibiotic therapy can be switched to targeted oral medication after few days (1–2 days parenterally only).

How might this impact on clinical practice or future developments?

- Future patients with septic (hand and wrist) arthritis and a good evolution after surgical drainage might profit from significantly less antibiotics.
- This might limit potential adverse events, costs and complications of (parenteral) antibiotic therapy.

INTRODUCTION

Native joint bacterial arthritis is frequent and usually associated with considerable morbidity, need for hospitalisation and substantial financial costs.[1–3] While the need for surgical drainage of these infections has been well established,[4 5] the ideal duration and route of administration of antibiotic therapy remains unknown. For almost 40 years, the recommended total duration of postsurgical systemic antibiotic therapy has been 3–6 weeks, with most clinicians prescribing 4 weeks for adults.[3] Unfortunately, this recommendation is based on expert opinion and individual experience, rather than on research studies. Furthermore, clinicians often treat bacterial arthritis at all anatomic sites in the same way, with no distinction between small and large joints.

In the current era of critical shortages of effective antibiotics, antimicrobial stewardship principles suggest that a shorter treatment duration and early switch to targeted oral agents could decrease antibiotic-related adverse events,[1 3 6] costs and possibly emergence of antimicrobial resistance. Retrospective studies,[2] reviews[7] and our own retrospective data[3] suggest that just 2 weeks of targeted systemic antibiotic therapy after surgical drainage may be sufficient, especially for hand joints. In an attempt to provide an evidence base for antibiotic treatment for adult native joint arthritis,[5] we undertook

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a prospective, randomised trial to determine if a postsurgical antibiotic course of 2 weeks, together with an early switch to oral targeted systemic antibiotics,[3–5] is non-inferior in inducing remission to the currently recommended 4 weeks.[3]

METHODS

We performed a single-centre (at Geneva University Hospitals), prospective, unmatched, unblinded, randomised, interventional study from 1 March 2015 to 10 March 2018, with database closure on 18 May 2018. Inclusion criteria among patients diagnosed with native joint bacterial arthritis were: age ≥18 years; underwent at least one articular lavage (by arthrotomy or arthroscopy; with or without synovectomy); and treatment with systemic antibiotic therapy. Of note, we allowed the inclusion of episodes with previous antibiotic therapy during at maximum 48 hours prior to the first lavage and episodes with concomitant crystalline disease. Patients with an abscess in surrounding soft tissue were equally allowed to participate, but only if the abscess could be excised or drained in toto. Exclusion criteria were: recurrent bacterial arthritis; non-bacterial arthritis; history of allergies to multiple antibiotics; receiving long-term antibiotic prophylaxis because of immune-suppression; presence of left-side endocarditis; incomplete joint lavage or >4 surgical lavages; plan for repetitive arthrocentesis as a therapeutic approach[5]; concomitant spondylodiscitis or osteomyelitis;[8] plan for arthrodesis or amputation; foot arthritis in a patient with diabetes mellitus[9]; presence of necrotiing fasciitis; intravertebral arthritis[8]; patient with or planned for a Girdlestone procedure[10 11]; patient who underwent a bone marrow transplantation; undergoing active oncological chemotherapy; or presence of foreign material in close vicinity to the infected joint. We also excluded infections for which published literature recommends either an unusually long, or a short duration of antibiotic therapy, for example, infection with mycobacteria,[12] actinomycetes, gonococci, meningococci, fungi, brucellosis, mycoplasma or nocardia.[13]

We defined bacterial arthritis as the presence of: clinical findings of joint infection (wound discharge, redness, warmth and new pain); and, at least two positive microbiological culture or Gram-strained smear[14] or PCR of joint, pus or synovial tissues. Elevated serum inflammatory markers, positive histology of synovial tissue, elevated intra-articular white cell counts[11] or abnormal radiological images were not required. We defined immune-suppression as the patient having undergone an organ transplantation, taking chronic corticosteroid medication equivalent to ≥15 mg prednisolone daily, requiring renal dialysis, having diabetes mellitus, active cancer, liver cirrhosis of at least CHILD class C, severe chronic alcoholism, untreated HIV disease and agranulocytosis.

We defined sequelae as persisting non-infectious handicaps after adequate physiotherapy, ergotherapy (sensory-integrative therapy) and analgesia that was not pre-existent and attributed to the recent infection. We asked all patients to indicate their pain on a Likert scale, with responses ranging from 0 (least) to 10 (greatest).

Study conduct

We assigned eligible hospitalised patients, following a specified randomisation procedure (1:1, by computer-generated random numbers) to therapy with systemic antibiotic agents for either 2 weeks (±3 days) or 4 weeks (±3 days). Treating surgeons, in consultation with an infectious diseases expert,[15] selected the agents in the antibiotic regimen from a list of options we provided them. Initial therapy was empirical and was started after collecting intraoperative samples for culture. Treating clinicians could amend the antibiotic regimen (agent(s) or route of administration) according to the microbiological results and clinical evolution of infection. During the first days of therapy, the most frequently administered (empiric) antibiotic regimens were of the following given by intravenous route: co-amoxiclav; cefazolin; cefuroxime; cefepime; ertapenem; imipenem; piperacillin/tazobactam; vancomycin; or daptomycin. Definitive therapy was based on of culture and sensitivity results, with a targeted oral antibiotic regimen selected from among the following agents: ciprofloxacin; levofloxacin; clindamycin; co-trimoxazole; doxycycline; linezolid; rifampicin; or fusidic acid. The timing for switching to oral therapy was at the discretion of the treating clinicians and depended on the global evolution of the patient and the infection. The treating surgeons decided on: the arthrotomy or arthroscopy techniques; the timing of any emergency intervention[4]; whether to do synovectomy; and when to repeat joint lavages. We avoided using intrasynovial antibiotic infusions or irrigations with local antiseptics. Twelve months after enrollment of the last patient, we searched the hospital databases to determine if any of the enrolled patients had been seen in outpatient clinics for problems related to the operative site. Online supplementary appendix S1 shows the original protocol (in French).

Patient and public involvement

We involved all study participants on hospitalisation for surgical drainage of septic arthritis by asking them directly. We informed them orally in their native language and provided French-language study documents. We moreover accompanied all patients until the test-of-cure visit, and beyond, if necessary. During accompaniment, the patients were always free to ask for additional information and clinical results. This was also the case for the study team that was free to ask to the patients. No information was withheld. Although not a formal part of the study protocol, we think that the patients will help and promote the study idea in their respective communities. On a larger scale, the study team shall inform the public, that is, with interviews in the local press. In contrast, patients and public had not been involved in the design of the study. The study team had developed this prospective-randomised study according to their clinical experience, academic interest, retrospective own data and on a scientific literature research. According to national ethical requirements, we will not provide a scientific copy of the final publication to every study participant, unless he or she wished to receive it specifically.

Sample size considerations, outcome parameters and statistical analyses

The primary outcome for this study was the rates of remission of infection. Remission was defined as the complete absence of clinical, laboratory or radiological findings after a minimal follow-up of 2 months after treatment. Secondary endpoints were: remission in the subgroup of hand and wrist septic arthritis; the occurrence of adverse events related to antibiotic therapy; and the development of non-infectious sequelae of bacterial arthritis. Using a non-inferiority design, with an alpha level of 5%, a power of 80%, expected remission rates of 96% in both study arms,[2 3] we calculated that we needed 48 patients in each group to establish a non-inferiority margin of 10%. We planned interim analyses beginning after enrolment of the first 40 cases. Our intention-to-treat (ITT) population was composed of all randomised patients, while the per-protocol (PP) population

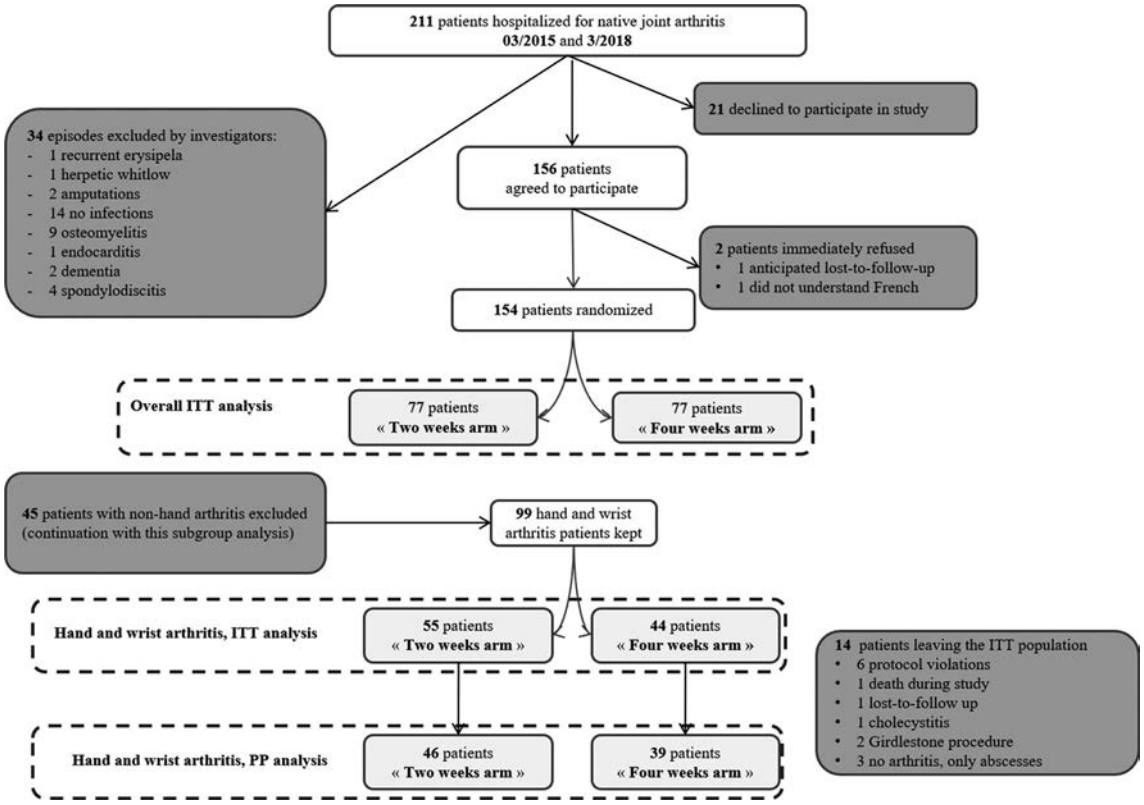

Figure 1 Study flow chart of patients. ITT, intention-to-treat.

included patients who completed the study without any major protocol deviation. Due to the predominance of hand arthritis cases, we performed a subgroup analysis for hand arthritis only, which included the fingers, hand and wrists. We compared treatment groups using the Pearson χ^2 or the Wilcoxon rank-sum test. Due to the small number of failures, we elected not to perform multivariate analyses. We used STATA software (V.9.0) and considered p values (two tailed) ≤0.05 as significant.

RESULTS

Patients (entire study population; ITT analysis)

Among 211 native joint arthritis cases, 154 (analysis in 154 different patients) were eligible for inclusion for the ITT analyses. Overall, we excluded 34 of 211 patients and episodes due to various exclusion criteria, while the number of patients refusing to participate in the study was only 21 (21/211; 10%) (see figure 1).

Clinically, these refusing patients and those excluded by investigators were a very heterogeneous group without clear anamnestic patterns or objectively common comorbidities (*data not shown*). Among the study patients, 77 were randomised to the 4-week treatment arm and 77 to the 2-week treatment arm. Demographic characteristics were similar for the patients in the two groups (see table 1, left part).

The actual median duration of antibiotic therapy in the 2-week arm was 14 days, and in the 4-week arm it was 28 days. Overall, 59 patients were women (38%), and the median age of all patients was 51 years. While many patients lacked comorbidities, 15 (10%) were chronically immune-compromised (due to diabetes mellitus (n=13), advanced cirrhosis (n=2), active cancer (n=3), solid organ transplant (n=1), steroid medication (n=2) or a combination of immune-suppressive conditions. In addition, six patients had an active psychiatric comorbidity and nine illicitly used drugs. The median American Society of Anesthesiologists' Score[16] for all enrolled patients was 2 points (IQR: 2–2 points).

Infections

The origin of infection varied greatly, including: surgical site infections[17] (n=12); cat bites (n=16); cat scratches (n=2); dog bites (n=14); rat bite (n=1); human bites (n=3); infection of gouty tophus (n=1); intravenous drug abuse (n=9); direct trauma (n=48)[18]; and Baker cyst rupture (n=1). In 47 cases, the origin was unknown. Bacteraemia was documented in six episodes (4%). There were no apparent seasonality[19] or outbreak situations. On admission, the overall median serum C reactive protein level was 30 mg/L and the median pain score 5 points (out of 10). The joints infected were: finger (n=95); wrist (n=3); knee (n=14); shoulder (n=7); ankle (n=3); elbow (n=1); hip (n=1); metatarsal (n=28); combination of wrist and ankle (n=1); and another combination of ankle and elbow (n=1). In 16 of the 154 episodes (10%), the infected joints had a prior known pathology: osteoarthritis (arthrosis) (n=6), cyst (n=2), rheumatological inflammation (n=4), psoriasis (n=1), meniscal lesion (n=1), recurrent subluxation (n=1) and chronic rotator calf injury (n=1). The infected joints also harboured intraarticular crystals in six cases (4%)[11]: calcium pyrophosphate in four, calcium apatite in one and urate in one. The median preoperative intracellular leucocyte count was 41 200 cells/mm^3, and the percentage of polynuclear leukocytes was 95%.[11] Culture results of intraoperative specimens revealed 31 different microbiological patterns. *Staphylococcus aureus* was the most commonly isolated pathogen, found in 48 episodes (31%); none of the strains was a methicillin-resistant strain. Streptococci were involved in 22 cases,[20] Gram-negative pathogens in 35 episodes (including 19 due to *Pasteurella* spp,[18] and skin commensals (coagulase-negative staphylococci, micrococci, corynebacteria or cutibacteria) in seven cases and the rest consisted of other

Table 1 Characteristics of patients treated with 2 weeks versus 4 weeks of systemic antibiotic therapy after surgical drainage of native joint septic arthritis (all arthritis cases on the left vs hand and wrist arthritis only on the right; both populations are intention-to-treat)

	Duration of antibiotic therapy				Duration of antibiotic therapy		
All arthritis cases n=154	**4 weeks n=77**	**2 weeks n=77**	**P value ***	**Only hand arthritis cases n=99**	**4 weeks n=44**	**2 weeks n=55**	**P value ***
Female sex	31 (40%)	28 (36%)	0.62	Female sex	17 (35%)	20 (42%)	0.82
Median age	52 years	48 years	0.23	Median age	50 years	45 years	0.49
Immune-suppression†	7 (9%)	8 (10%)	0.79	Immune suppression†	4 (9%)	5 (9%)	1.0
Bacteraemia	2 (3%)	4 (6%)	0.37	Bacteraemia	0 (0%)	0 (0%)	–
Median ASA Score[16]	2 (IQR: 1–2)	2 (IQR: 1–2)	0.13	Median ASA Score[16]	2 (IQR: 1–2)	2 (IQR: 1–2)	0.05
Pain score on admission (median)	five points	five points	0.33	Pain score on admission (median)	five points	five points	0.31
Antibiotics before first surgery	21 (27%)	21 (27%)	0.95	Antibiotics before first surgery	12 (28%)	15 (29%)	0.92
Duration of presurgical antibiotics (median)	0 day	0 day	0.90	Duration of presurgical antibiotics (median)	0 day	0 day	0.49
Staphylococcus aureus	23 (30%)	25 (32%)	0.73	*Staphylococcus aureus* infection	11 (25%)	18 (33%)	0.40
Streptococci	19 (25%)	12 (16%)	0.16	Streptococci	12 (27%)	8 (16%)	0.12
Gram negative(s)	19 (25%)	16 (21%)	0.56	Gram-negative pathogens	15 (34%)	14 (25%)	0.35
Number of surgical lavages (median)	1 (IQR: 1–3)	1 (IQR: 1–3)	0.13	Number of surgical lavages (median)	1 (IQR: 1–3)	1 (IQR: 1–3)	0.11
Duration of intravenous therapy (median)	2 days	1 day	0.01	Duration of intravenous therapy (median)	2 days	1 day	0.02
Complete microbiological remission	75 (97%)	76 (99%)	0.56	Complete microbiological remission	42 (95%)	53 (96%)	0.82
Duration of sick leave (median)	36 days	28 days	0.31	Duration of sick leave (median)	36 days	27 days	0.29
Number of outpatient attendances (median)	6 controls	7 controls	0.05	Number of outpatient attendances (median)	7 controls	7 controls	0.70
Length of hospital stay (median)	6 days	4 days	0.01	Length of hospital stay (median)	4 days	3 days	0.01
Mechanical or neurological sequelae	33 (54%)	27 (47%)	0.47	Mechanical or neurological sequelae	21 (53%)	23 (48%)	0.67
Antibiotic-related adverse events	5 (6%)	2 (3%)	0.25	Antibiotic-related adverse events	2 (5%)	2 (4%)	0.82

*Pearson χ^2 test or Wilcoxon rank-sum tests, as appropriate.
†Immune-suppression=diabetes mellitus, active cancer, cirrhosis CHILD C, organ transplant and steroid medication equivalent to ≥15 mg prednisolone daily.
ASA, American Society of Anesthesiologists.

pathogens. Twenty-one infections were bimicrobial, and there were no polymicrobial cases.

Treatment

In all patients, we performed surgical drainage of the affected joint and administered systemic antibiotic therapy. Surgery was performed by open arthrotomy in all but seven cases (5%), which were drained by arthroscopy. table 1Enrolled patients were treated with 13 different initial parenteral regimens and 11 different oral follow-on regimens. The most frequently administered parenteral antibiotics were co-amoxiclav (n=71), ceftriaxone (n=3) and vancomycin (n=4). Overall, we administered parenteral β-lactam antibiotics in 107 (69%) cases. In three episodes, the entire antibiotic course was given intravenously, while in 32 cases treatment was with an oral agent from the start. The oral antibiotics prescribed were co-amoxiclav (74), quinolones (26), clindamycin (19), doxycycline (2), co-trimoxazole (3) or a combination of above. The table 2 resumes the antibiotic classes and administration forms, stratified on the study arm and key pathogen groups.

Overall, these allocations were balanced between both arms. We did not prescribe corticosteroids for the treatment of arthritis in any patient, but some were treated for pain with paracetamol, ibuprofen and opioids (in limited cases). The median number of surgical lavages was 1 in both groups. The overall median length of hospital stay was 4 days (IQR: 3-8 days), and it was longer in the short duration treatment arm (6 days vs 4 days; see table 1).

Remission

Of the 154 episodes in the ITT population, 148 (96%) were microbiologically cured after an active median follow-up of 0.5 years (IQR: 0.3–1.1 years) and a passive median follow-up of 2.2 years (IQR: 1.6–2.8 years). Among the six patients who ultimately failed on therapy, three patients (one in the 4-week arm, and two in the 2-week arm) finally revealed new pathogens completely different from the initial agents. We interpreted these episodes as novel episodes in terms of surgical site infections.[17] Hence, the number of true microbiology-based recurrences was three (3/154; 2%) and these occurred after a median delay of 32 days after the end of treatment of the previous episode. There was no difference in the rate of microbiological recurrences between the two treatment groups (see table 1); they occurred in 2 of 77 in the 4-week arm compared with 1 of 77 in the 6-week arm (p=0.58). The three cases with microbiological recurrences involved one case caused by *S. pyogenes*, and two episodes caused by *S. aureus*. Formally, the 90% CIs (two tailed) regarding clinical remission were within the interval (between 0 and the 10% margin) and did not include the margin

Table 2 Main pathogen groups linked to corresponding selected antimicrobial substances and classes (overall ITT analysis)

n=154 *Pathogen group*	6 weeks Parenteral antibiotics	*Oral antibiotics*	4 weeks Parenteral antibiotics	*Oral antibiotics*
Staphylococcus aureus, n=48	Co-amoxiclav (n=14) Clindamycin (n=1) Cefuroxim (n=10)	*Co-amoxiclav (n=17)* *Clindamycin (n=5)* *Levofloxacin (n=3)*	Co-amoxiclav (n=13) Cefazolin (n=2) Cefuroxim (n=7)	*Co-amoxiclav (n=17)* *Clindamycin (n=5)* *Levofloxacin (n=3)*
Streptococci, n=31	Co-amoxiclav (n=11) Penicillin (n=1) Cefuroxim (n=6)	*Co-amoxiclav (n=9)* *Clindamycin (n=3)* *Levofloxacin (n=3)*	Co-amoxiclav (n=7) Cephalosporins (n=3) Cefuroxim (n=4)	*Co-amoxiclav (n=5)* *Clindamycin (n=3)* *Levofloxacin (n=1)*
Gram negatives, n=35	Co-amoxiclav (n=12) Cephalosporins (n=6)	*Quinolones (n=9)* *Co-amoxiclav (n=10)*	Co-amoxiclav (n=8) Cephalosporins (n=8)	*Quinolones (n=3)* *Co-amoxiclav (n=12)*
Pasteurella spp, n=17	Co-amoxiclav (n=6) Cefuroxim (n=2)	*Co-amoxiclav (n=7)* *Levofloxacin (n=1)*	Co-amoxiclav (n=6) Cefuroxim (n=2)	*Co-amoxiclav (n=8)* *Levofloxacin (n=1)*

Of note, the parenteral antibiotics are mostly empirical.
*Infections may be polymicrobial and thus the main antibiotics might be larger in spectrum than the main pathogens require.

(−0.7 percentage points (90% CIs −8.5% to +7.0%)). Idem for microbiological remissions (4.3 percentage points (90% CI −1.1% to +9.8%)).

Adverse events and complications

Overall, only eight patients (5%) reported adverse events that were attributed to antibiotic therapy: superficial fungal infection (n=3), allergic rash to amoxicillin or levofloxacin (n=3), severe diarrhoea due to co-amoxiclav (n=1) and dizziness due to clindamycin (n=1). As a consequence of adverse events, we changed the antibiotic or added fluconazole and probiotics in three cases. No patient developed *Clostridium difficile*-associated colitis and none left the study because of an adverse event. One patient developed a urinary tract infection, which we treated with another antibiotic agent inactive against the pathogen causing her bacterial arthritis. The major non-antibiotic-related complications were: haematoma needing revision (n=3), hospital-acquired fall (n=2), new giant cell tumour (n=1), Raynauds' phenomenon (n=1), stroke (n=1), scaphoid necrosis (n=1), influenza (n=1) and basilica vein thrombosis (n=1). The number of complications was similar in patients in the two study arms. Formally, the 90% CI (two tailed) regarding adverse events also excluded the margin (3.8% percentage points (90% CI −1.7% to +9.5%)).

Sequelae

Overall, in 60 episodes (39%), there were mechanical or neurological non-infectious sequelae, including: stiffness (n=16), flexion incapacity and adhesions (n=13; flexion contraction ranging between 5° and 30°), persistent pain (n=12), trigger zone (n=2), persistent dry wound (n=2), scaphoid necrosis (n=1), hypoesthesia (n=1) and Raynaud's phenomenon (n=1). Many of these sequelae did not require any specific therapy, but a minority were severe enough to require corrective actions: tenolysis (n=4), surgical closure of persistent wound (n=1), immunological treatment (n=1), psychiatric follow-up (n=1), persistent vacuum-assisted suctions (n=1), prolonged sensory-integrative therapy (n=1) or prolonged physiotherapy (n=4). Overall, 46 (of the 154) patients had follow-up radiographs and 20 (43%) of these had evidence of 'secondary osteoarthritis' after a median delay of 2 months. The median duration of sick leave (officially granted by the treating physicians or surgeons to the affected patient) was 33 days. Of note, the 90% CI regarding the outcome substantial sequelae failed to fulfil the statistical non-inferiority requirements (6.7% percentage points (90% CI −8.6% to +22.1%)).

Hand and wrist arthritis (ITT analysis)

Among the 154 enrolled cases, 99 (64%) involved the hands or the wrist. Therefore, we decided to perform a subgroup analysis for these patients, of whom 44 were randomised to the 4-week arm and 55 to the 2-week arm. The actual median duration of postlavage antibiotic therapy was 14 days for the 2-week arm and 28 days for the 4-week arm. The interphalangeal joints affected by bacterial arthritis were: thumb (n=15), index finger (n=29), middle finger (n=24), ring finger (n=7) and little finger (n=4). Metacarpal regions involved were: thumb (n=5), index finger (n=9), middle finger (n=1), ring finger (n=0) and little finger (n=1). In four cases, the wrist was involved. This subgroup's patient characteristics (see table 1, right part) and outcomes were similar to those in the overall arthritis population. The most frequent oral antibiotic agents used for hand cases were co-amoxiclav, levofloxacin or clindamycin. The median number of surgical lavages was one. Microbiological recurrence was documented in three patients (4%): one in the 2=week arm and two in the 4-week arm (p=0.44). The proportion of sequelae and adverse events were equal between both arms in the hand arthritis group. However, the duration of intravenous therapy and the total length of hospital stay were shorter in the 2-week arm (see table 1).

PP analysis

Among the 99 episodes composing the ITT population of hand arthritis cases, we removed 17 (17/99; 17%), when constituting the PP population (see figure 1) because of unintended protocol violations or being lost to follow-up. There were no significant differences between patients in the two study arms in the PP population (see table 3 and figure 2).

The median age was 48 years; 32 (38%) were women, and 9 were immune-suppressed, and the main cause of infection was bites (20 episodes). The predominant pathogens in these infections were *S. aureus* and *Pasteurella multocida* (20 and 12 episodes, respectively). Overall, we treated 39 patients with a 2-week course of antibiotics and 46 with a 4-week course, of which a median of 1 and 2 days was administered intravenously. In the two study arms, the median number of surgical lavages was 1 and the adverse events related to antibiotics were similar. We noted recurrence of bacterial arthritis after stopping antibiotic treatment in the same three patients as in the ITT population (4%): 1 in the 2-week arm (98% remission) and 2o in the 4-week arm (95%; p=0.46). Various sequelae occurred in 50% of the subjects in the 2-week treatment arm compared with 55% in the 4-week treatment arm. Only five (13%) and

Table 3 Characteristics of patients treated with 2 versus 4 weeks of systemic antibiotic therapy after surgical drainage of native joint bacterial arthritis of the hand and the wrist (per-protocol population only)

Hand arthritis (per-protocol analysis) n=85	4 weeks n=39	2 weeks n=46	P value*
Female sex	14 (36%)	18 (39%)	0.76
Median age	52 years	46 years	0.33
Immune-suppression†	4 (10%)	5 (11%)	0.93
Median ASA Score[16]	2 (IQR: 1–2)	2 (IQR: 1–2)	0.05
Serum C reactive protein at admission (median)	15 mg/L	8 mg/L	0.45
Pain score on admission (median)	4 points	5 points	0.15
Antibiotics before first surgery	10 (26%)	14 (30%)	0.63
Delay onset of infection surgery (median)	2 days (IQR: 1–3)	1 day (IQR: 0–2)	0.10
Staphylococcus aureus infection	10 (26%)	16 (35%)	0.36
Streptococci	9 (23%)	7 (15%)	0.36
Gram-negative pathogens	12 (31%)	13 (28%)	0.80
Number of surgical lavages (median)	1 (IQR: 1–1)	1 (IQR: 1–1)	0.23
Treatment with β-lactam antibiotics	28 (72%)	25 (76%)	0.65
Duration of intravenous therapy (median)	2 days	1 day	0.01
Complete microbiological remission	37 (95%)	45 (98%)	0.46
Duration of sick leave (median)	36 days	27 days	0.33
Number of outpatient attendances (median)	7 controls	8 controls	0.77
Length of hospital stay (median)	4 days	3 days	0.01
Mechanical or neurologic sequelae	21 (55%)	23 (50%)	0.52
Antibiotic-related adverse events	2 (5%)	2 (4%)	0.87

*Pearson χ^2 test or Wilcoxon rank-sum tests, as appropriate.
†Immune-suppression=diabetes mellitus, active cancer, cirrhosis CHILD C, organ transplant and chronic steroid medication.

six (13%) sequelae, respectively, required further and specific interventions (p=0.43). Statistically, our results equally fulfilled the non-inferiority requirements regarding the 10% margin for clinical (two tailed −0.8% percentage points (90% CI −8.5% to +7.0%)) and microbiological remission (4.3% (90% CI −1.1% to +9.8%)), as well as adverse events (0.8% (90%CI −7.0% to +8.5%)); but not regarding substantial sequelae (5.2% (90%CI −13.1% to +23.4%)).

DISCUSSION

In this randomised trial with 154 adult cases of native joint bacterial arthritis, we found no significant difference in rates of clinical remission, adverse events or sequelae in patients treated with 2, compared with 4, weeks of antibiotic therapy after surgical drainage. Moreover, we found no differences in these two arms in analyses of both the ITT and the PP populations, as well as the subgroup of hand arthritis compared with cases in other anatomical sites in group comparisons. Statistically speaking, our study population fulfilled the formal non-inferiority requirements regarding the outcome 'remission' (primary outcome). This was valid for both the ITT and PP analyses, the whole arthritis population and equally separated for the hand arthritis group only. Regarding the secondary outcomes 'adverse events' and 'sequelae', these statistical requirements were only fulfilled for adverse events in all analyses, whereas for substantial sequelae, for example, those with the need for further interventions, our sample size was formally too small. The only exceptions to the similarity in the two treatment arms is that patients in the 4-week arm had a significantly longer length of hospital stay and received a longer duration of parenteral antimicrobial therapy. Only 10% of potentially eligible patients refused to participate in the study, which equally good participation. Moreover, clinically and anamnestically, the refusing patients and those excluded by investigators were a very heterogeneous group without clear patterns of objective common co-morbidities, avoiding an overt refusal bias.

In our review of the literature, we found no randomised clinical trials in adult patients regarding the duration of postsurgical antibiotic therapy in patients with septic arthritis. Various expert groups have recommended different regimens for these cases, such as 2 weeks intravenous therapy for streptococci, 3–4 weeks intravenous for staphylococci and Gram-negative bacteria[21 22] and >4 weeks for immune-suppressed patients or those with

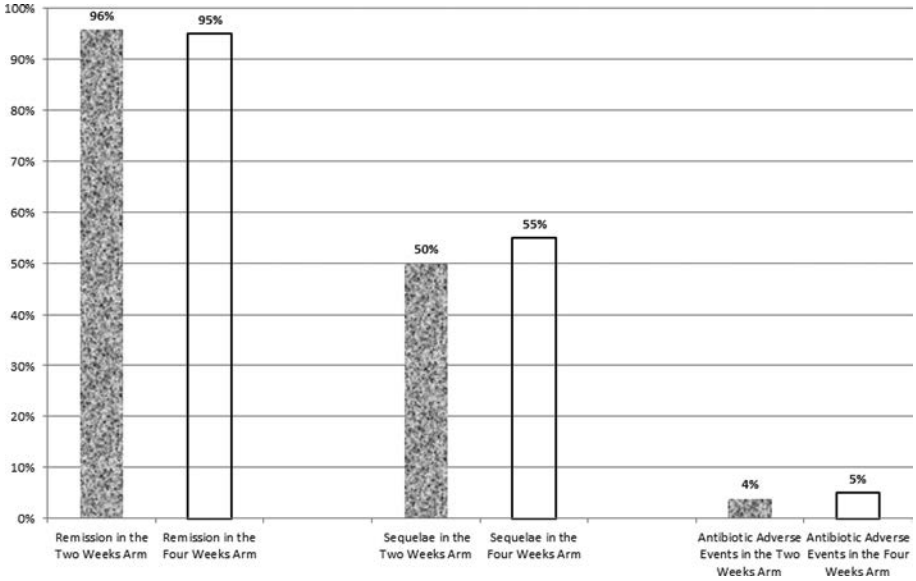

Figure 2 Outcomes of arthritis therapy of the hands and wrists (subgroup analysis). X-axis: study outcome parameters. Y-axis: number of corresponding episodes with proportions and absolute number of cases.

abnormal joints.[21] Others have recommended parenteral treatment for 2 weeks followed by another 2 weeks of oral treatment,[23] or just for a total 4 weeks.[24] Some surgeons prescribe antibiotics for even longer periods without further justification.[25] In the paediatric orthopaedic literature, recommendations are often for a total antibiotic duration of just 2–3 weeks[26–29] or even 10 days.[30] The only investigation of hand bacterial arthritis in the adult population we found was a retrospective study by Meier *et al*[2] that found good outcomes with no more than two surgical interventions and a median antibiotic duration of 14 days in 79% of episodes, similar to what we found in our study.

The results of our trial also support our long-standing policy of providing only a short duration of initial parenteral antibiotic therapy for bacterial arthritis.[3] This contrasts with widespread suggestions for several weeks of parenteral therapy for adult septic arthritis.[21 22] We have found no evidence supporting the need for intravenous antibiotic administration for intrasynovial infections, especially for cases involving smaller joints, such as hand arthritis, that can be drained in toto without missed reservoirs of infection. Even in cases of bone infection, administration of highly bioavailable oral antibiotics can provide acceptable penetration levels,[25] and paediatric arthritis data suggest that an early switch to oral antibiotics is as effective as prolonged parenteral regimens.[26–30] In the aforementioned Meier study of 101 cases of hand arthritis in adults, the transition from the parenteral to oral route occurred 3–5 days after definite surgery in the vast majority of the cases.[2] Finally, very recently, colleagues from Oxford published a large multicentre prospective randomised study with various types of osteoarticular infections, mostly prosthetic joint infections. They could demonstrate the non-inferiority of an early switch to oral targeted medication after 7 days postoperatively.[31] In our study, we switched much earlier after 1–2 days. The lack of prospective randomised trials had made it difficult to change the view of experts' that an initial 2 weeks of parenteral antibiotic therapy is required for all moderate to severe orthopaedic infections. We hope that these recent prospective data, including ours, may make a start in revising that view, at least with hand bacterial arthritis.

Our study has limitations. The majority of arthritis cases involved the hand and wrist (n=99), with only 55 affecting larger joints. While the overall study population entirely fulfilled the sample size requirements for a non-inferiority trial, the subgroup of these 55 patients with larger joint infections did not, making the study underpowered for conclusions regarding non-hand episodes. With this issue in mind, we performed a second interim analysis and considered it unethical to continue the trial just to ensure sufficient numbers (≥250 cases in each study arm) for all subsets of joint infections. Another limitation is that we only compared treatment durations of 2 weeks versus 4 weeks, so we cannot comment on the possible value of shorter (or longer) durations of therapy. While there appears to be little benefit for treating native joint infections with longer than 4 weeks, there are almost no data favouring antibiotic courses shorter than 2 weeks. The third limitation is that the study is performed in a single centre and that it was unblended, demanding for further confirmations in other settings, for example, regarding surgical techniques and approaches.

CONCLUSION

In adult patients who have undergone drainage of native joint bacterial arthritis, we found no difference in rates of clinical remission, adverse events or postinfectious sequelae for those treated with only 2 weeks, compared with 4 weeks' of antibiotic therapy. Our data have statistical validity for hand arthritis cases but are underpowered for other anatomic localisations. As there are many clinical and economic advantages, and apparently no disadvantages, to shorter courses of antibiotic therapy, patients with hand bacterial arthritis might benefit from shorter treatment courses. Similarly, our data support an earlier switch from parenteral to oral antibiotic therapy for these patients, which could help reduce financial costs, length of hospital stay and potentially intravenous line-related complications.

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Competing interests None declared.

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

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EPIDEMIOLOGICAL SCIENCE

Association between inactivated influenza vaccine and primary care consultations for autoimmune rheumatic disease flares: a self-controlled case series study using data from the Clinical Practice Research Datalink

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Objectives To examine the association between inactivated influenza vaccine (IIV) administration and primary care consultation for joint pain, rheumatoid arthritis (RA) flare, corticosteroid prescription, vasculitis and unexplained fever in people with autoimmune rheumatic diseases (AIRDs).

Methods We undertook within-person comparisons using self-controlled case-series methodology. AIRD cases who received the IIV and had an outcome of interest in the same influenza cycle were ascertained in Clinical Practice Research Datalink. The influenza cycle was partitioned into exposure periods (1–14 days prevaccination and 0–14, 15–30, 31–60 and 61–90 days postvaccination), with the remaining time-period classified as non-exposed. Incidence rate ratios (IRR) and 95% CI for different outcomes were calculated.

Results Data for 14 928 AIRD cases (69% women, 80% with RA) were included. There was no evidence for association between vaccination and primary care consultation for RA flare, corticosteroid prescription, fever or vasculitis. On the contrary, vaccination associated with reduced primary care consultation for joint pain in the subsequent 90 days (IRR 0.91 (95% CI 0.87 to 0.94)).

Conclusion This study found no evidence for a significant association between vaccination and primary care consultation for most surrogates of increased disease activity or vaccine adverse-effects in people with AIRDs. It adds to the accumulating evidence to support influenza vaccination in AIRDs.

Key messages

What is already known about this subject?

- Inactivated influenza vaccine is recommended in patients with autoimmune rheumatic diseases to minimise the increased risk of influenza and its complications in this population.
- Concerns about influenza vaccine associating with increased risk of autoimmune rheumatic disease (AIRD) activity and anecdotal reports of the influenza vaccine triggering diseases such as vasculitis are barriers to seasonal influenza vaccination.

What does this study add?

- Seasonal influenza vaccination is not associated with AIRD flare and vasculitis.

How might this impact on clinical practice or future developments?

- This study provides new data on the safety of influenza vaccine in people with AIRDs and adds to the accumulating evidence to support seasonal influenza vaccination in this population.

INTRODUCTION

Autoimmune rheumatic diseases (AIRDs) such as rheumatoid arthritis (RA) associate with increased risk of influenza and its complications.[1] Even though inactivated influenza vaccine (IIV) has clinical and serological effectiveness in AIRDs, its uptake remains suboptimal.[2–4] For instance, when AIRDs were the sole indication for vaccination, 49% and 59% people aged 18–44 and 45–64 years, respectively, were vaccinated in the 2015–2016 influenza season in the UK,[5] with lower vaccine uptake reported in Germany.[6]

Barriers to IIV include scepticism about effectiveness, concerns about side effects or disease flare and reports of vaccination triggering AIRDs such as vasculitis.[7–11] Trials assessing serological response to IIV report stable disease activity following vaccination provided disease-modifying antirheumatic drugs (DMARDs) treatment is continued.[12 13] However, these studies typically include people with stable disease, and, to the best of our knowledge, a real-world study evaluating the effect of IIV on AIRDs has not been performed. Thus, the objectives of this study were to investigate the association between IIV administration and primary care consultation for joint pain, RA flare, new oral corticosteroid prescription and potential vaccine adverse effects, such as vasculitis and non-infective fever.

METHODS

Data source

Data were extracted from the Clinical Practice Research Datalink (CPRD).[14] CPRD is a longitudinal database of anonymised health records of >15 million people registered in >700 general

practices in the UK. Participants are representative of the UK population.[14] It contains details of demographics, diagnoses, immunisations, prescriptions and lifestyle factors.

Study design

Self-controlled case series (SCCS) was developed for assessing associations between exposures and outcomes using data from participants who develop an outcome of interest and is an accepted methodology for vaccine safety studies.[15 16] It has the advantage of being unaffected by between-person confounding as each participant acts as their own control. However, it does not account for time varying covariates such as season which vary between the unexposed and exposed periods.

Source population

Adults aged ≥18 years with RA, spondyloarthritis (SpA) or systemic lupus erythematosus (SLE) and prescribed DMARDs.[3]

Study period

The study period was 1 September 2006 to 31 of August 2016. This was partitioned into 10 influenza cycles, beginning on 1 September of 1 year and ending on 31 August the subsequent year. Due to the use of non-standard monovalent vaccine alongside trivalent IIV during 2009–2010 pandemic year, data for this year were excluded. Observation periods for each year were censored if death, emigration from general practice or last collection of data from general practice occurred before 31 August of the subsequent year.

Exposure and outcomes

Vaccination, the exposure of interest, was defined using Read codes[5] and event dates. Influenza cycles in which a patient was coded as having received the IIV elsewhere, for example, at work for health care professionals or in community pharmacies were excluded from the analysis as the date of vaccination outside primary care is not recorded in the CPRD. For cases with >1 entry for vaccination in an influenza cycle, the earliest record was retained.

The outcomes were primary care consultation for:

A. Surrogates of disease activity: joint pain, flare of RA and new corticosteroid prescriptions.
B. Vaccine hypersensitivity: vasculitis, non-infective fever. Only the first Read code for vasculitis was considered since it is a chronic illness, and, participants diagnosed with vasculitis before study entry were excluded.

See online supplementary material for details.

Exposure and unexposed periods

The influenza cycle was divided into unexposed and exposed periods, and the latter was further categorised into smaller time periods (figure 1). The first cut-off was selected at 14-day post-vaccination as it takes 2 weeks for the IIV to induce a serological response, and, we hypothesised that this period of immune reconstitution was most likely to associate with disease activity.[17] The 14-day period immediately preceding vaccination was excluded from the baseline period to minimise confounding due to healthy vaccinee effect.[18]

Statistical analyses

SCCS involves fitting a Poisson model conditioned on the number of events and calculates incidence rate ratios and 95% CI for each exposure period. Distinct SCCS analysis data sets were drawn for each outcome. Each participant contributed data from one randomly selected eligible influenza cycle in which both vaccination and an outcome of interest occurred (online supplementary table S1). We analysed data from single randomly selected influenza cycle since people who develop an adverse outcome temporally closely related to vaccination, for example, joint pain within 1–2 weeks of vaccination, will be less likely to attend for repeat vaccinations compared to those who have such an event after a longer time period. Thus, if vaccination was to cause an adverse effect, including data from all influenza cycles in which an outcome occurred for a study participant would introduce differential bias. Except for vasculitis, >1 event of the same type in an influenza cycle were considered as recurrent episodes provided the interval between any two consultations was ≥15 days.

As corticosteroids are prescribed for many reasons, we performed sensitivity analysis restricted to corticosteroid prescription on the same day on which there is a primary care consultation for joint pain or RA flare. All analyses were carried out using Stata V.14.

RESULTS

Data for 14 928 AIRD cases with ≥1 outcome of interest in an influenza cycle in which they received the IIV were included. Of these, 11 953 (80.07%) had RA, 2347 (15.72%) had SpA and 628 (4.21%) had SLE. The majority were female (68.5%) and

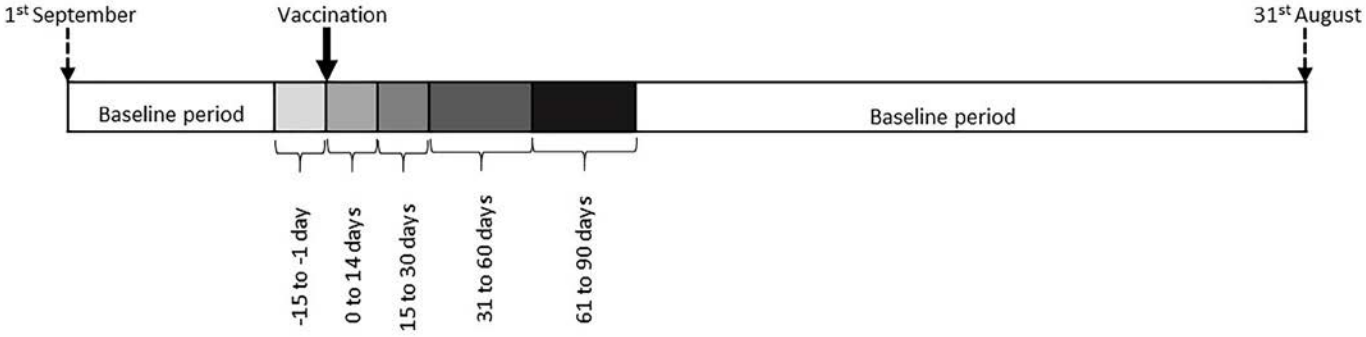

Figure 1 Influenza cycle divided into baseline, prevaccination and postvaccination periods. The baseline extended from latest of diagnosis date or 1st September in index year to 15 days pre-vaccination,and from 90 days post-vaccination to earliest of 31st August of the next year, date of leaving GP surgery, date of death, or latest date of data collection. Exposed period extended from vaccination to 90 days later,and was categorised as illustrated above.

Table 1 Association between IIV and consultation for joint pain, new corticosteroid prescription and RA flare

Outcome	Risk period	Events (n)	Person-time (days)	IRR (95% CI)*	P value*
Joint pain	Unexposed	9977	2 712 373	1.00	**<0.001**
	15 days prevaccination	788	160 775	**1.29 (1.20 to 1.39)**	
	Postvaccination intervals				
	0–14 days	479	150 314	**0.84 (0.77 to 0.92)**	**<0.001**
	15–30 days	567	160 842	0.94 (0.86 to 1.02)	0.127
	31–60 days	1121	321 024	**0.93 (0.88 to 0.99)**	**0.025**
	61–90 days	1069	319 890	**0.90 (0.84 to 0.96)**	**0.001**
Corticosteroid prescription	Unexposed	9470	2 266 070	1.00	<0.001
	15 days prevaccination	704	135 493	1.21 (1.12 to 1.31)	
	Postvaccination intervals				
	0–14 days	539	126 625	1.00 (0.91 to 1.09)	0.924
	15–30 days	554	135 515	0.96 (0.88 to 1.04)	0.338
	31–60 days	1151	270 109	1.00 (0.95 to 1.07)	0.876
	61–90 days	1182	268 968	1.04 (0.98 to 1.11)	0.184
RA flare	Unexposed	460	153 247	1.00	0.785
	15 days prevaccination	24	8957	0.95 (0.64 to 1.41)	
	Postvaccination intervals				
	0–14 days	27	8400	0.97 (0.65 to 1.45)	0.888
	15–30 days	29	9000	1.12 (0.78 to 1.62	0.525
	31–60 days	59	18 000	1.06 (0.80 to 1.39)	0.693
	61–90 days	46	17 978	0.81 (0.59 to 1.10)	0.180

*Statistically significant results are in bold (p<0.05).
IIV, inactivated influenza vaccine; IRR, incidence rate ratio; RA, rheumatoid arthritis.

their mean (SD) age was 59 (14) years. Vaccination associated with fewer primary care consultations for joint pain with the magnitude of reduction broadly consistent across the four risk periods (table 1). When data for the first 30 days were pooled together, vaccination associated with fewer primary care consultations for joint pain (IRR 0.89 (95% CI 0.84 to 0.9)). The 14-day prevaccination period associated with significantly more primary care consultations for joint pain and new corticosteroid prescriptions (table 1). The median (IQR) corticosteroid dose was 10 (5–30) mg/day prednisolone equivalents, data available for 54% prescriptions.

There were no significant associations between vaccination and other adverse outcomes in this study (tables 1 and 2). On sensitivity analysis, vaccination was not associated with new corticosteroid prescription on the same day on which there was a primary care consultation for either RA flare or joint pain (online supplementary table S2).

DISCUSSION

This study reports no significant associations between IIV administration and new primary-care corticosteroid prescriptions or primary care consultations for vasculitis and

Table 2 Association between inactivated influenza vaccine, vasculitis and unexplained fever*

Outcome	Risk period	Events (n)	Person-time (days)	Incidence rate ratio (95% CI)	P values
Vasculitis	Unexposed	89	30 936	1.00	0.906
	15 days prevaccination	5	1815	0.95 (0.38 to 2.33)	
	Postvaccination intervals				
	0–14 days	X[1]	1694	0.41 (0.10 to 1.65)	0.207
	15–30 days	5	1815	0.95 (0.38 to 2.33)	0.906
	31–60 days	8	3630	0.76 (0.37 to 1.56)	0.453
	61–90 days	12	3630	1.14 (0.62 to 2.08)	0.677
Unexplained Fever	Unexposed	92	30 735	1.00	0.507
	15 days prevaccination	X[1]	1819	0.71 (0.26 to 1.94)	
	Postvaccination intervals				
	0–14 days	X[1]	1708	0.71 (0.26 to 1.94)	0.589
	15–30 days	6	1830	1.06 (0.28 to 2.07)	0.887
	31–60 days	5	3650	0.45 (0.18 to 1.10)	0.079
	61–90 days	12	3630	1.09 (0.60 to 1.99)	0.780

X[1] fewer than five events in each cell, data suppressed according to Clinical Practice Research Datalink policy.
*Unexplained fever was defined as fever not due to a known cause, for example, infection.

non-infective fever. However, there was a negative association between vaccination and primary care consultations for joint pain upto 90 days postvaccination. Further research is required to understand the underlying mechanism. It is unlikely to result from contextual response or healthy vaccine effect as there is no prevalent belief that vaccination improves AIRD outcomes, and SCCS utilises within-person comparisons accounting for the latter. However, this observation could result from regression to the mean.

We observed increased primary care consultation for joint pain and new corticosteroid prescriptions in the 14 days preceding vaccination. This could indicate opportunistic vaccine promotion to people consulting for AIRD flare. Alternatively, this may be due to the fact that most influenza vaccinations occur in late autumn and winter months[5] that coincide with increased AIRD activity. It is therefore of interest that the 30-day postvaccination period, also in the late autumn and winter months, expected to have more consultations for joint pain, had significantly fewer consultations.

The potential for vaccines to elicit an immune-mediated adverse reaction has raised concerns about a link between vaccines and AIRDs.[9 10] However, our data do not identify a significant association between vaccination and vasculitis and are in line with other studies.[19] Similarly, there was no association between IIV and incident RA in the Epidemiological Investigation of Rheumatoid Arthritis cohort.[20]

The main strength of this study is its robust design, employing the SCCS method. By performing within-person comparisons, it minimises the influence of confounding between individuals, a serious problem in observational studies of adverse-effects following vaccination. Additionally, the use of consultation and prescription data minimised recall bias. The inclusion of a broad spectrum of AIRDs makes our findings generalisable. Additionally, we performed sensitivity analysis restricting to corticosteroid prescriptions on the same day as primary care consultation for either joint pain or RA flare.

However, there are several limitations to this study. First, data on disease activity is not recorded in the CPRD, and primary care consultations occur at least a few days after flare onset. Self-managed flares and those managed by rheumatologists are excluded. Thus, the use of consultation-based database underestimates the event rate. However, these caveats are unlikely to affect the validity of our findings as there is no reason for the ratio between events and primary care consultations to vary across the influenza-cycle. Our a priori decision to restrict the data analysis to one randomly selected influenza-cycle may have reduced the power for some outcomes such as new corticosteroid prescription which occurred in ≥2 influenza cycles for 50% participants. This does not apply to other uncommon outcomes, about 90% of which occurred in only one influenza cycle.

In conclusion, this study supports the safety of influenza vaccine in AIRDs. These data should be used to address the fear of adverse effects from vaccination, an important reason for suboptimal uptake of influenza vaccination in AIRDs.

Contributors AA conceived the idea for the study and all authors planned the study collaboratively. AA, MG and GN developed the analysis plan; data analysis was undertaken by GN and supervised by MG and AA. JSN-V-T and PRM provided influenza specific input and advised on the data analysis plan. CDM provided primary care input. AA together with GN wrote the first draft of the manuscript. All authors reviewed the results and critically reviewed the manuscript for intellectual content. All authors approved the final version of the manuscript.

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TRANSLATIONAL SCIENCE

Chromatin interactions reveal novel gene targets for drug repositioning in rheumatic diseases

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ABSTRACT

Objectives There is a need to identify effective treatments for rheumatic diseases, and while genetic studies have been successful it is unclear which genes contribute to the disease. Using our existing Capture Hi-C data on three rheumatic diseases, we can identify potential causal genes which are targets for existing drugs and could be repositioned for use in rheumatic diseases.

Methods High confidence candidate causal genes were identified using Capture Hi-C data from B cells and T cells. These genes were used to interrogate drug target information from DrugBank to identify existing treatments, which could be repositioned to treat these diseases. The approach was refined using Ingenuity Pathway Analysis to identify enriched pathways and therefore further treatments relevant to the disease.

Results Overall, 454 high confidence genes were identified. Of these, 48 were drug targets (108 drugs) and 11 were existing therapies used in the treatment of rheumatic diseases. After pathway analysis refinement, 50 genes remained, 13 of which were drug targets (33 drugs). However considering targets across all enriched pathways, a further 367 drugs were identified for potential repositioning.

Conclusion Capture Hi-C has the potential to identify therapies which could be repositioned to treat rheumatic diseases. This was particularly successful for rheumatoid arthritis, where six effective, biologic treatments were identified. This approach may therefore yield new ways to treat patients, enhancing their quality of life and reducing the economic impact on healthcare providers. As additional cell types and other epigenomic data sets are generated, this prospect will improve further.

Key messages

What is already known about this subject?
- There is a need to identify effective treatments for rheumatic diseases.
- Selecting drug targets with genetic evidence support can double the chance of success in clinical development.
- Genetic studies, while successful, have had limited impact in identifying genes and functional mechanisms which could contribute to treatment repositioning.

What does this study add?
- This study provides an approach, using Capture Hi-C data, to link genetic associations to potential causal genes and assess the potential of drug repurposing in three rheumatic diseases.

How might this impact on clinical practice or future developments?
- This has the potential to identify genes which are functionally relevant and the target of existing therapies, which could provide new ways to treat patients, enhancing their quality of life and reducing the economic impact on healthcare providers.

INTRODUCTION

Autoimmune rheumatic diseases such as rheumatoid arthritis (RA), juvenile idiopathic arthritis (JIA) and psoriatic arthritis (PsA) constitute a substantial socioeconomic burden estimated to cost more than €200 billion per year in Europe.[1] While a number of therapies are used to treat symptoms, around half of all patients fail to respond well, and currently there is no cure, in part due to the lack of understanding of the biology underlying the disease. Furthermore, biologics, the most effective drugs to treat these rheumatic diseases, are expensive and constitute a major burden for healthcare systems. Indeed, the greatest overall cost of medicines in 2015/2016 in the UK was for adalimumab (£416.6 million), one of the biologics used to treat RA. Although biosimilars are emerging, the need for novel, more effective targeted treatments is therefore imperative.

For the past three decades, genetics and genomics have been incorporated into pipelines for drug discovery with the rationale that understanding the genes that cause disease may lead to a shift from alleviating symptoms to modifying the underlying mechanisms of disease.[2] Indeed, in a review of AstraZeneca's small-molecule drug projects from 2005 to 2010, it was found that 73% of projects with some genetic linkage of the target to the disease were active or successful in phase II compared with 43% of projects without such data.[3] In addition, an extensive study has shown that selecting a drug target with direct genetic evidence supporting its role can double the chance of a drug's success in clinical development.[4]

In this regard, well-powered genome-wide association studies (GWAS) have successfully identified hundreds of single nucleotide polymorphisms (SNPs) that predispose to rheumatic diseases.[5] Some

of these findings have sparked the successful repositioning of drugs, for example the association of genes in the interleukin (IL)-23 pathway; biologic drugs targeting components of this pathway are now used routinely for psoriasis and PsA and have been shown to be effective in the treatment of ankylosing spondylitis and inflammatory bowel disease.[6–8] It has been proposed that the annual sales of these medications alone are likely to be greater than the total amount spent on GWAS in the past decade.[9] GWAS discoveries have also highlighted novel therapeutic targets, and several programmes are currently under way to develop drugs based on this evidence, for example protein arginine deiminase inhibitors in RA.[10 11]

Despite these successful examples, the use of GWAS findings in drug discovery programmes has been quite limited. This is due to the fact that, although GWAS have identified numerous genetic variants that predispose to disease, around 90% lie outside traditional protein coding regions of the genome, often at considerable genomic distances from annotated genes,[12 13] and therefore their potential role in pathological mechanisms is not obvious.[14 15]

Recently, functional genomics studies including chromosome conformation capture-based methods[16] have provided evidence that complex diseases might result from a dysregulated interplay between enhancers containing disease-associated SNPs and their target genes.[17–21] For example, in previous studies, we used Capture Hi-C (CHi-C) to characterise the chromatin interactions between all the regions of the genome associated with RA, JIA and PsA and their potential targets,[20] and then showed that an autoimmunity variant in the 6q23 chromosomal region regulated the closest gene, *TNFAIP3*, and *IL20RA* and *IFNGR1*. Interestingly, *IL20RA* is the target of an existing drug for RA, and therefore this shows how functional evaluation of disease risk loci can help translate GWAS findings into biologically meaningful mechanisms of disease and can validate therapeutic targets or suggest new ones.[22]

In this study, we aimed to systematically mine these existing chromatin interaction data[20] and integrate publicly available gene expression and epigenetics data to link GWAS variants to their potential target genes through physical contact. This has the potential to produce a more accurate disease gene list than simply annotating disease variants with genes in the traditional way, and therefore could identify potentially causal genes that are targets for existing drugs, which could be repositioned for use in RA, JIA and PsA.

METHODS

Capture Hi-C

CHi-C data were produced as part of a previous larger study targeting regions associated with four autoimmune diseases (RA, JIA, PsA and type 1 diabetes)[20] and analysed using Capture Hi-C Analysis of Genomic Organisation (CHICAGO) (online supplementary methods).[23]

Evaluation of reported GWAS genes

Reported GWAS hits are typically labelled according to the nearest genes; these designations were taken from the parent publications[24–27] used for the original CHi-C study and compared with the CHi-C genes showing interactions with the SNPs linkage disequilibrium (LD) block.

CHi-C filtering

ChromHMM chromatin state models for T helper naive, T helper memory and GM12878 lymphoblastoid cells from the Roadmap Epigenomics Project were used to filter CHi-C interactions between fragments showing enhancer states on one end and promoter states (transcription start site (TSS)) on the other end. Genes contained within the promoter state fragment (other end) were extracted and used for further analysis. Furthermore, gene lists were filtered to include only those genes expressed in either GM12878 or primary T cells (figure 1A and online supplementary methods).[28]

Identification of drug targets

Gene lists identified during CHi-C filtering were compared against existing drug targets from the DrugBank V.5.0.11 database (https://www.drugbank.ca/releases/5-0-11). Current treatments were identified by the presence of the relevant disease name (eg, rheumatoid arthritis) and variations (eg, juvenile arthritis) in the 'indication' field.

Refinement of drug targets using pathway analysis

Given that not all genes identified through the filtering step will be involved in the disease due to resolution limitation (~7 kb) of CHi-C, the approach was refined further. It was reasoned that genes belonging to enriched pathways, defined by Ingenuity Pathway Analysis (IPA), would be more likely to be involved in the disease and therefore more likely candidates. Enriched IPA pathways were identified using the core expression analysis method which uses Fisher's exact test to identify enriched pathways, followed by Benjamini-Hochberg false discovery rate (FDR) controlling procedure to account for multiple testing. Additional targets on the same enriched pathway could also be identified and evaluated using this revised approach, expanding the list of possible drugs available for repositioning.

RESULTS

Evaluation of reported GWAS genes

Our stringent filtering of the original CHi-C data identified potential causal genes for 54% of GWAS regions across all diseases, around 40% of which did not include the previously annotated causal gene (figures 1B and 2 and online supplementary table S1). Where the previously annotated gene was identified (32%), CHi-C showed evidence implicating the involvement of more than one causal gene for 44 out of 54 (81%) regions and more than five genes in 39% of cases, in addition to the previously annotated gene. This effect was largely driven by RA, which showed evidence for multiple genes at 38 out of 46 loci (83%) and more than 5 genes in 18 (39%).

Identification of drug targets

Overall, ChromHMM enhancer regions containing associated SNPs showed evidence of interacting with 408 genes which were expressed in either B cells or T cells, corresponding to 92 associations (54%) (table 1). Of these, 48 were existing approved drug targets, identified in DrugBank, for which 108 drugs are available (online supplementary tables S2-S4). CHi-C identified the most genes for RA (324), the most drugs (84) and 9 existing therapies used in the treatment of RA, 6 of which are effective biologic therapies (adalimumab, etanercept, rituximab, sarilumab, tocilizumab and tofacitinib). Thus 97 currently available therapies, 75 of which were for RA, not used in these diseases, were identified which could potentially be repositioned to provide effective treatment alternatives. By comparison, using the same method with RA reported genes from Okada *et al*[26] instead of CHi-C-identified genes, 24 existing drug targets were

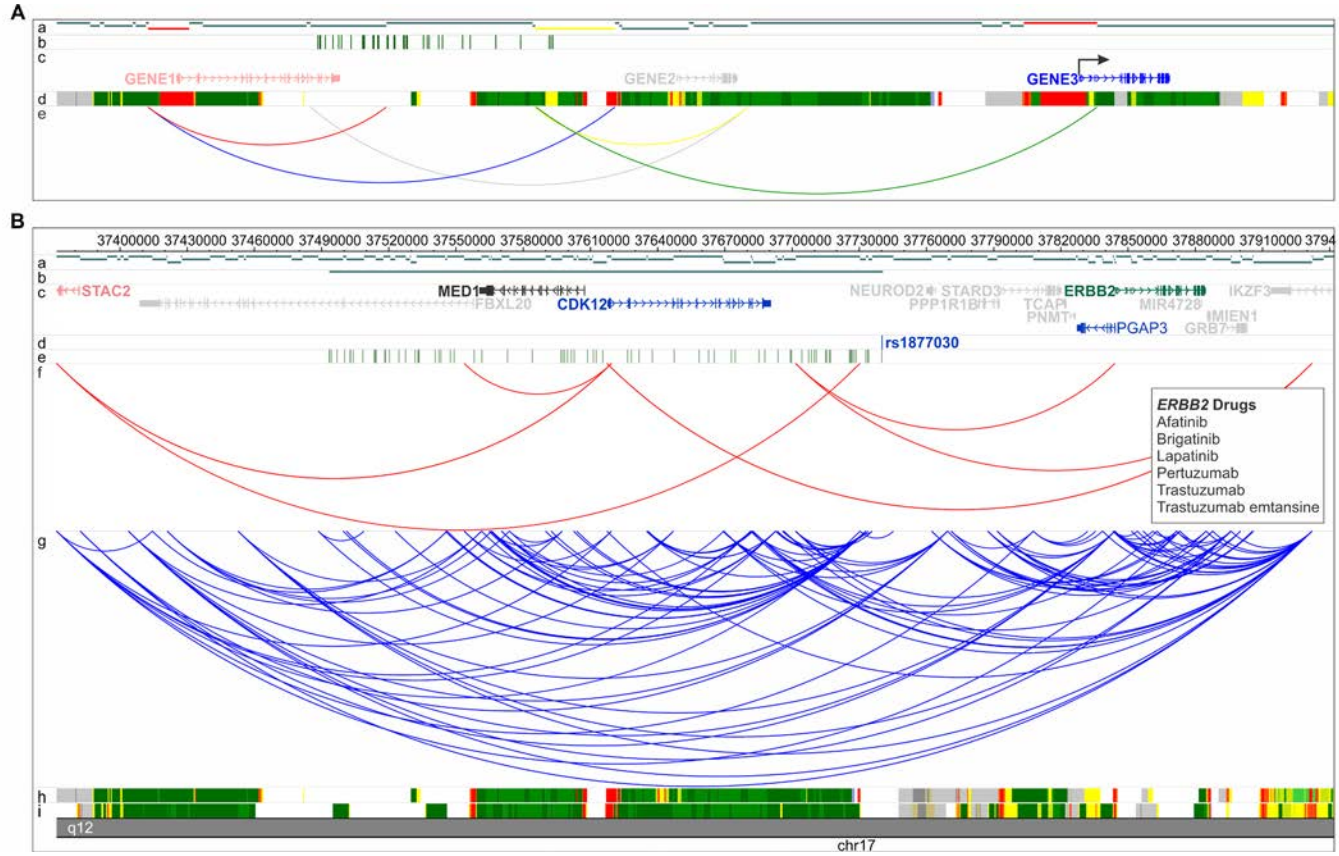

Figure 1 (A) CHi-C filtering strategy schematic. Tracks are labelled a–e: (a) HindIII restriction digest fragments. Promoter fragments are shown in red and enhancer fragments are shown in yellow. (b) SNPs in LD with index SNP. (c) Genes in the region. Genes showing no evidence of interacting or show no promoter and enhancer activity are shown in grey; CHi-C-filtered genes not expressed are shown in red; CHi-C-filtered genes which show evidence of promoter and enhancer activity and are expressed (arrow) are shown in blue. (d) Example 15-state ChromHMM states. (e) All CHi-C interactions within the region. Only interactions which are between promoter and enhancer fragments and are expressed (green) are used for further analysis. Interactions showing no promoter or enhancer states (grey), only promoter (red), only enhancer (yellow), or involving genes which are not expressed (blue) are removed. Therefore, using this filtering strategy, only GENE3 would be retained for further analysis. (B) Example of CHi-C region showing RA locus rs1877030. For this locus CHi-C did not identify the GWAS-reported gene, MED1, as a potential candidate, but did identify three other genes (CDK12, ERBB2 and Pgap3), one of which is a known drug target (ERBB2). Genomic coordinates are shown along the top of the region and tracks are labelled a–i: (a) HindII restriction digest fragments. (b) rs1877030 linkage disequilibrium region ($r^2 \geq 0.8$). (c) RefSeq genes from the UCSC Genome Browser, downloaded 1 January 2012. The GWAS-reported gene is shown in black; genes showing no evidence of interacting with rs1877030 are shown in grey; CHi-C-identified genes not expressed in B cells or T cells are shown in red; CHi-C-identified genes which are not drug targets are shown in blue and CHi-C-identified genes which are drug targets are shown in green. (d) rs1877030 location. (e) SNPs in LD with rs1877030 ($r^2 \geq 0.8$). (f) CHi-C interactions filtered to retain those between fragments showing enhancer states on one end and promoter states on the other end. (g) All CHi-C interactions from T cells and B cells (unfiltered). (h,i) 15-state ChromHMM states for B cells and T cells, respectively. Identified drug targets and drugs are shown in boxes. CHi-C, Capture Hi-C; GWAS, genome-wide association studies; LD, linkage disequilibrium; RA, rheumatoid arthritis; SNP, single nucleotide polymorphism; UCSC, University of California Santa Cruz.

identified, corresponding to 50 drugs including 7 currently used to treat RA.

Among the drugs identified by CHi-C, 23% are used in the treatment of various carcinomas, lymphomas, melanomas and leukaemia, 9% in the treatment of multiple sclerosis and psoriasis, and 7% in the treatment of hypertension across all diseases (online supplementary tables S2-S4). These include alemtuzumab, used in the treatment of chronic lymphocytic leukaemia and multiple sclerosis, and natalizumab and daclizumab, used in the treatment of multiple sclerosis. Daclizumab has been trialled for the treatment of JIA-associated uveitis and resulted in five out of six participants showing a two-step reduction in inflammation (NCT00130637; https://clinicaltrials.gov/ct2/show/NCT00130637).

Refinement of drug targets using pathway analysis

IPA of the CHi-C-identified genes resulted in 139 enriched pathways across the three diseases (online supplementary table S5). However after controlling for FDR ($p \leq 0.05$), 41 pathways remained significant (table 2 and online supplementary table S6); these included CD40 signalling, T helper cell differentiation, and the JAK1, JAK2 and TYK2 in interferon signalling pathways.

Considering only those drug targets identified through CHi-C that form part of an enriched pathway reduced the total number of genes from 408 to 59 and the number of potential drugs for repositioning to 31 (table 3 and online supplementary table S5). Additionally eight drugs currently used to treat RA were identified: six biologic therapies (adalimumab, etanercept, rituximab, sarilumab, tocilizumab and tofacitinib), one analgesic

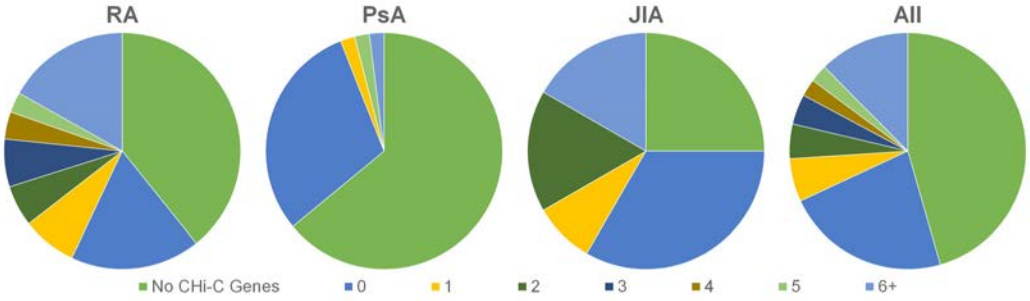

Figure 2 Comparison of genes identified for each SNP association loci by CHi-C and GWAS reported genes, by disease (RA, PsA, JIA) and overall (all). SNP associations where no genes were identified by CHi-C are labelled as 'no CHi-C genes'; category 0 shows SNP associations where CHi-C identified one or more genes but where the reported gene was not among them; category 1 shows SNP associations where CHi-C only identified the reported gene and none others; and categories 2–5 and 6+ represent the number of CHi-C genes identified where the reported gene was among those identified. CHi-C, Capture Hi-C; GWAS, genome-wide association studies; JIA, juvenile idiopathic arthritis; PsA, psoriatic arthritis; RA, rheumatoid arthritis; SNP, single nucleotide polymorphism.

(acetylsalicylic acid) and immune globulin human. Interestingly, only 42% of these genes represented reported GWAS genes. Expanding this gene list to include all genes involved in significant pathways resulted in 307 potential pathway target genes, corresponding to 412 drugs overall. Over 92% (283 of 307) of the potential pathway target genes and 96% of the potential pathway drugs were identified using RA GWAS associations. The approach also identified 95% (57 of 60) of existing drugs overall, including 54 of 57 drugs currently used in the treatment of RA.

Figure 3 shows the CD40 signalling pathway enriched for RA CHi-C genes, identifying 9 out of 34 possible targets. Two of these, *ICAM1* and *ATM*, are existing drug targets used in the treatment of multiple sclerosis, fatigue, orthostatic hypotension and osteoarthritis (natalizumab, caffeine and hyaluronic acid, respectively) and may provide potential targets for repositioning. This pathway shows the potential for the repositioning of existing targets (shaded in blue), such as *JAK3*, *MKK*, and *ERK1* and *ERK2*, and the possibility of novel targets (outlined in red), such as *CD40*, *TRAF1* and *TRAF6*. Indeed, an experimental drug targeting the *CD40* receptor is currently in phase II clinical trial for the treatment of lupus nephritis, caused by systemic lupus erythematosus (NCT02770170; https://www.boehringer-ingelheim.com/press-release/phase-ii-trial-now-enrolling-patients-lupus-nephritis).

Table 1 Number of drug target genes identified using disease associations interacting with CHi-C genes for each disease and the corresponding number of drugs

Disease	Genes identified by CHi-C (n)	Genes which are existing drug targets (n)	Drugs identified (n)	Drugs currently used (n)	Drugs for potential repositioning (n)
RA	324	39	84	9	75
PsA	110	10	28	1	27
JIA	37	3	8	0	8
All	408	48	108	11	97

Among the drugs currently in use, six effective biologic therapies (adalimumab, etanercept and rituximab (*FCGR2A*), sarilumab and tocilizumab (*IL6R*) and tofacitinib (*TYK2*)) were identified for RA, and a relatively recent treatment, apremilast (*PDE4A*), for PsA. Drugs with potential for repositioning included alemtuzumab (*FCGR2A*), natalizumab (*ICAM1*, *FCGR2A*) and daclizumab (*IL2RA*, *FCGR2A*).
CHi-C, Capture Hi-C; JIA, juvenile idiopathic arthritis; PsA, psoriatic arthritis; RA, rheumatoid arthritis.

DISCUSSION

We had previously generated data from chromosome conformation capture experiments to improve the assignment of genes to susceptibility variants identified through GWAS based on physical interactions rather than proximity for RA, JIA and PsA. For the first time, these data have been used to identify potential novel drug targets. Linking to DrugBank has allowed the identification of a number of existing drugs that could be tested for efficacy in these conditions.

The approach of using GWAS data to identify potential drug targets for complex diseases has been explored previously. In 2014, Okada *et al* assessed the potential of RA genetics in drug discovery based on the findings of a large transethic GWAS meta-analysis.[26] Using reported GWAS genes, or genes from a direct protein–protein interaction network, they tested 2430 genes against 871 drug target genes, defined as approved, in clinical trials or experimental, and found a significant 3.7-fold enrichment for approved RA drug targets. This corresponded to 67% of the approved RA target genes included in the analysis. However, the study was limited by their initial gene list of reported GWAS genes based purely on linear proximity on the chromosome of disease SNPs and genes.

A major strength of this study is the use of physical interactions of chromatin to identify genes directly affected by disease susceptibility variants. The advantage and validity of this approach were demonstrated by the increased ability to identify existing therapies (95% of all existing RA therapies). While this is promising, the method was, however, unable to identify three currently used RA drugs: prednisolone, methylprednisolone and methotrexate. Both prednisolone and methylprednisolone are corticosteroids which target the glucocorticoid receptor (*NR3C1*), which is located on 5q31.3, over 10 megabases from the nearest association, while the gene target for methotrexate (*DHFR*) is located over 15 megabases from the nearest association. The action of these drugs is therefore unlikely to be due to any disease susceptibility association; however, the efficacy of the drugs may still be influenced by genetics as previously shown.[29]

While other methods exist which predict enhancer targets, such as integrated method for predicting enhancer targets (IM-PET),[30] CHi-C has the potential to directly link disease associations to causal genes by interrogating the physical interactions between these regions. These interactions have been shown to play a role in gene regulation and can therefore link disease

Table 2 Pathways showing significant (p≤0.05) enrichment for CHi-C genes for each disease using the Benjamini-Hochberg FDR controlling procedure

Disease	Pathway	Molecules in pathway n)	Benjamini-Hochberg p value	SNP associations (n)	Interacting genes (n)	Interacting drug targets (n)	Cell type-specific significance
RA	CD40 signalling	81	7.08×10^{-4}	11	9	2	Both
	iNOS signalling	45	7.08×10^{-4}	7	7	2	Both
	T helper cell differentiation	73	1.38×10^{-3}	8	8	3	Both
	IL-12 signalling and production in macrophages	148	4.90×10^{-3}	10	10	3	Both
	Th17 activation pathway	91	4.90×10^{-3}	8	8	3	Both
	Toll-like receptor signalling	76	7.76×10^{-3}	7	7	0	Both
	Dendritic cell maturation	196	8.13×10^{-3}	12	11	3	Both
	April-mediated signalling	39	1.05×10^{-2}	6	5	0	Both
	Molecular mechanisms of cancer	789	1.05×10^{-2}	15	16	5	Both
	B cell activating factor signalling	41	1.20×10^{-2}	6	5	0	Both
	IL-1 signalling	91	1.23×10^{-2}	7	7	1	Both
	NF-κB signalling	187	1.23×10^{-2}	11	10	2	Both
	Role of JAK family kinases in IL-6-type cytokine signalling	25	1.23×10^{-2}	4	4	2	Both
	Th1 and Th2 activation pathway	187	1.23×10^{-2}	9	10	6	Both
	IL-10 signalling	69	1.29×10^{-2}	8	6	2	Both
	ErbB signalling	106	1.86×10^{-2}	8	7	3	Both
	RANK signalling in osteoclasts	104	1.86×10^{-2}	8	7	2	Both
	Th1 pathway	137	1.86×10^{-2}	7	8	5	Both
	TNFR2 signalling	30	1.86×10^{-2}	6	4	0	Both
	4-1BB signalling in T lymphocytes	32	2.14×10^{-2}	5	4	0	Both
	IL-17A signalling in airway cells	80	2.14×10^{-2}	7	6	2	Both
	IL-17A signalling in fibroblasts	35	2.82×10^{-2}	5	4	0	Both
	Small cell lung cancer signalling	87	2.82×10^{-2}	7	6	1	Both
	Th2 pathway	152	2.82×10^{-2}	7	8	4	Both
	Production of nitric oxide and reactive oxygen species in macrophages	196	3.63×10^{-2}	10	9	4	Both
	LPS-stimulated MAPK signalling	95	3.80×10^{-2}	7	6	2	Both
	NF-κB activation by viruses	95	3.80×10^{-2}	7	6	2	Both
	IL-8 signalling	204	4.07×10^{-2}	8	9	3	Both
	Role of PKR in interferon induction and antiviral response	41	4.07×10^{-2}	5	4	0	Both
	STAT3 pathway	132	4.17×10^{-2}	7	7	3	Both
	mTOR signalling	208	4.37×10^{-2}	8	9	4	Both
	IL-6 signalling	136	4.47×10^{-2}	8	7	2	GM12878
	PI3K signalling in B lymphocytes	135	4.47×10^{-2}	9	7	2	Both
PsA	Role of JAK1, JAK2 and TYK2 in interferon signalling	24	4.07×10^{-2}	3	3	1	
	Epithelial adherens junction signalling	149	4.47×10^{-2}	4	5	1	Both
	Interferon signalling	36	4.47×10^{-2}	3	3	1	GM12878
	Regulation of actin-based motility by rho	89	4.47×10^{-2}	3	4	0	Both
JIA	iNOS signalling	45	3.63×10^{-3}	3	3	1	Both
	Interferon signalling	36	3.63×10^{-3}	3	3	1	
	Th17 activation pathway	91	1.95×10^{-2}	3	3	1	GM12878
	Role of JAK1, JAK2 and TYK2 in interferon signalling	24	2.09×10^{-2}	2	2	1	Both
	Role of JAK family kinases in IL-6-type cytokine signalling	25	2.09×10^{-2}	2	2	1	Both
	IL-15 production	28	2.14×10^{-2}	2	2	1	Both
	PI3K/AKT signalling	129	2.14×10^{-2}	2	3	1	Both
	STAT3 pathway	132	2.14×10^{-2}	3	3	1	Both
	Th1 pathway	137	2.14×10^{-2}	2	3	2	Both
	Oncostatin M signalling	40	2.57×10^{-2}	2	2	1	Both
	Role of PKR in interferon induction and antiviral response	41	2.57×10^{-2}	2	2	0	Both
	Th1 and Th2 activation pathway	374	3.89×10^{-2}	2	3	2	Both
	Production of nitric oxide and reactive oxygen species in macrophages	196	4.07×10^{-2}	3	3	1	Both
	IL-8 signalling	204	4.27×10^{-2}	3	3	1	Both

The total number of molecules in the pathway is shown together with the number of SNP associations and the corresponding numberof genes and drug targets on the pathway. 'Cell type specific significance' specifies whether a pathway is significant using T cell (Jurkat) only or Bcell (GM12878) only genes (online supplementary methods). 'Both' states that the pathway was significant in both T cell-only genes and B cell-onlygenes

CHi-C, Capture Hi-C; FDR, false discovery rate; IL, interleukin; JIA, juvenile idiopathic arthritis; LPS, Lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PKR, protein kinase R; PsA, psoriatic arthritis; RA, rheumatoid arthritis; RANK, Receptor activator of nuclear factor κ B; SNP, single nucleotide polymorphism; STAT3, signal transducer and activator of transcription 3; iNOS, inducible nitric oxide synthase; mTOR, mechanistic target of rapamycin.

Table 3 Number of drug target genes identified for CHi-C genes enriched in significant pathways (Benjamini-Hochberg) for each disease, corresponding to the number of drugs and the potential for non-existing pathway gene targets for drug repositioning

Disease	Genes identified by CHi-C (n)	Genes which are existing drug targets (n)	Drugs identified (n)	Drugs currently used (n)	Drugs for potential repositioning (n)	Potential pathway targets (n)	Potential pathway drugs (n)
RA	50	13	38	8	30	283	398
PsA	9	2	2	0	2	47	87
JIA	10	2	4	0	4	205	325
All	59	14	39	8	31	307	412

CHi-C, Capture Hi-C; JIA, juvenile idiopathic arthritis; PsA, psoriatic arthritis; RA, rheumatoid arthritis.

enhancers to target genes. Additionally, using this method, we have shown evidence that one disease association may well affect more than one gene, something which is often not considered. There are, however, limitations to this approach dependent first on the completeness of the mapping of disease variants for a particular disease and second on the choice of cell type for generation of the chromosome conformation capture data. In this study the genetic architecture was better mapped for RA than for JIA or PsA, and the choice of cell type had a stronger evidence base for RA, potentially limiting the power of the analysis for PsA and JIA.

Due to the high degree of overlapping interactions, and therefore genes, between cell types and the shared protein functions (mainly general immune signalling-related proteins), many of the IPA pathways were significantly enriched in both cell types individually and therefore cannot explicitly implicate a cell type directly. However, certain pathways exhibited a higher significance in one cell type; for example, the IL-6 signalling and role of JAK family kinases in IL-6-type cytokine signalling pathways were both more associated in B cell-only genes, validating the approach. The integration of other epigenetic resources may help to further implicate a particular cell type and inform drug choice.

Furthermore, while most CHi-C interactions are shared between cell types, the failure to identify a putative causal gene for just under half of all disease regions may be due to the cell types studied as the associated variant may exhibit its effect in different cell types such as fibroblast-like synoviocytes, the major cell type present in inflamed joints. The selection of additional cell types, for each disease, should be informed by existing data:

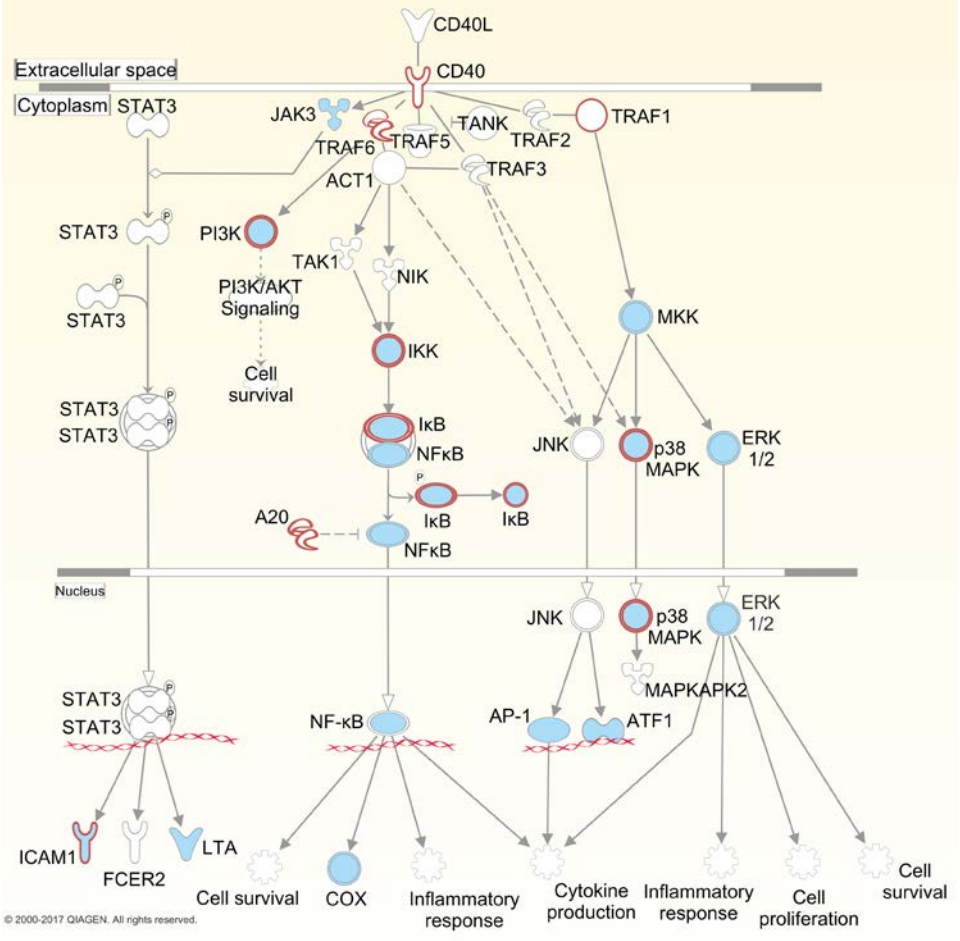

Figure 3 CD40 signalling pathway. Capture Hi-C-identified genes are outlined in red and existing drug targets are shaded in blue.

for example, while B cells and CD4+ T cells are important cell types in PsA, the decreased CD4+:CD8+ T cell ratio, the significant association with HLA-B*27 and the enrichment of associations to CD8+ H3K4me3 peaks suggest that CD8+ T cells are a major cell type in PsA,[27 31] and therefore the genes and/or interactions mediated by the associated variants may not fully be observed in this study. This may also explain the higher proportion of SNP associations where CHi-C was unable to identify any genes for PsA, compared with RA and JIA (figure 2). A limitation could also be the use of cell lines rather than primary cells for the generation of CHi-C data. This has implications, both in terms of the cells genetic and epigenetic profiles, which could influence the presence or strength of an interaction, confounding the results. Further experiments would therefore need to be performed in multiple, genetically diverse patient samples with integration of additional genetic and epigenetic data to control for this. Despite this, CHi-C provided evidence that almost half (49%) of all disease associations may either be incorrectly annotated or represent multiple potential gene targets in addition to the previously reported gene. CHi-C was more successful in identifying genes for RA, identifying potential target genes for over 60% of associations.

While these findings are promising and we have applied strict filtering of significant interactions, it should be noted that GWAS-based omics enrichment analyses can be biased towards false positives. However, due to the experimental aim of the initial CHi-C experiment, to target and investigate interactions between GWAS loci for the three rheumatic diseases (RA, PsA and JIA), it is not possible to fully correct for this. Further whole-genome CHi-C or validation experiments would assist in controlling for this limitation.

Additionally, while DrugBank provides the most comprehensive and accessible drug target resource, the exploration and integration of multiple drug target databases may identify additional, potentially useful drugs or help prioritise our existing drug targets. While our approach is unique in using CHi-C data to identify potential target genes, other target-searching methods, such as Open Targets Platform, exist.[32] Despite offering complementary approaches, the integration of these resources may further refine and prioritise any potential drug target candidates.

The experimental approach could be further refined and improved as, while CHi-C provides support for an involvement of a gene in disease, it is important to consider the effect of a variant in disease: for example, whether the gene is upregulated or downregulated and what implication this has on a drug's mode of action. It would therefore be important to incorporate gene expression data which would provide this information. Furthermore, studies using the CRISPR-Cas9 genome editing system would provide an independent validation of the associated variant effect at a pathway level and test the result of perturbations to this pathway.

Using existing data, we have evaluated the potential of CHi-C to identify new therapeutic targets or existing drugs which could be repurposed to treat rheumatic diseases. It is, however, important that any drugs identified by this or similar approaches are presented in full and evaluated extensively by biopharmaceutical industry professionals to confirm their potential for repurposing. It should also be noted that, despite this, not all drugs identified using these approaches will be successfully repurposed. For example, alemtuzumab, natalizumab and daclizumab, identified during this study, have been trialled for use in RA, but either showed little benefit (alemtuzumab and natalizumab) or did achieve Food and Drug Administration approval (daclizumab),[33] but was withdrawn due to unacceptable side effects including liver damage, encephalitis and meningoencephalitis.[34] This illustrates that while genetic evidence can provide support for drugs targets, trials are clearly required to confirm efficacy and safety.

Drug development is an expensive and time-consuming procedure, costing pharmaceutical companies an estimated $2.6 billion per drug and taking at least 10 years to develop. Additionally about 9 out of 10 drugs fail to make it to market either due to lack of efficacy or unacceptable adverse events.[35 36] Using genetics to inform drug development has the potential to dramatically cut costs and improve the likelihood of success. Drug repositioning is a complementary approach that removes the time spent on drug development and safety considerations entirely, and instead allows the drug to proceed straight to a tolerance/efficacy clinical trial.

We have therefore shown that CHi-C has the potential to identify existing drug targets which could be repositioned to treat rheumatic diseases. This was particularly successful for RA, where six effective, biologic treatments were initially identified, followed by 95% of existing therapies. Furthermore, while this analysis was limited to the three diseases selected (RA, PsA and JIA), this approach could be applied to other disease-specific or whole-genome promoter CHi-C data sets to identify potential drugs for further diseases. As more is known about the genetic component of PsA and JIA, coupled with further, more relevant CHi-C and epigenomic data sets, this approach may yield new ways to treat patients with rheumatic diseases, enhancing their quality of life and reducing the economic impact on healthcare providers.

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

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Efficacy and safety data based on historical or pre-existing conditions at baseline for patients with active rheumatoid arthritis who were treated with baricitinib

Patients with rheumatoid arthritis (RA) have a high prevalence of comorbidities.[1] This post-hoc analysis investigated the effect of select comorbidities (depression, osteoporosis, hepatic, cardiovascular or pulmonary disorders) on the efficacy and safety of baricitinib 4 mg once daily in patients with moderate-to-severe active RA and an inadequate response to conventional synthetic disease-modifying antirheumatic drugs (csDMARDs).

Data from the placebo-controlled periods of five baricitinib studies[2–6] were pooled for baricitinib 4 mg; data for baricitinib 2 mg were not analysed due to low (n=302) patient numbers. Additional data for all baricitinib-treated patients with a median exposure of 2 years were derived from an ongoing open-label, long-term extension (LTE) study that included patients from phase II and III studies (RA-BEYOND; NCT01885078).[7] Efficacy outcomes were evaluated at week 12 (vs placebo). Interaction of comorbidity-by-treatment was analysed using logistic regression or analysis of covariance. Safety observations up to week 16 (vs placebo) and during the LTE were summarised by the Medical Dictionary for Regulatory Activities preferred term.

Data from 1684 patients (803, baricitinib 4 mg; 881, placebo) from the placebo-controlled periods were analysed. The mean (SD) age was 52.7 (12.1) years, with only 38 (2.3%) patients aged ≥75 years; most patients (1506, 89.4%) were receiving background methotrexate, alone or in combination with another csDMARD. The numbers of patients receiving placebo and baricitinib 4 mg combined (with or without each comorbidity, respectively) were 133/1551 for depression, 247/1437 for osteoporosis, 424/1260 for hepatic disorders, 731/953 for cardiovascular disorders, and 166/1518 for pulmonary disorders. Demographic and clinical characteristics within each comorbidity subgroup were similar between patients randomised to baricitinib or placebo.

Higher proportions of patients achieved all clinical endpoints with baricitinib 4 mg than with placebo at week 12 across subgroups (table 1). Response rates in patients with or without each comorbidity who received baricitinib were generally close to overall response rates, with the exception of depression, where numerically lower response rates were observed in patients with versus without depression and overall (table 1). Responses to baricitinib versus placebo were similar between patients with or without comorbidity within each comorbidity subgroup, even for those with/without depression (p>0.1).

The safety analysis set from the placebo-controlled periods included 1683 patients (802, baricitinib; 881, placebo) with 235.2 and 246.9 patient-years of exposure (PYE) to baricitinib 4 mg or placebo, respectively. Similar proportions of patients experienced ≥1 treatment-emergent adverse events (TEAEs) between baricitinib and placebo across comorbidity subgroups (table 1). The most common TEAEs for the overall population were nasopharyngitis (5.6%, baricitinib vs 5.2%, placebo), upper respiratory tract infections (4.5% vs 3.5%), urinary tract infections (3.5% vs 2.6%) and bronchitis (2.9% vs 2.6%). Serious adverse events (SAEs) and discontinuations were infrequent, occurring at similar rates across subgroups for baricitinib and placebo. SAE and discontinuation rates for baricitinib-treated patients with/without each comorbidity were generally close to overall rates, although numerical differences were observed between patients with depression (lower rates), osteoporosis (higher rates) or pulmonary disorders (higher rates) and those without these comorbidities.

In the LTE study, safety data were available from 3439 patients with a total of 6633 PYE to baricitinib. Overall, the incidence of TEAEs, SAEs, discontinuations and reported deaths was similar between patients with or without comorbidity within each comorbidity subgroup (table 1). Further, the exposure-adjusted incidence rates for all patients were lower in the LTE than during the placebo-controlled period for all safety outcomes assessed. The numerical differences between patients with/without each comorbidity observed after 16 weeks (see above) were not observed in the LTE study.

This is the first analysis assessing the impact of select comorbidities on the efficacy and safety of baricitinib 4 mg. Limitations of this post-hoc analysis include the analysis of patient data derived from randomised clinical trials rather than real-world patient data, such that patients with certain acute or historical conditions, unstable illness, or certain laboratory abnormalities were excluded from the trials (see online supplementary text); the limited number of patients in each comorbidity subgroup and the limited duration of analyses; the non-inclusion of all known comorbidities in RA; the mix of comorbidities included in each subgroup, which could vary in severity and overall impact on patients; the inability to assess adverse events of specific interest and whether comorbidity severity improved or worsened following baricitinib treatment; and the inability to conclude on the efficacy and safety of baricitinib in patients aged ≥75 years and/or exposed to baricitinib 2 mg due to low patient numbers. Planned analyses of safety data from registries or healthcare databases in the USA and Europe are expected to address many of the current limitations.

Baricitinib 4 mg showed similar efficacy and safety during placebo-controlled and LTE observation periods regardless of the presence or absence of select comorbidities in patients with moderate-to-severe active RA and an inadequate response to csDMARDs. No trends for increased risk of safety events related to comorbidity were observed across comorbidity subgroups for baricitinib during a median exposure of 2 years. Further studies are needed to confirm the data presented, which provide a hypothesis only.

Trial registration numbers: NCT01185353, NCT01469013, NCT00902486, NCT01710358 and NCT01721057.

Table 1 Efficacy and safety data reported by select comorbidities in patients treated with baricitinib or placebo*

	Depression	No depression	Osteoporosis	No osteoporosis	Hepatic disorders	No hepatic disorders	CV disorders	No CV disorders	Pulmonary disorders	No pulmonary disorders	Overall
Baricitinib 4 mg											
Efficacy outcomes at week 12											
Patients, N	64	739	113	690	222	581	350	453	77	726	803
ACR20 response (%)†	59.4	68.2	65.5	67.8	67.1	67.6	68.3	66.9	66.2	67.6	67.5
ACR50 response (%)†	34.4	41.7	40.7	41.2	40.5	41.3	40.9	41.3	40.3	41.2	41.1
DAS 28-hsCRP ≤3.2 response (%)†	31.3	44.9	46.9	43.3	42.8	44.2	44.9	43.0	37.7	44.5	43.8
Change in HAQ-DI‡	−0.46	−0.54	−0.36	−0.55	−0.58	−0.53	−0.54	−0.52	−0.51	−0.53	−0.53
Safety outcomes at week 16											
Patients, N	64	738	113	689	222	580	350	452	77	725	802
Any TEAE, n (%), (EAIR)	45 (70.3), (246)	450 (61.0), (208)	70 (61.9), (216)	425 (61.7), (210)	152 (68.5), (232)	343 (59.1), (202)	227 (64.9), (221)	268 (59.3), (202)	62 (80.5), (284)	433 (59.7), (203)	495 (61.7), (210)
SAE, n (%), (EAIR)	0, (0.0)	25 (3.4), (11.5)	9 (8.0), (27.8)	16 (2.3), (7.9)	8 (3.6), (12.2)	17 (2.9), (10.0)	14 (4.0), (13.6)	11 (2.4), (8.3)	4 (5.2), (18.3)	21 (2.9), (9.8)	25 (3.1), (10.6)
Discontinuation due to AEs, n (%), (EAIR)	1 (1.6), (5.5)	24 (3.3), (11.1)	7 (6.2), (21.6)	18 (2.6), (8.9)	9 (4.1), (13.7)	16 (2.8), (9.4)	14 (4.0), (13.6)	11 (2.4), (8.3)	3 (3.9), (13.8)	22 (3.0), (10.3)	25 (3.1), (10.6)
Deaths, n (%), (EAIR)	0, (0.0)	0, (0.0)	0, (0.0)	0, (0.0)	0, (0.0)	0, (0.0)	0, (0.0)	0, (0.0)	0, (0.0)	0, (0.0)	0, (0.0)
Placebo											
Patients, N	69	812	134	747	202	679	381	500	89	792	881
Efficacy outcomes at week 12											
ACR20 response (%)†	31.9	39.8	32.1	40.4	37.1	39.8	40.7	38.0	32.6	39.9	39.2
ACR50 response (%)†	8.7	14.9	11.9	14.9	11.4	15.3	14.4	14.4	12.4	14.6	14.4
DAS 28-hsCRP ≤3.2 response (%)†	13.0	16.9	12.7	17.3	12.9	17.7	15.7	17.2	16.9	16.5	16.6
Change in HAQ-DI§	−0.20	−0.26	−0.08	−0.27	−0.28	−0.26	−0.27	−0.23	−0.17	−0.26	−0.25
Safety outcomes at week 16											
Any TEAE, n (%), (EAIR)	57 (82.6), (301)	437 (53.8), (192)	91 (67.9), (251)	403 (53.9), (191)	119 (58.9), (207)	375 (55.2), (198)	228 (59.8), (212)	266 (53.2), (191)	59 (66.3), (238)	435 (54.9), (196)	494 (56.1), (200)
SAE, n (%), (EAIR)	6 (8.7), (31.7)	25 (3.1), (11.0)	9 (6.7), (24.9)	22 (2.9), (10.4)	5 (2.5), (8.7)	26 (3.8), (13.7)	16 (4.2), (14.9)	15 (3.0), (10.8)	2 (2.2), (8.1)	29 (3.7), (13.1)	31 (3.5), (12.6)
Discontinuation due to AEs, n (%), (EAIR)	3 (4.3), (15.9)	21 (2.6), (9.2)	9 (6.7), (24.9)	15 (2.0), (7.1)	6 (3.0), (10.4)	18 (2.7), (9.5)	12 (3.1), (11.1)	12 (2.4), (8.6)	4 (4.5), (16.1)	20 (2.5), (9.0)	24 (2.7), (9.7)
Deaths, n (%), (EAIR)	1 (1.4), (5.3)	0, (0.0)	1 (0.7), (2.8)	0, (0.0)	0, (0.0)	1 (0.1), (0.5)	1 (0.3), (0.9)	0, (0.0)	0, (0.0)	1 (0.1), (0.5)	1 (0.1)§, (0.4)
Baricitinib safety outcomes after median exposure of 2 years											
Patients, N	342	3097	500	2939	810	2629	1535	1904	373	3066	3439
Any TEAE, n (%), (EAIR)	318 (93.0), (51.4)	2592 (83.7), (43.1)	444 (88.8), (45.9)	2466 (83.9), (43.5)	711 (87.8), (45.1)	2199 (83.6), (43.5)	1351 (88.0), (44.8)	1559 (81.9), (43.1)	343 (92.0), (45.7)	2567 (83.7), (43.6)	2910 (84.6), (43.9)
SAE, n (%), (EAIR)	73 (21.3), (11.8)	489 (15.8), (8.1)	132 (26.4), (13.7)	430 (14.6), (7.6)	168 (20.7), (10.7)	394 (15.0), (7.8)	348 (22.7), (11.5)	214 (11.2), (5.9)	102 (27.3), (13.6)	460 (15.0), (7.8)	562 (16.3), (8.5)

Continued

Table 1 Continued

	Depression	No depression	Osteoporosis	No osteoporosis	Hepatic disorders	No hepatic disorders	CV disorders	No CV disorders	Pulmonary disorders	No pulmonary disorders	Overall
Discontinuation due to AEs, n (%), (EAIR)	36 (10.5), (5.8)	283 (9.1), (4.7)	71 (14.2), (7.3)	248 (8.4), (4.4)	93 (11.5), (5.9)	226 (8.6), (4.5)	173 (11.3), (5.7)	146 (7.7), (4.0)	35 (9.4), (4.7)	284 (9.3), (4.8)	319 (9.3), (4.8)
Deaths, n (%), (EAIR)	3 (0.9), (0.5)	10 (0.3), (0.2)	7 (1.4), (0.7)	6 (0.2), (0.1)	1 (0.1), (0.1)	12 (0.5), (0.2)	9 (0.6), (0.3)	4 (0.2), (0.1)	4 (1.0), (0.5)	9 (0.3), (0.2)	13 (0.4), (0.2)

Comorbidities and associated terms are based on the Medical Dictionary for Regulatory Activities classification system. Terms for depression included depressed mood, depression postoperative, depression, depressive symptoms, dysthymic disorder, major depression, suicidal ideation and suicide attempt. Terms for osteoporosis included low bone density (bone density decreased), non-traumatic bone fracture (osteoporotic fracture), osteopaenia, osteoporosis postmenopausal, osteoporosis and senile osteoporosis. Terms for hepatic disorders included alanine aminotransferase increased, aspartate aminotransferase increased, biliary cirrhosis primary, blood alkaline phosphatase increased, cholestasis, chronic hepatitis, drug-induced liver injury, gamma-glutamyltransferase increased, hepatic enzyme abnormal, hepatic enzyme increased, hepatic function abnormal, hepatic lesion, hepatic steatosis, hepatitis, hepatitis acute, hepatitis toxic, hepatomegaly, hypertransaminasaemia, hypoalbuminaemia, jaundice, liver disorder, liver function test abnormal, liver injury, liver operation, non-alcoholic steatohepatitis, serum hepatitis B virus core antibody positive, serum hepatitis B virus surface antibody positive, spider nevus, transaminases increased and varices oesophageal. Terms for cardiovascular disorders included acute myocardial infarction, blood cholesterol increased, blood glucose abnormal, blood glucose increased, cerebral infarction, cerebrovascular accident, coronary artery bypass, diabetes mellitus, diabetes mellitus inadequate control, diabetic ketoacidosis, essential hypertension, gestational diabetes, glucose tolerance impaired, hypercholesterolaemia, hyperglycaemia, hyperlipidaemia, hypertension, hypertensive cardiomyopathy, hypertensive heart disease, intermittent claudication, ischaemic stroke, labile hypertension, lacunar infarction, low-density lipoprotein increased, myocardial infarction, peripheral arterial occlusive disease, peripheral artery stenosis, subarachnoid haemorrhage, subdural haematoma, systolic hypertension and transient ischaemic attack. Terms for pulmonary disorders included allergic bronchitis, apnoea, asbestosis, aspirin-exacerbated respiratory disease, asthma, asthma exercise-induced, atelectasis, bronchial hyper-reactivity, bronchiectasis, bronchiolitis, bronchitis chronic, bronchospasm, bullous lung disease, chronic obstructive pulmonary disease, cor pulmonale chronic, diffuse panbronchiolitis, dyspnoea, dyspnoea exertional, emphysema, idiopathic pulmonary disease, infantile asthma, interstitial lung disease, Langerhans cell histiocytosis, lung consolidation, lung cyst, obstructive airways disorder, pneumoconiosis, pneumonitis, pulmonary bulla and pulmonary fibrosis. The number of patients with idiopathic pulmonary disease, interstitial lung disease or pulmonary fibrosis was low (6 and 8 patients for baricitinib 4 mg and placebo, respectively); thus, the impact of these conditions on baricitinib treatment outcomes cannot be concluded with certainty.

*Efficacy outcomes were assessed at week 12 for baricitinib and placebo; safety outcomes were recorded from baseline to week 16 for baricitinib and placeboin the randomised controlled trials and for all patients receiving baricitinib after a median exposure of 2 years in the long-term extension study.

†Percentage of patients achieving ACR20, ACR50 and DAS 28-hsCRP ≤3.2.

‡Least squares mean changes in HAQ-DI scores from baseline to week 12; negative changes indicate improvement from baseline.

§One death occurred in a patient receiving placebo; this patient had depression, osteoporosis and a cardiovascular disorder.

ACR, American College of Rheumatology; ACR20, 20% improvement from baseline in ACR criteria; ACR50, 50% improvement from baseline in ACR criteria; AE, adverse event; CV, cardiovascular; DAS28-hsCRP, Disease Activity Score for 28-joints based on the level of high-sensitivity C reactive protein; EAIR, exposure-adjusted incidence rate; HAQ-DI, Health Assessment Questionnaire–Disability Index; N, number of patients randomised and treated in each comorbidity subgroup; n, number of patients with event; SAE, serious adverse event; TEAE, treatment-emergent adverse event.

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Patient consent Obtained.

Ethics approval All studies were conducted in accordance with the ethical principles of the Declaration of Helsinki and Good Clinical Practice guidelines. Studies were approved by the following review boards: NCT01469013: Parexel #205204; NCT01185353: Schulman Associates IRB, IRB #10-5255-0; NCT00902486: Copernicus Group IRB #PAR1-09-143; NCT01710358 (RA-BEAM): Quorum Review IRB #27257; NCT01721057 (RA-BUILD): Quorum Review IRB #27258. In addition, studies were approved by each participating centre's institutional review board or ethics committee.

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First cardiovascular MRI study in individuals at risk of rheumatoid arthritis detects abnormal aortic stiffness suggesting an anti-citrullinated peptide antibody-mediated role for accelerated atherosclerosis

Patients with rheumatoid arthritis (RA) are at greater risk of major cardiovascular (CV) events, predominantly due to accelerated atherosclerosis, underpinned by inflammation and RA disease factors and also heart failure.[1] Overall, modest event rate has necessitated the use of surrogate CV abnormalities of increased CV risk including arterial stiffness. Increased arterial stiffness is well recognised in established RA,[2] with early, similar reports using comprehensive and reliable cardiac MRI (CMRI) in our treatment-naïve, early onset RA cohort.[3] Autopsy, histopathological and clinical studies in general population and RA cohorts with and without CV disease (CVD) suggest citrullinated proteins as a mechanism for atherosclerosis, including presence of citrullination within the atherosclerotic plaque of subjects without RA.[4 5] We hypothesised that individuals with circulating anti-cyclic citrullinated peptide (CCP) but no systemic inflammation (of RA typically associated with increased CV risk) also demonstrate CV abnormalities.

Anti-CCP-positive individuals with any new musculoskeletal symptoms but no clinical synovitis (subsequently termed at-risk individuals), and no prior history of CVD, were recruited from a tertiary centre rheumatology outpatient clinic. Following informed consent, 18 at-risk individuals and 30 healthy controls (HC), matched for age and gender, underwent multiparametric 3.0T CMRI with late Gadolinium enhancement (Achieva, Philips, Best, The Netherlands) in an academic CMRI centre. As part of our previously described, cohort studies at at-risk

Table 1 Baseline cardiac MRI findings for at-risk individuals and HCs

Variable	HCs (n=30)	All at-risk individuals (n=18)	P value (controls vs all at risk)	<50% At-risk individuals (n=8)	P value (controls vs <50% risk)	>50% At-risk individuals (n=10)	P value (controls vs >50% risk)	Progressors to RA within 1 year (n=5)	P value (controls vs progressors)	Nonprogressors to RA within 1 year (n=13)	P value (controls vs non progressors)
Aortic stiffness											
Aortic distensibility (10^{-3}mm Hg^{-1})	4.9±2.1	3.6±1.3	0.001	4.2±1.7	0.35	3.1±0.6	0.001	3.2±0.7	0.002	3.8±1.5	0.048
Aortic compliance	17.4±4.2	14.3±3.6	0.15	15.2±3.5	0.15	13.6±3.8	0.15	13.8±5.1	0.20	14.5±3.1	0.017
Aortic strain	0.25±0.08	0.20±0.05	0.001	0.21±0.08	0.23	0.19±0.02	0.001	0.18±0.02	0.001	0.21±0.06	0.05
Aortic stiffness index (β)	2.7±0.9	3.4±0.9	0.005	3.1±1.0	0.29	3.7±0.8	0.005	4.0±1.1	0.048	3.2±0.7	0.06
LV structure											
LV mass/BSA (g/m^2)	49±8	46±10	0.72	44±7	0.16	48±11	0.72	45±13	0.57	46±9	0.39
Measures of fibrosis											
Native T1 (ms)	1199±35	1212±34	0.39	1214±40	0.37	1210±32	0.39	1200±21	0.98	1217±38	0.19
ECV (%)	25.4±2.5	27.7±3.6	0.16	28.0±3.7	0.1	27.4±3.8	0.16	26.3±1.7	0.35	28.3±4.1	0.04
LGE	0/30	1/18	0.59	0/8	–	1/10 (10%)	0.59	0/5	–	1/13 (8%)	0.68
Function											
S′ ($seconds^{-1}$)	1.16±0.14	1.12±0.12	0.23	1.16±0.08	0.92	1.10±0.14	0.23	1.12±0.17	0.67	1.12±0.10	0.31
LVEF (%)	62±5	62±4	0.95	61±4	0.48	62±4	0.95	63±5	0.84	61±4	0.49
LVEDV/BSA (mL/m^2)	78±10	83±12	0.42	84±9	0.17	83±15	0.42	80±16	0.88	84±11	0.11
Torsion (degrees)	15.1±−4.7	16.3±4.6	0.76	17.1±4.6	0.39	15.7±4.8	0.76	13.7±3.5	0.44	17.6±4.7	0.19
Twist (degrees)	15.8±4.6	17.1±4.8	0.43	16.9±5.2	0.66	17.3±4.9	0.43	17.6±5.5	0.53	16.9±4.8	0.54

Mean (±SD) values presented unless otherwise stated.
BSA, body surface area; ECV, extracellular volume; HCs, healthy controls; LGE, late gadolinium enhancement; LVEDV, left ventricular end diastolic volume; LVEF, left ventricular ejection fraction.

individuals were classified as 'low' (<50%) or 'high' (>50%) risk of developing RA using a published clinical risk model[6] and followed up for 12 months to assess for progression to a clinical diagnosis of RA. CMRI analysis was performed blinded to patient details. Using SPSS V.22, unpaired Student t-tests and Mann-Whitney tests compared continuous variables.

Of the at-risk individuals, 4 (22%) were males, the mean (±SD) age was 53±15 years, anti-CCP 136±136 IU/mL and predicted absolute risk of RA 49%±17%. There were no differences between at-risk individuals and HC for age, gender, blood pressure, CV risk factors (hypertension, diabetes, hypercholesterolaemia) and active smoking status, although at-risk individuals had a higher body mass index (29±5 and 25±5 kg/m^2, respectively) and proportion of ex-smokers (56% and 17%, respectively). Online supplementary table S1 details baseline demographic data of at-risk RA individuals and HC. Five of the 18 recruited patients progressed to RA over 12 months.

Analyses (table 1) revealed aortic distensibility was notably lower (indicating greater arterial stiffness) in at-risk individuals compared with HC (3.6±1.3 vs 4.9±2.1×10^{-3} mm Hg^{-1}, respectively); a finding most pronounced in the high-risk individuals (n=8) compared with low-risk individuals (n=10) (3.1±0.6 and 4.2±1. × 10^{-3}mm Hg^{-1}, respectively), and in those who progressed to RA over 12 months (see table 1). Similarly, sizeable differences in all other measures of aortic stiffness, including aortic compliance and aortic strain and aortic stiffness (β) were observed, again with greater aortic stiffness in high versus low at-risk individuals and in at-risk individuals progressing to RA.

To our knowledge, this is the first study showing subclinical increase in aortic stiffness in at-risk individuals for RA, with values numerically close to those seen in early, treatment-naïve RA. Our use of CMRI as a research tool offers a particularly sensitive assessment of structural and functional changes to reflect microvascular and macrovascular pathological processes of RA. The key limitation of this pilot study is absence of control groups. Nevertheless, the abnormal aortic stiffness measures were most pronounced in the high at-risk cohort and those progressing to RA (although with a trend for greater stiffness also seen in low-risk patients), implying a particular role of CCP antibodies.

These data advance the concept of anti-CCP-mediated atherosclerosis and support additional investigation in larger, and both anti-CCP-positive and anti-CCP-negative control populations.

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Presence of anti-rituximab antibodies predicts infusion-related reactions in patients with systemic lupus erythematosus

Rituximab (RTX) is a chimeric monoclonal anti-CD20 antibody used in the treatment of various rheumatic diseases.[1] Although generally well tolerated, infusion-related reactions (IRR) represent the most common adverse event associated with treatment and are difficult to predict. In patients undergoing treatment for rheumatoid arthritis, the incidence of IRR is quoted as 3%–4%[2 3]; however, in systemic lupus erythematosus (SLE) this is significantly higher at 19%.[4] To date, few studies have assessed the role antidrug antibodies (ADA) play in the lack of response or development of IRR to RTX in SLE. Here, we investigate how the presence of ADA relates to IRR and effectiveness of RTX therapy in SLE.

Fifty-seven patients fulfilling American College of Rheumatology criteria were recruited from the lupus clinic at University College London Hospital, UK. All patients were receiving RTX for active SLE (British Isles Lupus Assessment Group [BILAG] A or 2B scores) for the first time. Confirmed IRR were recorded in electronic health records. Baseline characteristics including complement C3 (C3), double-stranded DNA antibody titres (dsDNA) and BILAG score were recorded at the time of treatment and at each subsequent clinic visit. CD19 positive lymphocyte (CD19) levels were measured at 1 and 6 months following treatment. IRR were classified in accordance with Common Terminology Criteria for Adverse Events v4 criteria (online supplementary table 1).[5] Presence of ADA was assessed via a bridging electrochemiluminescence assay using biotinylated and ruthenylated RTX as capture and detection. X^2 with Bonferroni correction was used to compare categorical differences between ADA^+ and ADA^- groups. Paired t-test was used to assess for differences immediately prior to and at 6 months following treatment.

As shown in table 1, ADA were identified in 37% of patients following treatment. ADA^+ patients were younger both at diagnosis (p=0.03) and at the time of first treatment with RTX (p<0.001). In spite of low overall numbers, ADA were more commonly seen in males (p=0.04). There was no significant difference in concomitant treatment, disease manifestation and ethnicity. At the time of treatment, there was no difference in C3, dsDNA titres or BILAG. Figure 1 demonstrates that at 6 months post-treatment, ADA^- patients show a significant increase in C3 levels (p=0.003) and reduction in dsDNA antibody binding (p=0.008) in keeping with effective response to treatment. In ADA^+ patients, although normalisation of C3 was seen at 6 months (p=0.007), there was no observed improvement in dsDNA titres (p=0.96). Both ADA^+ and ADA^- patients displayed a significant improvement in global BILAG score 6 months after treatment (p<0.0001). There was no difference in CD19 between ADA^+ and ADA^- patients at either 1 or 6 months post-treatment. Of the 57 patients recruited, 25 patients underwent retreatment with RTX (18 ADA^+ and 7

Table 1 Patient characteristics

	ADA positive N=21	ADA negative N=36	P value
Age at diagnosis, years (SD)	22.0 (7.9)	30.0 (11.8)	**<0.001*** **
Age at time of treatment, years (SD)	31.9 (9.1)	42.9 (11.1)	**0.03***
Disease duration, months (SD)	119 (76)	151 (108)	NS
Gender (Female:Male) (%)	16:5 (76:24)	34:2 (94:6)	**0.04***
Ethnicity, n (%)			
Afro-Caribbean	5 (24)	6 (17)	NS
Caucasian	9 (43)	19 (53)	NS
South Asian	3 (14)	6 (17)	NS
East Asian	3 (14)	2 (5)	NS
Other	1 (5)	3 (8)	NS
Indication for treatment, n (%)			
Nephritis	10 (48)	7 (19)	NS
Arthritis	5 (24)	11 (31)	NS
Haematological	3 (14)	2 (6)	NS
Neuro-psychiatric	0 (0)	1 (3)	NS
Serositis	1 (5)	3 (8)	NS
Cutaneous	6 (29)	9 (25)	NS
Other	3 (14)	3 (8)	NS
Additional treatment, n (%)			
Hydroxychloroquine	10 (48)	20 (56)	NS
Azathioprine	5 (24)	5 (14)	NS
Mycophenolate mofetil	4 (19)	4 (11)	NS
Methotrexate	0 (0)	4 (11)	NS
Prednisolone	17 (81)	27 (75)	NS
Cyclophosphamide†	15 (71)	22 (61)	NS
Disease activity at the time of treatment			
Complement C3 (g/L) (SD)	0.76 (0.29)	0.69 (0.24)	NS
Anti-dsDNA titres (IU) (SD)	411.7 (651.6)	699.8 (874.2)	NS
Global BILAG score (SD)	15.8 (10.2)	13.5 (5.6)	NS
Disease activity 6 months post-treatment			
Complement C3 (g/L) (SD)	0.91 (0.27)	0.83 (0.24)	NS
Anti-dsDNA titres (IU) (SD)	201.4 (369.6)	682.0 (1009.0)	**0.04***
Global BILAG score (SD)	6.4 (5.4)	7.4 (5.4)	NS

*p<0.05; ***p<0.001.
†Given in combination with rituximab therapy.
ADA, antidrug antibody; BILAG, British Isles Lupus Assessment Group; dsDNA, double-stranded DNA; NS, not significant.

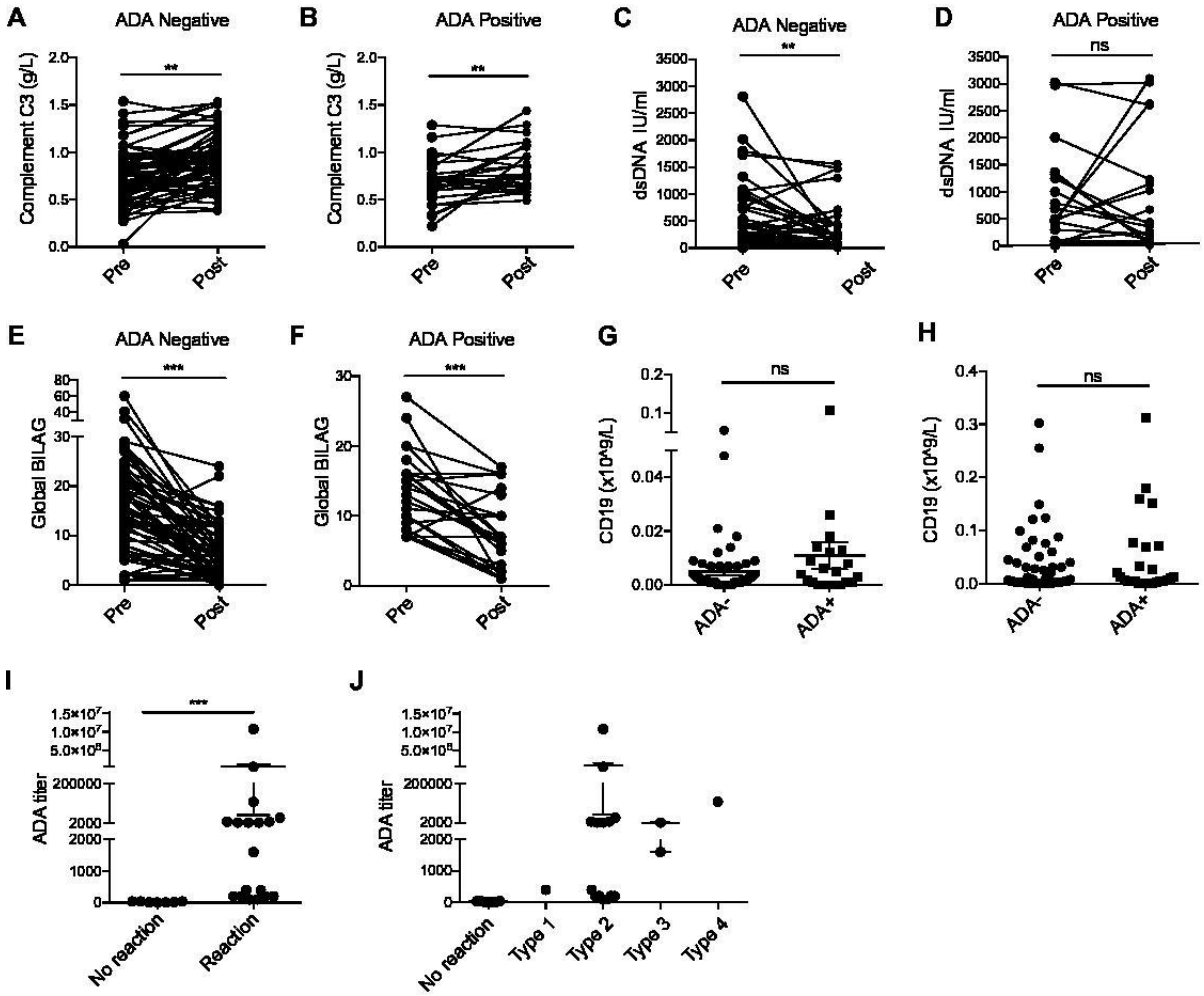

Figure 1 Complement C3 levels significantly improve at 6 months post-treatment with RTX in ADA$^-$ (A) and ADA$^+$ patients (B). Although a significant improvement in dsDNA binding was seen in in those who are ADA$^-$ at 6 months post-treatment (p=0.008) (C), this was not seen in those who were ADA$^+$ (p=0.96) (D). A significant improvement in global BILAG score was seen in both (E) ADA$^-$ (p<0.0001) and (F) ADA$^+$ patients (p<0.0001). No significant difference in CD19 positive lymphocyte count was seen at either 1 (G) and 6 (H) months post-treatment regardless of ADA status. ADA$^+$ patients undergoing retreatment with RTX all developed IRR (I). The most common type of reaction observed was CTCAE type 2 with more severe reactions seen in those with higher ADA titres (J). ADA, antidrug antibody; CTCAE, Common Terminology Criteria for Adverse Events; IRR, infusion-related reactions; RTX, rituximab.

ADA$^-$ patients). All ADA$^+$ patients developed IRR, whereas no IRR was reported in those who were ADA$^-$ (p<0.001). Severe reactions resulting in hospitalisation were seen in three cases in which ADA titres were >1500 IU. In one such case, subsequent treatment with ofatumumab (a fully humanised anti-CD20 monoclonal antibody) was well tolerated without the occurrence of further IRR.

We demonstrate that ADA to RTX are common in those undergoing treatment for SLE and have a clear association with subsequent IRR. Contrary to previous studies,[6] our findings suggest that CD19 count is not affected by ADA, however the presence of ADA appeared to impair normalisation of dsDNA titres following treatment. If validated, these findings may support routine screening for ADA prior to treatment with RTX, thus potentially identifying patients at risk of developing IRR and prompting greater caution and enhanced surveillance. In the context of high ADA titres, this may necessitate the use of an alternate B-cell depleting agent (such as ofatumumab).

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Short lymphocyte, but not granulocyte, telomere length in a subset of patients with systemic sclerosis

Interstitial lung disease (ILD) affects up to 40% of patients with systemic sclerosis (SSc) and is the leading cause of death.[1] The risk of ILD is increased in SSc patients who are older and antitopoisomerase-1 antibody (ATA) positive.[2] SSc-ILD shares features with idiopathic pulmonary fibrosis (IPF), although these two fibrosing lung diseases have different natural histories. Telomere shortening is the best-defined risk factor for IPF. In familial IPF, mutations in seven telomerase and telomere-related genes explain familial clustering in 30% of cases.[3] Sporadic forms of IPF are also associated with telomere shortening in 50%–60% of cases.[4 5] In IPF, short telomeres are found across cell types, which is consistent with an inherited and systemic defect in telomere maintenance.[4] Previous studies of telomere length (TL) in patients with SSc have yielded conflicting results, with some studies showing longer and others shorter TL compared with age-matched controls.[6 7] A major caveat in these studies is that TL was measured using techniques that are both highly variable and fail to distinguish TL among leucocyte subsets. Moreover, these studies failed to examine the impact of ILD on TL.We therefore sought to determine TL in patients with SSc and associated ILD using a clinically validated robust assay that allows accurate measurement of TL in lymphocytes and granulocytes by flow cytometry and fluorescence in situ hybridisation (flow-FISH).[8 9]

Subjects were evaluated at the Northwestern Scleroderma Program between 2013 and 2014 and and fulfilled 2013 American College of Rheumatology criteria for SSc. Clinical and demographic data, including smoking history, disease duration (from first non-Raynaud disease manifestation), modified Rodnan skin score (mRSS, range 0–51), treatment history and laboratory studies, were collected. Pulmonary function testing and high-resolution chest CT imaging were performed by standard methods.[10] CT scans were scored by an expert chest radiologist. The diagnosis of SSc-ILD disease required the presence of bilateral pulmonary reticulations and/or honeycombing, and ILD severity was scored using the Kazerooni method.[11] Peripheral blood cells were isolated by Ficoll centrifugation and pellets were suspended in freezing media and preserved in liquid nitrogen until analysis. TL was measured by flowFISH as described.[8 9] DeltaTL was defined as the difference from

Table 1 Demographic and clinical characteristics of the study population

	Patients with SSc without ILD (n=18)	Patients with SSc with ILD (n=15)
Age median (range), years	53 (28–71)	52 (32–70)
Gender	15F/3M	10F/5M
Race	16 Caucasian, 2 Hispanic	10 Caucasian, 2 African American, 1 Hispanic, 2 Asian
Smoking history	Six former (33%)	Four former (26%)
Disease duration* median (range), months	102 (32–291)	87 (15–313)
Disease subtype	9 lcSSC, 8 dcSSc, 1 SSS	5 lcSSc, 10 dcSSc
Autoantibodies	2 ATA, 7 ACA, 5 RNA Pol III, 1 None, 3 not determined	7 ATA, 2 RNA Pol III, 2 none, 3 not determined
Disease-modifying treatment history	4 MMF, 1 MTX, 1 prednisone, 1 chloroquine plus MMF, 11 none	3 MMF, 2 prednisone, 1 tocilizumab, 9 none

*From first non-Raynaud disease manifestation.
ACA, anticentromere antibodies; ATA, antitopoisomerase I antibodies; MMF, mycophenolate mofetil; MTX, methotrexate; RNA Pol III, anti-RNA polymerase III antibodies; SS, systemic sclerosis; dcSSc, diffuse cutaneous SSc; lcSSc, limited cutaneous SSc.

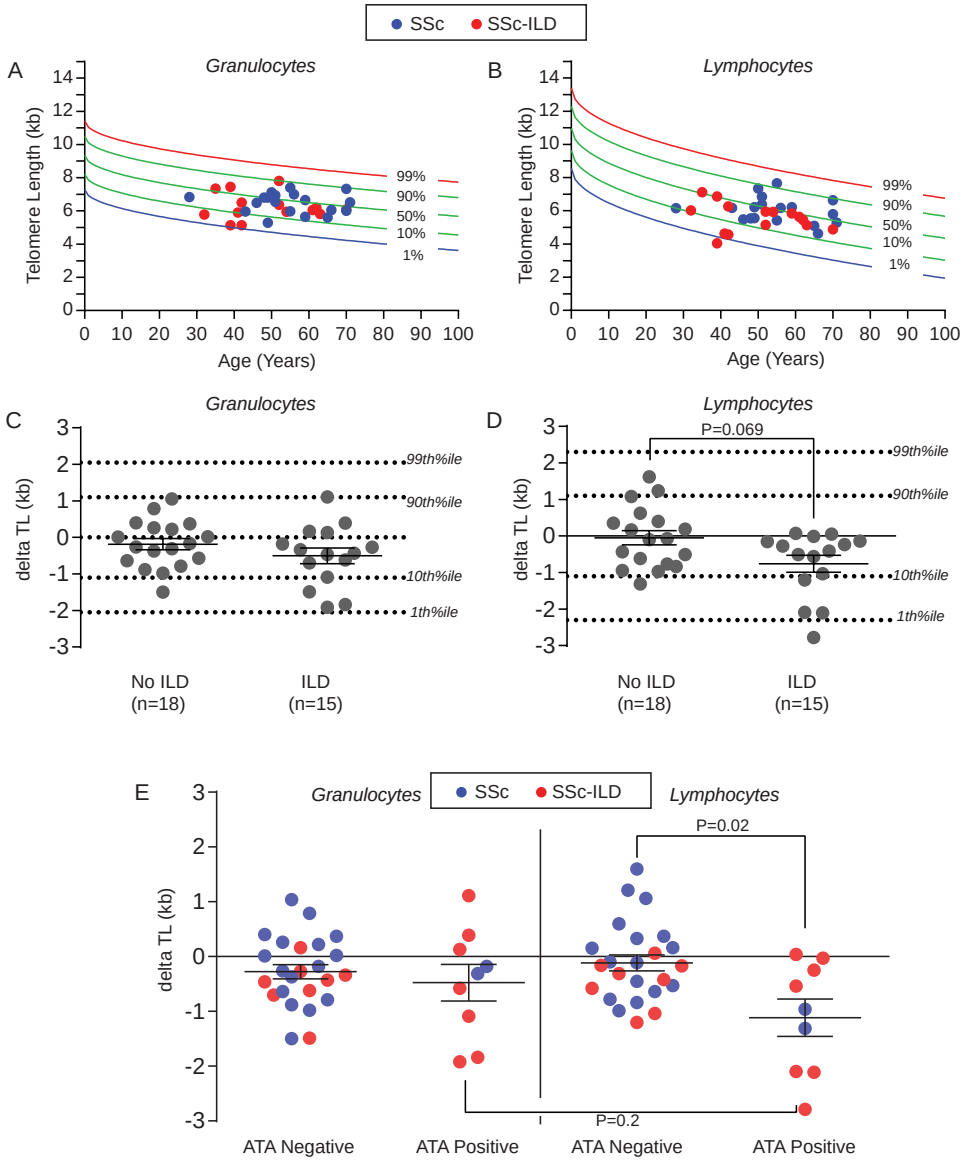

Figure 1 Telomere lengths in patients with SSc with and without interstitial lung disease (ILD). (A and B) Telograms showing telomere lengths (TL) in granulocytes and lymphocytes in 33 patients. This telomere length nomogram was derived from 192 healthy controls and is clinically validated across populations.(C, and D) Delta TL in granulocytes and lymphocytes. Note lymphocyte-specific TL shortening in patients with SSc-ILD. (E) Delta TL in granulocytes and lymphocytes in antitopoisomerase (ATA)-positive and ATA-negative patients. P values calculated by Mann-Whitney U test (two-sided).

the age-adjusted median.[9] Comparisons were made relative to a validated TL nomogram of healthy controls.[9] For patients with the shortest TL (defined as <10th age-adjusted percentile) genomic DNA was sequenced using an Illumina-based Truseq platform for variants in *TERT*, *TR*, *DKC1*, *TINF2*, *NAF1* and *RTEL1* as previously described.[12 13] Statistical analysis was run on GraphPad Prism.

The clinical characteristics of prospectively recruited SSc patients with (n=15) and without (n=18) ILD are summarised in table 1. The two groups were well matched in age. When studied together, lymphocyte and granulocyte TL were similar to healthy controls in both groups (figure 1A–B). This contrasts with patients with IPF, who have significantly shorter TL systemically than age-matched controls including lymphocytes, granulocytes, lung epithelial cells .[4 14] These results indicate that TL in SSc is not a significant determinant of disease onset, in contrast to patients with IPF.

We next examined whether TL among the SSc patients correlated with the presence of SSc-ILD. In granulocytes, there were no differences in TL (delta TL −0.5 kb vs −0.2 kb in patients with ILD (n=15) and without ILD (n=18), respectively, p=0.3, Mann-Whitney U test, figure 1C). In contrast, we noted a trend towards shorter TL in lymphocytes in patients with SSc-ILD compared with those without ILD (delta TL −0.8 kb vs 0.0 kb, p=0.069, Mann-Whitney U test, figure 1D). Three of 15 patients with SSc-ILD (20%) had lymphocyte TL below the age-adjusted 10th percentile, while in contrast, none of the 18 patients with SSc without ILD fell within that range (p=0.08, Fisher's exact test). DNA sequence analysis indicated that none of the three SSc patients with the shortest telomeres (each below the 10th age-adjusted percentile) carried germline mutations in telomerase or telomere-related genes. These results suggested that lymphocyte-specific telomere shortening in patients with SSc may correlate with the presence of ILD.

We next investigated whether lymphocyte-specific telomere shortening in SSc correlated with the presence of ATA. Compared with ATA-negative SSc patients (n=24), those who were ATA positive (n=9) had significant shortening in lymphocytes (−0.96 kb vs −0.16 kb in ATA positive vs ATA negative, respectively, p=0.02, Mann-Whitney U test). In contrast, short TL was not detected in SSc patients with other autoantibodies including anticentromere, anti-RNA polymerase III antibodies and patients who had no detectable antibodies. Moreover, the ATA-positive short lymphocytes TL association was not seen in other subsets of SSc patients when the data were analysed for disease duration, smoking status or medications used. These data suggested that the ATA-positive status in SSc has a sizeable and measurable effect on lymphocyte TL.

The present results are notable for several reasons. First, the fact that SSc patients with ILD had normal TL in granulocytes is distinct from the systemic telomere shortening seen in all cell types assessed for IPF patients including lymphocytes, granulocytes, alveolar epithelial cells and other cell types.[4] This distinction suggests that although the clinical phenotype of SSc-ILD and IPF may show partial overlap, the susceptibility factors for these two fibrosing lung diseases are distinct[15]. Second, we found lineage-specific TL shortening in lymphocytes in both SSc-ILD and ATA-positive patients. This pattern is also distinct from IPF, where TL shortening is concordant in both lymphocytes and granulocytes. One potential model to explain the observed lymphocyte-specific TL shortening in SSc is that ILD might be associated with, or caused by, excessive lymphocyte proliferation that in turn leads to telomere shortening. This phenomenon is likely not specific, as acquired lymphoid disorders conditions that affect lymphocyte survival or proliferation may also show a similar pattern (Armanios *et al*, unpublished). The present results highlight the potential utility of leucocyte subset analysis of TL using flowFISH in understanding disease pathogenesis and indicate that in contrast to IPF, in SSc-associated ILD, acquired lineage-specific TL shortening in lymphocytes may be a consequence of lung disease rather than its pathogenic driver.

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Contributors MA and JV designed the study. KL, VSH, RA and MC collected the data. RA and VSH analysed the data. MA and KL conducted statistical analyses. KL, MA and JV interpreted the data and wrote manuscript. All authors reviewed the manuscript and approved final version.

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Association of *MUC5B* promoter polymorphism with interstitial lung disease in myeloperoxidase-antineutrophil cytoplasmic antibody-associated vasculitis

Interstitial lung disease (ILD) is a major complication and a prognostic factor of antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV).[1] The prevalence of ILD is higher in Japanese AAV as compared with European populations.[1] In a Japanese inception cohort study, 45.0% of patients with microscopic polyangiitis (MPA) were complicated by ILD.[2] Such ethnic difference suggests a role for genetic background in the development of AAV-associated ILD (AAV-ILD).

Single nucleotide polymorphism (SNP) rs35705950(G/T) in the promotor region of *MUC5B,* encoding mucin 5B, is a strong genetic factor for idiopathic pulmonary fibrosis (IPF).

Table 1 Case-control association study of *MUC5B* rs35705950 with AAV

		Genotype count and frequency				Dominant model (vs healthy controls)		
	n	G/G	T/G	T/T	MAF	OR (95% CI)	P value	FDR Q
EMEA classification								
MPA	291	284 (0.976)	7 (0.024)	0 (0)	0.012	2.23 (0.79 to 6.06)	0.115	
GPA	95	95 (1)	0 (0)	0 (0)	0	0.46 (0.03 to 7.96)*	0.610*	
EGPA	56	56 (1)	0 (0)	0 (0)	0	0.78 (0.04 to 13.5)*	1.00*	
Unclassifiable	32	31 (0.969)	1 (0.031)	0 (0)	0.016	2.97 (0.16 to 16.6)	0.309	
MPO-AAV	382	374 (0.979)	8 (0.021)	0 (0)	0.010	1.96 (0.73 to 5.17)	0.170	
PR3-AAV	60	60 (1)	0 (0)	0 (0)	0	0.73 (0.04 to 12.6)*	1.00*	
AAV-ILD	163	156 (0.957)	7 (0.043)	0 (0)	0.021	3.93 (1.38 to 10.7)	0.008	0.040
AAV without ILD	264	263 (0.996)	1 (0.004)	0 (0)	0.002	0.35 (0.02 to 1.86)	0.317	
MPO-AAV-ILD	149	142 (0.953)	7 (0.047)	0 (0)	0.023	4.34 (1.52 to 11.9)	0.004	0.040
MPO-AAV without ILD	198	197 (0.995)	1 (0.005)	0 (0)	0.003	0.47 (0.03 to 2.54)	0.479	
Healthy controls	842	833 (0.989)	9 (0.011)	0 (0)	0.005	ref		

ORs, 95% CIs and p values were adjusted for sex using logistic regression analysis under the dominant model for T allele.

*In these comparisons, because T/G and T/T genotypes were absent, Fisher's exact test was performed under the dominant model for T allele. For calculation of OR and 95% CI, 0.5 was added to each cell (Haldane-Anscombe correction).

AAV, ANCA-associated vasculitis; EGPA, eosinophilic granulomatosis with polyangiitis; EMEA, EuropeanMedicines Agency; FDR, false discovery rate; GPA, granulomatosis with polyangiitis; ILD, interstitial lung disease; MAF, minor allele frequency; MPA, microscopic polyangiitis; MPO, myeloperoxidase; PR3, proteinase 3.

The risk allele T was associated with overexpression of mucin 5B in the lung.[3] Recently, rs35705950 has been associated with rheumatoid arthritis-associated ILD (RA-ILD).[4]

Here, we examined whether rs35705950 is associated with AAV-ILD in case-control and case–case association studies. About 474 Japanese patients with AAV and 842 healthy Japanese controls were recruited at rheumatology or nephrology centres based on the clinical diagnosis of AAV, and classified according to the European Medicines Agency algorithm.[5] The patients were examined for the presence or absence of ILD using CT or high-resolution CT (HRCT) by site investigators. 163 patients were reported positive and 264 negative for ILD (see online supplementary table S1,S2). This study was conducted in accordance with the Declaration of Helsinki.

Genotyping was performed using TaqMan SNP Genotyping Assays (Assay ID: C___1582254_20) (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Odds ratios (ORs), 95% confidence intervals (CIs) and p values adjusted for sex were calculated by logistic regression analysis using R software, unless otherwise noted. Because of the lack of T/T genotype, dominant model for T allele was employed. Correction for multiple testing was performed using false discovery rate (FDR) method. Q values <0.05 were considered significant.

As shown in table 1, the allele frequency of rs35705950T was substantially lower in the healthy Japanese controls (0.5%) compared with Caucasian populations (~10%),[3 4] and individuals with genotype T/T were not present. All patients with genotype T/G were positive for myeloperoxidase (MPO)-ANCA, but none was positive for proteinase 3 (PR3)-ANCA.

Significant association was observed in AAV-ILD, but not in AAV patients without ILD, when compared with healthy controls. Significant association was also detected when only the patients with MPO-ANCA and ILD (MPO-AAV-ILD) were compared with healthy controls.

To confirm whether the rs35705950T is associated with presence of ILD rather than AAV itself, we next compared the genotype frequencies between AAV-ILD and AAV without ILD. rs35705950T was significantly associated with AAV-ILD with the OR of 11.6 (95% CI 2.04 to 218.6, p=0.023). The association was also observed when MPO-AAV-ILD was compared with MPO-AAV without ILD (OR 9.47, 95% CI 1.65 to 178.3, p=0.037). The association was also observed when the subjects were limited to those diagnosed by HRCT (online supplementary table S3, S4).

To our knowledge, this is the first to report the association of *MUC5B* variant with AAV-ILD. ILD is classified into subgroups according to the histopathological and radiographic patterns. Similarly to IPF and RA-ILD, AAV-ILD most commonly exhibit usual interstitial pneumonia (UIP) pattern,[1 4] while ILD in systemic sclerosis and myositis are predominantly associated with non-specific interstitial pneumonia pattern. A recent study reported that rs35705950T was more strongly associated with UIP than with ILD inconsistent with UIP.[6]

In addition to the small sample size, this study suffers from some limitations (online supplementary text). However, the striking observation that seven out of eight patients with AAV carrying rs35705950 exhibited ILD warrants further studies on the role of *MUC5B* in AAV-ILD.

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Gout is associated with increased healthcare utilization after knee arthroplasty

Total knee arthroplasty (TKA) is an effective surgery for patients with advanced knee arthritis refractory to medical treatment. Gout, the most common inflammatory arthritis in adults, was associated with a 40% increased TKA risk in women in a recent population-based study.[1] This was attributable possibly to accelerated cartilage wear associated with soluble urate or urate crystals. It is not known if post-TKA healthcare utilisation and complications are higher in people with gout. We assessed whether gout was associated with a higher risk of post-TKA healthcare utilisation and in-hospital complications.

We used the 1998–2014 US National Inpatient Sample data, a 20% stratified sample of discharges from US community hospitals.[2] We used validated International Classification of Disease, ninth revision, common modification (ICD-9-CM)

Table 1 Demographic and other cohort characteristics

	Entire cohort N=8 127 182*	No gout N=7 895 812*	Gout N=231 470*
	N (%), unless specified otherwise		
Age, mean (SE); median	66.4 (0.03); 66.5	66.5 (0.02); 66.5	68.1 (0.05); 68.0
Age category			
<50	430 140 (5.3%)	423 713 (5.4%)	6427 (2.8%)
50–64	2 872 619 (35.3%)	2 800 798 (35.5%)	71 821 (31.0%)
65–79	3 969 942 (48.8%)	3 842 081 (48.7%)	127 861 (55.2%)
≥80	850 132 (10.5%)	824 780 (10.4%)	25 352 (11.0%)
Gender			
Female	5 126 808 (63.1%)	5 050 791 (64.0%)	76 017 (32.8%)
Male	2 985 796 (36.7%)	2 830 384 (35.8%)	155 412 (67.1%)
Race			
White	5 507 281 (67.8%)	5 346 818 (67.7%)	160 463 (69.3%)
Black	472 392 (5.8%)	453 237 (5.7%)	19 155 (8.3%)
Hispanic	340 292 (4.2%)	333 934 (4.2%)	6358 (2.7%)
Other/missing	1 807 102 (22.2%)	1 761 613 (22.3%)	45 489 (19.7%)
Primary diagnosis			
Rheumatoid arthritis	64 126 (0.8%)	63 330 (0.8%)	796 (0.3%)
Aseptic bone necrosis	21 031 (0.3%)	20 698 (0.3%)	333 (0.1%)
Osteoarthritis	7 866 436 (96.8%)	7 640 179 (96.8%)	226 257 (97.7%)
Other	173 672 (2.1%)	169 632 (2.1%)	4040 (1.7%)
Fracture	1904 (0.0%)	1865 (0.0%)	39 (0.0%)
Hospital location/teaching			
Rural	1 041 160 (12.8%)	1 012 017 (12.8%)	29 143 (12.6%)
Urban	3 665 765 (45.1%)	3 564 877 (45.1%)	100 888 (43.6%)
Urban teaching	3 398 113 (41.8%)	3 297 365 (41.8%)	100 748 (43.5%)
Insurance			
Medicaid	226 917 (2.8%)	222 499 (2.8%)	4418 (1.9%)
Medicare	4 631 192 (57.0%)	4 485 862 (56.8%)	145 330 (62.8%)
Other	267 624 (3.3%)	261 284 (3.3%)	6340 (2.7%)
Private	2 947 675 (36.3%)	2 873 513 (36.4%)	74 162 (32.0%)
Self	36 588 (0.5%)	35 831 (0.5%)	757 (0.3%)
Income category			
0–25th percentile	1 511 692 (18.6%)	1 464 907 (18.6%)	46 785 (20.2%)
25–50th percentile	2 156 223 (26.5%)	2 094 636 (26.5%)	61 587 (26.6%)
50–75th percentile	2 158 011 (26.6%)	2 097 245 (26.6%)	60 766 (26.3%)
75–100th percentile	2 151 430 (26.5%)	2 093 366 (26.5%)	58 064 (25.1%)
Hospital bed size			
Small	1 408 128 (17.3%)	1 366 931 (17.3%)	41 197 (17.8%)
Medium	2 107 250 (25.9%)	2 047 151 (25.9%)	60 099 (26.0%)
Large	4 589 661 (56.5%)	4 460 177 (56.5%)	129 484 (55.9%)
Hospital region			
Northeast	1 359 230 (16.7%)	1 321 311 (16.7%)	37 919 (16.4%)
Midwest	2 269 799 (27.9%)	2 207 287 (28.0%)	62 512 (27.0%)
South	2 957 629 (36.4%)	2 869 525 (36.3%)	88 104 (38.1%)
West	1 540 624 (19.0%)	1 497 690 (19.0%)	42 934 (18.5%)
Deyo-Charlson Score			
0	4 104 090 (50.5%)	4 019 384 (50.9%)	84 706 (36.6%)
1	2 064 888 (25.4%)	2 005 515 (25.4%)	59 373 (25.7%)
≥2	1 958 304 (24.1%)	1 870 913 (23.7%)	87 391 (37.8%)
Gout	231 470 (2.8%)	–	231 470 (100.0%)
In-hospital post-TKA complications			
Transfusion	1 288 544 (15.9%)	1 251 071 (15.8%)	37 473 (16.2%)
Revision	15 310 (0.2%)	14 843 (0.2%)	467 (0.2%)
Infection	8165 (0.1%)	7938 (0.1%)	227 (0.1%)
Died during hospitalisation	7875 (0.1%)	7709 (0.1%)	166 (0.1%)
Post-TKA healthcare utilisation			
Length of hospital stay, mean (SE); median	3.53 (0.01); 2.70	3.54 (0.01); 2.70	3.59 (0.01); 2.69
Length of hospital stay category			
≤3	5 218 769 (64.2%)	5 068 813 (64.2%)	149 956 (64.8%)
>3	2 908 513 (35.8%)	2 826 999 (35.8%)	81 514 (35.2%)
Discharge status			
Rehabilitation facility†	4 965 279 (61.1%)	4 827 422 (61.1%)	137 857 (59.6%)
Home	3 130 289 (38.5%)	3 037 405 (38.5%)	92 884 (40.1%)

*US National estimates were based on the following in the 20% US National Inpatient Sample: all, N=1 690 531; no gout, N=1 642 585; gout, N=47 946.
†Rehabilitation facility included short-term or long-term care hospital, skilled nursing facility, intermediate care facility or a certified nursing facility.
LOS, length of stay; TKA, total knee arthroplasty.

code to identify the primary TKA cohort (81.54)[3], with TKA as the primary procedure and among these people with and without gout (274).[4] Study outcomes post-primary TKA were (1) healthcare utilisation, length of hospital stay (>3 days) and the discharge disposition, that is, to home versus rehabilitation/in-patient facility; and (2) in-hospital postoperative complications identified by respective ICD-9-CM codes for transfusion, revision or infection, and in-hospital mortality. Separate multivariable-adjusted logistic regression analyses controlled for

Table 2 Multivariable-adjusted association of gout with healthcare utilisation and in-hospital complications after primary TKA

	Main model*	Sensitivity analysis†: main model+hospital characteristics
	OR (95% CI)	OR (95% CI)
Length of hospital stay more than median (>3 days)‡	**1.08 (1.06 to 1.10)**	**1.08 (1.06 to 1.10)**
Discharge to a rehabilitation or skilled nursing facility	**1.18 (1.15 to 1.20)**	**1.19 (1.16 to 1.21)**
In-hospital complications§		
Transfusion	**1.15 (1.12 to 1.18)**	**1.15 (1.12 to 1.18)**
Revision	1.05 (0.85 to 1.29)	1.04 (0.85 to 1.29)
Infection	0.97 (0.72 to 1.30)	0.97 (0.72 to 1.30)
Death	**0.51 (0.36 to 0.72)**	**0.51 (0.36 to 0.72)**

Bold represents statistically significant ORs that do not include unity in the 95% CI.
*Main model was run separately for each outcome and was adjusted for demographics (age, race/ethnicity, gender), the Deyo-Charlson Comorbidity Index, the underlying diagnosis for TKA, the insurance payer type and the annual household income.
†Sensitivity model additionally adjusted the main model for the hospital variables including the region, the bed size and location/teaching status.
‡Median length of hospital stay was 2.7 days, rounded off to 3 days for categorisation of the length of hospital stay variable.
§ICD-9-CM codes for select in-hospital complications were as follows: (1) transfusion, 99.0x; (2) revision, 81.53, 00.70, 00.72, 00.73, 84.56, 84.57 or 80.05; and (3) infection, 711.xx, 730.xx, 996.66 or 996.67.
TKA, total knee arthroplasty.

covariates/potential confounders: age, race, gender, underlying primary diagnosis for TKA, Deyo-Romano comorbidity index, insurance payer and income. We present odds ratios (OR) and 95% confidence intervals (CI). Sensitivity analyses additionally adjusted for hospital location/teaching status, hospital bed size and region.

Of the 8 127 182 primary TKAs from 1998 to 2014, 231 470 primary TKAs were performed in people with gout. Compared with no gout, people with gout were significantly older, more likely to be male, Black, have higher medical comorbidity and be in the lowest income quartile (table 1). After multivariable adjustment, gout was associated with statistically significant higher OR (95% CI) of the following: discharge to a non-home setting, 1.18 (95% CI, 1.15 to 1.20); the length of hospital stay above the median of 3-days, 1.08 (95% CI, 1.06 to 1.10); and transfusion, 1.15 (95% CI, 1.12 to 1.18) (table 2). No differences were seen in infection or revision. Mortality was lower in people with gout versus non-gout, 0.51 (95% CI, 0.36 to 0.72). Sensitivity analyses confirmed the findings from the main analyses with minimal attenuation of odds ratios (table 2).

Gout was independently associated with 18%, 8% and 15% higher risk of discharge to a non-home setting, the length of hospital stay above median and in-hospital transfusion. Chronic inflammation and oxidative stress due to hyperuricemia and urate crystals in gout, hypothesised to underlie the increased risk of rotator cuff surgery in gout,[5] might prolong the postoperative recovery and partially explain this association. Gout-associated metabolic syndrome, hyperlipidaemia and other comorbidities may also have contributed.[6] Our study findings are limited due to residual confounding bias and the lack of longitudinal data. We were unable to assess the underlying mechanisms of these associations including the effect of gout duration or gout treatment. Surgeons and policy-makers can incorporate these findings in the informed consent discussion and discharge planning/resource assessment. Whether better gout management can reduce the increased healthcare utilisation and transfusion risk post-TKA needs to be examined in future studies.

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'Not all drugs (and route) are same'

I read with great interest, study by French vasculitis group, the *MAINRITSAN 2* trial.[1] The results of this trial again underline our limitations for search of biomarker, which can predict relapse of antineutrophil cytoplasmic autoantibodies (ANCA)-associated vasculitis. I have few concerns about details and analysis regarding induction regime used in the patient study group.

First is about the data provided by authors about the use of cyclophosphamide for remission induction. Previous study has shown that route and cumulative dose used for remission induction can affect relapse in long term.[2] Risk of relapse is significantly lower when daily oral cyclophosphamide was used for remission induction as against pulse intravenous cyclophosphamide. It would have been clearer if authors had given and compared details of cyclophosphamide therapy used for the last flare, especially when more than 60% of group of participants have received the drug.

Second, a previous study has suggested that ANCA titres predicts relapse in patients in whom rituximab was used for remission induction but this observation was not seen in patient treated with cyclophosphamide.[3] In current study group, use of cyclophosphamide was more frequent than rituximab, which may have affected results of study. May be subset analysis between these two groups can be more useful.

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Response to: 'Not all drugs (and routes) are same' by Zanwar and Gandhi

We would like to thank Zanwar and Gandhi[1] for their comments regarding our paper.[2] We are aware of the long-term results of the European Vasculitis Society (EUVAS) trial: patients treated with daily oral (DO) cyclophosphamide experienced fewer relapses than those given pulse intravenous cyclophosphamide for induction therapy.[3] At 18 months, no significant between-group differences were seen and long-term follow-up indicated fewer relapses only for the DO cyclophosphamide group. However, as mentioned by the authors, the study was retrospective and approximately 10% of the data were missing, which might have modified their results. The DO versus pulse difference in relapse rates could be explained by those groups' different cumulative (median (IQR) cyclophosphamide doses, respectively: 15.9 (11–22.5)g vs 8.2 (5.95–10.55)g). The excess relapses in the pulse arm were not associated with higher mortality or more renal damage. In light of the well-known increased risk of adverse events when DO cyclophosphamide is prescribed (infertility, malignancies, infections), we no longer recommend it as first-line therapy. Since the demonstration of rituximab non-inferiority to oral cyclophosphamide to induce antineutrophil cytoplasm antibody (ANCA)-associated vasculitis remission,[4] we have modified our cyclophosphamide use strategy. We prescribe DO cyclophosphamide only for patients with refractory disease, after pulse cyclophosphamide and rituximab have failed. In the MAINRITSAN2 trial, only one patient in the fixed-schedule infusion arm received oral cyclophosphamide for induction versus none in the individually tailored arm.

Based on the post-hoc analysis of the RAVE trial data, Fussner *et al*[5] reported that higher antiproteinase-3 ANCA titres were associated with relapses only in patients who had received rituximab as induction therapy; this group of patients did not receive any maintenance therapy. No ANCA-titre–relapse association was found for patients taking DO cyclophosphamide followed by azathioprine. We do not think that their observed association could affect MAINRITSAN2 results because, after remission was obtained with different induction regimens, all patients received rituximab for maintenance at randomisation, thereby rendering the population homogeneous.

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Antinuclear antibody as entry criterion for classification of systemic lupus erythematosus: pitfalls and opportunities

Antinuclear antibodies (ANAs) are helpful to support the diagnosis of ANA-associated systemic rheumatic diseases (AASRD). Pisetsky *et al* recently reported on the variability of ANA detection, with differences observed between assay platforms (indirect immunofluorescence (IIF) vs solid phase) and kits in patients with established systemic lupus erythematosus (SLE).[1] Variation of ANA detection has also been shown for automated IIF systems.[2] Initiatives to better understand the variability of ANA tests are needed.[3] Pisetsky *et al*[1] also pointed out that ANA negativity occurs in established SLE, thereby complicating screening for patients for clinical trials.[1] Yet, an Italian study reported a high sensitivity of ANA for established SLE.[4]

Testing for ANAs is complex and accurate interpretation of test results might be difficult. A task force of the European League Against Rheumatism (EULAR) has recently been installed that will address these issues in conjunction with other international committees.[5] In this context and of particular interest is that new criteria for the classification of patients with SLE are being developed under the umbrellas of the EULAR and the American College of Rheumatology (ACR).[6] In these criteria, a history of ANAs ≥1:80 by HEp-2 IIF will be the entry criterion (ie, must be present to be considered for classification as SLE).[6] The ≥1:80 cut-off was chosen in order to ensure high sensitivity.[6]

We evaluated the performance of ANA for SLE diagnosis on 9851 unique consecutive patients tested for ANA (for description of the population, see Willems *et al*[7]). All patients were tested for ANA by IIF (HEp-2000; ImmunoConcepts) and by solid-phase assay (EliA CTD screen; Thermo Fisher).[7] The clinical diagnosis was documented for 2475 patients, including (1) all patients who tested positive for IIF (cut-off 1:80) and/or CTD screen (cut-off ratio 0.7) and (2) a selection of 500 patients who tested double negative (including 150 patients with IIF titre 1:40).[7] This allowed us to calculate the positive predictive value (PPV) of IIF for SLE. As all samples were also tested by CTD screen, we could document SLE cases that tested negative by IIF but positive by CTD screen. Patients with SLE were divided into newly diagnosed SLE, established SLE and patients who did not fulfil the classification criteria.[8]

The titre-specific PPV of IIF 1:80 for SLE fulfilling the ACR classification criteria[8] was 1%, which is low and comparable with the estimated prevalence of SLE in the entire population (0.9%). The estimated likelihood ratio (LR) associated with IIF 1:80 was 1.16, indicating almost no difference in pretest to post-test probability. Of note, IIF 1:80 accounted for 37% of all positive ANA IIF results. The titre-specific PPV for SLE increased with increasing antibody levels and was 3.5%, 5.8%, 8.7%, 11.8% and 16.8% for, respectively, IIF titre 1:160, 1:320, 1:640, ≥1:1280 and reactivity to overexpressed SSA on the HEp-2000 substrate. The estimated titre-specific LRs were, respectively, 4.1, 7.0, 10.8, 14.7 and 21.8. Newly diagnosed patients with SLE had IIF results ≥1:160, whereas 10% (8/83) of patients with established SLE were IIF negative. Of note, six of the eight IIF-negative patients with established SLE tested positive with CTD screen.

ANAs are also associated with cutaneous lupus, mixed connective tissue disease (MCTD), systemic sclerosis (SSc), Sjögren's syndrome (SS) and idiopathic inflammatory myopathy (IIM). The PPV for AASRD (SLE, SSc, SS, IIM, MCTD and cutaneous lupus) was 2%, 6.8%, 15%, 31.7%, 47.6% and 50% for, respectively, IIF 1:80, 1:160, 1:320, 1:640, ≥1:1280 and reactivity to the overexpressed SSA. Thus, SLE has to be distinguished from other AASRDs.

The PPV for SLE of IIF 1:80 combined with a positive CTD screen was 5.6% (estimated LR: 6.8) compared with 1% for IIF 1:80 alone. It was 0.4% for IIF 1:80 combined with a negative CTD screen (estimated LR: 0.4). Similar findings (ie, increased PPV for double positivity and decreased PPV for singly positivity) were found when CTD screen was combined with higher IIF titres (see table 1 for an overview of the PPVs). For AASRD, an analogous increase in PPV was observed when IIF was combined with solid phase assay (see table 1 and Willems *et al*[7]).

Taken together, we found (1) that the titre-specific PPV of low-titre ANA for SLE is low, (2) that the PPV for SLE increases with increasing IIF titre and (3) that combining IIF with solid-phase assay adds value.

This implies that a low-positive ANA IIF titre (1:80) does not significantly increase the post-test probability for SLE (as the PPV is comparable with the PPV for the entire population tested for ANA). Thus, in those cases, classification will have to rely on clinical manifestations/characteristics. The downsides of the low PPV include potential false diagnoses and inappropriate treatment by clinicians not familiar with rheumatic diseases or inappropriate referrals to rheumatologists. It is important that clinicians are acquainted with the clinical manifestations/characteristics of SLE.

It is valuable to distinguish a low positive IIF titre (1:80) from a negative IIF result, as a negative result is useful to exclude SLE, whereas a low-positive result is not. It is also valuable to distinguish a low-positive IIF titre from a high-positive IIF titre, as a high titre has a higher PPV for SLE than a low titre. Therefore, an IIF result should not be seen as a dichotomous result (positive vs negative) but as a result with titre-specific LRs for disease. A potential danger of the new classification criteria is that clinicians not familiar with systemic rheumatic diseases will overestimate the PPV of a low-positive IIF ANA, as a cut-off of 1:80 is explicitly mentioned.

New classification criteria should recognise the high (but not absolute) negative predictive value of IIF and also that a low-positive IIF ANA has a lower PPV than a high-positive IIF ANA. Different weights could be assigned to an IIF result depending on the level of positivity. Furthermore, combining IIF with solid-phase assay can help to better stratify patients, especially in case of low-positive IIF titre.[9–11]

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Contributors PW reviewed all the medical records of the patients included and performed the analysis. EDL, RW, SV and DB took care of the patients and helped with clinical classification of the patients. XB wrote a draft of the manuscript. All authors reviewed the draft and approved the submission of the manuscript.

Table 1 Clinical diagnoses in patients tested for antinuclear antibodies (ANAs)

Disease	IIF negative	IIF 1:80	IIF 1:160	IIF 1:320	IIF 1:640	IIF ≥1:1280	IIF SSA	Total
SLE (newly diagnosed)			3 (1,0,2)	1 (0,1,0)	2 (1,0,1)	6 (1,0,5)	3 (0,0,3)	15
SLE (established)	8 (2,0,6)	6 (2,1,3)	14 (8,0,6)	11 (4,2,5)	7 (2,0,5)	6 (0,0,6)	16 (0,0,16)	68
SLE (not fulfilling classification criteria)	6 (3,2,1)	2 (0,0,2)	2 (2,0,0)	1 (0,0,1)	1 (1,0,0)	3 (1,1,1)	5 (0,0,5)	20
Cutaneous lupus	4 (4,0,0)	3 (1,0,2)	4 (2,0,2)	1 (1,0,0)			10 (0,0,10)	22
Systemic sclerosis (newly diagnosed)			3 (1,0,2)	5 (0,0,5)	9 (2,0,7)	19 (3,0,16)		36
Systemic sclerosis (established)			5 (1,0,4)	5 (1,1,3)	11 (0,0,11)	6 (1,0,5)		27
Systemic sclerosis (not fulfilling classification criteria)	1 (0,1,0)	3 (3,0,0)	1 (0,0,1)	2 (0,0,2)	4 (0,0,4)	2 (1,0,1)	1 (0,0,1)	14
Polymyositis/dermatomyositis (newly diagnosed)	2 (0,0,2)		1 (0,0,1)	2 (1,1,0)	2 (1,0,1)	2 (0,0,2)		9
Polymyositis/dermatomyositis (established)			1 (1,0,0)	2 (1,1,0)	1 (0,0,1)	3 (1,1,1)	1 (0,0,1)	8
Polymyositis/dermatomyositis (not fulfilling classification criteria)	2 (0,0,2)	1 (0,1,0)	2 (2,0,0)	4 (3,0,1)	2 (0,0,2)		1 (0,0,1)	12
Sjögren's syndrome (newly diagnosed)	4 (0,0,4)	1 (0,0,1)				1 (0,0,1)	13 (0,0,13)	19
Sjögren (established)	2 (0,1,1)	2 (2,0,0)	1 (0,0,1)	3 (0,0,3)	1 (0,0,1)	1 (0,0,1)	16 (0,0,16)	26
Sjögren (not fulfilling classification criteria)	2 (1,1,0)	1 (1,0,0)					1 (0,0,1)	4
Mixed connective tissue disease (newly diagnosed)						4 (0,0,4)		4
Mixed connective tissue disease (established)			1 (0,0,1)	1 (0,0,1)		2 (0,0,2)		4
Mixed connective tissue disease (not fulfilling classification criteria)			1 (0,1,0)			3 (0,0,3)		4
Not differentiated (doubtful)	1 (1,0,0)		3 (3,0,0)	2 (1, 1)	1 (1,0,0)		1 (0,0,1)	8
Non-AASRD	47 (30,3,14)	30 (22,0,8)	18 (17,01)	8 (6,0,2)	2 (1,0,1)	2 (2,0,0)	3 (0,0,3)	110
Rheumatic disease	49 (25,11,13)	42 (37,1,4)	52 (48,0,4)	23 (20,1,2)	6 (5,1,0)	4 (4,0,0)	7 (0,0,7)	183
Inflammatory disease	76 (39,15,22)	64 (54,4,6)	44 (37,2,5)	27 (3,15,9)	12 (10,1,1)	12 (10, , 2)	6 (0,0,6)	241
No inflammatory disease	655 (395,96,164)	439 (399,10,30)	332 (296,13,23)	109 (92,9,8)	43 (33,4,6)	29 (18,1,10)	34 (4,2,28)	1641
Total	859 (500,130,229)	594 (521,17,56)	488 (419,16,53)	207 (145,19,43)	104 (56,5,43)	105 (42,4,59)	118 (4,2,112)	2475
Positive predictive values (PPVs)								
PPV of IIF for SLE	0.001	0.010	0.035	0.058	0.087	0.118	0.168	
PPV of IIF/EliA(–) SLE		0.004	0.022	0.028	0.055	0.024		
PPV of IIF/EliA(+)* SLE		0.056	0.116	0.131	0.125	0.180	0.174	
PPV of IIF for AASRD		0.020	0.069	0.157	0.344	0.515	0.541	
PPV of IIF/EliA(–) for AASRD		0.010	0.034	0.057	0.109	0.150		
PPV of IIF/EliA(+)* for AASRD	0.040	0.100	0.284	0.404	0.659	0.772	0.562	
PPV of IIF/EliA(+)† for AASRD	0.058	0.111	0.365	0.447	0.750	0.796	0.573	
Estimated likelihood ratios (LRs)								
Estimated LR of IIF for SLE	0.096	1.2	4.1	7.0	10.9	15.1	23.0	
Estimated LR of IIF/EliA(–) for SLE		0.44	2.5	3.2	6.6	2.8		
Estimated LR of IIF/EliA(+) for SLE		6.8	14.9	17.1	16.2	25.0	24.0	

9851 consecutive patients were tested for ANA by indirect immunofluorescence (IIF) (HEp-2000; ImmunoConcepts) and by solid phase (EliA CTD screen, detecting antibodies to dsDNA, SSA/Ro 52, SSA/Ro 60, SSB/La, U1-RNP (RNP-70, A, C), Sm, Jo-1, Scl-70, CENP, fibrillarin, RNA Pol III, PM-Scl, Mi-2, Rib-P and PCNA). The table gives an overview of the clinical diagnoses and the test results in consecutive patients who tested positive for ANA by IIF and/or CTD screen and in a selection on 500 patients who tested negative by both assays (total n=2475; 325 patients were excluded because there were insufficient data for proper clinical categorisation). The values indicate the number of patients with a particular IIF result. The values in parentheses indicate the number of patients who tested negative (first number), equivocal (second number) or positive (third number) with CTD screen. The population has been described in Willems *et al*.[7] Non-AASRD (non-ANA-associated systemic rheumatic disease) includes, for example, different types of vasculitis, polymyalgia rheumatica and sarcoidosis. Rheumatic diseases include, for example, rheumatoid arthritis and psoriatic arthritis. Inflammatory diseases include, for example, colitis ulcerosa, Crohn's disease, autoimmune hepatitis, autoimmune thyroiditis, psoriasis and immune thrombocytopenic purpura.

Newly diagnosed: tested on a diagnostic sample (ie, at the time of diagnosis).

Established: tested on a follow-up sample (most of these patients had received immunosuppressive therapy and had been diagnosed in another centre).

Not fulfilling classification criteria: the clinician strongly considered the presence of an AASRD and initiated immunosuppressive therapy, but the patient did not fulfil the classification criteria. For description of the classification criteria, see Willems *et al*.[7]

For the estimation of the PPV of a negative IIF result for systemic lupus erythematosus (SLE), we only took into account the SLE cases documented (1) in the patients who were single positive for CTD screen and (2) in a selection of 500 patients who were negative for IIF and CTD screen. This probably is an underestimation as we did not check the medical records of all double-negative patients. Patients with AASRD were checked whether they fulfilled the classification criteria of Sjögren's syndrome, systemic sclerosis, dermatomyositis/polymyositis, mixed connective tissue disease and SLE as described in Willems *et al*.[7] For estimation of the PPV, we excluded patients who did not fulfil the classification criteria. For estimation of the PPV for AASRD, we included cutaneous lupus as an AASRD.

*Including equivocal results.

†Excluding equivocal results.

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Response to: 'Antinuclear antibody as entry criterion for classification of systemic lupus erythematosus: pitfalls and opportunities' by Bossuyt *et al*

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We very much appreciate the comments of Willems *et al*[1] on our article on the variability of antinuclear antibody (ANA) testing for patients with established systemic lupus erythematosus (SLE).[2] Others have provided their perspective on test variability.[3–6] In their letter, Willems *et al* discuss the importance of antibody titre especially in the context of patient classification. In the new classification criteria under development, a positive ANA at a titre of 1:80 or higher is required.[7] In view of our findings on assay variability, we have also commented on the use of serology as the initial element in classification.[8] Without specification of the kit used and its performance characteristics, uncertainty about this key element of classification can occur.

The study presented by Willems *et al* provides valuable data on ANA detection by two assay formats—an immunofluorescence assay or IFA and a solid phase assay—for the broad range of patients with autoantibody-associated systemic rheumatic diseases (AASRD). Of note, among their patients with established SLE, 10% showed negative results, consistent with our findings[2]; interestingly, six out of eight of the IFA-negative patients showed antibody positivity with the solid phase assay. Together, these studies highlight the complexity of serological testing and emphasise that, whatever the purpose of such determinations—disease classification or clinical trial eligibility—it is important to take into account the performance characteristics of any particular assay, including the titres obtained.

Serological testing is a key element in the diagnosis and management of patients with AASRD because of the utility of the ANA as a biomarker especially when coupled with assays for antibodies to specific antigens. Unfortunately, standardisation remains an issue and we look forward to further studies like those of Willems *et al* to alert the field to a problem that is often not adequately recognised especially if the IFA is called the 'gold standard'. Despite decades of investigation, the field is not yet at the point where any one kit or assay is uniformly successful and has achieved the 'gold standard' status. Pending more technological advances, the use of a combination of formats (ie, IFA and solid phase) may be the best solution to fulfil the need to classify patients and identify those who are eligible for new treatments.

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Association between use of non-steroidal anti-inflammatory drugs and risk of myocardial infarction in patients with spondyloarthritis and osteoarthritis

Non-steroidal anti-inflammatory drugs (NSAIDs) have anti-inflammatory, antipyretic and analgesic effects and are widely used clinically for the treatment of osteoarthritis (OA), rheumatoid arthritis (RA) and many other inflammatory diseases.[1] At present, NSAIDs are the first-line therapy for psoriatic arthritis and axial spondyloarthritis (SpA),[2–4] but the use of NSAIDs may be related to the risk of myocardial infarction (MI).[5 6] Recently, we read with great interest the paper entitled 'Risk of myocardial infarction with use of selected non-steroidal anti-inflammatory drugs in patients with spondyloarthritis and osteoarthritis' published online in 19 April 2018 in *Annals of the Rheumatic Diseases*. Dubreuil *et al* concluded that compared with remote use of any NSAIDs, the use of diclofenac in SpA was related to twofold to threefold risk of MI, but the use of naproxen did not increase the risk of MI in OA or SpA.[7] Certainly, the findings of Dubreuil *et al* will be significant for clinicians, while there are still several questions that we would like to communicate with the authors.

First, the diagnostic criteria of MI are not clearly described in the study. According to the third universal definition of MI,[8] we can diagnose the patient as MI if one of the conditions is met. (1) To detect a rise and/or fall of cardiac biomarker values (preferably cardiac troponin (cTn)) and at least one value above the 99th percentile upper reference limit (URL). (2) Cardiac death with symptoms suggestive of myocardial ischaemia and presumed new ischaemic ECG changes or new left bundle-branch block, but death occurred before cardiac biomarkers were obtained or increased. (3) Percutaneous coronary intervention correlated MI: a rise of cTn values (>5×99th percentile URL) in patients with normal baseline values (≤99th percentile URL). (4) Stent thrombosis related to MI when detected by coronary angiography. Therefore, providing a uniform and accurate diagnostic criteria for MI will help us to know the selection criteria for patients.

Second, the definition of OA is not described in this article. At present, there is no consensus about the classification criteria of OA. American College of Rheumatology (ACR) classification criteria[9] and the Kellgren and Lawrence (K-L) system[10] are the most frequently used criteria. The ACR classification criteria are based on clinical manifestation (pain or stiffness in joint), laboratory and radiographic aspects of OA, while the K-L system identifies and grades OA depend on radiographs. However, there are subgroups of patients who have only radiographic but not symptomatic OA and vice versa owing to the heterogeneity of OA,[11] so it might be better to clarify the definition of OA in this report.

Third, the age span of patients in the study is too large (18–89 years old). The incidence of MI is different in different age groups. According to previous study, the risk of MI among older patients was greater and the size of this population was increasing.[12] Moreover, patients aged 65 years and older with MI are a heterogeneous group by age.[13] The author does not seem to consider that the prevalence of MI in patients of different ages before taking medicine is not the same.

Fourth, the information about the use of NSAIDs was not shown in detail in this paper. NSAIDs play an important role in the remission treatment of OA so that a large number of guidelines have provided relevant therapeutic opinions on the treatment of OA by NSAIDs and recommended topical NSAIDs as first-line drugs for OA (table 1).[14–16] The authors have pointed out that some patients may take NSAIDs inconsistently, only on an as-needed basis for pain. We considered whether the participants can be divided into different subgroups according to their drug use frequency or dosage in order to avoid this limitation. Therefore, the detailed usage of NSAIDs may be of great importance.

Table 1 International osteoarthritis diagnosis and treatment guidelines

Guidelines	Recommended usage
AAOS	Patients with knee OA and high risk of gastrointestinal tract: topical NSAIDs and non-selective oral NSAIDs combined with gastrointestinal protection agents.
AGS	Patients with local non-neuronal persistent pain: topical NSAIDs.
EULAR	Patients with hand osteoarthritis: topical medication is better than systemic treatment. Patients with knee osteoarthritis: topical NSAIDs are effective and safe.
NICE	Patients with knee or hand OA: to add topical NSAIDs based on core therapy to relieve pain. Topical NSAIDs should be used prior to oral NSAIDs, cyclo-oxygenase-2 inhibitors or opioids.
OARSI	Patients with knee OA: topical NSAIDs are effective adjuvant therapy and alternative treatment for oral analgesic/anti-inflammatory treatment.

AAOS, American Academy of Orthopaedic Surgeons; AGS, American Geriatrics Society; EULAR, The European League Against Rheumatism; NICE, The National Institute for Health and Care Excellence; NSAID, non-steroidal anti-inflammatory drug; OA, osteoarthritis; OARSI, Osteoarthritis Research Society International.

Last but not least, many drugs are used for the treatment of OA at present including glucosamine drugs (such as chondroitin sulfate (CS)), corticosteroid drugs (such as glucocorticoids (GCs)) and so on. CS can significantly reduce serum total cholesterol, low-density lipoprotein and triglyceride levels, which may be associated with the risk of MI.[17] Besides, GCs also may influence the risk of MI,[18] which can be mediated by its deleterious effects on hypertension, hyperlipaemia, glucose tolerance, accelerated atherosclerosis and coagulation disturbances.[19–21] How does the author rule out the effect of other osteoarthritis medications which the patients may take on MI?

We utterly respect the great contributions of the authors. Meanwhile, we would be very interested in the authors' response regarding the above issues.

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Response to: 'Association between use of non-steroidal anti-inflammatory drugs and risk of myocardial infarction in patients with spondyloarthritis and osteoarthritis'

We wish to thank Zhou and colleagues for their letter.[1] As outlined in the published paper,[2] the myocardial infarction (MI) case definition was based on the diagnosis being recorded as a MI Read code in The Health Improvement Network (THIN), a validated means of identifying MI in pharmacoepidemiological studies.[3] Because THIN is based on general practitioner's coded records, the additional information Zhou *et al* suggested[1] is not systematically available. This was already acknowledged in the discussion section as a limitation, and we additionally described our internal MI validation study in the discussion section, in which 89% of MI cases had an administrative code supporting the occurrence of an MI; these codes included a hospitalisation, having had an ECG or angiogram performed and a referral to a cardiologist. Similarly, the definition of osteoarthritis is also outlined in the Methods section.

We recognise the importance of considering age as an effect modifier related to non-steroidal anti-inflammatory drug (NSAID) use and attempted to partly address this through the sensitivity analysis restricted to subjects aged 55–70.

The most frequent NSAID prescriptions are described in online supplementary table 1 from our paper.[2] The topic of NSAID dosage and frequency certainly deserves further study.

Finally, we are not aware of data demonstrating a relation of chondroitin to risk of MI. Systemic glucocorticoids are not recommended for the treatment of knee or hip osteoarthritis nor for spondyloarthritis according to the most recent American College of Rheumatology and European League Against Rheumatism guidelines; therefore, we expect low prevalence of use among subjects in this study. Further, these medications would be considered to be intermediates in the causal pathway, and therefore adjustment for any of those types of medications would induce bias.

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Protective effects of antimalarials in Chinese patients with systemic lupus erythematosus

We read with great interest the article by Müller-Calleja *et al*[1] in which a novel mechanism of hydroxychloroquine (HCQ) was discovered, which might explain well-established anti-inflammatory effects of antimalarials.[2–4] HCQ and chloroquine (CQ), both known as antimalarial drugs, have become fundamental therapeutic elements in systemic lupus erythematosus (SLE) in these decades. However, their specific benefits on organ involvement and long-term outcome remain to be elucidated.

To examine the role of antimalarials in different clinical aspects of Chinese patients with SLE, we retrieved the medical records of 1372 patients with SLE who experienced their first hospitalisation at 26 centres across Jiangsu, an eastern province of China, between January 1999 and December 2009 from a longitudinal SLE database collected by the Jiangsu Lupus Collaborative Group.[5] Demographics of the enrolled patients are summarised in table 1. All the patients were followed up in 2015 to check for their survival status. Based on Kaplan-Meier survival analysis, the survival proportions for antimalarial users were 92.6%, 90.3% and 87.8% at 5, 10 and 16 years, significantly higher than that of non-users (83.1%, 78.9% and 76.0%, p=0.000) (figure 1A), which was consistent with previous reports in Latin American, African-American and Caucasian patients with SLE.[6–8] There was no difference in survival proportions between HCQ users and CQ users.

In previous studies, the protective role of antimalarials was mainly concentrated on lupus nephritis.[9] To further clarify this question, we performed univariate Cox proportional hazards regression analysis according to antimalarial exposure status on various clinical features. As shown in figure 1B, antimalarial drugs could exert their protective roles on a wide range of aspects, including but not limited to female gender, longer disease duration, a variety of organ involvement, hypocomplementemia and administration of glucocorticoids as well as cyclophosphamide. Besides renal involvement, patients with cardiopulmonary, gastrointestinal or haematological impairments also benefited from antimalarial treatment (all p<0.0001 by Kaplan-Meier survival analysis, figure 1C). Since antimalarials could

Table 1 Baseline characteristics of the patients with systemic lupus erythematosus by usage of antimalarials

Characteristic	Antimalarial use		P values
	User	Non-user	
Demographics			
Numbers of patients	562 (41.0%)	810 (59.0%)	
Women	526 (93.6%)	740 (91.4%)	ns
Age (years)	32.7 (12.1)	36.1 (12.6)	<0.0001
Disease characteristics			
Disease duration (years)*	3.1 (5.0)	3.7 (5.4)	ns
SLEDAI on admission	14.4 (7.4)	14.6 (8.8)	ns
SDI (≥1) on admission	1.4 (0.61)	1.6 (1.0)	ns
Organ involvements†			
Mucocutaneous	420 (74.7%)	494 (61.0%)	<0.0001
Neuropsychiatric	26 (4.6%)	66 (8.1%)	0.0113
Musculoskeletal	325 (57.8%)	419 (51.7%)	0.0276
Cardiopulmonary	91 (16.2%)	191 (23.6%)	0.0009
Gastrointestinal	114 (20.2%)	156 (19.3%)	ns
Ocular	5 (0.9%)	4 (0.5%)	ns
Renal	270 (48.0%)	431 (53.2%)	ns
Haematological	234 (41.6%)	383 (47.3%)	0.0412
Serology			
Anti-dsDNA positive	265 (50.4%)	376 (54.7%)	ns
Anti-Sm positive	164 (32.6%)	239 (34.3%)	ns
Anti-cardiolipin positive	69 (29.2%)	77 (29.7%)	ns
RF positive	112 (30.0%)	131 (27.7%)	ns
Low C3 complement‡	395 (76.6%)	489 (72.4%)	ns
Low C4 complement‡	343 (76.6%)	427 (76.8%)	ns
Medications			
Glucocorticoids	541 (96.3%)	723 (89.3%)	<0.0001
Cyclophosphamide	224 (40.0%)	358 (44.2%)	ns
Other immunosuppressants	104 (18.5%)	129 (15.9%)	ns
Comorbidities			
Hypertension	9 (1.6%)	22 (2.7%)	ns
Diabetes mellitus	26 (4.6%)	39 (4.8%)	ns
Infection	15 (2.7%)	22 (2.7%)	ns

*From the date of symptom onset to the date of first admission.
†Were assessed as Feng *et al*[5] described.
‡Complement C3 <0.8 g/L, complement C4 <0.2 g/L.
ns, non-significant; RF, rheumatoid factor; SDI, Systemic Lupus International Collaborating and Clinics /American College of Rheumatology Damage Index ; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index.

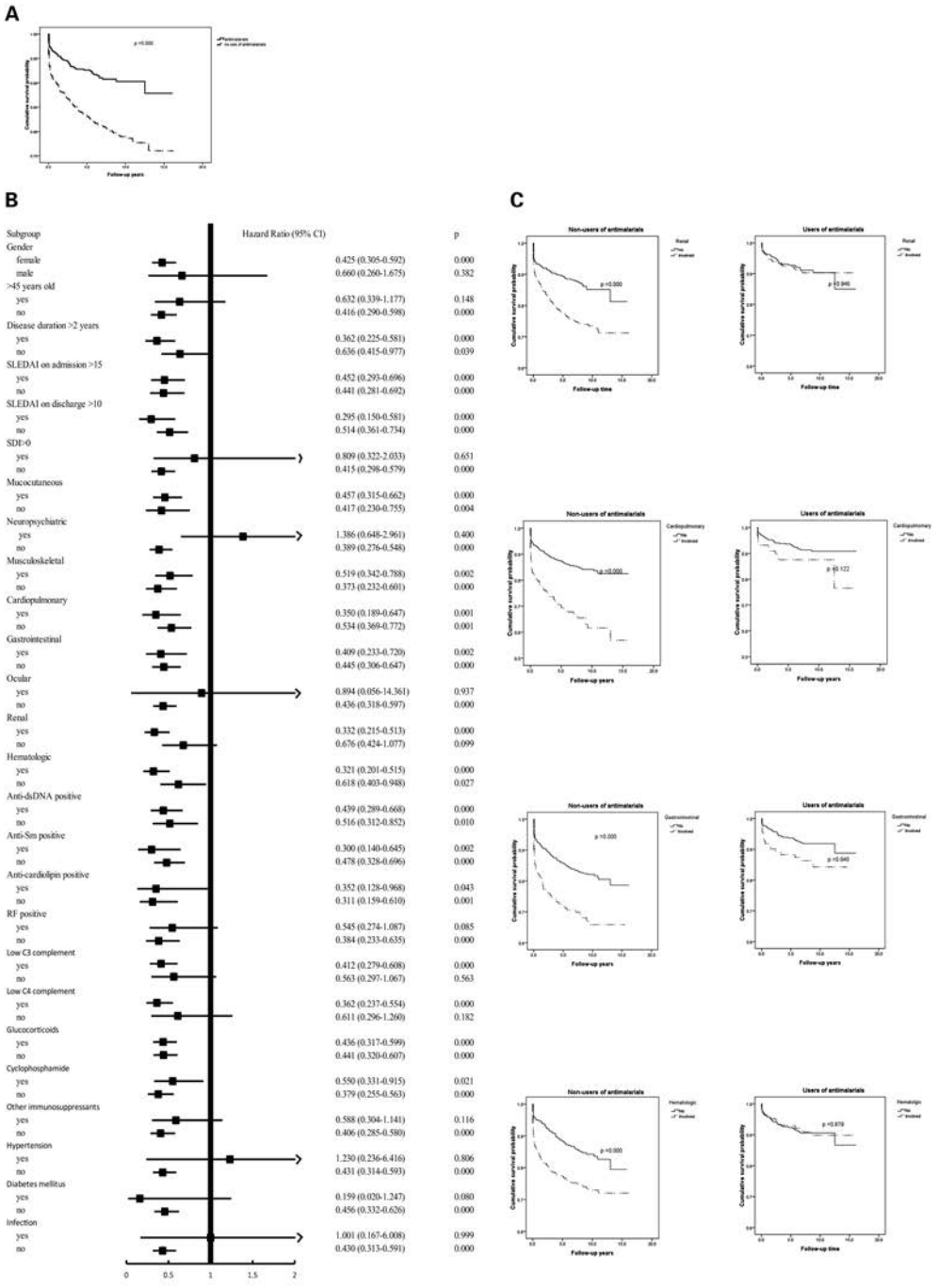

Figure 1 (A) Survival curves of patients with systemic lupus erythematosus (SLE) divided by whether using antimalarial drugs. (B) Examinations of HRs of SLE patients' characters in using antimalarials. HRs are shown with 95% CI. (C) Comparisons of survival according to SLE patients' organ involvements and antimalarial usage. SDI, Systemic Lupus International Collaborating and Clinics /American College of Rheumatology Damage Index ; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index.

interference with the assembly of endosomal NADPH oxidase to inhibit the overactivity of immune system,[1] users were expected to have better outcomes than non-users when they were accompanied with vital organ involvements.

The effect of antimalarials on alleviating organ damage may also be related to lipid lowering,[2] glycaemic controlling[3] and thrombosis prevention.[4] In this cohort, 376 patients had the record of second hospitalisation before 2015, of which 165 received antimalarial treatment. Blood level of total cholesterol was lower in antimalarial treating group than the non-treated group (4.47±1.25 mmol/L vs 5.04±2.17 mmol/L, $p<0.05$), and there was no difference between the two groups in the first hospitalisation. Other metabolic data, such as systolic and diastolic blood pressure, fasting blood sugar, triglyceride and uric acid, were similar between the two groups in two times of hospitalisation. Although percentages of leucopenia, anaemia and thrombocytopenia were similar between antimalarial users and non-users during the first hospitalisation, non-users were more likely to be anaemia on their second inpatients visits (46.5% vs 58.2%, $p<0.05$).

In conclusion, our data show that the use of antimalarials could improve long-term outcome of Chinese patients with SLE. These drugs possess protective effects on a wide range of clinical aspects. Patients with vital organ involvements, especially cardiopulmonary, gastrointestinal, renal or haematological impairments, may gain more benefits from antimalarial treatments.

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Contributors FW and WZ collected data, analysed results and FW wrote the manuscript. SW, WP, LL, MW, FD, HH, XD, HW, YZ, XQ, MW, JW, JTao, JTan, ZD, MZ and JL acquired clinical data. LS and XF designed the study. XF edited the manuscript. LS supervised the project. All authors made substantial intellectual contributions to conception of the work, the interpretation of data and approval of the final manuscript.

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Response to: 'Protective effects of antimalarials in Chinese patients with systemic lupus erythematosus' by Wang *et al*

In their letter, Wang *et al*[1] present further data showing impressively the beneficial effects of antimalarials and in particular hydroxychloroquine (HCQ) in patients with systemic lupus erythematosus. They refer to our article on the interference of HCQ with proinflammatory signalling pathways.[2] We showed that HCQ prevents activation of endosomal NADPH-oxidase (NOX) by cell surface receptors, for example, the receptors for tumour necrosis factor α (TNFα) or interleukin-1β (IL-1β). Since signalling of both receptors is mediated in large part but not exclusively by endosomal NOX, HCQ can be regarded as an inhibitor of TNFα and IL-1β. While we would like to agree with Wang *et al* that this pharmacological effect of HCQ may be an explanation of its therapeutic efficacy, their data do not provide any clue to the potential mechanisms of the observed effects. Numerous other potentially beneficial properties of HCQ have been described in the past.[3] Recently, Schreiber *et al* reported in a small cohort of patients with the antiphospholipid syndrome that treatment with HCQ for 3 months significantly reduced the amount of soluble tissue factor (TF) in plasma.[4] Since TF can be induced by antiphospholipid antibodies directly by activation of endosomal NOX[5] or indirectly via TNFα, this provides some indirect evidence that inhibition of endosomal NOX may be relevant in vivo in humans. Interestingly, beneficial metabolic effects of HCQ have been described including lowering of low-density lipoprotein cholesterol and improving insulin resistance.[6] The latter effect would be compatible with an anti-TNFα effect of HCQ. In summary, having identified many pharmacological effects of HCQ, we now need to understand which of them is responsible for the improved patient outcome. This will require clinical studies focused on the different known targets of HCQ.

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Eosinophilic granulomatosis with polyangiitis (Churg-Strauss) induced by immune checkpoint inhibitors

We read with great interest the articles by Kostine *et al*[1] (and subsequent correspondence by Arnaud *et al*[2]) and by Belkhir *et al*.[3] The prognosis of various cancer types has dramatically improved since the advent of immune checkpoint inhibitors (ICIs). Yet, ICI therapy is associated with frequent and potentially organ or life-threatening immune-related adverse events (irAEs), generally mimicking autoimmune or inflammatory conditions.[4] Rheumatic disorders have been reported in this setting, mainly rheumatoid arthritis, polymyalgia rheumatica and systemic lupus erythematosus.[1–3] Vasculitis seems to occur more seldom, with predominantly medium-vessel to large-vessel involvement.[5] Here, we report on a patient with eosinophilic granulomatosis with polyangiitis (EGPA, formerly Churg-Strauss syndrome) following treatment with ICI for a stage IV melanoma.

A 34-year-old non-smoking female patient with stage IV melanoma was treated with ipilimumab (a monoclonal antibody targeting the cytotoxic T–lymphocyte associated antigen 4) and nivolumab (a monoclonal antibody targeting the programmed death protein 1) every 3 weeks. After three infusions of combined therapy, the patient developed a severe cough associated with progressive shortness of breath due to bronchial hyper-reactivity. Biological tests showed marked hypereosinophilia (up to 2.2 g/L) and high IgE level (763 IU/L). An extensive diagnostic work-up (including HIV, toxocarosis and aspergillosis serologies, tryptase and vitamin B_{12} dosages) was unremarkable. Antineutrophil cytoplasmic antibody (ANCA) serology was negative. T-lymphocyte phenotyping showed no aberrant (eg, $CD3^{-}CD4^{+}$) phenotype and no clonal T-cell receptor gene pattern was evidenced. Troponin and brain natriuretic peptide (BNP) levels as well as ECG, echocardiography and cardiac MRI were normal. Her condition improved rapidly after treatment with inhaled corticosteroids (ICSs) and long-acting beta-agonist therapy (LABA). Routine PET imaging showed a dramatic tumour response.

After four additional infusions of nivolumab maintenance therapy, the patient had recurrence of dry cough, shortness of breath, wheezing, decreased oxygen saturation (93%) and hypereosinophilia up to 1.76 g/L. A high-resolution chest CT showed ground-glass opacities of the right lung apex and bronchial wall thickening. Bronchoscopy and bronchial biopsies were unremarkable, yet bronchoalveolar lavage could not be performed due to the bronchospasm. Nivolumab was paused and the patient's condition improved within days. When nivolumab was reintroduced, eosinophil level increased again, and asthma symptoms worsened despite of continuation of ICS/LABA, resulting in definitive discontinuation of nivolumab (figure 1). Two months after stopping nivolumab, the patient developed joint pain and swelling of both knees, whose aspiration revealed 8100 leucocytes/mm^3 (24% of which were granulocytes) without evidence of bacterial infection nor microcrystalline arthropathy and negative immunological work-up (including ANCA, rheumatoid factor, antinuclear and anticitrullinated protein autoantibodies). Concomitantly, brain MRI performed for melanoma follow-up showed evidence of pansinusitis (mucosal thickening and opacification of sinuses). Hence, in the context of asthma, hypereosinophilia, pulmonary infiltrates, arthritis and paranasal sinus abnormalities, the diagnosis of EGPA (formerly Churg-Strauss syndrome) was retained[6] and the patient was treated with ICS/LABA and intraarticular corticosteroid injection.

Moderate asymptomatic hypereosinophilia has been reported in up to 3% of patients treated with ICI.[7] To our knowledge, this is the first case report of a patient with ICI-induced EGPA. Since the patient showed no evidence of genuine vasculitis, one might also classify him as having hypereosinophilic asthma with systemic manifestations.[8] The pathophysiological process leading to eosinophilia remains unknown, but the high level of serum IgE strongly suggests Th2-mediated reactive hypereosinophilia induced by ICI.

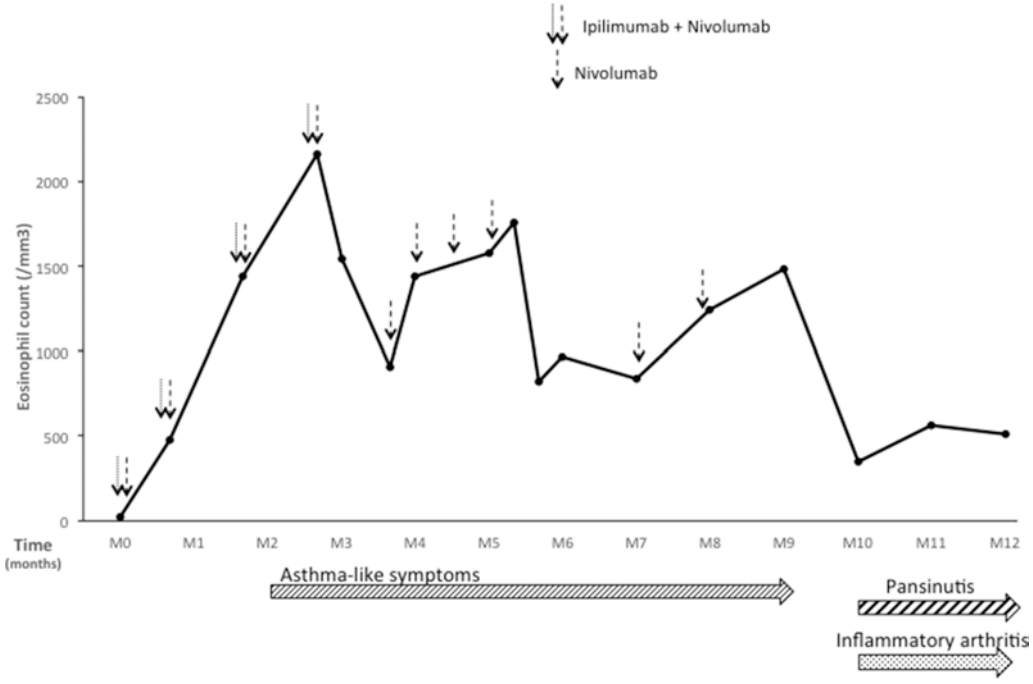

Figure 1 Timeline showing the relationship between serum eosinophil count and treatment with immune checkpoint inhibitors (double arrow: nivolumab 1 mg/kg and ipilimumab 3 mg/kg infusions; single arrow: nivolumab 3 mg/kg infusion).

Kostine *et al*[1] suggest that patients with rheumatic irAEs are more likely to respond to ICI, and increased eosinophil counts also correlate with improved survival in patients with metastatic melanoma treated with ICI,[9] possibly due in part to the antitumour effect of eosinophil granulocytes.[10] In this line, a complete tumour response persisted 12 months after nivolumab discontinuation in our patient.

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Author note MG recently moved to the Department of Internal Medicine, National Referral Centre for Hypereosinophilic Syndromes (CEREO), Hopital Foch, Suresnes, France.

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Response to: 'Eosinophilic granulomatosis with polyangiitis (Churg-Strauss) induced by immune checkpoint inhibitors' by Delyon *et al*

We thank Delyon *et al* for their interest in our original paper[1] and for reporting on a patient with immune checkpoint inhibitors (ICI)-induced eosinophilic granulomatosis with polyangiitis (EGPA).[2] Since the first description in 2014 of giant cell arthritis (GCA) following treatment with ipilimumab (anti-CTLA-4 agent) in two patients with melanoma,[3] several vasculitis cases have been reported, as recently highlighted by Daxini *et al* in a systematic literature review.[4] Overall, they compiled 20 cases of confirmed ICI-induced vasculitis, which were classified using the revised International Chapel Hill Consensus Conference nomenclature. The most commonly reported types of vasculitis were large vessel vasculitis and vasculitis of the nervous system, with a median exposure time to ICI of 3 months. The majority of patients received oral or intravenous corticosteroids and ICI was discontinued. Of note, a granulomatosis with polyangiitis occurred 1 week following anti-programmed cell death 1 (PD-1) agent for advanced melanoma, requiring methylprednisolone and oral daily cyclophosphamide.[5] Unfortunately, data on cancer and vasculitis outcomes are often missing.

By adding the first case report of EGPA, Delyon *et al* complete the unique and expanding list of ICI-related adverse events (irAEs). Interestingly, antineutrophil cytoplasmic antibodies were negative, similar with findings in other rheumatic irAEs such as myositis and inflammatory arthritis where autoantibodies are often negative, which should not exclude the diagnosis. Therefore, rheumatologists as well as other organ specialists involved in the diagnosis and management of irAEs have to consider vasculitis when evaluating patients receiving ICI. Notably, since polymyalgia rheumatica-like syndrome is frequently reported with anti-PD-(L)1 therapies,[6] we should pay particular attention to GCA symptoms.

On the other hand, Zhang *et al* reported an insufficient negative signalling by the immune checkpoint PD-1/PD-L1 in the pathogenesis of GCA.[7] Indeed, they demonstrated low expression of PD-L1 in GCA dendritic cells compared with healthy arteries, unleashing PD-1^{high} T cells to infiltrate and damage the walls of large arteries. These data shed light on the association between vasculitis and the downregulation of PD-1/PD-L1 pathway, either spontaneous or induced by ICI, and may suggest future therapeutic immunomodulatory approaches in our classical vasculitis.

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Inconsistency between supplement and article?

In their recent article in the *Annals of the Rheumatic Diseases*, van der Heijde D *et al*[1] evaluated the effect of certolizumab pegol (CZP) on radiographic progression in patients with ankylosing spondylitis and non-radiographic axial spondyloarthritis (nr-axSpA). They suggested that CZP-treated patients with axSpA had less modified Stokes Ankylosing Spondylitis Spinal Score (mSASSS) change and spinal progression at the end of 4 years. I read this fascinating article with interest. There is not an extra word to say about the design of the study, data presentation and valuable conclusion. But I noticed a conflict between table 1 (supplementary table 1) and supplementary table 2.

According to results of the study, there are 196 patients who had ≥1 mSASSS assessment, which is consistent with Supplementary table 2. But, according to table 1 and Supplementary table 1 of the study, there are 190 patients who have baseline mSASSS assessment, which is inconsistent with supplementary table 2. According to supplementary table 2, there is 193 patient who have baseline mSASSS assessment. I did not notice any explanation of this situation in the article. Although I am aware that three patients would not change the results of the study, I think this should be clarified.

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REFERENCE

1 van der Heijde D, Baraliakos X, Hermann KA, *et al*. Limited radiographic progression and sustained reductions in MRI inflammation in patients with axial spondyloarthritis: 4-year imaging outcomes from the RAPID-axSpA phase III randomised trial. *Ann Rheum Dis* 2018;77:699–705.

Response to: 'Inconsistency between supplement and article?' by Babaoglu H

We thank Dr Babaoglu for his question about our manuscript entitled 'Limited Radiographic Progression and Sustained Reductions in MRI Inflammation in Patients with Axial Spondyloarthritis: Four-Year Imaging Outcomes from the RAPID-axSpA Phase 3 Randomised Trial.'[1 2] In his response, he refers to an apparent discrepancy between information in the main manuscript and supplemental material. He is correct in his statement that 196 patients had ≥1 modified Stoke Ankylosing Spondylitis Score (mSASSS) assessment (online supplementary table 2) whereas 190 had an mSASSS assessment at baseline (table 1 and online supplementary table 1); that is, six patients who had ≥1 mSASSS assessment did not have an assessment at baseline. These tables are correct. Of these six patients without baseline assessments, three had mSASSS assessments at both week 12 and week 204, and three had mSASSS assessments at both week 96 and week 204.

For three of the six subjects without an available mSASSS assessment at baseline, radiographs and MRI were conducted at week 12. Since negligible or no changes in mSASSS are expected within 3 months, these data from week 12 were used in the mixed-model repeated measures (MMRM) analysis of mSASSS change and multiple imputation analysis of mSASSS progression (defined as a ≥2 point increase). In online supplementary table 2, which provides supporting information to these model-based analyses, we labelled these three patients as having available mSASSS assessments at baseline. However, these data were not used for any descriptive analyses involving baseline data. We acknowledge that the information currently provided is somewhat inconsistent and more details could have been given about these data, but the number of patients that were used for the various analyses is correct.

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Comment on: 'Aberrant tRNA processing causes an autoinflammatory syndrome responsive to TNF inhibitors' by Giannelou *et al*: mutations in *TRNT1* result in a constitutive activation of type I interferon signalling

We read with great interest the paper of Giannelou *et al*[1] reporting, for the first time, the efficacy of tumour necrosis factor (TNF) inhibitors in sideroblastic anaemia with immunodeficiency, fevers and developmental delay (SIFD). These authors also demonstrated high levels of interleukin (IL)-6, IL-12p40, IL-18, interferon (IFN)-γ and IFN-induced chemokines (IP-10 and MIG) in two patients. Herein, we wish to highlight that an activation of the type I IFN pathway may also be observed in SIFD.

Patient 1 (P1) was a 12-month-old girl referred because of recurrent attacks of fever from the age of 2 months, with or without documented infections, and failure to thrive. C-reactive protein (CRP) levels were elevated during each episode. Infections consisted of recurrent septicaemias. Aseptic febrile manifestations included vulvitis, parotiditis, adenitis and neutrophilic panniculitis. A chronic microcytic anaemia and low levels of serum IgG, IgA and IgM were noted from 2 months of age. She developed progressive lymphopenia with undetectable levels of B lymphocytes and $CD27^+$ B memory cells by age 15 months, so that intravenous immunoglobulin (IVIG) (400 mg/kg/month) was initiated at this time. At last follow-up, aged 6 years, her height and development were normal and she was no longer subject to recurrent infectious episodes. However, she continued to experience three to four febrile attacks, lasting for 1–2 days, each month, associated with elevated CRP levels. Whole-exome sequencing identified compound heterozygous mutations in the *TRNT1* gene (c.1213G>A, p.G405R / c.1057–7C>G) encoding tRNA nucleotidyl transferase.

Patient 2 (P2), a girl born at term to first-cousin parents of African ethnicity, presented with intrauterine growth retardation (IUGR) and severe neonatal anaemia necessitating blood transfusion on the first day of life. During infancy, she required regular blood transfusions because of sideroblastic anaemia, which resolved spontaneously at the age of 4 years. She also demonstrated developmental delay and severe disease flares associated with fever, diarrhoea and dehydration. CRP levels were elevated during these episodes. Mild T $CD8^+$ and natural killer lymphopenia, together with a profound B cell defect (B lymphocytes: 0.062×10^9/L, normal range 0.273–0.86×10^9/L), were evident at 4 years of age. Sanger sequencing of *TRNT1* identified a homozygous mutation in exon 7 (c.977T>C, p.I326T). The family history was notable for a first female child deceased at 29 weeks of gestation with IUGR, severe anaemia and respiratory and cardiac failure. Autopsy revealed pericardial effusion, cardiomegaly and congestion of the spleen and the liver. No DNA testing was performed.

Using an ultrasensitive digital ELISA combined with a high specificity pan-IFN-α antibody pair,[2] we observed an increased concentration of IFN-α protein in the serum from P1 (246.44 fg/mL, healthy controls <10 fg/mL) and P2 (108.18 and 38.05 fg/mL, healthy controls <10 fg/mL), comparable to the levels measured in certain monogenic type I interferonopathies.[2] Consistent with these data, we also recorded an increased expression of IFN stimulated genes (ISGs) in the whole blood of P1 and P2 on three occasions. Furthermore, in an *ex vivo* flow cytometry assay, STAT1 and STAT3 were constitutively phosphorylated in T $CD3^+$ lymphocytes and monocytes from the whole blood of P2. Both patients also displayed a negative IFN score on one occasion, suggesting a fluctuating biological process. A high daily variability of IFN scores has been reported in patients with mutations in the immunoproteasome.[3]

These observations indicate a constitutive activation of the type I IFN pathway in patients with biallelic mutations in *TRNT1* and thus suggest a possible role of type I IFN in the pathogenesis of SIFD. An increase in serum IFN-α protein, measured by standard ELISA, and an enhanced expression of ISGs have been previously reported in one patient with SIFD.[4] Mitochondrial (mt) reactive oxygen species (ROS) have recently emerged as critical factors in the regulation of immune signalling pathways. By triggering NLRP3 inflammasome activation,[1] Giannelou *et al* suggest that ROS accumulation observed in cultured TRNT1-deficient fibroblasts might explain the associated autoinflammatory manifestations. Indeed, a high production of IL-1 in the supernatant of stimulated monocyte-derived macrophages and in colon biopsy tissue was observed in two patients. Of note, anti-IL1 agents were not tested in these patients, who responded to TNF inhibitors. Mitochondrial ROS impact immunoregulatory functions in plasmacytoid dendritic cells (pDCs), both in the early and late type I IFN response.[5] Agod *et al* demonstrated that the RIG-I-mediated late phase of type I IFN production is intensified by elevated mtROS levels in pDCs.[5] On the other hand, toxic ROS levels in mixed-Lineage-Leukemia-5 (Mll5)-deficient mice are critically dependent on type I IFN signalling, which triggers mitochondrial accumulation of full-length Bid.[6] Thus, it might be hypothesised that a mutual activation of ROS and type I IFN results in type I IFN-mediated autoinflammatory disease. Moreover, NLRP3 recruitment via mitochondrial antiviral-signaling (MAVS)-RIG-I could occur in the mitochondrial outer membrane,[7] suggesting that the autoinflammation ascribed to NLRP3 in SIFD may be initiated through the activation of RIG-I. RIG-I encodes a helicase, responsible for the recognition of foreign dsRNAs and the subsequent induction of a type I IFN response through MAVS, a facilitator of the activation of the TBK1 kinase and the IRF3 transcription factor after RNA detection. Overall, our data suggest that constitutive activation of the type I IFN pathway could be relevant to the pathophysiology of SIFD, although how aberrant tRNA processing secondary to TRNT1 dysfunction might mediate enhanced type I IFN production and signalling is unclear at this time.

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Multimodal analgesia to reduce NSAID induced myocardial Infarction

We read with great interest the extended report by Dubreuil *et al* on 'Risk of myocardial infarction with use of selected non-steroidal anti-inflammatory drugs in patients with spondyloarthritis and osteoarthritis'.[1] This study raises interesting point that the use of certain non-steroidal anti-inflammatory drugs (NSAIDs) such as diclofenac increases the risk of myocardial infarction (MI) in patients with spondyloarthritis by twofold to threefold.

The treatment goal for patients with ankylosing spondylitis should be to maximise quality of life through: symptom control, minimising inflammation, prevention of structural damage and preservation/normalisation of function and social participation.[2 3] NSAIDs are recommended by the European League against Rheumatism (EULAR) as first-line therapy in patients with spondyloarthritis in both short term and long term.[4–6]

The analgesic benefit of NSAIDs appears to be dose dependent, interestingly, so too is the risk of MI.[7 8] Furthermore, Bhala and colleagues demonstrated that use of certain NSAIDs increase the risk of MI in people without a history of cardiovascular disease.[7 9] Currently, EULAR recommends physicians to consider additional analgesics for residual pain after previous suboptimal treatments. Given the likely dose-dependent risk of MI, it may also be necessary to recommend the use a multimodal analgesic approach in an effort to reduce the required NSAID dosing.

Previously, a Cochrane review found that there was no added benefit when comparing monotherapy versus combination therapy in adults aged 18 years or older with diagnosis of inflammatory arthritis: rheumatoid arthritis, ankylosing spondylitis, psoriatic arthritis and other spondyloarthritis. However, previous studies do not report on the ability of additional analgesic agents to reduce the required dose of NSAIDs. Within the Cochrane review, three studies showed some benefit with the addition of paracetamol.[10]

It is worth noting that none of these studies assess the value of combination therapy for patients with inflammatory arthritis who have persistent pain despite optimal disease suppression. In this situation, it may be found that anti-inflammatory analgesic may be of no greater benefit than other simple analgesics such as paracetamol. Additional agents that may prove beneficial in reducing the required doses of NSAIDs, if not improving pain control in acute flares include pregabalin,[11] duloxetine,[11] tramadol,[12] tapentadol[13] and buprenorphine.[14]

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Correction: *Development and validation of the Scleroderma Clinical Trials Consortium Damage Index (SCTC-DI): a novel instrument to quantify organ damage in systemic sclerosis*

Ferdowsi N, Huq M, Stevens W, *et al*. Development and validation of the Scleroderma Clinical Trials Consortium Damage Index (SCTC-DI): a novel instrument to quantify organ damage in systemic sclerosis. Ann Rheum Dis 2019;78:807–16. doi:10.1136/annrheumdis-2018-214764

Figures 3 and 4 have been placed the wrong way around. The citations to the figures in the text and the figure legends are correct in the article.

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